Chittaranjan Kole *Editor*

Wild Crop Relatives: Genomic and Breeding Resources

Plantation and Ornamental Crops

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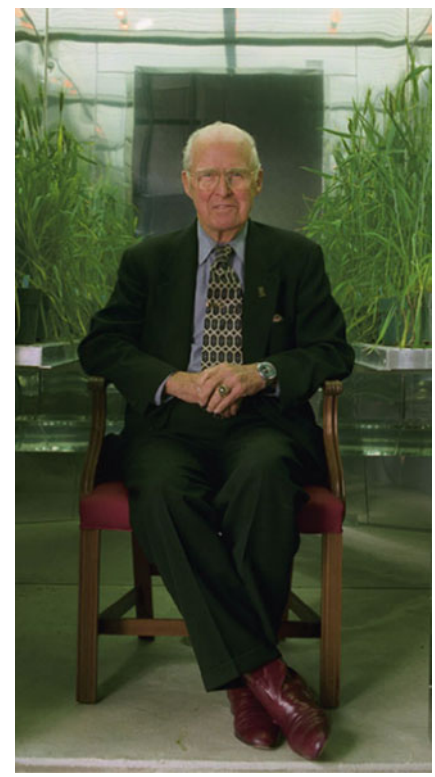
Dedication



Dr. Norman Ernest Borlaug,[1] the Father of Green Revolution, is well respected for his contributions to science and society. There was or is not and never will be a single person on this Earth whose single-handed service to science could save millions of people from death due to starvation over a period of over four decades like Dr. Borlaug's. Even the Nobel Peace Prize he received in 1970 does not do such a great and noble person as Dr. Borlaug justice. His life and contributions are well known and will remain in the pages of history of science. I wish to share some facets of this elegant and ideal personality I had been blessed to observe during my personal interactions with him.

It was early 2007 while I was at the Clemson University as a visiting scientist one of my lab colleagues told me that "somebody wants to talk to you; he appears to be an old man". I took the telephone receiver casually and said hello. The response from the other side was – "I am Norman Borlaug; am I talking to Chitta?" Even a million words would be insufficient to define and depict the exact feelings and thrills I experienced at that moment!

[1]The photo of Dr. Borlaug was kindly provided by Julie Borlaug (Norman Borlaug Institute for International Agriculture, Texas A&M Agriculture) the granddaughter of Dr. Borlaug.

I had seen Dr. Borlaug only once, way back in 1983, when he came to New Delhi, India to deliver the Coromandal Lecture organized by Prof. M.S. Swaminathan on the occasion of the 15th International Genetic Congress. However, my real interaction with him began in 2004 when I had been formulating a 7-volume book series entitled *Genome Mapping and Molecular Breeding in Plants*. Initially, I was neither confident of my ability as a series/book editor nor of the quality of the contents of the book volumes. I sent an email to Dr. Borlaug attaching the table of contents and the tentative outline of the chapters along with manuscripts of only a few sample chapters, including one authored by me and others, to learn about his views as a source of inspiration (or caution!) I was almost sure that a person of his stature would have no time and purpose to get back to a small science worker like me. To my utter (and pleasant) surprise I received an email from him that read: "May all Ph.D.'s, future scientists, and students that are devoted to agriculture get an inspiration as it refers to your work or future work from the pages of this important book. My wholehearted wishes for a success on your important job". I got a shot in my arm (and in mind for sure)! Rest is a pleasant experience – the seven volumes were published by Springer in 2006 and 2007, and were welcome and liked by students, scientists and their societies, libraries, and industries. As a token of my humble regards and gratitude, I sent Dr. Borlaug the Volume I on *Cereals and Millets* that was published in 2006. And here started my discovery of the simplest person on Earth who solved the most complex and critical problem of people on it – hunger and death.

Just one month after receiving the volumes, Dr. Borlaug called me one day and said, "Chitta, you know I cannot read a lot now-a-days, but I have gone through only on the chapters on wheat, maize and rice. Please excuse me. Other chapters of these volumes will be equally excellent, I believe." He was highly excited to know that many other Nobel Laureates including Profs. Arthur Kornberg, Werner Arber, Phillip Sharp, Günter Blobel, and Lee Hartwell also expressed generous comments regarding the utility and impact of the book series on science and the academic society. While we were discussing many other textbooks and review book series that I was editing at that time, again in my night hours for the benefit of students, scientists, and industries, he became emotional and told me to forget about my original contributions and that I deserved at least the World Food Prize, if not Nobel Prize for peace like him. I felt honored but really very ashamed as I am aware of my almost insignificant contribution in comparison to his work, and was unable to utter any words for a couple of minutes!

In another occasion he wanted some documents from me. I told him that I will send them as attachments in emails. Immediately he shouted and told me: "You know, Julie (his granddaughter) is not at home now and I cannot check email myself. Julie does this for me. I can type myself in type writer but I am not good in computer. You know what, I have a Xerox machine and it receives fax also. Send me the documents by fax." Here was the ever-present child in him.

Another occasion is when I was talking with him in a low voice, and he immediately chided me: "You know that I cannot hear well now-a-days; I don't know where Julie has kept the hearing apparatus, can't you speak louder?" Here was the fatherly figure who was eager to hear each of my words!

I still shed tears when I remember during one of our telephone conversations he asked: "You know I have never seen you, are you coming to Texas in the near future by chance?" I remember we were going through a financial paucity at that time and I could not make a visit to Texas to see him, though it would have been a great honor.

In late 2007, whenever I tried to talk to Dr. Borlaug, he used to beckon Julie to bring the telephone to him, and in course of time Julie used to keep alive communications between us when he slowly succumbed to his health problems.

The remaining volumes of the Genome Mapping and Molecular Breeding in Plants were published in 2007, and I sent him the volumes. I wished to learn about his views. During this period he could hardly speak and write. Julie prepared a letter on his behalf that read: "Dear Chitta, I have reviewed the seven volumes of the series on Genome Mapping and Molecular Breeding in Plants, which you have authored. You have brought together genetic linkage maps based on molecular markers for the most important crop species that will be a valuable guide and tool to further molecular crop improvements. Congratulations for a job well done."

During one of our conversations in mid-2007, he asked me what other book projects I was planning for Ph.D. students and scientists. I told him that the wealth of wild species already utilized and to be utilized for genetic analysis and improvement of domesticated crop species have not been deliberated in any book project. He was very excited and told me to take up the book project as soon as possible. By that time I had a huge commitment to editing book volumes and could not start the series he was so interested about.

His sudden demise in September 2009 kept me so morose for a number of months that I did not even communicate my personal loss to Julie. But in the meantime, I formulated a ten-volume series on *Wild Crop Relatives: Genomic and Breeding Resources* for Springer. And whom else to dedicate this series to other than Dr. Borlaug!

I wrote to Julie for her formal permission and she immediately wrote me: "Chitta, Thank you for contacting me and yes I think my grandfather would be honored with the dedication of the series. I remember him talking of you and this undertaking quite often. Congratulations on all that you have accomplished!" This helped me a lot as I could at least feel consoled that I could do a job he wanted me to do and I will always remain grateful to Julie for this help and also for taking care of Dr. Borlaug, not only as his granddaughter but also as the representative of millions of poor people from around the glove and hundreds of plant and agricultural scientists who tries to follow his philosophy and worship him as a father figure.

It is another sad experience of growing older in life that we walk alone and miss the affectionate shadows, inspirations, encouragements, and blessings from the fatherly figures in our professional and personal lives. How I wish I could treat our next generations in the same way as personalities like Dr. Norman Borlaug did to me and many other science workers from around the world!

During most of our conversations he used to emphasize the immediate impact of research on the society. A couple of times he even told me that my works on molecular genetics and biotechnology, particularly of 1980s and 1990s, have high fundamental importance, but I should also do some works that will benefit people. This advice elicited a change in my approach to science and since then I have been devotedly endeavored to develop crop varieties enriched with phytomedicines and nutraceuticals. Inspiration, advices, and blessings of Dr. Borlaug have influenced both my personal and professional life, particularly my approach to science, and I dedicate this series to him as a token of my regards and gratitude, and in remembrance of his great contribution to science and society and above all his personal affection for me.

I emailed the above draft of the dedication page to Julie for her views and I wish to complete my humble dedication with great satisfaction with the words of Julie who

served as the living ladder for me to reach and stay closer to such as great human being as Dr. Borlaug and expressing my deep regards and gratitude to her. Julie's email read: "Chitta, Thank you for sending me the draft dedication page. I really enjoyed reading it and I think you captured my grandfather's spirit wonderfully. . .. So thank you very much for your beautiful words. I know he would be and is honored."

Clemson, USA Chittaranjan Kole

Preface

Wild crop relatives have been playing enormously important roles both in the depiction of plant genomes and the genetic improvement of their cultivated counterparts. They have contributed immensely to resolving several fundamental questions, particularly those related to the origin, evolution, phylogenetic relationship, cytological status and inheritance of genes of an array of crop plants; provided several desirable donor genes for the genetic improvement of their domesticated counterparts; and facilitated the innovation of many novel concepts and technologies while working on them directly or while using their resources. More recently, they have even been used for the verification of their potential threats of gene flow from genetically modified plants and invasive habits. Above all, some of them are contributing enormously as model plant species to the elucidation and amelioration of the genomes of crop plant species.

As a matter of fact, as a student, a teacher, and a humble science worker I was, still am and surely will remain fascinated by the wild allies of crop plants for their invaluable wealth for genetics, genomics and breeding in crop plants and as such share a deep concern for their conservation and comprehensive characterization for future utilization. It is by now a well established fact that wild crop relatives deserve serious attention for domestication, especially for the utilization of their phytomedicines and nutraceuticals, bioenergy production, soil reclamation, and the phytoremediation of ecology and environment. While these vastly positive impacts of wild crop relatives on the development and deployment of new varieties for various purposes in the major crop plants of the world agriculture, along with a few negative potential concerns, are envisaged the need for reference books with comprehensive deliberations on the wild relatives of all the major field and plantation crops and fruit and forest trees is indeed imperative. This was the driving force behind the inception and publication of this series.

Unlike the previous six book projects I have edited alone or with co-editors, this time it was very difficult to formulate uniform outlines for the chapters of this book series for several obvious reasons. Firstly, the status of the crop relatives is highly diverse. Some of them are completely wild, some are sporadically cultivated and some are at the initial stage of domestication for specific breeding objectives recently deemed essential. Secondly, the status of their conservation varies widely: some have been conserved, characterized and utilized; some have been eroded completely except for their presence in their center(s) of origin; some are at-risk or endangered due to genetic erosion, and some of them have yet to be explored. The third constraint is the variation in their relative worth, e.g. as academic model, breeding resource, and/or potential as “new crops.”

The most perplexing problem for me was to assign the chapters each on a particular genus to different volumes dedicated to crop relatives of diverse crops grouped based on their utility. This can be exemplified with Arabidopsis, which has primarily benefited the Brassicaceae crops but also facilitated genetic analyses and improvement in crop plants in other distant families; or with many wild relatives of forage crops that paved the way for the genetic analyses and breeding of some major cereal and millet crops. The same is true for wild crop relatives such as Medicago truncatula, which has paved the way for in-depth research on two crop groups of diverse use: oilseed and pulse crops belonging to the Fabaceae family. The list is too long to enumerate. I had no other choice but to compromise and assign the genera of crop relatives in a volume on the crop group to which they are taxonomically the closest and to which they have relatively greater contributions. For example, I placed the chapter on genus Arabidopsis in the volume on oilseeds, which deals with the wild relatives of Brassicaceae crops amongst others.

However, we have tried to include deliberations pertinent to the individual genera of the wild crop relatives to which the chapters are devoted. Descriptions of the geographical locations of origin and genetic diversity, geographical distribution, karyotype and genome size, morphology, etc. have been included for most of them. Their current utility status – whether recognized as model species, weeds, invasive species or potentially cultivable taxa – is also delineated. The academic, agricultural, medicinal, ecological, environmental and industrial potential of both the cultivated and/or wild allied taxa are discussed.

The conservation of wild crop relatives is a much discussed yet equally neglected issue albeit the in situ and ex situ conservations of some luckier species were initiated earlier or are being initiated now. We have included discussions on what has happened and what is happening with regard to the conservation of the crop relatives, thanks to the national and international endeavors, in most of the chapters and also included what should happen for the wild relatives of the so-called new, minor, orphan or future crops.

The botanical origin, evolutionary pathway and phylogenetic relationship of crop plants have always attracted the attention of plant scientists. For these studies morphological attributes, cytological features and biochemical parameters were used individually or in combinations at different periods based on the availability of the required tools and techniques. Access to different molecular markers based on nuclear and especially cytoplasmic DNAs that emerged after 1980 refined the strategies required for precise and unequivocal conclusions regarding these aspects. Illustrations of these classical and recent tools have been included in the chapters. Positioning genes and defining gene functions required in many cases different cytogenetic stocks, including substitution lines, addition lines, haploids, monoploids and aneuploids, particularly in polyploid crops. These aspects have been dealt in the relevant chapters. Employment of colchiploidy, fluorescent or genomic in situ hybridization and Southern hybridization have reinforced the theoretical and applied studies on these stocks. Chapters on relevant genera/species include details on these cytogenetic stocks.

Wild crop relatives, particularly wild allied species and subspecies, have been used since the birth of genetics in the twentieth century in several instances such as studies of inheritance, linkage, function, transmission and evolution of genes. They have been frequently used in genetic studies since the advent of molecular markers. Their involvement in molecular mapping has facilitated the development of mapping

populations with optimum polymorphism to construct saturated maps and also illuminating the organization, reorganization and functional aspects of genes and genomes. Many phenomena such as genomic duplication, genome reorganization, self-incompatibility, segregation distortion, transgressive segregation and defining genes and their phenotypes have in many cases been made possible due to the utilization of wild species or subspecies. Most of the chapters contain detailed elucidations on these aspects.

The richness of crop relatives with biotic and abiotic stress resistance genes was well recognized and documented with the transfer of several alien genes into their cultivated counterparts through wide or distant hybridization with or without employing embryo-rescue and mutagenesis. However, the amazing revelation that the wild relatives are also a source of yield-related genes is a development of the molecular era. Apomictic genes are another asset of many crop relatives that deserve mention. All of these past and the present factors have led to the realization that the so-called inferior species are highly superior in conserving desirable genes and can serve as a goldmine for breeding elite plant varieties. This is particularly true at a point when natural genetic variability has been depleted or exhausted in most of the major crop species, particularly due to growing and promoting only a handful of so-called high-yielding varieties while disregarding the traditional cultivars and landraces. In the era of molecular breeding, we can map desirable genes and poly-genes, identify their donors and utilize tightly linked markers for gene introgression, mitigating the constraint of linkage drag, and even pyramid genes from multiple sources, cultivated or wild taxa. The evaluation of primary, secondary and tertiary gene pools and utilization of their novel genes is one of the leading strategies in present-day plant breeding. It is obvious that many wide hybridizations will never be easy and involve near-impossible constraints such as complete or partial sterility. In such cases gene cloning and gene discovery, complemented by intransgenic breeding, will hopefully pave the way for success. The utilization of wild relatives through traditional and molecular breeding has been thoroughly enumerated over the chapters throughout this series.

Enormous genomic resources have been developed in the model crop relatives, for example Arabidopsis thaliana and Medicago truncatula. BAC, cDNA and EST libraries have also been developed in some other crop relatives. Transcriptomes and metabolomes have also been dissected in some of them. However, similar genomic resources are yet to be constructed in many crop relatives. Hence this section has been included only in chapters on the relevant genera.

In this book series, we have included a section on recommendations for future steps to create awareness about the wealth of wild crop relatives in society at large and also for concerns for their alarmingly rapid decrease due to genetic erosion. The authors of the chapters have also emphasized on the imperative requirement of their conservation, envisaging the importance of biodiversity. The importance of intellectual property rights and also farmers' rights as owners of local landraces, botanical varieties, wild species and subspecies has also been dealt in many of the chapters. I feel satisfied that the authors of the chapters in this series have deliberated on all the crucial aspects relevant to a particular genus in their chapters.

I am also very pleased to present many chapters in this series authored by a large number of globally reputed leading scientists, many of whom have contributed to the development of novel concepts, strategies and tools of genetics, genomics and breeding and/or pioneered the elucidation and improvement of particular plant

genomes using both traditional and molecular tools. Many of them have already retired or will be retiring soon, leaving behind their legacies and philosophies for us to follow and practice. I am saddened that a few of them have passed away during preparation of the manuscripts for this series. At the same time, I feel blessed that all of these stalwarts shared equally with me the wealth of crop relatives and contributed to their recognition and promotion through this endeavor.

I would also like to be candid with regard to my own limitations. Initially I planned for about 150 chapters devoted to the essential genera of wild crop relatives. However, I had to exclude some of them either due to insignificant progress made on them during the preparation of this series, my failure to identify interested authors willing to produce acceptable manuscripts in time or authors' backing out in the last minute, leaving no time to find replacements. I console myself for this lapse with the rationale that it is simply too large a series to achieve complete satisfaction on the contents. Still I was able to arrange about 125 chapters in the ten volumes, contributed by nearly 400 authors from over 40 countries of the world. I extend my heartfelt thanks to all these scientists, who have cooperated with me since the inception of this series not only with their contributions, but also in some cases by suggesting suitable authors for chapters on other genera. As happens with a mega-series, a few authors had delays for personal or professional reasons, and in a few cases, for no reason at all. This caused delays in the publication of some of the volumes and forced the remaining authors to update their manuscripts and wait too long to see their manuscripts in published form. I do shoulder all the responsibilities for this myself and tender my sincere apologies.

Another unique feature of this series is that the authors of chapters dedicated to some genera have dedicated their chapters to scientists who pioneered the exploration, description and utilization of the wild species of those genera. We have duly honored their sincere decision with equal respect for the scientists they rightly reminded us to commemorate.

Editing this series was, to be honest, very taxing and painstaking, as my own expertise is limited to a few cereal, oilseed, pulse, vegetable, and fruit crops, and some medicinal and aromatic plants. I spent innumerable nights studying to attain the minimum eligibility to edit the manuscripts authored by experts with even life-time contributions on the concerned genera or species. However, this indirectly awakened the "student-for-life" within me and enriched my arsenal with so many new concepts, strategies, tools, techniques and even new terminologies! Above all, this helped me to realize that individually we know almost nothing about the plants on this planet! And this realization strikingly reminded me of the affectionate and sincere advice of Dr. Norman Borlaug to keep abreast with what is happening in the crop sciences, which he used to do himself even when he had been advised to strictly limit himself to bed rest. He was always enthusiastic about this series and inspired me to take up this huge task. This is one of the personal and professional reasons I dedicated this book series to him with a hope that the present and future generations of plant scientists will share the similar feelings of love and respect for all plants around us for the sake of meeting our never-ending needs for food, shelter, clothing, medicines, and all other items used for our basic requirements and comfort. I am also grateful to his granddaughter, Julie Borlaug, for kindly extending her permission to dedicate this series to him.

I started editing books with the 7-volume series on Genome Mapping and Molecular Breeding in Plants with Springer way back in 2005, and I have since

edited many other book series with Springer. I always feel proud and satisfied to be a member of the Springer family, particularly because of my warm and enriching working relationship with Dr. Sabine Schwarz and Dr. Jutta Lindenborn, with whom I have been working all along. My special thanks go out to them for publishing this "dream series" in an elegant form and also for appreciating my difficulties and accommodating many of my last-minute changes and updates.

I would be remiss in my duties if I failed to mention the contributions of Phullara – my wife, friend, philosopher and guide – who has always shared with me a love of the collection, conservation, evaluation, and utilization of wild crop relatives and has enormously supported me in the translation of these priorities in my own research endeavors – for her assistance in formulating the contents of this series, for monitoring its progress and above all for taking care of all the domestic and personal responsibilities I am supposed to shoulder. I feel myself alien to the digital world that is the sine qua non today for maintaining constant communication and ensuring the preparation of manuscripts in a desirable format. Our son Sourav and daughter Devleena made my life easier by balancing out my limitations and also by willingly sacrificing the spare amount of time I ought to spend with them. Editing of this series would not be possible without their unwavering support.

I take the responsibility for any lapses in content, format and approach of the series and individual volumes and also for any other errors, either scientific or linguistic, and will look forward to receiving readers' corrections or suggestions for improvement.

As I mentioned earlier this series consists of ten volumes. These volumes are dedicated to wild relatives of Cereals, Millets and Grasses, Oilseeds, Legume Crops and Forages, Vegetables, Temperate Fruits, Tropical and Subtropical Fruits, Industrial Crops, Plantation and Ornamental Crops, and Forest Trees.

This volume "Wild Crop Relatives: Genomic and Breeding Resources – Plantation and Ornamental Crops" includes 13 chapters dedicated to *Antirrhinum*, *Camellia*, *Coffea*, *Cola*, *Digitallis*, *Elaeis*, *Euphorbia*, *Gladiolus*, *Lilium*, *Nicotiana*, *Petunia*, *Rosa*, and *Theobroma*. The chapters of this volume were authored by 65 scientists from 18 countries of the world, namely Belgium, Brazil, Bulgaria, France, Germany, India, Malaysia, Mexico, Netherlands, People's Republic of China, Poland, Republic of Korea, Romania, South Africa, Spain, Sweden, the USA and West Indies.

It is my sincere hope that this volume and the series as a whole will serve the requirements of students, scientists and industries involved in studies, teaching, research and the extension of plantation and ornamental crops with an intention of serving science and society.

Clemson, USA Chittaranjan Kole

Contents

Abbreviations

2,4-D	2,4 Dichloromethene
2iP	2-Dimethylallylamino-purine
3β-HSD	Δ^5-3β-Hydroxysteroid dehydrogenase/reductase
5b-POR	Progesterone 5b-reductase
ACC	1-Aminocyclopropane-1-carboxylic-acid
AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
APM	Amiprophosmethyl
ARS	Agricultural Research Service
AuH	*L. auratum* × *L. henryi* hybrid
BA	Benzyl-adenine
BAC	Bacterial artificial chromosome
BAP	Benzyl amino purine
BC_1	Backcross 1
BC_2	Backcross 2
BCCCA	The Biscuit, Cake, Chocolate and Confectionary Alliance (UK)
BE	Belem (Brazil)
BLAST	Basic local alignment search tool
BSA	Bulked segregant analysis
BYMV	Bean yellow mosaic virus
CA	Careiro Island (Brazil)
CAB	Cacau da Amazonia Brasileira (Brazil)
CaMV	Cauliflower mosaic virus
CAS	Controlled atmospheric storage
CATIE	Centro Agronómico Tropical de Investigación y Enseñanza (Costa Rica)
cDNA	Complementary-DNA
CEPLAC	Comissão Executiva do Plano da Lavoura Cacaueira (Brazil)
CFH	Cardenolide β-D-fucohydrolase
CGH	Cardenolide 16′-*O*-glucohydrolase
CHS	Chalcone synthase
CIM	Composite interval mapping
CIRAD	French Agricultural Research Centre for International Development (France)
CITES	Convention on International Trade in Endangered Species of Wild Flora and Fauna
CJ	Cachoeira do Jari (Brazil)

cM	CentiMorgan
CMS	Cytoplasmic male sterility
CMV	Cucumber mosaic virus
CoA	Coenzyme A
CORESTA	Cooperation Centre for Scientific Research Relative to Tobacco
Cp-DNA	Chloroplast-DNA
CPO	Crude palm oil
CRIN	Cocoa Research Institute of Nigeria
CRU	Cocoa Research Unit of the University of West Indies (Trinidad)
CSUL	Cruseiro do Sul (Brazil)
DAT	Digitoxin 15′-O-acetyltransferase
DFR	Dihydroflavonol-4-reductase
DFT	Digitoxigenin 3-*O*-fucosyltransferase
DGT	Digitoxin 16′-O-glucosyltransferase
DHZR	Dihydrozeatin riboside
DMSO	Dimethyl sulfoxide
DMT	3,5-Dimethoxytoluene
DOE-JGI	Department of Energy- Joint Genome Institute
*Dp*21MaT	21-Hydroxypregnane 21-*O*-malonyltransferase
DPGT	Digiproside 4′-O-glucosyltransferase
DW	Dry weight
EET	Estacion Experimental Tropical (Ecuador)
ELP	Euleupousing (French Guiana)
EMBRAPA	Empresa Brasileira de pesquisa Agropecuária (Brazil)
EMS	Ethylmethane sulfonate
ERJOH	Estação de Recursos Genéticos do Cacau José Haroldo (Brazil)
EST	Expressed sequence tag
EU	European Union
FAC	Fatty acid composition
FAO	Food and Agriculture Organization (United Nations)
FCM	Flow cytometry
FDR	First division restitution
FELDA	Federal Land Development Authority
FFB	Fresh fruit bunch
FIS	Inbreeding coefficients
FISH	Fluorescent in situ hybridization
FW	Fresh weight
GA_3	Gibberellic acid
GCW	Global Compendium of Weeds
GFP	Green fluorescent protein
GISH	Genomic in situ hybridization
GM	Genetically modified
GMS	Genic male sterility
GOFB	Global Oil and Fats Business Magazine
GRIN	Germplasm Resources Information Network
GU	Guyane
GUBQ1	Polyubiquitin promoter isolated from Gladiolus
GUS	β-Glucuronidase

*gus*A	Glucuronidase A gene
HMGR	Hydroxy-3-methyl-glutaryl coA reductase
HPLC	High performance liquid chromatography
IAA	Indole acetic acid
IARI	Indian Agricultural Research Institute
IBA	Indole butyric acid
IBPGR	International Board for Plant Genetic Resources (Italy)
IC3	International CATIE Cacao Collection (Costa Rica)
ICG, T	International Cacao Genebank, Trinidad
ICQC, R	International Cocoa Quarantine Centre, University of Reading (UK)
ICT	Instituto de Cultivos Tropicales (Peru)
IIHR	Indian Institute of Horticultural Research (India)
IMC	Iquitos Mixed Calabacillo (Peru)
IMR	Intermediate division restitution
INGENIC	International Group for Genetic Improvement of Cocoa
INIAP	Instituto Nacional Autónomo de Investigaciones Agropecuarias (Ecuador)
iPA	Isopentenyl adenosine
IPT	Isopentenyl transferase
ipt	Isopentenyl transferase gene
ISSR	Intersimple sequence repeat
ISTR	Inverse sequence tagged repeat
ITS	Internal transcribed spacer
IUCN	International Union for Conservation of Nature
IV	Iodine value
KER	Kérindioutou (French Guiana)
KIN	Kinetin
KSI	Δ^5-3-Ketosteroid isomerase
LA	Longiflorum $\times$ Asiatic hybrid
LAE	Lanatoside 15′-O-acetylesterase
LCT EEN	The London Cocoa Trade - Estacion Experimental Napo (expedition)
LEA	Late embryogenesis abundant
LG	Linkage group
LII	Leaf injury index
LN	Liquid nitrogen
MA	Modified atmosphere packaging
MAO	Monoamine oxidase
MARDI	Malaysian Agricultural Research and Development Authority
MAS	Marker-assisted selection
MAX4	More axillary growth4 (gene)
MMCT	Microprotoplast-mediated chromosome transfer
MO	Morona (Peru)
MPOB	Malaysian Palm Oil Board
mRNA	Messenger-RNA
MS	Murashige and Skoog (medium)
mtDNA	Mitochondrial-DNA
Mya	Million years ago

NA	Nanay (Peru)
NAA	Naphthalene acetic acid
NAD	Nicotinamide-adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NBS	Nucleotide binding site
NCBI	National Center for Biotechnology Information
NCGRP	National Center for Genetic Resources Preservation
*ndh*F	Nicotinamide adenine dinucleotide dehydrogenase subunit F (gene)
NIFOR	Nigerian Institute for Oil Palm Research
NIL	Near-isogenic lines
NOR	Nucleolar organizing region
NPGS	National Plant Germplasm System
OA	Oriental × Asiatic hybrid
OPGC	Ornamental Plant Germplasm Center
OT	Oriental × Trumpet hybrid
OYA	Oyapok (French Guiana)
P_{450}scc	Cholesterol side-chain cleaving enzyme
PA	Parinari (Peru)
PCO	Principle coordinate analysis
PCR	Polymerase chain reaction
PEG	Polyethylene-glycol
PEM	Pro-embryogenic mass
PK	Protein kinase
PKO	Palm kernel oil
PMC	Pollen mother cell
PMPSC	Pollen-mediated pseudo-self-compatibility
PNG	Papua New Guinea
PPT	Phosphinothricin
PSC	Pseudo-self compatibility
QTL	Quantitative trait loci
RAPD	Random(ly) amplified polymorphic DNA
RB	Rio Branco (Brazil)
*rbc*L	Ribulose 1,5-biphosphate carboxylase oxydase subunit L (gene)
r*Dl*3β-HSD	Recombinant Δ^5-3β-Hydroxysteroid dehydrogenase/reductase enzyme from *Digitalis*
r*Dl*5b-POR	Recombinant progesterone 5b-reductase enzyme from *Digitalis*
rDNA	Ribosomal-DNA
RFLP	Restriction fragment length polymorphism
RGA	Resistance gene analog
RNase	Ribonuclease
ROS	Reactive oxygen species
*rps*2	Resistance to *Pseudomonas syringae* pv. tomato (gene)
RRD	Rose rosette disease
SC	Self-compatible
SCA	Scavina (Peru)
SCAR	Sequence-characterized amplified region
SDR	Second division restitution
SDR	Short chain dehydrogenase

SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SEMARNAT	Secretaría de Medio Ambiente y Recursos Naturales
SGN	SOL (Solanaceae family) Genomics Network
SI	Self-incompatibility/self-incompatible
SNP	Single nucleotide polymorphism
SSH	Suppression subtractive hybridization
SSR	Single sequence repeat
STMS	Sequence tagged microsatellite site
TAZ1	Tapetum development zinc finger protein 1
TGI	Tobacco Genome Initiative
TLC	Thin layer chromatography
TMB	1,3,5-Trimethoxybenzene
TMV	Tobacco mosaic virus
*trn*L-F	Transfer RNA genes comprising the *trn*L intron and *trn*L-F spacer
TSA	Trinidad selected Amazon (Trinidad)
TSH	Trinidad selected hybrids (Trinidad)
UCBG	University of California Botanical Garden
UDP	Uridine diphosphate
UF	United Fruit Company (Costa Rica)
*uid*A	Gene coding for GUS
UPGMA	Unweighted pair group method with arithmetic mean
USDA	United States Department of Agriculture
UWI	University of West Indies
VIGS	Virus-induced gene silencing
VOCs	Volatile organic compounds
WBD	Witches' broom disease
WPM	Woody plant medium
YAL	Yaloupi (French Guiana)
ZR	Zeatin riboside

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Chapter 1
Antirrhinum

Janakiram Tolety and Anuradha Sane

1.1 Introduction

"*Antirrhinum* has always allowed new, and frequently surprising, insights to be made into the nature, variability and manifestation of genetic substance and, even today, the rich variety of appearance in the genus *Antirrhinum* offers an inexhaustible resource for genetics-based studies in developmental biology, biochemistry, and evolution" (Stubbe 1966). This statement regarding *Antirrhinum* is as valid today as it was half a century ago. In fact, *Antirrhinum* was used in the earliest studies of inheritance by Mendel and Darwin, and became established as a model by Erwin Baur during the first decades of the twentieth century. Despite the tremendous success of *Arabidopsis thaliana*, no single model can represent the vast range of form that is seen in the ~250,000 existing species of flowering plants (angiosperms). *Antirrhinum* is considered as an alternative angiosperm model. Wide diversity of *Antirrhinum* species, combined with classical and molecular genetics – the two traditional strengths of *Antirrhinum* – provide an opportunity for developmental, evolutionary, and ecological approaches (Schwarz-Sommer et al. 2003a).

The cultivated *Antirrhinum majus* is grown in tropical, subtropical, and temperate countries. Magnificent and charming flowers are borne on terminal long spikes of many colors except blue, and with numerous shades. It is one of the excellent cut flowers, which have long lasting qualities. These are also used as garden plants, bedding plants, in rockeries or herbaceous borders and as potted plants. Plants can be grown for cut flowers in an open field or under protection depending on the climate. The varieties in plant height in different types and groups widen its scope for use in the garden for different purposes.

1.1.1 Uses

An edible oil is extracted from the seeds and has been cultivated in Russia for this purpose (Grieve 1984). The leaves and flowers are antiphlogistic, bitter, resolvent, and stimulant (Chiej 1984). They have been employed in poultices on tumors and ulcers. Also effective in the treatment of all kinds of inflammation and is also used on hemorrhoids. The plant is harvested in the summer when in flower and is dried for later use. A green dye is obtained from the flowers, which does not require a mordant. Dark green and gold can also be obtained if a mordant is used.

1.1.2 Scope for Domestication and Commercialization

The Russians used to cultivate snapdragons on a large scale to extract oil from its seeds, which is equal to olive oil (Genders 1969). If women want to restore their beauty and youthfulness, washing the face with the water in which snapdragon seeds are soaked is recommended. The plants possess bitter and stimulant properties. They are used as diuretic, for scurvy,

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tumor, as detergent and astringent. In olden times, it was valued as a preservative against witchcraft.

1.1.3 Distribution

A. majus (Snapdragon) is a species of *Antirrhinum* (Family Scrophulariaceae) native to the Mediterranean region, from Morocco and Portugal north to southern France, and East to Turkey and Syria. Most species are found around the Mediterranean Sea and in North America (Stubbe 1966). Among them, only *A. majus* has been domesticated as an ornamental.

The progenitor of the modern snapdragon is from the Mediterranean region and, more specifically, it is native to southern France. It was believed that the snapdragon reached Britain with the Romans at an early date, where it has been naturalized on mountainous regions. Thereafter, it spread to different parts of the world from Britain. *Antirrhinum glandulosum* is a native of California.

Antirrhinum siculum Miller and *A. tortuosum* Bosc, are widespread taxa, distributed through a vast area in the Mediterranean basin, while *A. latifolium* Miller, *A. litigosum* Pau, *A. cirrhigherum* welw ex rothm., *A. majus* L, and *A. linkianum* Boiss. et Reut. have small ranges. While *A. cirrhigherum* grows on sands, all other species grows on limestone crevices, roadsides, walls, and roofs. The genus *Antirrhinum* (Thompson 1988) consists of 36 species in three sections. In section *Saerorhinum*, 15 small-flowered, mostly annual species are distributed in western North America. Section *Antirrhinum*, which includes the model organism *A. majus*, comprises 19 perennial species with relatively large flowers that are native to the western Mediterranean regions, with most of the species occurring as narrow endemics in the Iberian Peninsula (Tutin et al. 1972). The two species in section *Orontium* also occur in the Mediterranean region. The species in section *Saerorhinum* are geographically centered in California, though some species occur as far South as Baja California Sur, as far North as southern Oregon, and as far East as western Utah. They are found on a variety of substrates including shale, serpentine, salt flats, and recently burned soils.

1.2 Basic Botany of the Species

1.2.1 Taxonomy and Relatives of A. majus

Antirrhinum is a member of the asterid clade of flowering plants. The more commonly used model species *Arabidopsis* is a member of the second major clade of broad-leaved plants – the rosids – from which asterids diverged an estimated 120 million years ago (Mya). Within the asterids, *Antirrhinum* belongs to the order Lamiales, a close relative of the order Solanales, which includes other model species such as petunia and tomato. *Antirrhinum* was recently placed in the family Plantaginaceae (syn. Veronicaceae) following a revision of the classical family Scrophulariaceae based on DNA sequence variation (Olmstead et al. 2001).

Other aspects of *Antirrhinum* taxonomy remain controversial. The generic epithet "*Antirrhinum*" is now usually reserved for the monophyletic group of Old World perennials with a diploid chromosome number of 16. However, it is still applied to a broader monophyletic group that includes species with different chromosome numbers, such as the New World *Sairocarpus* and the annual *Misopates*, with which *Antirrhinum* species are unable to form fertile hybrids (Oyama and Baum 2004). Within *Antirrhinum sensu stricto*, a variable number of different species have been proposed, and relationships between taxa are currently unresolved. These taxonomic problems largely reflect the young age of the genus (<5 million years) (Gübitz et al. 2003) and the effects of hybridization (see, e.g., Whibley et al. 2006); thus, attempts to reconstruct phylogenies based on nuclear or chloroplast DNA sequence variation have so far been unsuccessful (Jiménez et al. 2005). In the absence of a taxonomic revision based on a resolved phylogeny, the descriptions of approximately 20 species and their likely hybrids in *Flora Europaea* (Webb 1972) provide a realistic working guide.

The genus has traditionally been divided into three subsections or morphological groups (Rothmaler 1956) that have received support from studies of isozyme and DNA polymorphism. The subsection *Antirrhinum* includes the close relatives of *A. majus* and consists of species with similar upright growth,

large organs, and pink or yellow flowers. These tend to be geographically widespread and grow in a variety of habitats. *A. majus* was probably domesticated in northeastern Spain or southwestern France from *Antirrhinum pseudomajus* (also known as *Antirrhinum majus* subspecies *pseudomajus*), from which it differs by having more darkly pigmented flowers, although traits such as flower color variation might have been introduced by introgression from other species.

Further, the taxonomy of this genus is disputed at present. At one extreme, Integrated Taxonomic Information System (ITIS) recognizes only the Old World species of sect. *Antirrhinum* in the genus, listing only the Garden Snapdragon *A. majus* (the only species in the section naturalized in North America). At the other, Thompson (1988) places 36 species in the genus; many modern botanists accept this circumscription.

Recent research in the molecular systematics of this group, and related species, by Oyama and Baum (2004), has confirmed that the genus as described by Thompson is monophyletic, provided that one species (*A. cyathiferum*) is removed to a separate genus, and two others (previously listed as *Mohavea confertiflora* and *M. breviflora*) are included. It is widely agreed that this broad group should be subdivided into three or four subgroups, but the level at which this should be done, and exactly which species should be grouped together, remain unclear. Some authors continue to follow Thompson in using a large genus *Antirrhinum*, which is then divided into several sections; others treat Thompson's genus as a tribe or subtribe, and divide it into several genera.

If the broad circumscription is accepted, its sections are as follows:

- Section *Antirrhinum*: about 20 Old World species of perennial plants, the type *A. majus*, mostly native to the western Mediterranean region with a focus on the Iberian Peninsula.
- Section *Orontium*: two to six species, also Mediterranean. The species in this section, including the type Lesser Snapdragon *A. orontium*, are often treated in the genus *Misopates*.
- Section *Saerorhinum*: about 16 New world species, mostly annual plants and mostly native to California, though species are found from Oregon to Baja California Sur and as far East as Utah. Like other authors, Thompson placed *A. cyathiferum* in this section, but Oyama and Baum, following earlier authors, suggest that it should be reclassified in genus *Pseudorontium,* while *Mohavea confertiflora* and *M. breviflora* should be included. Some authors classify the species in this section into the genera *Sairocarpus*, *Howelliella*, and *Neogaerrhinum*.

1.2.2 Family and Related Taxa

Antirrhinum belongs to the family Scrophulariaceae Juss. Forty to forty-two species have been reported under the genus *Antirrhinum*. Other important genera in this family are *Calaceolaria*, *Hebe*, *Penstemon*, *Verbascum*, *Nemesia*, *Veronica*, *Castilleja*, *Pedicularis*, *Digitalis*, *and Minulus* (Vinod Kumar and Bhattacarjee 2006).

1.2.3 Classification and Morphology

Classification

Kingdom	Plantae – Plants
Subkingdom	Tracheobionata – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Asteridae
Order	Scrophulariales
Family	Scrophulariaceae – Figwort Family
Genus	*Antirrhinum* L. – Snapdragon
Species	*Antirrhinum majus L.* – Garden snapdragon

Antirrhinum majus subsp. *Linkianum*. There are five subspecies:

- ***Antirrhinum majus* subsp. *majus*** southern France, Northeast Spain.
- ***Antirrhinum majus* subsp. *cirrhigerum*** (Ficalho) Franco. southern Portugal, Southwest Spain.
- ***Antirrhinum majus* subsp. *linkianum*** (Boiss. & Reut.) Rothm. Western Portugal (endemic).
- ***Antirrhinum majus* subsp. *litigiosum*** (Pau) Rothm. southeastern Spain.
- ***Antirrhinum majus* subsp. *tortuosum*** (Bosc) Rouy. throughout the species' range.

The species vary considerably in morphology, size, and habitat and include both small shrubs and herbs. All behave genetically as diploids, share the chromosome number of $2n = 16$, and form fertile hybrids

when artificially cross-pollinated (Rieger 1957; Stubbe 1966). The genus also includes *A. majus,* which has been domesticated as an ornamental over two millennia and adopted as a genetic model. The species is often planted in gardens for its flowers. Although perennial, it is often treated as annual plant, particularly in colder areas where they may not survive the winter. Numerous cultivars are available, including plants with lavender, orange, pink, yellow, or white flowers, and also plants with peloric flowers, where the normal flowering spike is topped with a single large, symmetrical flower. It often escapes from cultivation, and naturalized populations occur widely in Europe north of the native range, and elsewhere in temperate regions of the world. The inflorescence is a terminal raceme with numerous perfect flowers. Stamens are four and not winged. Closely allied to *Linaria*, from which it differs in the spur-less flowers. Each flower is borne on a short pedicel along the stem with five lobed petals; the corolla is fused into a tube. Three lower lips are typically spreading, the two upper lips are erect and form a "mouth"; hence the name snapdragon. Leaves are lanceolate, entire, dark green to purplish and mainly opposite (Baiely and Baiely 1976).

The descriptions of some of the species are given below.

Antirrhinum microphyllum Rothm. (Scrophulariaceae) is an herbaceous perennial listed as "vulnerable" (Gómez-Campo 1987) according to IUCN (International Union for Conservation of Nature) categories, and protected by the regional legislation of Junta de Castilla-La Mancha (Anonymous 1998) due to its narrow distribution, an area of approximately 30 km^2. *Antirrhinum microphyllum* is a self-incompatible species (Torres et al. 2002) and flowers are pollinated mainly by a solitary bee, *Rhodanthidium sticticum* (Megachilidae) (Torres et al. 2001).

Antirrhinum blanquetti Rothm: This is a perennial and glabrous, except for glandular pubescent inflorescence. Plants are erect, branched, having a height of 120 cm. Leaves are linear oblong to elliptic, acute, opposite below, alternate above. Inflorescence is 5–20 cm and flowers are 5–30 in number. Pedicles are 3–6 mm long. Calyx lobes are ovate, lanceolate, acute, and up to 1 cm long. Corolla is yellow in color and 3–4 cm long. Capsule is oblong, glandular, hairy, and 1.5 cm in size.

Antirrhinum calycnium: This is a glabrous annual having a height of 45 cm. Leaves are narrowly elliptic and 5 × 2.5 cm in size.

Antirrhinum coulterianum: Annual erect or scandent with a plant height of 1 m. Lateral branches are tendril like. Leaves are ovate to elliptic with a size of 38 cm. Raceme is pubescent. Corolla is 9.5–13 mm, which is white with pale yellow plate.

Antirrhinum filipes: This is a slender, glabrous, scandent annual with a height of 1 m. Upper leaves are linear to lanceolate while leaves are ovate to oblong. Pedicel is tendril-like with a length of 10 cm. Flowers are yellow with lower lip golden and palate speckled maroon.

Antirrhinum glandulosum: Annual species, plant height is 60 cm and branches are reddish and glandular. Rose colored flowers with lower portion white yellow and produced on leafy raceme.

Antirrhinum glutinosum: This is a native of Spain, woody and perennial species. Branches are fragile; leaves are alternate and oblong to ovate. Flowers are large, erect, and solitary in leaf axil.

Antirrhinum hispanicum: Dwarf shrub with much branched stem, which is glandular, pubescent, procumbent to ascending. Plant height is 20–60 cm. Leaf size is 5–35 × 2–20 mm, which is lanceolate to orbicular. Calyx is ovate to lanceolate. Corolla is either white or pink in color and palate is often yellow. Corolla lobes are 6–8 cm. Leaves are lanceolate to broadly ovate.

Antirrhinum latifolium: This is a perennial species, which is erect with a height up to 1 m. Leaves are ovate, obtuse broadly truncate having a size of 20–70 × 8–32 mm. Bracts are ovate. Calyx lobes are broadly ovate. Corolla is pale yellow in color.

Antirrhinum majus: This is a perennial species, native of the Mediterranean region. Stem is glabrous, erect, reaching up to 2 m. Leaves are oblong to lanceolate, glabrous and size is 10–70 × 2–25 cm. Pedicles are 2–15 mm straight or curving. Corolla is 3.4–5 cm, usually purple or pink in color. Flower is glandular, oblong, 10–14 mm in diameter.

Antirrhinum molle: Dwarf shrub, which is native to the Pyrenes. Branches are decumbent, slender, and woody. Leaves are 8.2 × 0.5 × 2 cm opposite, small, and glandular. Bracts are leaf-like. Peduncles are 3–22 cm erect or spreading. Calyx lobes are lanceolate-acute. Corolla is 2.5–3.5 cm, which is pale pink or white in color. Inflorescence is raceme, bears few flowers which are produced at the end of branches.

Antirrhinum multiflorum: This is an herbaceous perennial species which is erect, pubescent, having a height of 1–5 cm. Leaves are 2.5–3.8 cm, usually

alternate, lanceolate to linear, sessile, obtuse or acute at apex. Calyx is pubescent, 2 mm in size while corolla is 13–18 mm, pale pink to carmine in color. Palate is white, becoming brown to tan as flowers open.

Antirrhinum nuttallinum: This is an annual erect, delicate, pubescent miniature snapdragon up to 2 m tall. Branchlets are tendril-like. Leaves are 6–5 cm in size and acute. Flowers are solitary, axillary or on branchlets. Corolla is lavender to blue-purple. Tube and palate is white in color. Flower is oblique-ovoid and glandular to pubescent.

Antirrhinum orontium: This is an annual herb. Stems are erect, sparingly branched, glandular pubescent in upper parts. Leaves are linear to oblong elliptic, 20–50 mm long, 2.7 mm wide, subsessile. Flowers are borne on terminal raceme. Bracts are similar to leaves but much smaller. Calyx is 10–17 mm long, lobes are linear. Corolla is pink and rarely white in color, 10–15 mm long, as long as or slightly shorter than the calyx.

Antirrhinum ovatum: This is an annual species having a height of 60 cm; many branched, spreading to penultimate node on the main stem. Basal leaves are opposite, lanceolate-oblanceolate or ovate, others alternate ovate, obtuse. Corolla is 17–20 mm, cream, flushed pink on upper lip and tube. Flowers are oblique to ovoid.

Antirrhinum pulverulentum: This species is similar to *Antirrhinum sempervirens*, differing only in larger hair. Upper leaves are alternate. Corolla is pale yellow in color.

Antirrhinum sempervirens: This is a dwarf woody, evergreen shrub with a height of 40 cm. It is a native of the Pyrenes. Leaves are oblong to broadly elliptic, obtuse, cuneatte, mostly opposite, having a size of 5–3 × 4–17 mm. Petioles are 2–8 mm. Inflorescence is some times pubescent. Calyx lobes are 5–6 mm and lanceolate, acuminate, pubescent. Corolla is 2–2.5 cm, cream or white in color. Violet patch on upper lip. Palate is yellow or white. Flower is whitish, subglobose, glandular, pubescent, and 6 mm in diameter.

Antirrhinum siculum: This is a perennial herb with a height of 50 cm, glabrous, erect, free branching. Leaves are linear or linear elliptic, acute, sessile or subsessile, becoming bract-like. Calyx lobes are ovate to lanceolate, acute or subacute. Corolla is pale yellow in color and is 1.7–2.5 cm long.

1.3 Genetic and Genomics Resources

A. majus is amenable to classical genetics. It has a relatively short generation time of ~4 months, is diploid ($2n = 16$), and is easily self- and cross-pollinated (Hudson et al. 2008). The wide diversity of *Antirrhinum* species, combined with classical and molecular genetics – the two traditional strengths of *Antirrhinum* – provide an opportunity for developmental, evolutionary, and ecological approaches. These factors make *A. majus* an ideal comparative angiosperm (Schwarz-Sommer 2003a).

1.3.1 Cytogenetics

In contrast to the significant advances in the genetic and molecular study of *A. majus*, very limited work has been done on the karyotype and cytogenetic structure of the *A. majus* genome. The genetic linkage groups (LGs) have not been integrated into individual chromosomes. In recent years, extensive molecular and genomic resources have been established in *A. majus* (Schwarz-Sommer et al. 2003a). Two tandem repetitive sequences, CentA1 and CentA2, were isolated from the centromeric regions of *Antirrhinum* chromosomes. A standard karyotype has been established by anchoring these centromeric repeats on meiotic pachytene chromosome using fluorescent in situ hybridization (FISH). An ideogram based on the DAPI-staining pattern of pachytene chromosomes was developed to depict the distribution of heterochromatin in the *Antirrhinum majus* genome. To integrate the genetic and chromosomal maps, we selected one or two molecular markers from each LG to screen an *Antirrhinum* transformation-competent artificial chromosome (TAC) library. These genetically anchored TAC clones were labeled as FISH probes to hybridize to pachytene chromosomes of *A. majus*. As a result, the relationship between chromosomes and the LGs in Antirrhinum has been established (Zhang et al. 2005). Bacterial artificial chromosome (BAC) and TAC libraries representing the *Antirrhinum* genome are available now. An expressed sequence tag (EST) database has been created in this species containing ~12,000 unique sequences. These resources provide us with an opportunity to investigate the *A. majus*

genome using a cytogenetic approach that has been established in the model cytogenetic species *A. thaliana* (Fransz et al. 1998) and rice (Cheng et al. 2001).

1.3.2 Role in Development of Cytogenetic Stocks and Their Utility

1.3.2.1 Self-Incompatibility

As a model system in classical plant genetics, the genus *Antirrhinum* has been well studied, especially in gametophytic self-incompatibility, flower development biology, and transposon-induced mutation. Whereas cultivated *A. majus* and some wild species (e.g., *Antirrhinum valentinum*, *Antirrhinum subbaeticum*, and *Antirrhinum siculum*) are self-fertile, the majority of *Antirrhinum* species show gametophytic self-incompatibility that is determined by a single, complex *S*-locus. Individuals of self-incompatible species reject pollen carrying an *S*-allele that corresponds to one of their own alleles and are therefore obligate outbreeders. Through studies of relatives in the family Solanaceae, rejection of pollen was known to involve an *S*-encoded RNase that was expressed in the pistil (Mcclure et al. 1989) although the pollen-expressed component had remained elusive. Mapping and sequence analysis of the active *S*-locus of *Antirrhinum hispanicum*, followed by expression and functional studies, identified the likely pollen component to be an F-box protein involved in targeting the RNase for degradation (Lai et al. 2002).

Cultivated varieties and wild progenitors of *A. majus* and *A. siculum* are able to self-pollinate whereas all other members of the genus show gametophytic self-incompatibility and therefore are largely, or entirely, outbreeding. Self-incompatibility is determined by a single *S*-locus in *Antirrhinum* and involves rejection of pollen grains carrying an *S*-allele that is also present in the female (Gruber 1932; East 1940; Herrmann 1973).

Molecular studies have demonstrated that the *S*-RNase and *SLF*/*SFB* genes encoded by the single polymorphic *S*-locus. They control the pollen and pistil functions of SI in three distantly related families, Solanaceae, Scrophulariaceae, and Rosaceae, are organized in a haplotype-specific manner. Previous work suggested that the haplotype structure of the two genes is probably maintained by recombination suppression at the *S*-locus. To examine features associated with this suppression, first mapped the *S*-locus of *A. hispanicum,* a member of the Scrophulariaceae, to a highly heterochromatic region close to the distal end of the short arm of chromosome 8. Both leptotene chromosome and DNA fiber FISH analyses showed an obvious haplotype specificity of the *Antirrhinum S*-locus that is consistent with its haplotype structure. A chromosome inversion was also detected around this region between *A. majus* and *A. hispanicum*. These results revealed that DNA sequence polymorphism and a heterochromatic location are associated with the *S*-locus. Possible roles of these features in maintenance of the haplotype specificity involved in both self- and non-self-recognition are discussed by Yang et al. (2007). A range of self-compatible (SC) transposable element (Tn) mutants of *A. majus*, were generated in which the different domains of the *S*-locus are affected in isolation. The inheritance of self-incompatibility was studied, putative *S*-allele products in the pistil were identified and lines were generated, which were suitable for transposon tagging (McCubbin et al. 1992).

In many flowering plants, self-fertilization is prevented by an intraspecific reproductive barrier known as self-incompatibility (SI) that, in most cases, is controlled by a single multiallelic *S*-locus. So far, the only known *S*-locus product in self-incompatible species from the Solanaceae, Scrophulariaceae, and Rosaceae is a class of ribonucleases called *S*-RNases (ribonucleases). Molecular and transgenic analyses have shown that *S*-RNases are responsible for pollen rejection by the pistil but have no role in pollen expression of SI, which appears to be mediated by a gene called the pollen self-incompatibility or *Sp* gene. To identify possible candidates for this gene was investigated the genomic structure of the *S*-locus in *Antirrhinum* spp. (*A. majus* and *A. hispanicum*), a member of the Scrophulariaceae. A novel F-box gene, *AhSLF-S2*, encoded by the *S2* allele, with the expected features of the *Sp* gene was identified. *AhSLF-S2* is located 9 kb downstream of *S2* RNase gene and encodes a polypeptide of 376 amino acids with a conserved F-box domain in its amino-terminal part. Hypothetical genes homologous to *AhSLF-S2* were apparent in the sequenced genomic DNA of *A. thaliana* and rice. Together, they define a large gene family, named *SLF* (*S*-locus F-box) family. *AhSLF-S2* is highly polymorphic and is specifically

expressed in tapetum, microspores and pollen grains in an allele-specific manner. The possibility that *Sp* encodes an F-box protein and the implications of this for the operation of self-incompatibility were discussed. The nucleotide sequence data reported will appear in the EMBL nucleotide data library under the accession numbers AJ300474 (S2BAC), AJ297974 (*AhSLF-S2*) and AJ297975 (*AhSLF-S2L*) (Lai et al. 2002).

Most species of the genus *Antirrhinum* are characterized by gametophytic self-incompatibility, regulated by a single multiallelic *S*-locus (East 1940), which has been used as a model system for studying self-incompatibility (Xue et al. 1996; Lai et al. 2002; Ma et al. 2002; Zhou et al. 2003; Qiao et al. 2004).

1.4 Classical Breeding

Classical breeding together with the most advanced techniques in molecular biology have been applied to a wide variety of species, contributing to the elucidation of molecular mechanisms in plant development. The recent discoveries of the mechanisms of flowering in the model plants *A. majus, A. thaliana*, and *Petunia hybrida* allowed new sets of genes to be available. These include genes, which affect flowering time and flower architecture, commonly named: flowering-time genes, meristem-identity genes and organ-identity genes. The possibility of using molecular techniques to transfer genes in major ornamentals has greatly increased the resources available to plant breeders. Flower induction genes could affect flowering in species unrelated to the plant from which they were isolated. Moreover, native genes can be over expressed or suppressed. In the near future plant biotechnologists and plant breeders will continue to work alongside to improve floricultural plants (Giovannini 2007).

Seven related species of *Antirrhinum* (*A. siculum*, *A. majus*, *A. latifolium*, *A. linkianum*, *A. litigiosum*, *A. cirrhigherum*, and *A. tortuosum*) were studied in order to compare levels of genetic variation and its partitioning in them, and to check relationships between genetic patterns and the reproductive system. Genetic variability in *A. siculum* was found to be the lowest known in the genus. Mean values of FIT and FIS were mostly positive and not significantly different from zero. Population differentiation (FST) ranged between 6.1 in *A. tortuosum* and 17.6 in *A. linkianum*. The inbreeding coefficient within populations ranged between FIS $= -0.5$ in *A. tortuosum* and FIS $= 1$ in *A. siculum*. Estimates of gene flow ranged between Nm $= 15$ in *A. majus* (considered as very high) and Nm $= 0.42$ in *A. siculum* (considered as low). Correlation was found between levels of diversity and differentiation on one hand, and the reproductive system of the studied taxa on the other. Striking differences among species in the inbreeding coefficient (FIS) show different reproductive systems, which mostly support previous reports.

1.5 Classical and Molecular Genetic Studies

1.5.1 Ecology and Population Genetics

The *Antirrhinum* species group comprises approximately 20 morphologically diverse members that are able to form fertile hybrids. It includes the cultivated snapdragon *A. majus*, which has been used as a model for biochemical and developmental genetics for more than 75 years. The research infrastructure for *A. majus*, together with the interfertility of the species group, allows *Antirrhinum* to be used to examine the genetic basis for plant diversity. The garden snapdragon *A. majus* has several centuries' history of cultivation as a flowering ornamental. The *Antirrhinum* species group also has a history of use in studies of natural variation. The close relatives of *A. majus* form a monophyletic group of approximately 20 species native to the Mediterranean region, particularly southwestern Europe and northern Africa. The species vary widely in morphology and ecology and are adapted to different – often extreme – habitats. However, all are able to form fertile hybrids with one another and with *A. majus*, allowing the identification of genes that underlie their differences (see, e.g., Langlade et al. 2005). Population genetic studies that have been applied to *Antirrhinum* species show different population sizes, geographic distributions, and breeding systems, from self-fertility to obligate outcrossing (Jiménez et al. 2002; De Paco and Mateu-Aandres 2006).

Antirrhinum species have been the subject of population genetic studies, including those aimed at

assessing genetic diversity in order to inform conservation strategies for rare endemic species (Mateu-Andres 2004). As with other taxa, genetic diversity has generally been found to be lower in smaller populations, and the distribution of genetic variants within and between populations has been correlated with their level of self-incompatibility. In addition, several genetically well-characterized aspects of *Antirrhinum* development and physiology are relevant to their reproductive ecology, including petal cell morphology, genetic self-incompatibility, flower color, and scent production.

It emerged as a model organism during early studies of inheritance and mutation because of its diploid inheritance, ease of cultivation, and variation in morphology and flower color. Laboratory lines of *A. majus* were produced from cultivars, and a substantial collection of mutants had amassed during the course of the twentieth century. This collection included lines with unstable mutations in pigment genes, which produced variegated flowers.

1.5.1.1 Allozyme and RAPD Markers

Fifty-two populations were sampled in order to establish the taxonomic delimitation and relationships of eight taxa belonging to *A. majus* L. and *A. siculum* Miller groups. The genetic distances, together with the lack of morphological differences and the sympatric distribution ranges, support the inclusion of *A. australe* into *A. tortuosum*, *A. dielsianum* into *A. siculum*, and *A. latifolium* subsp. *intermedium* as a synonym of *A. latifolium*. The results support separation of the taxa studied into two groups, coinciding with series Sicula Rothm. and Majora, but disagreeing with the arrangement of species into them. According to results, Sicula consist of *A. siculum* and *Majora* consists of *A. latifolium*, *A. majus*, *A. tortuosum*, *A. linkianum*, *A. cirrigherum*, *A. litigiosum*, and *A. barrelieri* (De Paco and Mateu-Aandres 2006).

Thirteen allozyme loci and 68 random amplified polymorphic DNA (RAPD) markers were analyzed to assess the genetic diversity and population structure of threatened *A. microphyllum*, a narrow endemic of central Spain known from only four populations. According to allozyme data, species genetic diversity ($p = 46.15\%$, $A = 2.61$, and \$H_e = 0.218\$), as well as within-population genetic diversity ($p = 44.23\%$, $A = 2.10$, and \$H_e = 0.204\$), were high when compared to average estimates for other narrowly distributed plant species. Ninety-four percent of species genetic diversity corresponded to within-population genetic diversity. Nevertheless, significant differences were found among populations in allele frequencies of four of the six polymorphic loci, and three private alleles were detected. Inbreeding coefficients (FIS) suggest that populations are structured in genetic neighborhoods. The RAPDs also showed high levels of genetic diversity ($p = 89.71\%$ and \$H_e = 0.188\$ at the species level, and $p = 67.65\%$ and \$H_e = 0.171\$ at the population level). Nei's genetic distances estimated both from allozymes and RAPDs indicated low differentiation among populations. In spite of this, the low frequencies of certain alleles and the presence of private alleles indicate that efforts should be made to conserve all four remaining populations (Torres et al. 2003).

1.5.2 Molecular Characterization and Linkage Mapping

1.5.2.1 Cp DNA Markers

A molecular analysis of 24 taxa of *Antirrhinum* was undertaken using cpDNA sequences from the *trn*T (UGU)–*trn*L (UAA) 5′ exon region. The Kimura two-parameter model was chosen to calculate pairwise nucleotide divergence values between cpDNA sequences, and a bootstrapped neighbor-joining dendrogram was constructed from the nucleotide divergence distance matrix. Eighteen sites were variable across the studied samples and the position of seven indels, ranging from 1 to 7 bp, was inferred from the sequence alignment. Several *trn*T–*trn*L sequences are identical: some members of subsection *Kickxiella* (*A. subbaeticum*, *A. valentinum*, *A. mollisimum*, *A. charidemi*, and *A. hispanicum*); subsection *Antirrhinum* (*A. australe*, *A. graniticum*, and three subspecies of *A. majus*); and some species from subsection *Kickxiella* (*A. lopesianum* and *A. molle*) together with subsection *Streptosepalum* (*A. braunblanquetii*). Few supported clades were recovered from distance- or parsimony-based methods, all of which conflicted with traditional infrageneric splitting. cpDNA results are congruent with, but do not prove, the hypothesis of

reticulation as one of the major processes to have occurred in the evolution of snapdragons, and suggest that other species in addition to those belonging to the *A. majus* group may also have been involved in the reticulation scenario (Jiménez et al. 2005). As a model system in classical plant genetics, the genus *Antirrhinum* has been well studied, especially in gametophytic self-incompatibility, flower development biology, and transposon-induced mutation.

In contrast to the advances in genetic and molecular studies, little is known about *Antirrhinum* cytogenetics. Two tandem repetitive sequences were isolated, CentA1 and CentA2, from the centromeric regions of *Antirrhinum* chromosomes. A standard karyotype has been established by anchoring these centromeric repeats on meiotic pachytene chromosome using FISH. An ideogram based on the DAPI-staining pattern of pachytene chromosomes was developed to depict the distribution of heterochromatin in the *A. majus* genome. To integrate the genetic and chromosomal maps, we selected one or two molecular markers from each LG to screen an *Antirrhinum* TAC library. These genetically anchored TAC clones were labeled as FISH probes to hybridize to pachytene chromosomes of *A. majus*. As a result, the relationship between chromosomes and the LGs in *Antirrhinum* has been established (Zhang et al. 2005).

1.5.3 Linkage Map

To increase the utility of *Antirrhinum* for genetic and evolutionary studies, a molecular linkage map for an interspecific *A. majus* × *A. molle* F_2 population ($n = 92$) genotyped at a minimum of 243 individual loci was constructed. Although distorted transmission ratios were observed at marker loci throughout the genome, a mapping strategy based on a fixed framework of codominant markers allowed the loci to be placed into eight robust LGs consistent with the haploid chromosome number of *Antirrhinum*. The mapped loci included 164 protein-coding genes and a similar number of unknown sequences mapped as amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), inverse sequence tagged repeat (ISTR), and inter-simple sequence repeat (ISSR) markers. Inclusion of sequences from mutant loci allowed provisional alignment of classical and molecular LGs. The total map length was 613 cM with an average interval of 2.5 cM, but most of the loci were aggregated into clusters reducing the effective distance between markers. Potential causes of transmission ratio distortion and its effects on map construction were investigated. This first molecular linkage map for *Antirrhinum* should facilitate further mapping of mutations, major quantitative trait loci (QTL), and other coding sequences in this model genus (Schwarz-Sommer et al. 2003a, b).

In *Antirrhinum*, reproductive systems range from self-compatible to self-incompatible, but the actual outcrossing rates of self-compatible populations are not known. Thus the extent to which levels of variability and inbreeding differ among *Antirrhinum* populations is not known. To address this issue we isolated nine *Antirrhinum* nuclear microsatellite loci. In contrast to several nuclear genes that show low levels of sequence variation, six of the microsatellite loci indicate high levels of variability within and between *Antirrhinum* species. The highly self-compatible *A. majus* subsp. *cirrhigerum* population has high level of variability and no significant deviation from Hardy–Weinberg equilibrium, suggesting substantial rates of outcrossing (Zwettler et al. 2002).

1.5.4 Genetic Map

A classical genetic map for *A. majus*, produced from mutant crosses, was composed of 57 morphological markers in eight LGs with a total length of 420 cM (Stubbe 1966). Cytologically visible deletions that unmasked recessive mutations allowed two LGs to be assigned tentatively to chromosomes distinguishable by size and by morphological features (Ohlendt 1942). Linkage analyses in crosses between *A. majus* mutants and several other species, including *A. molle*, revealed conservation of gene order within a substantial part of two chromosomes (Stubbe 1966), but estimates of map distances differed according to the species used as parents (Hoffmann 1949).

A molecular marker-based genetic linkage map has been constructed recently on the basis of an F_2 population of 92 individuals derived from an interspecific hybrid *A. majus* × *A. molle* (Schwarz-Sommer et al. 2003b). These maps, together with its relatively small genome size will make positional cloning feasible in *Antirrhinum* (Lai et al. 2002).

A molecular recombination map, currently comprising more than 250 loci with an average distance between loci of ~2.0 cM has been produced for *Antirrhinum* using hybrids between *A. majus* and wild species, and the map is maintained at http://www.antirrhinum.net/. It has been aligned with a map of classical mutants and with the eight *Antirrhinum* chromosomes using FISH (Zhang et al. 2005). Maps constructed using hybrids between different *Antirrhinum* species are largely colinear, suggesting an absence of extensive chromosomal rearrangements, although distorted transmission of some genomic regions in interspecies hybrids can hinder genetic mapping (Schwarz-Sommer et al. 2003b). Recombinant inbred lines (RILs) and near-isogenic lines (NILs) have been produced from hybrids between different *Antirrhinum* species, allowing the genes that underlie differences between species to be identified.

1.5.5 Gene Expression

Cells of the petal epidermis of *Antirrhinum*, like those of many flowering plants, have a conical shape that is dependent on activity of the MYB transcription factor encoded by *MIXTA*. These conical cells intensify flower color by reducing reflection, as revealed by *MIXTA* mutants with flat epidermal cells, and are important in attracting pollinating bees (Noda et al. 1994). In addition to having a role in the reproductive ecology of *Antirrhinum*, the misexpression of *MIXTA* or related genes can give rise to the formation of epidermal hairs (trichomes), indicating a common regulation of these two cell types and a role for other *MIXTA*-like genes in regulating multicellular trichome development in *Antirrhinum*. This latter function of *MIXTA*-like genes does not appear to be conserved in *Arabidopsis*, which has unicellular trichomes.

1.6 Role in Crop Improvement Through Traditional and Advanced Tools

About 18 wild *Antirrhinum* species are found in Southwest Europe and northern Africa: these display considerable variation in traits, including flower color patterns, flower and leaf shape and size and plant growth habit. Usefully, most of the species are interfertile, and so amenable to genetic analysis. This makes it feasible to identify and isolate genes that underlie species differences and to test the adaptive importance of traits.

1.6.1 Interspecific Hybrids

The interspecific hybrids between *A. majus* and *A. molle* have been reported. There was intergrading variation in characters such as plant height, habit of branching and corolla form. From the progeny of this specific hybrid, new class of *Antirrhinum* has been developed. These hybrids have creeping habit and are highly suitable for covering the grounds as carpet and hence, the group is called as miniature or magic carpet. These hybrids are also suited to rock gardens.

The fertile hybrids between *A. majus* × *A. linkianum* have also been produced. Mahal (1972) intercrossed eight species, namely, *A siculum* Mill., *A. linkianum* Boiss., *A. tortuosum* Bosc., *A. cirrhigerum* Linn., *A. molle* Linn., *A. glutinosum* Boiss. et Reut., *A. majus* Linn., and *A. orontium* in nearly all directions. The crosses *A majus* × *A cirrhigerum*, *A majus* × *A tortuosum*, *A majus* × *A glutinosum*, and *A. cirrhigerum* × *A. linkianum* were successful in both the directions, whereas, crosses, *A. majus* × *A. linkianum*, *A. majus* × *A. siculu*, *A. molle* × *A. tortuosum* and *A. tortuosum* × *A. linkianum* were successfully only in one direction. Crosses involving *A. orontium* and *A. siculum* with other species (except *A. siculum* with *A. majus*) failed altogether. Hybrids of successful crosses were all fertile. Hybrids produced were mostly intermediate, but dominance of some characters of one of the parents was also noted. At meiosis, 16 chromosomes were seen to form bivalents, which indicated genetic homology of the species involved. Some of the disharmonious gene combinations recorded in F_2 plants, which were manifested by abnormal growth and flower forms that are radically different from either of the parents, suggested evolutionary potentialities through transgressive gene.

1.6.2 Heterosis

Genetic studies on diploid and tetraploid progenies in *Antirrhinum* revealed appreciable heterosis in F_1

hybrids for all the characters studied (Malik 1979). Crossing inbred lines of *A. majus* with a dwarf species *A. glutinosum* produced F_1 dwarf hybrid *Antirrhinum* (Mahal 1972). Hybrids were uniform with semi-dwarf habit and large number of tillers with prominently held inflorescences bearing closely set reasonably large flowers with perfect synchronous flowering.

1.6.3 Polyploidy

Antirrhinum is well known for its lack of natural polyploidy. The genus has been divided into two sections *Antirrhinum* and *Sarrrhinum*, which are distributed in the Mediterranean region of Europe and south-western parts of North America, respectively. So far from the European element of the genus 16 species have been worked out cytogenetically. All these are diploid ($n = 8$). Furthermore, there is predominance of self-incompatible and cross-fertilized species. However, gene flow between species is restricted by bee specification in relation to color, size, and structure of flower and ecological isolation. As a result there is a mixed population of different species like *A. majus* and *A. glutinosum*. There is ordinarily little interspecific hybridization although the species cross readily when pollinated by hand. There appears to be no barrier to recombination once hybridization between the subspecies or species is effected. Fertile and segregating progenies are known to result from hybrids involving *A. majus* and *A. glutinosum*. European species of *Antirrhinum* have an ecospecific pattern in which species differentiation is maintained at diploid level by ethological and eco-geographic isolation. Accordingly any polyploids would have auto or segmental alloploid characters. Such polyploids, unaided by other supportive mechanism, have inherent limitations and are unable to establish in nature, although autoploids of the ornamental *A. majus* are a tremendous success under garden conditions. Tetraploid snapdragon lines were also developed, which are of high performance. The autoploids are hardier, more vigorous, stouter, and possess larger flowers than corresponding diploids.

Induced colchiploids by treating the seeds of the variety "Katrain Local" with 0.4% colchicine were reported (Jain et al. 1962). The most characteristic features of the induced tetraploids, which distinguished them from the diploids, were the increased size of individual flowers, which resulted in a more compact form of inflorescence and the increased length of the main floral branch. Flowers in the tetraploids had a more ruffled corolla marked by the presence of small papillae-like structure not found in the diploids.

Colchiploids of *Antirrhinum majus*, *Antirrhinum siculum*, *Anttirhinum linkianum*, and two varieties of *A. orontium* were raised (Mahal et al. 1968; Mahal 1972). These autotetraploids were hardier; more stout; slower in growth and shorter with larger flowers, pollen grains, fruits and seeds than their diploid forms. Meiotic behavior was found to be typical autotetraploid with as many as maximum possible number of quadrivalents in a large number of cases. In all the autotetraploids, pollen and seed fertility was quite high. A plant in autotetraploid population showed cytomixis. All pollen cells in this plant had a varying number of chromosomes from the enucleate condition to a very high number ($2n = 72$). The plant was totally pollen- and seed-fertile. Crosses between diploids and tetraploids were made in both the directions. It was found that this cross succeeds to a slight extent only when a tetraploid is used as seed parent. The triploids produced were better morphologically than both the parents, being faster in growth and longer in flowering duration. At meiosis, the three sets of chromosomes were found to form as many as eight trivalents in a number of cells. Anaphase segregation was highly irregular, but pollen and seed fertility was appreciably high. As a result of these studies, the National Botanical Research Institute, Lucknow, India, released one variety named "Tetra Giant." The resultant tetraploid is hardier, sturdier, stockier, and shorter than the corresponding diploids. It has a higher number of flowering stems, with conspicuously larger, deeper colored flowers, which are long lasting than their diploid counterparts.

1.7 Conservation Initiatives

One feature of progress in the sciences related to crop domestication is the availability of well-characterized germplasm resources in the global network of genetic resources centers (gene banks). Germplasm in gene banks is providing research materials for understanding domestication as well as for plant breeding. The

Ornamental Plant Germplasm Center (OPGC) at the Ohio State University is a new germplasm repository of the United States Department of Agriculture (USDA)/National Plant Germplasm System (NPGS). The OPGC's mission encompasses a priority list of 30 genera formulated by the USDA Herbaceous Ornamental Crop Germplasm Committee. In 2005, the OPGC distributed 54 accessions of *Antirrhinum* and the taxa held by the Ornamental Plant Germplasm Center, Ohio are given below:

- *Antirrhinum barrelieri* (1 accession)
- *Antirrhinum braun-blanquetii* (1 accession)
- *Antirrhinum graniticum* (1 accession)
- *Antirrhinum hispanicum* (1 accession)
- *Antirrhinum majus* (93 accessions)
- *Antirrhinum majus* subsp. *cirrhigerum* (2 accessions)
- *Antirrhinum majus* subsp. *linkianum* (3 accessions)
- *Antirrhinum meonanthum* (2 accessions)
- *Antirrhinum molle* (1 accession)
- *Antirrhinum siculum* (1 accession)

Isabel Mateu-Andres (2004) suggested implications for conservation of the species while comparing the levels of genetic variation and its partitioning in three related species of *Antirrhinum*, *A. subbaeticum*, *A. pertegasii*, and *A. pulverulentum*. The genetic variabilities in *A. subbaeticum* and *A. pertegasii* were found to be the lowest known for the genus, the within population genetic diversity being correlated with population size in both species. Levels of total genetic diversity suggest that species with small total population size have lower levels of genetic variability than those with bigger ones. Strategies for the conservation of these species are recommended, such as preservation of natural populations and avoidance of possible causes of threat, as well as ex situ preservation of seeds, reinforcement of small populations of *A. subbaeticum* with plants or seeds from the same population, and avoidance of translocations among populations.

In another study, Isabel De Paco and Mateu-Aandres (2006) studied levels of genetic variation in seven related species of *Antirrhinum* including *A. siculum*, *A. majus*, *A. latifolium*, *A. linkianum*, *A. litigiosum*, *A. cirrhigherum*, and *A. totusum* and recommended strategies for the conservation of *A. siculum* such as preservation of natural populations as well as ex situ preservation of seeds from different populations.

1.8 Some Dark Sides and Their Addressing

A. orontium (lesser snapdragon) is an herbaceous plant native to southwestern and central Europe and is listed as an invasive plant in Alaska (USGS 2002). In Hawai'i, this plant has become naturalized in dry areas of O'ahu, Maui, and Kaho'olawe (Wagner et al. 1999; Staples et al. 2002; Starr et al. 2002). On Maui, *A. orontium* was collected about 25 years ago in the Pu'u o Kali area. *A. orontium* is currently spreading on Maui and has recently been documented from Lualailua Hills and Kahikinui on the south slope. It has also become established in the Makawao and Ukalani areas. In these areas, *A. orontium* can form dense patches on lava, cinder, or rocky substrates. While *A. orontium* is fairly well established on Maui, it appears that it has not reached its full potential distribution, and will likely continue to spread.

1.8.1 Control Methods

Physical control: Plants can be hand-pulled. This does cause disturbance and follow-up control will likely be needed to pull new seedlings as they come up.

Chemical control: Apparently, in the Pacific Northwest, *A. orontium* is increasingly becoming a roadside weed due to its tolerance for many herbicides.

Cultural control: These plants are not large and may be kept from invading by having healthy ecosystems with plenty of plant cover and minimizing disturbance. Plants in the Lualailua cinder pit could be controlled to curb long distance dispersal.

Global Compendium of Weeds also listed *A. barrelieri*, *A. siculum*, also as weeds.

1.9 Recommendations for Future Actions

In addition to *Antirrhinum* continuing its role as a genetic model for plant biology and development, several new experimental avenues are to be explored with *Antirrhinum* and related species.

Furthermore, given the tremendous morphological and ecological diversity in this crop, a phylogeny

within the genus *Antirrhinum* with an emphasis on the New World section *Saerorhinum* may help to pinpoint interesting patterns that warrant further study.

To initiate network of cooperation among the horticultural industry, universities, botanical gardens and arboreta, crop specific societies and individuals for promoting conservation and utilization of wild relatives.

To establish a system that promotes industry oriented collaborative research to enhance germplasm utilization and conservation.

The wild relatives of crops continue to be an important reservoir of genes for potential use in agriculture. Sometimes, the genes furnished have had a dramatic effect on improvement and contributed in evolutionary studies. Therefore, there is a continued urgency to conserve these wild genetic resources appropriately, both in situ and ex situ, and to characterize them for future crop improvement.

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Chapter 2
Camellia

Tapan Kumar Mondal

2.1 Introduction

The genus *Camellia* includes shrubs and trees belonging to the family Theaceae and is native to eastern Asia. The genus was named by Linnaeus in the honor of Jesuit missionary G. J. Kamel, who first recommended to grow the Japanese rose in Europe. In his systema Naturae of 1735, Linnaeus gave the name *Camellia tsubaki* to the plant found in Japan as the tsubaki and it only acquired its present systematic name, *Camellia japonica* in his *Species Plantarum*.

The center of origin of the genus *Camellia* is in South and southwestern China, centering the provinces of Yunnan, Guangxi and Guangdong straddling the Tropic of Cancer, the area bounded by longitude 85°W and 150°E and latitudes 37°N and 10°S, but its distribution within this area is not uniform. Apparently 90% of *Camellia* species originated in South and Southeast China though some species, viz. *C. japonica* and *C. lancelolata* originated in Japan and Philippines as well as Indonesia, respectively.

In the East, different species of *Camellia* were spread from country to country by Buddhist monks. Their introduction into Europe began some 300 years ago, when the first attempt was to cultivate ornamental *Camellia* plant brought by sea from the East. Those *Camellia* flourished all over the Europe from England to France, Belgium, and Italy and from Portugal to Spain in their acid soil and temperate humid climate. *Camellia* was introduced to the USA at the beginning of the eighteenth century. From England, ornamental *Camellia* was introduced to Australia during the nineteenth century.

The economic importance of the genus *Camellia* is largely due to *C. sinensis*, whose young leaves are used to prepare tea. This single species is the economic backbone of several South Asian countries including India, China, Japan, Korea, Sri Lanka, Indonesia, erstwhile USSR, and African countries such as Malawi, Kenya, etc. Amidst the wild species, economic value of *C. japonica* ranks the highest due to its beautiful ornamental flowers so much so that more than 3,000 cultivars are available now worldwide. Other wild species with ornamental values are *C. reticulata*, *C. sasanqua*, and *C. saluensis*. A few wild species such as *C. oleifera*, *C. semiserrata*, and *C. chekiangolomy* are used to produce oil from their seeds, which is used in Chinese cookery and has pharmaceutical value. Finally, *Camellia* leaves contain a number of substances used in the pharmaceutical industry, including xanthine, teophiline, teabromine, adenine, tearine, and oleic acid.

2.2 Botany

2.2.1 Morphology

Camellias are broad-leaved, evergreen shrubs, which may grow up to a height of 25 ft, but more often to 6–12 ft. It has a spread of 6–10 ft. The dark-green leathery leaves are 4 in. long. The flowers, which

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range in color from white to pink and red, are 3–5 in. in diameter. The flowers generally bloom between September and April either as single, semi-double, or double and are pollinated mainly by bees. Botanically, leaves are coriaceous, pinnately veined, often serrated, petiolated, rarely sessile, and amplexicaul. Flowers are hermaphroditic, solitary or in clusters at the branch terminals or leaf axils, pedicellate or sessile, bracts usually 2–8, sepals usually 5–6, sometimes the differentiation between bracts and sepals is indistinct, becomes perulate to 21 perules, deciduous or persistent, corollas white, red, or yellow, petals 5–12, basically connate, stamens numerous in 2–6 series, outer filament whorl often connate into filament tube adnate to petal bases, anthers dorsifixed or occasionally basifixed, 2-locular, longitudinally cleft, ovaries superior, 3–5 locular, sometimes unilocular, 3–5 valvate usually dehiscent from the top, columella persistent or lacking; seed globose or polygonal, seed coast corneus (Chang and Bartholomew 1984).

2.2.2 Conventional Propagation

Camellia plants are propagated either through seeds or cuttings. Usually mature seeds are collected from mother plant, pre-soaked for 24 h in water and the hard covering around the micropyle should be filed down to leave a thin covering and then sown in polythene sleeves in the nursery where it takes 12–18 months to attain more than 15 cm height before transferring to the field. Nevertheless, seed-grown plants show a high degree of variability. Alternatively, it can be multiplied vegetatively wherein cuttings with an axillary bud are planted in moss peat under shade followed by the transfer of the rooted plants to the field. Two other methods known as layering and grafting are also used for propagation. Layering can be done with the plants of more than 2 year old where leaves and shoots of the branch hanging down are removed. Then using a sharp knife, a small incision is introduced in a single place and the branch down is trained to the ground in such a way to secure the tip in the soil with the clip, which is then covered with wet, moist peat soil. Roots formed after 2 weeks from the incision allow the shoot tip to be separated from the parent plant to be planted individually.

In the nineteenth century, when modern methods of root cutting were not developed, it was common to propagate Camellias by grafting. Normally, crown grafts were made at the base of young *C. japonica* stocks, though different kinds of side cleft graft and approach graft could be used (Laborey 1986). Juvenile grafting onto hypocotyls of seedling has proved to be both very effective and simple to practice (Vieitez and Vieitez 1983). In Japan, particularly in the Camellia Centre of Kyuohu Island, it is common for highly prized bonsai Camellias to be created by grafting onto segments of root 50–60 cm long and 2–3 cm in diameter taken from wild *C. japonica*; flowering 3–4 years later (Vieitez et al. 1991).

2.2.3 Genetic Diversity

The genus *Camellia* had reportedly 40 species in 1920. The number of species was increased to 87 by Sealy in 1958 (Sealy 1958) and more than 267 species were registered in 1982 (Chang and Bartholomew 1984). Presently, this genus is believed to comprise more than 300 species (Prince and Parks 2000) that indicates genetical instability and high outbreeding nature of the genus. In a conservative estimation, there are more than 3,000 cultivated varieties of ornamental *Camellia* worldwide of which more than 2,500 have been registered in the American Camellia Society.

The *Camellia* is the largest genus of the family Theaceae. The genus is valued for tea due to the content of caffeine and apurine alkaloids, which act as stimulus for central nervous system of human being. Nagata and Sakai (1984) reported the distribution of caffeine in 23 species of the genus *Camellia*. The caffeine content on a dry weight basis in some of them was as follows: *C. sinensis* var. *sinensis* (2.78%), *C. sinensis* var. *assamica* (2.44%), *C. taliensis* (2.54%), and *C. kissi* (0.02%). *C. kissi* belongs to the section *Paracamellia* and the other genera belong to the section *Thea*.

The other three genera in the family are *Eurya* with 140 species, *Ternstroemia* with 130 species, and *Adinandra* with 100 species. Chang and Bartholomew (1984) divided the whole *Camellia* genus into 4 subgenera and 20 sections totally, which are depicted below with the example of some prominent species in each section.

I Subgenus	*Protocamellia*
Section I	*Archecamellia*
	Camelia granthamiana
	Camellia albogigas
	Camellia pleurocarpa
Section II	*Stereocarpus*
	Camellia krempfii
	Camellia dormoyana
	Camellia yunnanensis
	Camellia liberistyla
	Camellia liberistyloides
Section III	*Piquetia*
	Camellia piquetiana
II Subgenus	*Camellia*
Section IV	*Olifera*
	Camellia gauchowensis
	Camellia sasanqua
	Camellia vietnamensis
	Camellia oleifera
Section V	*Furfuracea*
	Camellia integerrima
	Camellia polypetala
	Camellia latipetiolata
	Camellia crapnelliana
	Camellia furfuracea
	Camellia oblate
	Camellia gaudichaudii
	Camellia parafurfuracea
Section VI	*Paracamellia*
	Camellia grijsii
	Camellia confuse
	Camellia kissii
	Camellia lutescens
	Camellia fluviatilis
	Camellia brevistyla
	Camellia obtusifolia
	Camellia maliflora
	Camellia miyagii
	Camellia shensiensis
	Camellia brevissima
	Camellia puniceiflora
	Camellia tenii
	Camellia microphylla
	Camellia phaeoclada
	Camellia weiningensis
Section VII	*Pseudocamellia*
	Camellia szechuanensis
	Camellia chungkingensis
	Camellia trichocarpa
	Camellia ilicifolia
	Camellia henryana
Section VIII	*Tuberculata*
	Camellia tuberculata
	Camellia anlungensis
	Camellia obovatifolia
	Camellia rhytidocarpa
	Camellia litchi
	Camellia parvimuricata
Section IX	*Luteoflora*
	Camellia luteoflora
Section X	*Camellia*
	Camellia omeiensis
	Camellia polydonta
	Camellia lapidea
	Camellia mairei
	Camellia villosa
	Camellia kweichowensis
	Camellia albovillosa
	Camellia albescens
	Camellia tunganica
	Camellia trichosperma
	Camellia phellocapsa
	Camellia semiserrata
	Camellia multiperulata
	Camellia lungshenensis
	Camellia reticulate
	Camellia pitardii
	Camellia hiemalis
	Camellia uraku
	Camellia edithae
	Camellia xylocarpa
	Camellia hongkongensis
	Camellia cryptoneura
	Camellia oviformis
	Camellia compressa
	Camellia setiperulata
	Camellia saluenensis
	Camellia boreali-yunnanica
	Camellia lucidissima
	Camellia magnocarpa
	Camellia japonica
	Camellia subintegra
	Camellia longicaudata
III Subgenus	*Thea*
Section XI	*Corallina*
	Camellia coralline
	Camellia tonkinensis
	Camellia wardii
	Camellia pilosperma
	Camellia fleuryi
	Camellia nitidissimia
	Camellia paucipunctata
	Camellia lienshanensis
	Camellia pentamera
	Camellia scariosisepala
	Camellia acutiserrata
Section XII	*Brachyandra*
	Camellia muricatula
	Camellia szemaoensis
	Camellia pachyandra
	Camellia xanthochroma
	Camellia amplexifolia
	Camellia brachyandra
	Camellia nervosa
	Camellia nematodea
	Camellia gilbertii
	Camellia crassipetala
	Camellia yangkiangensis
	Camellia parviflora
Section XIII	*Longipedicellata*

(*continued*)

(*continued*)

	Camellia amplexicaulis
	Camellia petelotii
	Camellia longipedicellata
	Camellia indochinensis
Section XIV	*Flavae*
	Camellia flava
	Camellia aurea
Section XIV	*Chrysantha*
	Camellia chrysantha
	Camellia flavida
	Camellia impressinervis
	Camellia chrysanthoides
	Camellia tunghinensis
	Camellia pingguoensis
	Camellia pubipetala
Section XV	*Calpandria*
	Camellia lanceolata
	Camellia connata
Section XVI	*Thea*
	Camellia crassicolumna
	Camellia pentastyla
	Camellia taliensis
	Camellia irrawadiensis
	Camellia crispula
Section XVII	*Longissima*
	Camellia longissima
Section XVIII	*Glaberrima*
	Camellia gymnogyna
	Camellia costata
	Camellia yungkiangensis
	Camellia leptophylla
	Camellia pubicosta
	Camellia angustifolia
	Camellia sinensis
	Camellia assamica
	Camellia pubilimba
	Camellia waldenae
	Camellia fangchensis
	Camellia ptilophylla
	Camellia parvisepala
	Camellia glaberrima
	Camellia kwangtungensis
IV Subgenus	*Metacamellia*
Section XIX	*Theopsis or Eriandra*
	Camellia macrosepala
	Camellia cuspidata
	Camellia grandiflora
	Camellia chekiangensis
	Camellia longicuspis
	Camellia crassipes
	Camellia longicalyx
	Camellia forrestii
	Camellia acutisepala
	Camellia buxifolia
	Camellia minutiflora
	Camellia parvicuspidata
	Camellia acutissima
	Camellia subacutissima
	Camellia callidonta
	Camellia handelii
	Camellia triantha
	Camellia costei
	Camellia tsaii
	Camellia synaptica
	Camellia transnokoensis
	Camellia rosthorniana
	Camellia lutchuensis
	Camellia euryoides
	Camellia trichoclada
	Camellia parvilimba
	Camellia brevipes
	Camellia elongate
	Camellia longicarpa
	Camellia parvilapidea
	Camellia stuartiana
	Camellia transarisanensis
	Camellia fraternal
	Camellia dubia
	Camellia percuspidata
	Camellia membranacea
	Camellia rosaeflora
	Camellia campannisepala
	Camellia lancilimba
	Camellia tsingpienensis
	Camellia pubisepala
	Camellia parviovata
	Camellia viridicalyx
	Camellia lancicalyx
	Camellia parvicaudata
	Camellia subglabra
	Camellia nokoensis
	Camellia tsofuii
	Camellia trichandra
	Camellia villicarpa
	Camellia cratera
	Camellia punctata
	Camellia lawii
	Camellia trigonocarpa
	Camellia cordifolia
	Camellia wenshanensis
	Camellia melliana
	Camellia candida
	Camellia caudate
	Camellia assimiloides
	Camellia assimilis
	Camellia edentate
	Camellia salicifolia

(*continued*)

2.2.4 Karyotype and Genome Size

Based upon the analysis by flow cytometry and staining by propidium iodide, the genome size of *Camellia japonica* ($2n = 30$; basic chromosome number, $x = 15$) was found to be 4G bases though the triploids have 1.5 times higher DNA than diploids (Tanaka et al. 2005). Generally, the chromosomes are small in size and tend to clump together due to stickiness. The length of *Camellia* chromosome ranges from 1.28 to 3.44 μm (Bezbaruah 1971). The *r* value (ratio of long

arm to short arm) for all the 15 chromosomes range from 1.00 to 1.91. The consistency in diploid chromosome number suggests a monophyletic origin of all *Camellia* species.

Cytological markers of the genus *Camellia* were elaborately studied in the early 1970s with many interesting features. Chromosome number has been established for the most available taxa of *Camellia* including tea (Beretta et al. 1987), which was reviewed by Kondo (1975).

Karyotypic data of *Camellia* had also been accumulated in past for several species (Fukushima et al. 1966; Ackerman 1971; Kondo 1975). Unfortunately, karyotype grouping by chromosome size was difficult in the *Camellia* taxa due to high stickiness of the chromosomes. Furthermore, even in the best preparation, homologous chromosome pairs could not appear identical in *Camellia* (Kondo 1975). Relatively little intraspecific karyotypic variation had been observed for the cultivated species of *Camellia* studied (Kondo 1975). Sat-chromosomes in karyotypes within mass accessions of certain *Camellia* species are morpholo gically and quantitatively variable. Thus, karyotypes including characteristics of sat-chromosomes are not of taxonomic significance for *Camellia* taxa. Among the diploid species of *Camellia* studied, *C. japonica* L. *sensu lato* showed the greatest karyotypic variation, many of the accessions studied indicated similar karyotypic patterns to each other (Kondo 1975). For instance, *C. japonica* L. var. *spontanea* (Makino), *C. japonica* L. var. *macrocarpa* Masamune, *C. japonica* L. subsp. *rusticana* (Honda) Kitamura and four cultivars including "Aka-Wabisuke," "Fukurin-Wabisuke," "Kuro-Wabisuke" and "Wabisuke" carried same, most common standard acetoorcein-stained karyotype if the presence of satellites is not considered; 16 metacentric, 8 submetacentric, and 6 subtelocentric chromosomes. Actually, *C. japonica* L. var. *macrocarpa* Masamune had satellites on four submetacentric chromosomes and the other accessions had satellites on two submetacentric chromosomes (Kondo and Parks 1980). Later, it was shown by Kondo and Parks (1979) that the C-banding method can be applied to the somatic mid-metaphase chromosomes in *Camellia* taxa. These differentially stained bands in somatic mid-metaphase chromosomes permit the identification of 238 individual chromosomes and make it possible to match the homologous pairs of chromosomes more precisely and possibly even measure chromosome divergence among different clones within the same species with same or similar karyotypes. Karyotypic variability and divergence among the seven accessions of *C. japonica* L. *sensu lato* with same acetoorcein-stained karyotype were revealed by C-banding method (Kondo and Parks 1980). By this way, the cytological marker was used to sort and classify the vast number of cultivars. However, due to the development of more sensitive biochemical techniques, attention was shifted toward the search of biochemical markers.

2.3 In Vitro Culture in *Camellia* Species

2.3.1 Micropropagation

Since the propagation of some *Camellia* species by conentional methods is difficult and slow, other means have been sought. Several reviews on micropropagation of *Camellia* including tea and related species have been published (Kato 1989a; Vieitez et al. 1991; Dood 1994; Das 2001; Mondal et al. 1998). It is evident from the literature that while Bennett (1977) was pioneer for initiation of tissue culture of ornamental Camellias yet, Vieitez et al. (1991) did a systematic study of micropropagation with *C. japonica,* which elaborately highlighted various factors that affect multiplication rate in in vitro and subsequent hardening processes. Depending on the species-specific requirements among the wild *Camellias*, various factors that influence the micropropagation are briefly reviewed below.

2.3.1.1 *C. japonica*

In the late 1970s, the use of in vitro culture methods was suggested as a means to solve the constraints in propagation mainly due to shy rooting in vegetative cuttings (Bennett and Scheibert 1982). Since then, several protocols have been described for the micropropagation of *C. japonica*. The first elaborate report to regenerate plants from shoot tips and axillary buds was made by Creze and Beauchesne (1980), who took meristems with one or two leaf primordial of 0.5 mm long from 1-year-old rooted cuttings or from 3- to 4-year-old seedling and cultured them on a MS medium supplemented with adenine (20 mg/l); IAA (0.1 mg/l); 1 mg/l each of kinetin, BAP, and GA_3; and polyvinylpyrrolidine (10 g/l). Although cultures were established and elongated to produce shoots more rapidly than shoot tips, no rooting and transfer to the soil were not described.

In *C. japonica*, buds of juvenile origin gave consistently better results in terms of both growth and vigor on MS as compared to other macronutrient formulae of Lepoivre (Quoirin and Lepoivre 1977), Knop (Tabachnik and Kester 1977), Schenk and Hildebrandt (1972), and modified Heller (1953). However, regeneration from adult material of *C. japonica* cv. Alba Plena was poor on MS (Vieitez et al. 1989a). In a series of shoot multiplication experiments, Vieitez et al. (1989a) found that WPM was the best among the six macronutrient formula tested (modified Heller 1953), MS, half-strength MS, WPM, Gresshoff and Doy (1972) and Anderson (1984). In contrast to Carlisi and Torres (1986), who found that MS and half-strength MS were the best for culturing of *C. japonica*, the observations recorded by Vieitez et al. (1989a) were poor in these media. The different response observed by Carlisi and Torres (1986) was probably genotype-dependent.

For *C. japonica*, the most widely used cytokinin was BAP (Table 2.1). However, Creze and Beauchesne (1980) reported the importance of 2-iP (1 mg/l) as an essential component for shoot proliferation, kinetin was also found to have no effect on shoot multiplication when used either alone or in combination with BA (Samartin et al. 1984). The GA_3 (5–10 mg/l) was also used for proliferation of shoots in cv. Purple Dawn (Carlisi and Torres 1986; Torres and Carlisi 1986). Among the auxins, IAA and IBA are used for shoot proliferation, but there are no reports on the use of NAA and 2,4-D for culture of *C. japonica* (Creze and Beauchesne 1980; Vieitez et al. 1989b). Apart from plant growth regulators (PGR), another factor, which was found to be important, is the physical condition of the media. In general, liquid medium was more suitable than solid medium for shoot proliferation in *C. japonica* (Carlisi and Torres 1986; Vieitez et al. 1989a).

2.3.1.2 *C. oleifera*

Very little work has been done on this important oil-yielding species. Tian-Ling (1982) used MS medium supplemented with BAP (4 mg/l) and NAA (2 mg/l) for induction of adventitious buds leading to plantlet regeneration. In another study, lateral buds of adult trees were also used by Yan et al. (1984) for induction of axillary bud proliferation.

2.3.1.3 *C. reticulata*

Heller's (1953) macroelements with the addition of $(NH_4)_2SO_4$ (0.13 mg/l) in combination with MS vitamins were found to be the best for induction of axillary buds. WPM was also found to be superior to modified Heller (1953) and the recipes of Anderson 1984 (San-Jose and Vieitez 1990). A combination of BAP and zeatin has also been successfully used for promoting the growth and proliferation of axillary shoots (San-Jose and Vieitez 1990; San-Jose et al. 1991). Multiplication rates in terms of both number of axillary buds and the length of shoots could further be improved by horizontal placement of the explants (San-Jose and Vieitez 1990).

2.3.1.4 *C. sasanqua*

While Torres and Carlisi (1986) preferred MS medium, Samartin (1991) found B_5 (Gamborg et al. 1968) macronutrients supplemented with micronutrients of MS to be suitable for the growth and proliferation of axillary shoots. A combination of BAP and NAA was found to be the most suitable for shoot multiplication in both of these studies.

2.3.1.5 *Camellia* Hybrids

Despite the availability of limited information, the medium of Tukey (1934) was found to be the best for in vitro seedling growth of three different interspecific hybrids including *C. japonica* × *C. cuspidata*, *C. japonica* × *C. reticulata*, and *C. japonica* × *C. saluenensis* (Lammerts 1958). Creze and Beauchesne (1980) made the first attempt to regenerate plants from shoot tips and axillary buds on *C. saluenensis* × *C. chrysantha,* details on which were mentioned in their report.

2.3.2 Rooting and Hardening

Like other woody plants, rooting is a major limitation in micropropagation of *Camellia*. Rooting of in vitro raised shoots was achieved either upon continuous

Table 2.1 Summary of micropropagation studies in *Camellia*

Species/cultivar	Explant	Medium				Remarks	Response time	Reference
		Initiation	Multiplication	Regeneration/ organogenesis	Rooting			
C. saluenensis × *C. japonica*	In vitro shoot	–	–	–	1/2 MS	–	–	Beretta et al. (1987)
C. japonica, Purple Dawn	Shoot tips and nodal segments	1/2 MS + BA (1)	1/2 MS + BAP (1) + GA_3(5)	–	–	–	–	Carlisi and Torres (1986)
C. saluenensis × *C. chrysantha*	Shoot tips of seedlings	MS + Kn (1) + 2ip (1) + GA_3(1) + IAA (1) + PVP (10 g/l)	MS + Kn (1) + 2ip (1) + GA_3 (1) + IAA (1) + PVP (10 g/l)	–	–	–	–	Creze and Beauchesne (1980)
C. japonica	Shoot tips and nodal segments of 3–4-year-old seedlings	MS + Kn (1) + 2ip (1) + GA_3(1) + BA (1) + IAA (1) + Adenine (20) + PVP (10 g/l)	MS + Kn (1) + 2ip (1) + GA_3 (1) + BA (1) + IAA (1) + Adenine (20) + PVP (10 g/l)	–	–	–	–	Creze and Beauchesne (1980)
C. japonica	Shoot tips and nodal segment	MS + modified vit + BA (1) + IAA (0.1)	Same basal medium (MS) + BAP (1)	–	1/2 MS with same supplement	–	10 weeks, 4 weeks	Samartin et al. (1984)
C. japonica	Shoot tips 2–3-month-old seedling	MS + BA (1)	–	–	1/2MS with modified vit + IBA (1 g/l)	18 days dark treatment before placing rooting media enhances rooting	16 days	Samartin et al. (1986)
C. sasanqua, Onigoromo Thunb.	Shoot tips and nodal segments	–	–	–	Gamborg's (B_5) medium	–	Gamborg (B_5) + modified vita + BAP (0.5) + NAA (0.1)	Samartin (1991)
C. reticulata, "Captain Rawes"	Terminal shoot tips and nodes	Heller's (1953) macro + $(NH_4)_2SO_4$ $_{(132.14)}$ + MS vit+ BAP (2) + Zeatin (2) + IBA (0.01) + 2ip (2)	WPM + BAP (2) + Zeatin (2) + 2ip (2) + IBA (0.01)	–	1/2 WPM + dipping in IBA solution (1 g/l) for 30 min	Time of explant collection influenced shoot multiplication	16 weeks, 4 weeks	San-Jose and Vieitez (1990)
C. reticulata, Captain Rawes	In vitro leaf	Heller's (1953) macro + $(NH_4)_2SO_{4(132.14)}$ + vit + BAP (2) + Zeatin (2) + IBA (0.01) + 2ip (2)	WPM + BAP (2)+ Zeatin (2)+ 2ip (2)+ IBA (0.01)	WPM + BAP (2) + IBA (1)	1/2 WPM + sucrose (6%) + agar (0.6%)	Shoots from adventitious origin rooted very poorly in comparison with those axillary origin, under same culture condition	2–3weeks, 10–12 weeks respectively	San-Jose and Vieitez (1992)

(*continued*)

Table 2.1 (continued)

Species/cultivar	Explant	Medium				Remarks	Response time	Reference
		Initiation	Multiplication	Regeneration/ organogenesis	Rooting			
C. reticulata cv. Captain Rawes	Shoot tips and nodes of adult trees	Heller's (1953) macro + 1 mm $(NH_4)_2SO_4$ + MS vit + BAP (2) + Zeatin (2) + IBA (0.01) + 2ip (2)	WPM + BA (2) + Zeatin (2) + 2ip (2) + IBA (0.01). Horizontal position were better than vertical position for shoot multiplication	–	1/2 macro WPM + full micro + vit + sucrose (6%)	–	8 weeks, 16 weeks respectively	San-Jose et al. (1991)
C. oleifera	Immature cotyledons and embryos	–	–	MS + BA (4) + NAA (2)	–	Liquid medium filter bridge support was better for rooting	–	Tian-Ling (1982)
C. sasanqua, Day Dream	Shoot tips, stem segment	MS + BAP (1) + NAA (0.1) for juvenile plant MS + BAP (1) for adult material	1/2 MS + modified MS vit + NAA (0.1) + BAP (2) + GA_3 (5–10) + sucrose (3%)	–	1/2 MS + modified vit	–	8 weeks for shoot proliferation and 8 weeks for plantlet regeneration	Torres and Carlisi (1986)
C. ×williamsii, Debbie	Internode	WPM macro + MS micro + MS vit + BAP (0.5) + IBA (0.01)	–	MS + IBA (0.1) + Phytagel (0.25%) + TDZ (2.75)	–	Phytotoxic levels of antibiotic kanamycin and cefotaxime have been detected	Callusing, plantlet regeneration	Tosca et al. (1996)
C. japonica, Alba Plena	Shoot tips and nodal segment	Vieitez et al. (1989a, b)	Vieitez et al. (1989a, b)	–	WPM macro after dipping in IBA 1 g/l for 15 min followed by 12 days darkness	Supporting media (agar or paper bridge) did not significantly affect rooting	4 weeks	Vieitez et al. (1989a)
C. japonica cv. Alba Plena	Shoot tip (2–4 cm), nodal segment, and whole shoots of field grown plant	Heller's (1953) macronutrient increased by factor 1.25 + 1 mM $(NH_4)_2SO_4$ + MS micronutrient+ BAP (1) + IBA (0.01) + *m*-inositol (100) + Jacquiots vit (Gautheret 1959)	WPM + BAP (2) + Zeatin (2) + 2ip (2) + IBA (0.01)	–	WPMO after giving IBA (1 g/l) treatment for 15 min	The rate of shoot proliferation depends upon the explant used	2–3 weeks for rooting	Vieitez et al. (1989b)
C. oleifera	Lateral buds of adult trees	–	–	–	–	–	–	Yan et al. (1984)

Figures in parenthesis denote concentration (mg/l) *TDZ* Thidiazuron; *vit* Vitamin

exposure to a low concentration of auxin or initially to a less exposure to a high auxin concentration followed by their transfer to an auxin-free medium. In vitro rooting of *Camellia* species has been reported by a number of workers (Table 2.1). Reduction of MS salt concentrations to half-strength favored both induction and elongation of rooting in *Camellia* species (Samartin et al. 1984, 1986; Kato 1985). However, Vieitez et al. (1989b) did not find any significant difference in rooting of in vitro raised shoots of *C. japonica* cv. Alba Plena using half-strength MS medium.

In *Camellia,* IBA (0.5–8 mg/l) has been shown to give better results than NAA for in vitro root initiation. Roots induced by NAA were shorter, thicker, and with accompanying calli, which were undesirable features for the subsequent transplanting. On the other hand, with IBA treatments, rooting occurred much later but were long and fibrous (Samartin et al. 1986).

Liquid medium with filter paper bridge was beneficial for rooting in *C. olerifera* (Tian-Ling 1982; Kato 1985; Nakamura 1987b). Torres and Carlisi (1986) reported that a pulse treatment of shoots with 500 mg/l IBA for 30 min before placing on a root induction medium gave best results in *C. sasanqua*. Beretta et al. (1987) obtained increased rooting in *C. saluenous* × *C. japonica* hybrids with 1–2 g/l IBA treatment for 15 min. In comparison to other woody species, the *Camellia* spp. require higher IBA concentration and longer immersion time (San-Jose et al. 1988) and such high treatments were not as deleterious for Camellias as in other woody species like *Prunus avium* (Riffaud and Cornu 1981). Dark treatment after dipping the shoots in auxin was reported to favor rooting in *C. japonica* (Samartin et al. 1986) and *C. reticulata* (San-Jose and Vieitez 1990). However, Samartin (1991), who also worked with *C. japonica,* did not find any significant difference between the effects of light and dark treatments on rooting.

Rooting mixture alone influences the survival rate at nursery. While 75% survival of *C. japonica* was obtained in petrite:soil (1:1) by Samartin et al. (1984), a higher survival rate of 70–90% of the same species was achieved in soil:quartz (1:1) mixture by Samartin et al. (1986) and Vieitez et al. (1989b). In *C. reticulate,* rooted shoots were transferred to pot containing 1:1 mixture of peat and quartz and placed in a plastic tunnel with mist/fog system in lamps to a give a 16-h photoperiod, which resulted 80% survival (San-Jose et al. 1991).

However, there is no report on either of any nursery performance or any field performance of micropropagated Camellias so far.

2.3.3 Somatic Embryogenesis

Somatic embryogenesis is considered to be the most efficient regeneration system of *Camellia* (Jain and Newton 1990). However, the efficacy of such a system for plant production depends on the multiplication and conversion rate of somatic embryo. The recent biotechnological advances, including gene cloning and gene transfer, offer great promise for rapid improvement of genotypes for desirable traits and integrate well with the technique of somatic embryogenesis. Although somatic embryogenesis has been fully exploited in herbaceous species, there remain difficulties with woody species like *Camellia*. However, it has a tremendous potential in clonal propagation and most importantly in genetic transformation (Mondal et al. 1999). In *Camellia*, it has been successfully used for artificial seed production (Ballester et al. 1997), cryopreservation for long-term storage of germplasm (Janeiro et al. 1996), and some interspecific crosses of *Camellia* (Nadamitsu et al. 1986), where immature somatic embryos were rescued and cultured before abortion. It can also be used for the production of disease-free and androgenic or haploid plants (Pedroso and Pais 1994). The various factors, which govern the somatic embryogenesis of *Camellia* species, are concisely summarized below.

2.3.3.1 Explant Choice

Although somatic embryogenesis has been reported from various explants of ornamental Camellias, most of the workers have, however, used mature cotyledon slices or zygotic embryos (Table 2.2). In *C. japonica* and *C. reticulata*, immature cotyledons and zygotic embryos, roots, stems, and leaves have been used for the induction of somatic embryogenesis (Plata and Vieitez 1990; Vieitez and Barciela 1990; Plata 1993; Pedroso and Pais 1993; Zhuang and Liang 1985a). Dark period of at least 14 weeks appeared to be necessary for somatic embryogenesis from in vitro leaf for *C. reticulata* (San-Jose and Vieitez 1993). Vieitez

Table 2.2 Summary of somatic embryogenesis in *Camellia*

Species and cultivar	Explant	Medium				Reference
		Induction	Maturation	Germination	Multiplication	
C. japonica	Mature cotyledon	MS + BAP (1)	–	–	–	Barciela and Vieitz (1993)
C. japonica	Immature Cotyledon	MS with modified vit + BAP (1–2) + IBA (0–2)	–	–	MS + modified vit + BAP (1)	Barciela and Vieitz (1993)
C. japonica	Mature cotyledon	Knop + BA (0.5–5)	–	–	–	Bennett and Scheibert (1982)
C. japonica	Mature cotyledon	MS + BA (0–5) + IBA (0–2)	–	MS + GA_3 (1)	–	Kato (1986a)
C. sasanqua	Mature cotyledon	MS + BAP (0–10) + IBA (0–2)	–	–	–	Kato (1986b)
C. japonica	Primary somatic embryo	MS + GA_3 (1) + colchicine (0.1%)	–	MS + GA_3(1)	–	Kato (1989b)
C. vietnamensis × *C. chrysantha*	Mature cotyledon	MS + BA (3) + NAA (1)	–	MS + GA_3(1) + Coconut milk (10%)	–	Nadamitsu et al. (1986)
C. sinensis with 13 cultivars, *C. japonica* 3 cultivars, *C. sasanqua*, *C. brevistela*, *C. nokoensis*, *C. japonica* (cv. Kosyougatu) × *C. granthamiana*	Mature sliced cotyledon	MS + BA (1–5)	–	–	–	Nakamura (1988a)
C. japonica cv. Elegans	In vitro leaf	1/2 MS + DTT (2.5) but ferrous sulfate was replaced by ferric citrate (2.5)	Same as induction medium	Full strength, induction media + D-glucose (25 g/l) + BA (1) + IBA or IAA (0.1)	–	Pedroso and Pais (1993)
C. reticulata	Mature and immature cotyledons	MS + IBA (0.5–1) MS + BA (1) + IBA (0.5)	–	MS+GA_3 (3–5) + IAA (1–2)	–	Plata and Vieitez (1990)
C. reticulata cv. Mouchang	Immature zygotic embryo	–	–	–	MS + Modified vit + BAP (0.5) + IBA (0.1)	Plata et al. (1991)
C. japonica "Alba Plena" and *C. retieulata* Mouchang	In vitro leaf	MS + BAP (8) + IBA (0.5)	MS + BAP (8) + IBA (0.5)	MS + $GA_{3(3)}$ + IAA (1)	Secondary somatic embryogenesis was multiplied in MS + 4.4 μM BAP + IBA (0.1)	San-Jose and Vieitez (1993)

C. japonica	Immature and mature zygotic embryo	MS with modified vit + BAP (1–2) + IBA (0–2)	MS with modified vit + BAP (1–2) + IBA (0–2)	MS with modified vit + BAP (1) + (0.1)IBA + GA_3(5) + IAA (2)	MS + GA_3 (1/2) generally induced secondary embryogenisis	Vieitez and Barciela (1990)
C. japonica cv. Alba Plena	In vitro roots	MS with thiamin (1) + nicotinic acid (0.1) + pyrodoxine-Hcl (0.1)	MS with thiamin (1) + nicotinic acid (0.1) +pyrodoxine-Hcl (0.1)	MS+GA_3 (5) + IAA (1)	–	Vieitez et al. (1991)
C. japonica "Alba Plena"	In vitro roots of juvenile origin	MS + Zeatin (1) + BA (0–2) + IBA (0–2)	MS + Zeatin (1) + BA (0–2) + IBA (0–2)	MS+GA_3 (5) + GA_3 (1–2)	MS + IBA (0.1) + BAP (1)	Vieitez et al. (1991)
C. japonica × *C. chrysantha*	Immature zygotic embryos	MS + Kn (0.1–0.5) + NAA (0.5–1) + YE (1)	–	Anderson (1984) basal medium + 2ip (0.2–0.5) + GA_3 (5) + PVP (5 g/l)	–	Yamaguchi et al. (1987)
C. oleifera	Mature cotyledons	–	–	–	–	Yan et al. (1984)
C. chrysantha	Mature cotyledons	MS + BA (1) + NAA (0.2–0.5)	–	–	–	Zhuang and Liang (1985b)
C. sasanqua	Mature cotyledons	MS + BA (1) + NAA (0.2–0.5)	–	MS + BA (2) + IAA (0.5) + ABA (0.2) + glutamine 500 or MS+GA_3 (1)	–	Zhuang et al. (1988)
C. reticulata	Mature cotyledon	MS + BA (1) + NAA (0.2)	–	B_5 or liquid MS + BA (0.1–0.2) + IAA (0.1–0.5)	–	Zhuang and Liang (1985a)

Figures in parenthesis denote concentration (mg/l) *DTT* Dithiothreitol, *PVP* Polyvinylpyrrolidone, *Vit* Vitamin, *YE* Yeast extract

et al. (1991) reported somatic embryogenesis from the in vitro roots of *C. japonica* clones cv. "Alba Plena."

2.3.3.2 Physiological Stage

Successful induction of somatic embryos from cotyledon explants depends upon the physiological maturity of the cotyledons. In *C. japonica,* Vieitez and Barciela (1990) collected seeds in July, September, and October to determine the right stage for induction of somatic embryogenesis. They achieved 94% embryogenesis in seeds collected in September in contrast to 20% of those collected in October under the climatic condition prevailing at Spain. The seeds collected in September were fully grown but were still immature suggesting a transitory dormancy stage.

2.3.3.3 Genotypic Variation

Genotype plays a major role in the induction of embryogenesis. Nakamura (1988a) screened four *Camellia* species including Japanese tea cultivars among which the embryogenic response varied between 0 and 50%. Of all the cultivars screened, the best response was reported in "Yabukita" and "Kurasawa" tea cultivars. Among the other *Camellia* species, high differentiation rates of somatic embryos were obtained with *C. japonica* (48–58%), *C. sasanqua* (9–81%), and *C. brevistela* (93%).

2.3.3.4 Basal Medium and Growth Regulators

The type, concentration, and time of application of different growth regulators in culture media have been extensively worked out. In general, a high cytokinin-to-low auxin or low cytokinin alone was found to be necessary for induction of somatic embryos in *Camellia*, but cytokinins have been often reduced or omitted in subsequent subculturing. In general, direct somatic embryos in *Camellia* can be obtained on a wide range of culture conditions: full to 1/3 strength modified MS, 15–30 g/l sucrose, D-glucose or combinations of both, 0–10 mg/l auxin and 0–10 mg/l cytokinin, in liquid, semi-solid, or solid medium. Light was an important parameter for somatic embryo formation, especially from stem and leaf explants (direct and indirect). Somatic embryos either did not form in the dark or their number was significantly lower than that for cultures under photoperiod. Successful conversion of cotyledon-derived embryos into plants ranged from 35 to 79%, depending on the culture medium used (Pedroso-Ubach 1994). Identical results were obtained for leaves, cultured on modified MS medium supplemented with 20 g/l sucrose or 25 g/l D-glucose, 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.1 mg/l kinetin (Pedroso-Ubach 1991). Leaves (31%) cultured in MS liquid medium with 1.0 mg/l BAP and 0.5 mg/l 2,4-D produced clusters of 3–23 somatic embryos/leaf. Less frequently (2–3%), globular embryos detached from the remaining differentiated leaf tissues and developed singularly in the liquid culture. Only the embryos arising in clusters developed into plantlets.

Among the PGRs, cytokinin such as BAP (0–10 mg/l) has been widely used for *Camellia* (Table 2.2), though Vieitez et al. (1991) claimed that zeatin (1 mg/l) in combination with BAP and IBA was essential for the induction of somatic embryos in *C. japonica* cv. Alba Plena. In auxins, IBA (0–2 mg/l) was widely used in the induction medium for *Camellia,* though NAA has also been used in different concentrations in different species such as 0–2 mg/l in *C. reticulata* (Zhuang and Liang 1985a), 0.2–0.5 mg/l in *C. sasanqua* (Yamaguchi et al. 1987), 0.5–1.0 mg/l in *C. japonica* × *C. chrysantha*, and 1 mg/l in *C. vietnamensis* × *C. chrysantha* (Nadamitsu et al. 1986).

The other species on which embryogenesis was carried out are *C. brevistela* (Nakamura 1988), *C. chrysantha* (Zhuang and Liang 1985b), *C. oleifera* (Yan et al. 1984), and *C. sasanqua* (Nakamura 1988; Zhuang et al. 1988), and with new hybrids whose development is hindered by poor fertility or embryo viability (Nadamitsu et al. 1986; Yamaguchi et al. 1987). In all these reports, somatic embryogenesis was achieved using MS medium containing a cytokinin (usually BAP) with or without auxin (usually NAA). However, none of these studies involved comprehensive experiments to determine optimum conditions for embryogenesis.

2.3.3.5 Secondary Embryogenesis

There are two kinds of growth patterns for secondary embryogenesis in *Camellia* (1) somatic embryo-to-somatic embryo, commonly known as repetitive

embryogenesis and (2) callus-to-somatic embryo in which multiplication of somatic embryos depends upon subculturing of callus (Vieitez 1994), although the former pathway is more frequent in *Camellia* (Plata and Vieitez 1990; Vieitez and Barciela 1990). The works on secondary embryogenesis in different species of *Camellia* are discussed here.

C. japonica

Secondary embryogenesis has been reported primarily from embryos derived from a wide range of initial explants, i.e., from cotyledons or from excised embryos (Kato 1986a; Vieitez and Barciela 1990), roots (Vieitez et al. 1991), or from in vitro leaves (San-Jose and Vieitez 1993). Primary embryo upon transfer to MS medium with or without growth regulators gave rise to secondary embryogenesis within 3–4 weeks. In general, growth regulators used for *C. japonica* were higher concentration of BAP along with lower concentration of IBA.

C. reticulata

High frequency (65%) secondary embryogenesis was reported in *C. reticulata* on cotyledonary and hypocotyl region of isolated primary embryos by Plata and Vieitez (1990). This response was evinced on a medium containing BAP (0.5 mg/l) and IAA (0.5 mg/l). According to these workers, although a combination of BAP (2 mg/l) and IBA (1 mg/l) provided more embryos per explant, this gave the lowest responsive explant for secondary embryos.

Plata et al. (1991) studied the anatomical sequence of events, which led to the differentiation of secondary embryogenesis in *C. reticulata* cv. Mouchang. They found that embryogenesis occurred mainly on the hypocotyl region of primary embryos. Histological monitoring revealed that secondary embryos apparently had a multicellular origin from embryogenic areas originating in both epidermal and subepidermal layers of hypocotyl region. This morphogenic competence was related to the presence of relatively undifferentiated cells in superficial layers of the hypocotyl of the primary embryo.

2.3.3.6 Developmental Biology of the Somatic Embryo

The origin of somatic embryo originates from either single cell or group of cells, which depends upon the plant. The histological and anatomical aspects of somatic embryogenesis in *Camellia* have been mentioned by Kato (1986), Plata and Vieitez (1990), and Vieitez et al. (1991) without giving details on the cellular events and anatomical changes that occurred during embryogeneis. Barciela and Vieitz (1993) made a detailed study on the origin and anatomical development of somatic embryos differentiated on *C. japonica* cotyledon sections. This study used computer-aided image analysis for cytological quantification, and the measurements of cell starch and protein contents as the stained cell areas by staining with periodic acid Schiff (PAS) stain and by mercuric bromophenol blue, respectively. Barciela and Vieitz (1993) observed that small protuberances or nodules began to appear on the abaxial epidermis of the cotyledons from 7-day-old in vitro tissue. The nodules continued to develop and by 30th day they were 4–6 mm in diameter and became moderately prominent, and bore embryos in several different stages of development. After 2 months of culture, embryos were 6–8 mm long and could be isolated either for germination or secondary embryogeneis. Only the abaxial surface of the cotyledon explants was morphologically competent and had multicellular origin. To determine whether the embryogenic nodules could be maintained indefinitely in culture, they were isolated from the initial cotyledons, removed from in vitro produced somatic embryos, and then cultured for 6 months with monthly transfer to a fresh medium. The parenchymatic tissue of the nodule failed to proliferate or grow and turned progressly necrotic.

The above histological analysis suggests that the nodules associated with the occurrence of somatic embryogenesis can be considered as small localized callus tissue, which is necessary for the redetermination of embryogenic cells. Histological observations of embryogenesis in *C. reticulate* (Plata and Vieitez 1990) suggested that somatic embryos develop directly from cotyledon without any apparent callus phase. However, the differentiation of the embryos was nevertheless related to the developing swollen parts

(Kato 1986), swollen whitish areas, or compact bulging tissue of cotyledon explant (Plata and Vieitez 1990). Such swellings might be equivalent to the nodules observed in *C. japonica* (Vieitez and Barciela 1990; Barciela and Vieitz 1993). Though morphologically visible, the nodules that develop on *C. japonica* cotyledons cannot be ascribed as true callus.

2.3.3.7 Morphology of Somatic Embryo

The morphology of the *Camellia* somatic embryos is influenced by the concentration of cytokinin in the medium. In case of *C. japonica* (Vieitez and Barciela 1990; Vieitez et al. 1991), most embryos could be classified into the following two clearly distinct types (1) seed like embryos, which were yellowish-white with large cotyledons alike to mature zygotic *Camellia* embryos; and (2) bud like embryos, which were green with cotyledons resembling true leaves. They generally developed in media with relatively high BAP concentration. Ammirato (1985) stated that reasonably high levels of cytokinins partially or totally inhibit the development of somatic embryo cotyledons and the shoot apex grows out to form the first mature leaves so that the somatic embryo looks more like a shoot. The observed bud-like embryos may be an example of such cytokinin-induced premature shoot emergence. Anomalies such as polycotyledonary cotyledon, hypertrophy, or fascination were also observed to various extents among both seed-like and bud-like embryos, but both kinds were genuinely bipolar having both shoot and root meristems.

2.3.4 Cold Storage and Cryopreservation

The potential of using in vitro systems for germplasm collection and conservation as well as for multiplication has been broadly discussed in several reviews and feature articles (Kartha 1985; Engelmann 1997). The application of in vitro techniques to germplasm storage is of particular interest for the conservation of plants such as *Camellia* species that are normally propagated vegetatively and/or have recalcitrant seeds.

The storage of *Camellia* seeds in genebanks is problematic because *Camellia* seeds are classified as recalcitrant (Pence 1995). They are sensitive to low temperatures and desiccation and are unable to retain their viability through long-term storage (Kato 1989). Even when they are maintained under moist conditions at 3–5°C, their viability is relatively short-lived (Salinero and Silva-Pando 1986). The most common method for preserving the genetic resources of species with recalcitrant seeds or those vegetatively propagated, is as plants in field genebanks. Limited work has been carried out on cold storage and cryopreservation of *Camellia* with both material obtained ex vitro (seeds) and material cultured in vitro (somatic embryos, embryonic axes, and shoot apices) and are presented in Table 2.3.

Table 2.3 Summary of cold storage and cryopreservation studies in wild *Camellia*

Species	Explant	Storage method	Response	Reference
C. japonica	Somatic embryo clusters	Short- to medium-term storage at 2–4°C	Reduced embryogenic competence after 6 months, improved germination capacity after 2 months	Janeiro et al. (1995)
	Encapsulated somatic embryos	Short- to medium-term storage at 2–4°C	Reduced embryogenic competence after 2 months, 30–40% plant recovery after 2 months	
	Somatic embryos and encapsulated somatic embryos	Cryopreservation	No survival of frozen material	Janeiro et al. (1995)
	Embryonic axes from mature seeds	Cryopreservation after 2–3 h desiccation	100% survival and 40% plant recovery, 18% somatic embryogenesis rate	Janeiro (1996)
C. reticulata	Somatic embryo clusters	Cold storage at 2–4°C	76% germination after 2 months	Chaudhury et al. (1991)

2.3.4.1 Short-Term Storage

The attempts of preserving the wild *Camellia* explants by short-term storage has been made with three different explants such as somatic embryos, shoot tips, and their encapsulated forms, which are elaborated here. The effects of short- to medium-term cold storage on the maintenance of embryogenic capacity and germination of somatic embryos of *Camellia* were investigated by Janeiro et al. (1995). Four embryogenic lines were used: three belonging to *C. japonica* (1, 2 and SY-89) and one to *C. reticulata* cv. Mouchang. Lines 1 and 2 of *C. japonica*, which were used to study survival and the preservation of embryogenic capacity of somatic embryos induced directly on the roots of in vitro grown plantlets (Vieitez et al. 1991). The effect of cold storage on the germination of *Camellia* somatic embryos into whole plants was studied in greater detail (Janeiro et al. 1995). Cold treatment for 8 weeks significantly improves the secondary embryogenesis, but it depends on genotypes. The shoot and root length of the germinated plantlets were also significantly increased by 2 months cold treatment, but it depends on genotypes. However, the incidence of secondary embryogenesis during germination also decreased after cold treatment.

Somatic Embryos

The potential uses for artificial seeds are numerous including storage, handling, and delivery of elite germplasm. The possibility of using cold storage to preserve synthetic *Camellia* seeds was investigated by Janeiro et al. (1996). In that study, the effects of cold storage of *C. japonica* somatic embryos on the maintenance of embryogenic competence and germination of encapsulated embryos were determined. Somatic embryos were encased in sodium alginate (3%) beads made in MS basal medium with 3% sucrose. The beads were then stored for 1–2 months in darkness at 2–4°C. After 1 month, the encapsulated embryos exhibited a significant reduction in both survival rate and competence for secondary embryogenesis, however, additional 1 month cold storage had further little reduction effect. The survival and secondary embryogenesis rates, 68% and 69% respectively, when placed in the maintenance medium following 60 days storage at 4°C were still acceptable. However, the productivity (number of secondary embryos per responsive encapsulated embryo) was dramatically reduced from 62.6 for unstored encapsulated embryos (control) to 5.4 secondary embryos indicating the negative influence of cold.

The reduced competence for secondary embryogenesis of cold-stored encapsulated embryos of *Camellia* appears to reflect increased maturity, since their capacity for germination is better preserved than their embryogenic competence. In this respect, short- or medium-term cold storage of synthetic *Camellia* seeds destined for germination appears to be feasible as long as a 30–50% fall in plant recovery rate could be tolerable. In contrast, cold storage alone cannot be used to maintain embryogenic competence, since the productivity of cold-stored encapsulated embryos is seriously reduced.

Shoot Tips

Ballester et al. (1997) reported almost 100% survival frequencies in seven of the eight clonal shoot cultures of *C. japonica* tested, when stored at 2–4°C for up to 12 months. Shoot tips of *C. japonica* encapsulated in alginate beads and stored at 2–4°C survived for a shorter period of time than uncapsulated ones. Encapsulated material had survival rates of 75, 50, and 10% on 30, 60 and 75 days, respectively.

2.3.4.2 Long-Term Storage and Cryopreservation

Since the embryogenic competence of *Camellia* somatic embryos is clearly not preserved during short-term cold storage under the conditions used in the studies described in previous sections, the feasibility of using cryopreservation techniques was investigated (Janeiro 1996; Janeiro et al. 1996).

In these studies, somatic embryos of *C. japonica* (2–5 mm size) were subjected to several protective pretreatments to prevent the formation of ice crystals inside the cells. Following pretreatment, half of the somatic embryos in each experiment (controls) were placed directly in maintenance medium (MS medium supplemented with 4.40 μM BA and 0.49 μM IBA) and the other half was placed in sterile 2 ml polypropylene cryovials and immersed in liquid nitrogen for

24 h. Somatic embryos were thawed (by immersing the cryovials in water for 1–2 min at 35–38°C) and transferred to the maintenance medium. Both treated and untreated embryos were cultured in a growth chamber under the standard conditions specified above. After 10 weeks, they found that no frozen somatic embryos survived regardless of the desiccation period; however, survival rate of unfrozen embryos was 100% after 15 min of desiccation treatment, which was further reduced to 53% after 2 h of desiccation indicating an acceptable tolerance of these somatic embryos to dehydration (Janeiro 1996).

Janeiro et al. (1996) also investigated the feasibility of cryopreservation of *C. japonica* embryonic axes. The explants were isolated from mature seeds. After sterilization of seeds, the embryonic axes were excised from the cotyledons with 1–2 mm of petiole to protect the plumule, and were either used as such or dehydrated for 1.5 or 3 h in sterile laminar air flow. Half the material was placed directly in MS maintenance medium (controls), and the other half was placed in cryovials and frozen in liquid nitrogen for 24 h before transfer to the same MS medium. They found that the capacity of *Camellia* embryogenic axes to produce somatic embryos, especially on the hypocotyl region, is maintained and even enhanced after the stress produced by cryoexposure.

2.3.5 Haploid Culture

Microspore culture presents a number of potential advantages mainly in relation to in vitro selection strategies and to genetic studies for developing doubled-haploid mapping population, etc. In *C. japonica*, embryogenesis was induced from microspore. Among the various media composition and PGR formulation, MS along with 2,4-D (4.5 μM) and kinetin (0.5 μM) were reported to be the best. The development of microspore derived proembryos was obtained in MS medium supplemented with 2.2 μM BAP and reached the highest level when the microspores were cultured in this medium. However, the development of microspore-derived embryos ceased at maturation stage (Pedroso and Pais 1994) and no further work on this area has been reported so far.

2.3.6 Embryo Rescue

Camellia breeders from all over the world have desired to develop yellow flowered *Camellia*. The discovery of *C. chrysantha* has generated great excitement among the *Camellia* growers and breeders as a potential source for a new range of *Camellia* floral colors. Although numerous interspecific hybridizations have been attempted, crossing of *C. chrysantha* with some other species is very difficult. In this regard, several cultivars of *C. japonica* that contributes to about 70% of the current horticultural needs and *C. chrysantha* with its potential for new color were thought to be especially important. However, probably due to the phylogenetic distance between the two species, the interspecific hybridization is extremely difficult (Yoshikawa and Yoshikawa 1990). Hwang et al. (1992), therefore, did a systematic investigation to understand the nature of reproductive barrier between *C. japonica* and *C. chrysantha* with intra- and interspecific crosses using two different lines of each species. They found that zygote formation and early embryo development were similar in intra- and interspecific crosses. Full size but empty ovules in mature capsules resulted from embryo abortion. Liang et al. (1986) reported that interspecific hybrid embryos of *C. pitardii* var. *yunnanensis* × *C. chrysantha* developed normally, reached torpedo stage, and differentiated normally. However, a complete successful protocol of embryo rescue will be immensely helpful to develop the long-waited yellow-colored *Camellia* using *C. chrysantha* as a source.

2.4 Employment of Markers

2.4.1 Morphological Markers

The progress of *Camellia* breeding has been slowed down due to the lack of reliable selection criteria (Kulasegaram 1980). Though a number of morphological and biochemical markers have been reviewed in the past mainly involving *Camellia sinensis* (Ghosh-Hazra 2001), yet they have only marginally improved the efficacy of selection for desired agronomic traits. Morphological marker such as pollen morphology of

eight species of *Camellia* was examined using light microscope and scanning electron microscope. Results showed that the pollen size in genus *Camellia* was moderate or big mostly showing prolate from the equatorial view and trioblate-circular from the polar view. The aperture was 3-colporate with fine and long colpi and multishaped. The muri and lumina varied in size and shape among the species. The pollen morphology of the genus was relatively identical indicating that the genus *Camellia* was a natural group (Chen et al. 1997). The phylogenic classifications under section *Thea* in genus *Camellia* were briefly reviewed based on flower morphology, tree habit, etc. The geographical distribution and the evolutionary tendency of the species and varieties were also described by Chen et al. (2001).

However, most of the morphological markers are influenced greatly by the environmental factors and hence show a continuous variation with a high degree of plasticity. Therefore, these markers cannot be used to discrete groups for taxonomic identification of tea (Wickremaratne 1981).

2.4.2 Biochemical Markers

Biochemical markers were widely used for characterization of different plant germplasm (Das et al. 2002). Presence of calcium oxalate crystals and their quantity in parenchymatous tissue of leaf petioles, nomenclatured as phloem index, have been suggested to be a suitable criterion for classifying tea hybrids (Wight 1958).

Paper chromatography was also used to investigate the taxonomy of different species of *Camellia* under the section *Thea*. It has been found that species within the *Thea* section of the genus *Camellia* are closely similar in chemical composition, the general pattern of which bears no obvious relationship to the chemical composition of non-*Thea* Camellias (Roberts et al. 1958).

Though *Camellia saluensis* is found to cross readily with *C. japonica,* identification of their F_1 hybrid known as *C.* ×*williamsii* was a challenge for breeders at the young stage. Parks and Case (1968) on the basis of flavor and other coloring compounds using paper chromatography were successful to identify the true hybrid seedlings.

2.4.3 Isozyme Markers

Genetic analysis of isozyme variation was used for cultivar identification in a wide range of plants (Hirai and Kozaki 1986). Similarly, in *Camellia*, isozymes have also been analyzed for studying the genetic tendencies, cultivar identification, and implication in hybrid breeding, which are discussed below.

Wendel and Parks (1982) analyzed 17 isozymes of different cultivars of *C. japonica*. They found that 15 isozymes produced two to nine polymorphic loci while two produced one to three monomorphic bands. Based on the segregation of 12 loci by eight enzymes, they postulated codominant inheritance of single-gene traits. They also suggested that two pairs of genes are linked, that is, aspartate amino-transferase with phosphoglucomutase and 6-phosphogluconate dehydrogenase with phosphoglucomutase. In a further study, the same authors (Wendel and Parks 1983) reported isozyme variations at 15 loci from 12 enzymes with 205 genotypes of *C. japonica*. All loci were polymorphic and a total of 64 alleles were detected. Peroxidase and 6-phophogluconate dehydrogenase (6-PGDN) isozyme were also used to differentiate between varieties of *C. sinensis* and *C. japonica* (Ikeda et al. 1991). They concluded that alcohol dehydrogenase isozymes in *C. japonica* are encoded by two genes *adh*-1 and *adh*-2. Both loci are expressed in seeds and their products are randomly associated with intra- and intergenic dimers. Electrophoresis of leaf extracts produced only the products of *adh*-2. Formal genetic analysis indicated that the two *adh* loci are tightly linked. Most segregations fit the expected Mendelian ratios but in some individuals distorted segregation was also observed (Wendel and Parks 1984). Starch gel electrophoresis was used to score allelic variation at 20 loci in seeds of *C. japonica* collected from 60 populations distributed throughout Japan. In comparison with other plant species, the genetic diversity within the population is very high, that is, 66.2% of loci were polymorphic per population, which gave an average number of 2.16 alleles per locus. They also reported genotypic proportions at most of the loci in majority of all the populations and found a good fit of the Hardy–Weinberg expectations (Wendel and Parks 1985).

2.4.4 Molecular Marker

Due to widespread cultivation of clonal tea by elite planting material, the genetic diversity is diminishing gradually. Therefore, germplasm characterization at molecular level of *Camellia* will help (1) varietal improvement of *Camellia* for agronomically important characters; (2) to preserve the intellectual property right of *Camellia* breeders; (3) identification of individual *Camellia* hybrid cultivar by making a molecular passport; (4) prevention of duplicate entry of different genotypes in *Camellia* gene pool; (5) efficient selection of the varieties for hybridization program, graft compatibility in composite plant production, and so on; and (6) taxonomic classification of *Camellia* genotypes on the basis of molecular markers which is still fragile. The various molecular markers are discussed below.

2.4.4.1 RAPD Markers

Since the discovery of random amplified polymorphic DNA (RAPD) marker (Williams et al. 1990), it is being used for a number of areas in plant taxonomy. In *Camellia*, a considerable amount of work has been carried out.

The genetic diversity and molecular phylogeny of 24 ornamental *Camellia* species and varieties were investigated by RAPD analysis. Fifteen decamer oligonucleotide primers were selected from the 61 screened, which generated a total of 95.3% polymorphism of the amplified bands. The molecular phylogenetic dendrogram of 24 species was constructed using UPGMA that generated two groups, corresponding to 3- and 5-locular ovary in morphology. The genetic relationship and the molecular phylogeny among section *Thea* were discussed by Chen and Yamaguchi (2002).

Maternal inheritance of chloroplast DNA (cpDNA) in some cross progenies between *C. vernalis* and *C. japonica* was investigated using the polymorphism of *atp*H–*atp*I region by RAPDs. The cpDNAs of all *C. vernalis* cultivars showed the same type as those of *C. sasanqua*, and all the progenies from *C. vernalis*, either open-pollinated or crossed, had the same cpDNA type as their maternal plants (Tateishi et al. 2007).

Internal transcribed spacer (ITS) of nrDNA has been widely employed for reconstructing phylogenetic relationships in plants, especially at the species level. In order to assess the efficacy of nrITS in elucidating the interspecific relationships of *Camellia*, Vijayan and Tsou (2008) conducted an experiment with seven closely or distantly related species. Extensive study of *Camellia,* based on *Pfu*-amplified ITS sequences, showed well-resolved interspecies relationships. Thus, the potential of nrITS in deducing the phylogenetic relationships in *Camellia* was demonstrated.

RAPD markers were used for identification of *C. japonica* and related species as well as their hybrids. A wide range of markers such as random 10-oligomer to chloroplast-specific sequences were used and checked with the previously published monogram on *Camellia* for phylogenetic relationship. Finally, the taxonomic classification as mentioned in the Chang's manual for different *Camellia* species was confirmed (Prince and Parks 1997, 2000; Thakor 1997; Tiao and Parks 1997, 2003; Yoshikawa and Parks 2001; George and Adam 2006; Orel et al. 2007).

2.4.4.2 ISSR Markers

Intersimple sequence repeat (ISSR) has been used for genetic characterization of various plant species (Tsumura et al. 1996). Because of the greater length of ISSR primers, they show greater repeatability and stability of map position in the genome while comparing genotypes of closely related individuals (Zietkiewicz et al. 1994).

C. euphlebia, a rare and endangered species of China, is distributed in a small region in the Guangxi province. Wei et al. (2005) studied the level and pattern of the genetic diversity of 84 individuals from natural populations by using 100 ISSRs. Their results indicated a relatively low level of genetic diversity in *C. euphlebia* at the species level and at population level and a relative degree of differentiation among populations. Gene flow among populations was also found to be low. Inbreeding and limited gene flow might be the key factors resulting in the observed genetic structure of *C. euphlebia*. Strategies are proposed for the genetic conservation and management of this species.

C. nitidissima Chi (Theaceae), with its golden-yellow flowers, is a popular ornamental species. Due to deforestation and collection of seedlings, its natural populations have receded greatly in recent decades. Genetic diversity and genetic differentiation of 12 natural populations and one ex situ conserved population of *C. nitidissima* in China were analyzed using ISSR markers. Their study indicated a low level of genetic diversity at both species as well as population levels and a relatively high degree of differentiation among populations in naturally occurring populations. In contrast, the ex situ population contained higher genetic variability compared to each natural population. Thus, they suggest that all the wild *C. nitidissima* populations should be protected in situ (Wei et al. 2008).

2.4.4.3 Microsatellite Markers

Simple sequence repeats (SSRs), known also as microsatellites, are tandemly repeated DNA sequence motifs (usually 2–5 bp long) that are highly polymorphic in plant genomes (Wu and Tanksley 1993). Due to their hypervariability, relative ease of scoring by PCR, codominant nature, and high reproducibility, they are now considered to be one of the most reliable genetic markers.

Ueno et al. (1999) were pioneer to develop the SSRs from *C. japonica*, a closely related species of tea. Out of the total 339 RAPD amplifications, 21 were found to contain microsatellite repeats. Finally, four primer pairs were developed, which yielded single-locus polymorphic amplification products. Using these primer pairs, 53 *C. japonica* ecotypes were genotyped and population genetic parameters were calculated. The following year, Ueno et al. (2000) investigated the spatial genetic structure of *C. japonica* using four of these microsatellite primers. Spatial distribution of individuals was also assessed to obtain an insight into spatial relationships between individuals and alleles. Morisita's index of dispersion plotted 518 individuals of *C. japonica* in a single clump and Moran's *I* spatial autocorrelation coefficient revealed weak genetic structure, indicating a low level of allele clustering among the individuals.

Recently, an initial study of sequence tagged microsatellite site (STMS) variation was undertaken by Matteo et al. (2010) with 132 accessions of *Camellia* spp., which included 24 accessions representing 22 different species or varieties as well as 63 cultivars of *C. japonica*, 33 cultivars of *C. sasanqua*, 7 cultivars of *C.* × *vernalis*, 3 cultivars of *C.* × *hiemalis*, and 2 cultivars of *C. hybrida*. The four primer sets used (MSCJAF37, MSCJAH46, MSCJAF25, and MSCJAH38) successfully amplified polymorphic alleles in all the species analyzed, showing cross-transferability. Overall, 96 alleles were scored. MSCJAH38 primers produced the highest number of bands (30), while MSCJAH46 primers yielded the lowest number (15). The genetic distance between pairs of accessions was estimated on the basis of the Nei coefficient and a principal coordinate analysis was performed. The plot revealed a main differentiation between the *C. japonica* cultivars and the winter Camellias. The distribution of the genetic variation, attributed by AMOVA, particularly highlighted genetic overlap among *C. sasanqua* cultivars and the cultivars belonging to *C.* × *vernalis*, *C.* × *hiemalis*, and *C. hybrida*. The study demonstrated that STMS markers offer a suitable method for detection of genetic variability and molecular study of camellia genotypes.

The two major molecular phylogenetic investigations of the genus *Camellia* (one by Vijayan et al. 2009 with nrITS sequences and the other with nDNA *RPB2* sequences by Xiao and Parks 2003) have provided considerable insight into the interspecies relationships of *Camellia*, which could not be provided by many previous attempts with use of cpDNA sequences (Orel et al. 2003; Yang et al. 2006). These two molecular phylogenetic investigations share many important findings. Both studies revealed the need to revise the existing classifications, both supported the monophyly of sections *Thea* and *Furfuracea* and that the species *C. hongkongensis* should be shifted from section *Camellia* to section *Furfuracea*, and both revealed that sections *Eriandra* and *Theopsis* were closely related and not separable and that species of sections *Tuberculata* and *Chrysantha*, as well as *C. szechuanensis* from section *Pseudocamellia*, were closely related. Finally, results of both studies equally supported the section *Camellia* as polyphyletic. The species from the section *Camellia* formed groupings based on geographical origin and distribution, and species in this section distributed in the southeastern and eastern China, Korea, and Japan are well separated from those in southern and southwestern China.

Nevertheless, both studies disagreed on many points. The most notable disagreement was the monophyly of the section *Paracamellia* defined by Ming (2000) and Sealy (1958), which was supported by Xiao and Parks (2003), but our study showed a bifurcation of the section *Paracamellia* and supported Chang's (1981) creation of the section *Oleifera* from the section *Paracamellia*. A recent study of leaf anatomical characters also supported the separation of *Oleifera* from the section *Paracamellia* (Lin et al. 2008). Other important differences are, first, species of the section *Eriandra* and *Theopsis* formed a monophyletic clade in our tree, but mixed together with species from the section *Camellia* and divided into two well-separated clades in the study by Xiao and Parks (2003). Second, the positions of some species in small sections and isolates differed; for example, *C. amplexicaulis* of section *Longipedicellata* was isolated and was a sister to the clade of *Eriandra* and *Theopsis* in our trees, but was associated with clades of species in sections *Camellia*, *Oleifera*, and *Paracamellia* in the Xiao and Parks (2003) dendrogram trees. Also, *C. yunnanensis* of section *Stereocarpus* was embedded in the clade consisting of sections *Chrysantha* and *Tuberculata* in our trees, but was allied to section *Furfuracea* in the Xiao and Parks (2003) dendrogram trees. These types of conflicts in results are not uncommon in molecular phylogeny and can arise from both analytical and biological factors (Rokas et al. 2003a). Analytical factors that generally affect phylogenetic reconstruction are choice of optimality criterion (Huelsenbeck 1995), data availability (Cummings et al. 1995), taxon sampling (Graybeal 1998), and specific assumptions in the modeling of sequence evolution (Yang et al. 1994). The major biological factor that affects phylogenetic reconstruction is the evolutionary dynamics that may cause the history of the genes under analysis to obscure the history of the taxa (Rokas et al. 2003b).

2.5 Genomic Resources

Genomics and its global expression profile (proteomics) offer an additional advantage for rapid identification of genes and pathway to control important plant traits. Multigenic characters, such as abiotic stress, particularly drought and frost, etc. on being the major production constraints in *Camellia* cultivation could be studied in-depth by employing genomics.

Caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine) are two purine alkaloids that are present in high concentrations in some species of *Camellia*. However, most members of the genus *Camellia* contain no purine alkaloids. Tracer experiments using [8-^{14}C] adenine and [8-^{14}C] theobromine showed that the purine alkaloid pathway is not fully functional in leaves of purine alkaloid-free species. In five species of purine alkaloid-free *Camellia* plants, sufficient evidence was obtained to show the occurrence of genes that are homologous to caffeine synthase. Recombinant enzymes derived from purine alkaloid-free species showed only theobromine synthase activity. Unlike the caffeine synthase gene, these genes were expressed more strongly in mature tissue than in young tissue (Mariko et al. 2009).

Among the wild species, *C. oleifera* yields 55% oil from its kernel, which is considered to be one of the best plant oils, as it contains ~90% unsaturated fatty acids (~80% oleic acid, ~10% linoleic acid and linolenic acid). As the enzyme controlling the first-step desaturation during the biosynthesis of plant unsaturated fatty acids, stearoyl-ACP desaturase (SAD) directly regulates the proportion of saturated fatty acids and unsaturated fatty acids by dehydrogenating saturated fatty acids bonded to ACP (acyl carrier protein) to form oleic acids. Therefore, Zhang et al. (2008) cloned the full-length cDNA of *C. oleifera* SAD (CoSAD) gene, which will help in future for genetic improvement on other oil plants.

Partial cDNA sequences of three anthocyanin biosynthetic genes (*F3H*, *flavanone 3-hydroxylase*; *DFR*, *dihydroflavonol 4-reductase*; *ANS*, *anthocyanidin synthase*) were isolated from the petals of *C. japonica*. Their deduced partial amino acid sequences shared high homologies with those of woody plant species (CjF3Ha, 98.0%; CjF3Hb, 91.2%; CjDFR, 99.0% with *Camellia sinensis*; CjANS, 90.3% with *Rhododendron* × *pulchrum*). Some important amino acid residues for enzymatic activities were also conserved in the isolated clones, suggesting that the genes were the homologs of *C. japonica* (Tateishin et al. 2010).

C. reticulata is found as diploid, tetraploid, as well as hexaploid and hence expected to have a complex genome. Physical maps of the 18S–26S rDNA ribosomal RNA genes (rDNA) were generated by fluorescent in situ hybridization (FISH) for *Camellia*

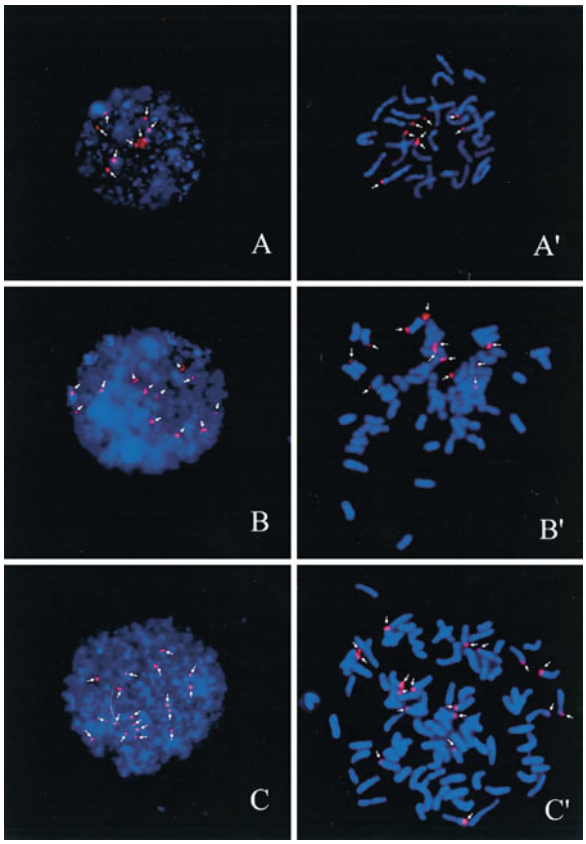

Fig. 2.1 Fluorescent in situ hybridization of *Camellia* interphase nuclei and metaphase chromosomes with the 18S–26S rDNA probe (*red color*) and blue fluorescence shows DNA counterstained with DAPI. The *white arrows* point the site of signals. (A, A′) The hybridization signals of diploid *C. reticulata*. (B, B′) The hybridization signals of tetraploid *C. reticulata*. (C, C′) The hybridization signals of hexaploid *C. reticulata* (Source: Gu and Xiao 2003)

reticulata polyploid complex, including three types of ploidy of *C. reticulata* and its related species, *C. japonica*, *C. yunnanensis*, *C. pitardii*, and *C. saluenensis*. An advanced method was used for preparing chromosome spreads (Fig. 2.1). Eight, twelve and eighteen rDNA sites were observed on the genomes of diploid, tetraploid, and hexaploid *C. reticulata,* respectively. Eight, four, five, and four rDNA loci were located on the chromosomes of *C. pitardii*, *C. japonica*, *C. saluenensis*, and *C. yunnanensis,* respectively. The number and position of rDNA sites in these species were compared for analysis. The results support some of the earlier phylogenetic speculation about this complex genome and suggest the occurrence of some structural rearrangements in chromosome (Gu and Xiao 2003).

2.6 Future Thrust

So far, genetic improvement of *Camellia* has mainly been achieved by conventional breeding. However, in the past three decades, serious attempts have been made to intervene in some of the conventional breeding approaches employing biotechnology. It is noteworthy to mention that since the work of Bennett (1977), the pioneer of *Camellia* micropropagation, the technique has been worked out well for various applications, albeit not commercially exploited. This is perhaps due to the fact that vegetative propagation techniques are well established and cost effective. On the other hand, many aspects of somatic embryogenesis have been studied in detail for ornamental *Camellia* including the regeneration pathway. Despite the fact that transgenic technology has tremendous scope for *Camellia*, surprisingly no transgenic plants have been developed so far. However, it is evident now among the different techniques of gene transfer, *Agrobacterium tumefacians*-mediated transformation has been attempted by different groups in tea, which therefore will be suitable for *Camellia* also. So far, DNA markers are concerned, several DNA markers have been used to make fingerprints, which need to be documented systematically and should be made available for public use to preserve the intellectual property rights of *Camellia* breeders. Although in several ways molecular biology of *Camellia* can be directed, yet priority should be given to the followings:

- Undertake a massive germplasm characterization effort across the world through a common "*Camellia* germplasm characterization consortium," which already exists for several similar crops
- DNA markers need to be identified to do early selection at nursery stage for various biotic (such as flower blight) and abiotic stresses (such as cold hardiness), which will revolutionize *Camellia* breeding where works suffer due to the lack of selection criteria and long gestation periods
- To develop the molecular markers for hybrid identification
- To generate and characterize the expressed sequence tags of *Camellia*

Lastly, the researches on transgenic *Camellia* need to be carried out to address some of the problems of the *Camellia* nursery growers by exploiting the highly regenerative system, which is fortunately available for *Camellia*.

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Chapter 3
Coffea and *Psilanthus*

F. Anthony, B. Bertrand, H. Etienne, and P. Lashermes

3.1 Introduction

Coffee is one of the most popular beverages in the world and represents a valuable agricultural export commodity, being the second leading export product from developing countries after oil. About 125 million people depend on coffee for their livelihoods in Latin America, Africa, and Asia (more details at http://www.ico.org). Up to 134 coffee species are now recognized (Davis et al. 2006; Davis and Rakotonasolo 2008), but commercial production relies mainly on two species, *Coffea arabica* L., which grows in highlands and *C. canephora* Pierre ex A.Froehn., which grows in lowlands. The cup quality (low caffeine content and fine aroma) of *C. arabica* makes it by far the most important species, representing 61.5% of world production in 2008. All coffee species are perennial woody bushes or trees that differ greatly in morphology, size, and ecological adaptation (Fig. 3.1).

Coffee cultivation is characterized by recentness, probably 1,500 years for *C. arabica* in Ethiopia (Wellman 1961) but only about 100 years for *C. canephora* in Indonesia (Charrier and Berthaud 1988). Consequently, genetic differentiation between wild and cultivated plants within the two cultivated species is rather low (Anthony et al. 2001; Dussert et al. 2003). During dissemination of *C. arabica*, successive reductions in genetic diversity occurred that reduced polymorphism of cultivated plants (Anthony et al. 2002). As a consequence, *C. arabica* cultivars present homogeneous agronomic behavior characterized by high yields and good cup quality, but high susceptibility to many pests and diseases, and low adaptability (Bertrand et al. 1999).

Breeding programs worldwide mainly rely on exploiting the genetic diversity of wild plants either within the target species (i.e., intraspecific strategy) or in other coffee species (i.e., interspecific or introgression strategies). Wild coffee species can be valorized in breeding programs since all coffee species share a common genome, which enables interspecific hybridizations and production of interspecific hybrids with variable levels of fertility (Charrier and Berthaud 1985). In the case of *C. arabica*, the intraspecific strategy is based on the use of wild plants from the center of diversity (i.e., Southwest Ethiopia), which is a valuable source of diversity (Anthony et al. 2001). Controlled crosses between wild Ethiopian progenitors and cultivars have produced high yielding F_1 hybrids, which can grow in both full-sun production systems and in agroforestry production systems (Bertrand et al. 2005a, 2006). However, the low diversity of *C. arabica* species (Lashermes et al. 1999) has considerably limited the success of breeding programs based on the intraspecific strategy. This explains why breeders have focused on the transfer of traits from other species, particularly for resistance to pests and diseases (Carvalho 1988; Van der Vossen 2001). To date, *C. canephora* is the main source of resistance traits not found in *C. arabica*, including coffee leaf rust (*Hemileia vastatrix*), coffee berry disease (*Colletotrichum kahawae*), and root-knot nematodes (*Meloidogyne* spp.) (Anthony et al. 1999). Other species of interest for the improvement of *C. arabica* are *C. liberica* Bull. ex. Hiern and *C. racemosa* Lour. for resistance to coffee leaf rust (Srinivasan and Narasimhaswamy 1975) and leaf miner (*Leucoptera*

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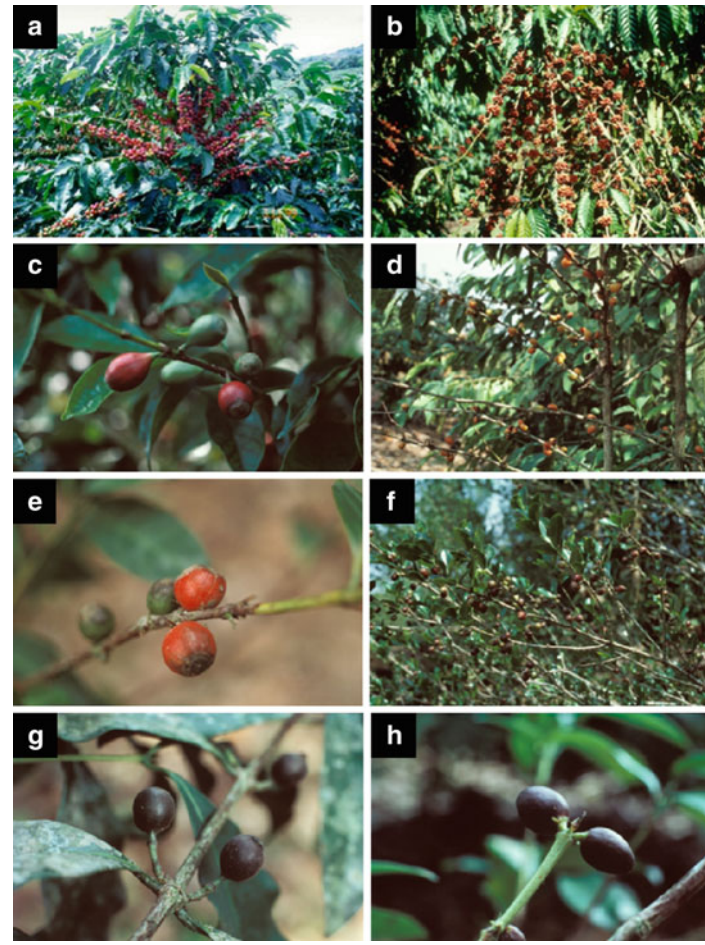

Fig. 3.1 Diversity of coffee fruits. (**a**) Cultivated *C. arabica*; (**b**) Cultivated *C. canephora*; (**c**) *C. heterocalyx*; (**d**) *C. congensis*; (**e**) *C. mayombensis*; (**f**) *C. racemosa*; (**g**) *C. pseudozanguebariae*; (**h**) *C. sessiliflora*

coffeella) (Guerreiro Filho et al. 1999), respectively. The development of molecular marker-assisted selection enables selection based on the presence of markers tightly linked to the genes of interest. Such a method of selection increases selection efficiency and can reduce the negative influence of undesirable genes, especially on liquor quality (Bertrand et al. 2003; Anthony and Lashermes 2005).

For the improvement of *C. canephora*, the intraspecific strategy uses wild plants from the center of diversity (i.e., West and Central Africa) that belong to six genetic groups (Dussert et al. 2003; Cubry 2008). A reciprocal recurrent selection program involving two of these groups produced intergroup hybrids with higher yield and vigor than intragroup hybrids (Leroy et al. 1993). The interspecific strategy has also been used to improve bean size and liquor quality of *C. canephora* cultivars by transferring genes from other coffee species.

In this chapter dedicated to wild coffee, we review information published on taxonomy, biology, conservation, and origin. We then describe the role of wild genotypes in genetic studies and crop improvement programs. We also describe the production of cytogenetic stocks and the development of genomic resources along with their applications.

3.2 Taxonomy and Biology

3.2.1 Taxonomy

The Coffeeae tribe belongs to the Ixoroideae monophyletic subfamily in the Rubiaceae family, and is close to the Gardenieae and Pavetteae tribes (Bremer and Jansen 1991; Davis et al. 2007). The Coffeeae

tribe consists of two genera, *Coffea* L. and *Psilanthus* Hook.f., which share the typical coffee bean morphology, i.e., a groove on the flat side of the seed, but differ in their floral morphology (Leroy 1980; Bridson 1987; Davis et al. 2005). Each genus has been divided into two subgenera, *Coffea* subgenus *Coffea* (103 species), *Coffea* subgenus *Baracoffea* (J.-F. Leroy) J.-F. Leroy (nine species), *Psilanthus* subgenus *Psilanthus* (two species), and *Psilanthus* subgenus *Afrocoffea* (Moens) (20 species) (Bridson 1987; Davis et al. 2005, 2006; Davis and Rakotonasolo 2008). Research has mainly focused on the *Coffea* subgenus *Coffea*, which comprises the majority of coffee species, including those of economic importance, i.e., *C. arabica* and *C. canephora*.

The number of coffee species has considerably increased since the creation of the genus *Coffea* L. in the eighteenth century, which included the only species known at that time, *C. arabica* (Fig. 3.2). Fifteen species had been described by the end of the nineteenth century, but 64 new species were added in the century that followed. The recent increase in the number of coffee species is due to intensive exploration of African and Madagascan forests (see Sect. 3.3.1; Collection of Genetic Resources), and to major revisions of herbarium samples from East Africa (Bridson and Verdcourt 1988), Central Africa (Stoffelen 1998), Madagascar (Davis and Rakotonasolo 2000, 2001a, b, 2003), and the Mascarenes (Dulloo et al. 1999). The inventory of coffee species is not yet complete as new species are still being described (Stoffelen et al. 2008, 2009). However, many characters used in coffee taxonomy are weak and variable, and many species have not been fully characterized, so it is hard to draw valid conclusions about their relationships (Bridson 1982).

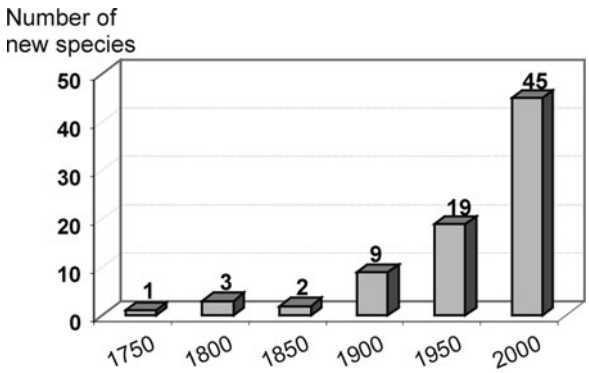

Fig. 3.2 Number of new *Coffea* species discovered per period of 50 years since 1750

3.2.2 Geographical Distribution and Diversity Hotspots

Both genera occur naturally in tropical Africa, *Coffea* also occurs in Madagascar, Grande Comore, and the Mascarenes, and *Psilanthus* in Southeast Asia, Oceania and northern Australia (Bridson 1987; Davis et al. 2006). The *Coffea* genus is represented by 41 species in Africa, 58 in Madagascar, one in Grande Comore and three in the Mascarenes, each area having 100% endemicity for its species (Davis et al. 2006; Davis and Rakotonasolo 2008). Except for *C. canephora* and *C. liberica*, from West and central Africa, and *C. eugenioides* S.Moore from East Africa, coffee species have a rather restricted distribution, sometimes only a few square kilometers. Three hotspots of species diversity have been identified in Madagascar (mainly in the evergreen humid forests of eastern Madagascar), Cameroon (14 species), and Tanzania (15 species, mainly in the eastern Arc Mountain) (Fig. 3.3) (Davis et al. 2006).

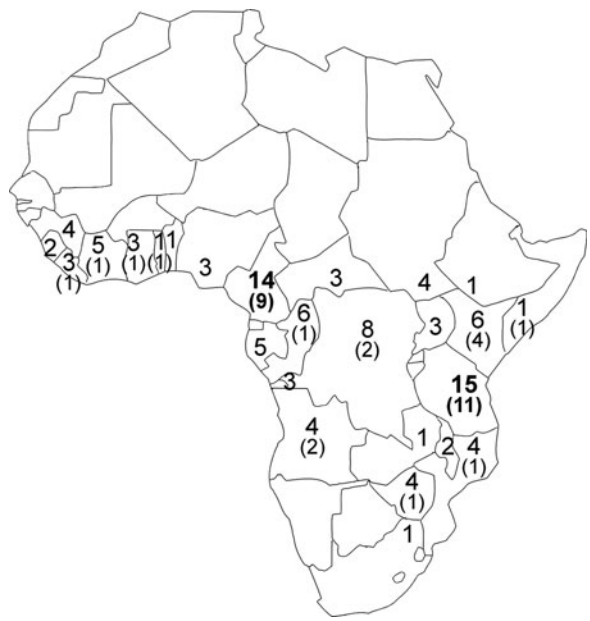

Fig. 3.3 Number of *Coffea* species recorded per country in Africa, according to Davis et al. (2006). The number of range-restricted species is in *brackets*

3.2.3 Habitat and Adaptation

Coffee trees have colonized various types of rainforest in Africa and Madagascar, but most species are found in humid, evergreen forest (Davis et al. 2006). The species widely distributed in Africa (i.e., *C. canephora*, *C. eugenioides*, *C. liberica*) are commonly found in humid, evergreen forest, gallery forest and seasonally dry, evergreen or mixed deciduous–evergreen forest (Table 3.1). In Africa, specific adaptations are observed in *C. congensis* in humid evergreen forest, either rheophytic (especially on sand banks) or in seasonally/temporarily flooded riparian forest, *C. fadenii* Bridson in cloud forest, *C. racemosa* in littoral forest on stabilized sand dunes (Davis et al. 2006), and *C. pseudozanguebariae* in littoral forest on a coral reef substrate (Anthony et al. 1987).

On the African mainland, mountainous areas have played a key role in flora and fauna diversification because of rainfall stability and habitat heterogeneity (Lovett and Friis 1996; Moritz et al. 2000; Linder 2001). Coffee species are found from sea level up to 2,300 m above sea level (*C. mufindiensis* Hutch. ex Bridson), but most species (67%) are adapted to a restricted range of altitude below 1,000 m (Fig. 3.4). Six species present a wide elevational distribution, from lowland (<500 m) up to 1,500 m (*C. brevipes* Hiern, *C. canephora*), 1,800 m (*C. liberica*, *C. salvatrix* Swynn. & Phillipson), 2,000 m (*C. mongensis* Bridson), and 2,200 m (*C. eugenioides*) (Davis et al. 2006).

3.2.4 Basic Genetics and Genomics

The basic chromosome number of coffee species is $n = 11$ chromosomes, which is typical for most genera of the family Rubiaceae. Somatic chromosomes are relatively small (1.5–3 μm) and morphologically similar to each other (Krug and Mendes 1940; Bouharmont 1959). All coffee species are diploid ($2n = 2x = 22$) and generally self-incompatible, except for *C. arabica*, which is tetraploid ($2n = 4x = 44$) and self-compatible (Charrier and Berthaud 1985). Self-compatibility has also been identified in two diploid species, *C. heterocalyx* Stoff. (Coulibaly et al. 2002) and *C. anthonyi* Stoff. & F. Anthony (Stoffelen et al. 2009). The genome of coffee species presents strong affinities, making it possible to produce crosses and hybrids at interspecific level (Charrier and Berthaud 1985) and also at intergeneric level (Couturon et al. 1998), which suggests low divergence between the two coffee genera. Moreover, the results of interspecific and intergeneric hybridizations indicate that divergence between coffee species is low, confirming the phylogenetic studies.

Table 3.1 Forest types colonized in Africa by *Coffea* species, according to Davis et al. (2006)

Forest type	*C. canephora*	*C. liberica*	*C. eugenioides*	Other species
Humid, evergreen forest	Commonly	Commonly	Commonly	*C. affinis*, *C. anthonyi*, *C. arabica*, *C. bakossii*, *C. brevipes*, *C. bridsoniae*, *C. carrissoi*, *C. charrieriana*, *C. congensis*, *C. dactylifera*, *C. fadenii*, *C. fotsoana*, *C. heterocalyx*, *C. humilis*, *C. kapakata*, *C. kihansiensis*, *C. kimbozensis*, *C. kivuensis*, *C. leonimontana*, *C. ligustroides*, *C. luluandoensis*, *C. magnistipula*, *C. mapiana*, *C. mayombensis*, *C. mongensis*, *C. montekupensis*, *C. mufindiensis*, *C. salvatrix*, *C. sessiliflora*, *C. stenophylla*, *C. togoensis*
Gallery forest	Sometimes	Sometimes	Sometimes	
Seasonally dry, evergreen forest	Sometimes		Sometimes	*C. pocsii*, *C. pseudozanguebariae*, *C. racemosa*, *C. schliebenii*
Seasonally dry, mixed deciduous–evergreen forest		Sometimes		*C. costatifructa*, *C. pseudozanguebariae*, *C. racemosa*, *C. salvatrix*, *C. schliebenii*
Seasonally dry, deciduous forest				*C. costatifructa*, *C. zanguebariae*
Savannah woodland and shrubland			Sometimes	*C. costatifructa*, *C. pseudozanguebariae*, *C. racemosa*, *C. rhamnifolia*, *C. schliebenii*

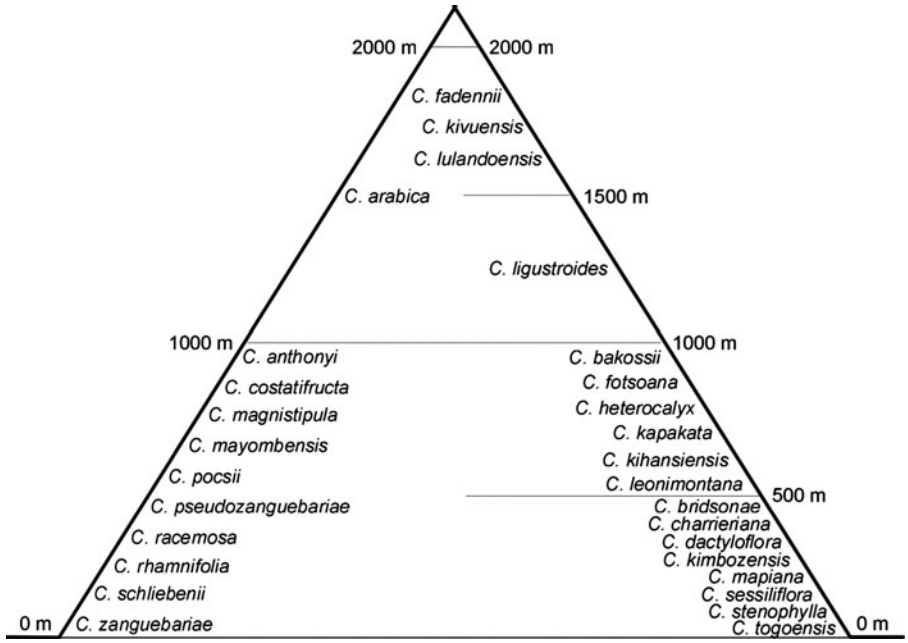

Fig. 3.4 Elevational distribution of 29 African coffee species, according to Davis et al. (2006). Species showing a wide elevational distribution (>1,000 m) are not included, i.e., *C. brevipes* (80–1,450 m), *C. canephora* (50–1,500 m), *C. eugenioides* (300–2,200 m), *C. liberica* (80–1,800 m), *C. mongensis* (400–2,000 m), *C. mufindiensis* (950–2,300 m), and *C. salvatrix* (400–1,850 m)

The origin of the cultivated species *C. arabica* was elucidated using complementary molecular approaches (Raina et al. 1998; Lashermes et al. 1999). *C. arabica* is an amphidiploid (i.e., C^aE^a genomes) resulting from hybridization between *C. eugenioides* (E genome) and *C. canephora* (C genome) or ecotypes related to these diploid species. Low divergence between the two constitutive genomes of *C. arabica* and those of its progenitor species suggests that speciation occurred very recently (Lashermes et al. 1999). Since cpDNA has a strict maternal inheritance in coffee (Lashermes et al. 1996b), a phylogeny based on plastid variations clearly indicated that *C. eugenioides* or a related species was the maternal progenitor species of *C. arabica* (Cros et al. 1998).

The nuclear DNA content of coffee species was estimated by flow cytometry. The DNA amount varied from 0.95 to 1.78 pg between diploid species (Cros et al. 1995; Noirot et al. 2003). The lowest values were found in East African species (e.g., *C. racemosa*), while the highest values were detected in West and central African species (e.g., *C. humilis* A.Chev.). The nuclear DNA content of coffee species appeared to be low in comparison to other plant species and similar to that of *Acacia heterophylla*, *Dioscorea alata*, and *Vigna unguiculata* (Cros et al. 1995). Based on flow cytometry data, the mean coffee haploid genome was estimated at around 700 Mb (Cros et al. 1995), which is rather low for angiosperms. Reduced fertility of certain interspecific F_1 hybrids appears to be associated with significant differences in the nuclear content of parental species (Barre et al. 1998).

3.3 Conservation of Genetic Resources

About 50% of known species have been collected and are now conserved in field genebanks (Dulloo et al. 2009). Of the 112 *Coffea* species recognized by Davis et al. (2006), 73 species (65.2%) are either critically endangered (14 species) or endangered (36 species) or vulnerable (23, including *C. arabica*).

3.3.1 Collection of Wild Coffee

Collection of wild coffee species received little attention until the second half of the twentieth century

when breeders became aware that deforestation was causing the erosion of coffee habitats, thereby threatening the conservation of wild coffee. In the 1960s, explorations in Madagascan forests were jointly initiated by the French Museum of Natural History (Paris) and the French research institutes CIRAD and IRD. Accessions of about 50 Madagascan species are now conserved in a unique field genebank at Kianjavato (Dulloo et al. 2009). Given the socio-economic importance of *C. arabica* cultivation, two large collections were then organized by FAO and IRD in Ethiopia, which is the primary diversity center of *C. arabica* species (Meyer et al. 1968; Guillaumet and Hallé 1978). The collected accessions were distributed worldwide and are now conserved in several field genebanks, mainly in Ethiopia, Kenya, Tanzania, Côte d'Ivoire, Costa Rica, and Brazil (Anthony et al. 2007a). In Africa, other survey missions were organized in Guinea (Le Pierrès et al. 1989), Côte d'Ivoire (Berthaud 1986), Cameroon (Anthony et al. 1985), Central African Republic (Berthaud and Guillaumet 1978), Republic of Congo (de Namur et al. 1987), Tanzania and Kenya (Anthony et al. 1987) (Fig. 3.5). About 4,700 *Coffea* accessions representing 15 African species were collected during these missions (Anthony et al. 2007a). The collected material was planted in field genebanks in Côte d'Ivoire and in each country visited. A core collection composed of 32 genetic groups (19 species) was conserved in vitro and planted in greenhouses in Montpellier, France (Dussert et al. 1997a). A duplicate of this collection was recently planted in a field genebank in Reunion Island (E. Couturon, personal communication). Finally, two large genebanks have been established in Madagascar and Côte d'Ivoire, and these conserve almost all the diploid genetic resources that

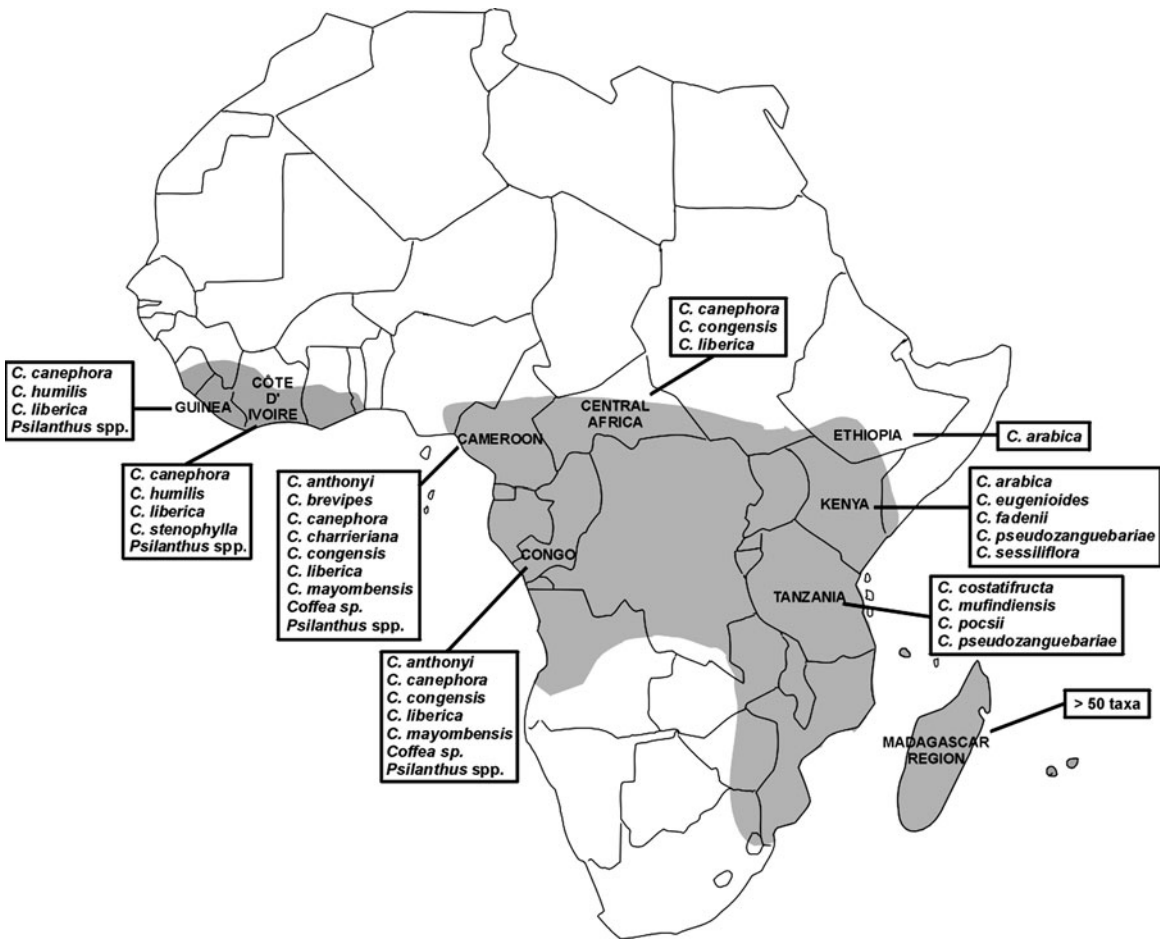

Fig. 3.5 Main collections of wild coffee in Africa and Madagascar (from Anthony et al. 2007a). The coffee distribution area is in *gray*

were collected. Some wild coffee specimens are also maintained in the large field genebanks created for cultivated species in Costa Rica, Colombia, Brazil, India, and Tanzania (Dulloo et al. 2009).

3.3.2 Modes of Conservation

Modes of conservation have been influenced by the non-orthodox nature of coffee seeds. Though coffee seeds can withstand desiccation down to 0.08–0.10 g H_2O g^{-1} dw water content (fresh weight basis), they cannot be considered orthodox because they remain sensitive to cold (Van der Vossen 1977; Becwar et al. 1983; Ellis et al. 1990). At 19°C and under 100% relative humidity, fully hydrated *C. arabica* seeds remained viable for 36 months and *C. canephora* and *C. stenophylla* G.Don seeds for 15 months (Couturon 1980). This explains why conservation of coffee genetic resources was organized firstly on farm, synchronously with coffee domestication in Ethiopia, then in field genebanks established in producer countries, following the worldwide extension of coffee cultivation. However plants conserved in the field are exposed to pests, diseases and other natural hazards such as drought, weather damage, human error, and vandalism. Field genebanks are costly to maintain and, as a consequence, are prone to economic decisions that may limit the level of replication of accessions, the quality of maintenance and even their very survival in times of economic stringency (Dulloo et al. 2009). In the large field genebank of Madagascar, 25% of the accessions and 50% of the genotypes are estimated to have been lost over a period of 20 years (Dulloo et al. 2001). In Costa Rica, genetic erosion in terms of lost trees is estimated at between 11.6 and 15.7% in different areas of the *C. arabica* genebank between 1993 and 2002 (Anthony et al. 2007b).

Alternative methods of ex situ conservation have been developed to overcome the limitations of field genebanks for medium- and long-term conservation. In vitro conservation of microcuttings was attempted under slow growth conditions (Kartha et al. 1981; Bertrand-Desbrunais et al. 1991, 1992), but adaptation to in vitro conditions could induce genotypic selection, causing genetic drift (Dussert et al. 1997a). Cryoconservation (i.e., storage at ultra-low temperature, −196°C in liquid nitrogen) have been performed using seeds, zygotic embryos, apices, and somatic embryos (Dussert et al. 1997b). A simple and efficient protocol for seed cryopreservation was defined for the cultivated species *C. arabica* (Dussert et al. 2007), then applied successfully to a subset of a core collection (Vasquez et al. 2005). Cryopreservation appears to be more cost-efficient and effective than field conservation (Dulloo et al. 2009), but its impact as an ex situ conservation method remains limited.

Projects for in situ conservation have been carried out in Mauritius Island and Ethiopia. In Mauritius, ten forest coffee conservation sites were selected, eight of them located in protected areas (Dulloo et al. 1998). However, protective measures have not really been applied so that conservation of wild coffee in its natural habitat is not guaranteed. In Ethiopia, three sites were identified in 1998 for forest coffee conservation, but the project produced very few concrete results mainly due to lack of efficient management and coordination between the institutional partners (M Westlake and R Rosskamp cited by Labouisse et al. 2008).

3.4 Origin and Evolution

3.4.1 Molecular Phylogenies

Molecular phylogenies of coffee species have been established based on variations in intergenic spacer sequences of plastid DNA (Lashermes et al. 1996b; Cros et al. 1998; Anthony et al. 2010), internal transcribed sequences (ITS) of rDNA (Lashermes et al. 1997), and a combination of four plastid regions and ITS (Maurin et al. 2007). Low sequence divergence was found between *Coffea* and *Psilanthus*, indicating that molecular data do not support the recognition of two genera (Lashermes et al. 1997; Cros et al. 1998). Enlarging the number of *Coffea* species and *Psilanthus* species did not resolve the relationship between the two genera (Maurin et al. 2007). At species level, a small number of parsimony-informative characters were found in molecular studies and the primary clades were weakly supported in the trees. This was attributed to the recent origin of the *Coffea* genus and a radial mode of speciation (Lashermes et al. 1997; Cros et al. 1998; Anthony et al. 2010). All the studies pointed to a

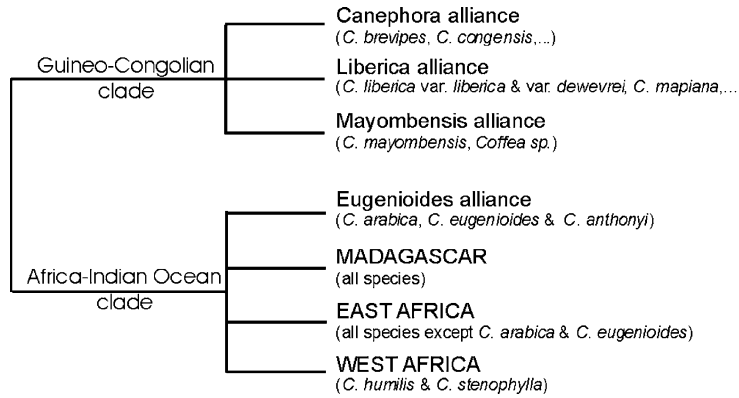

Fig. 3.6 Synthetic diagram of molecular phylogenies in coffee, adapted from Cros et al. (1998), Maurin et al. (2007) and Anthony et al. (2010)

link between the main groups of species and their geographical origin. The plastid data clearly revealed two lineages in coffee species, one spanning the entire geographical range of the *Coffea* genus, while the other is restricted to West and Central Africa. Within lineages, species were classified in subclades according to their biogeographic origin (i.e., East Africa, East-central Africa, Central Africa, West-central Africa, West Africa) (Fig. 3.6). The low divergence between Madagascan species indicated that colonization of Madagascar was doubtless the result of a single dispersal event from the African mainland, followed by insular speciation (Anthony et al. 2010). Overlap of coffee lineages in West-central Africa suggests that Lower Guinea could be the center of origin of the *Coffea* genus (Anthony et al. 2010). Coffee is thought to have spread radially from the center of origin, westwards up to West Africa and eastwards through Central Africa, including Grande Comore, Madagascar, and the Mascarenes. The evolutionary history of coffee probably occurred between 150,000 and 350,000 years ago (Anthony et al. 2010).

3.4.2 Biochemical Classifications

Biochemical markers were used to study chemotaxonomic relationships between coffee species, but the results were not congruent with molecular classifications. Analysis of several components absorbed at 276 nm (e.g., caffeine) and 313 nm (e.g., chlorogenic acids) by chromatography revealed two groups of coffee species corresponding to different metabolic pathways (Anthony et al. 1993). One led to the synthesis of small quantities of chlorogenic acids (<2.5% dry mass basis) and caffeine (<0.3% dmb) while the other produced large concentrations of chlorogenic acids (>4.5% dmb) and caffeine (>0.4% dmb). These groups do not correspond to the lineages revealed by non-coding plastid sequences. Chlorogenic acids were successfully used to separate *C. canephora*, *C. arabica* and lines derived from an interspecific hybrid (*C. arabica* × *C. canephora*), but characterization of accessions was weak within each genetic groups (Guerrero et al. 2001). The effectiveness of fatty acid and sterol composition was recently tested for coffee chemotaxonomy, but congruence with molecular classifications was low (Dussert et al. 2008).

3.5 Cytogenetic Stocks and Use

3.5.1 Doubled Haploids of C. canephora

Production of haploid plants followed by chromosome doubling offers the possibility of developing completely homozygous genotypes from heterozygous plants in a single generation. In woody plants, the use of doubled haploids has been limited principally because of the difficulty of producing sufficient numbers of (DH) doubled haploids. In *C. canephora*, doubled haploids were developed using haploid embryos that occur spontaneously in association with polyembryony (Couturon 1982). Haploid plants were obtained after grafting suspected haploid embryos extracted from immature polyembryonic seeds onto young diploid rootstocks (Couturon and Berthaud 1979). The frequencies of polyembryonic seeds varied

according to the parental genotype, between 0.37 and 1.27% (Couturon 1982). Haploid plants can be selected early using stomata density, 155–425 stomata mm^{-2} in haploids versus 80–150 stomata mm^{-2} in diploids (Couturon 1986). More than 750 doubled haploids isolated from a range of *C. canephora* genotypes were grown under field conditions and evaluated for different characters of agronomic importance (Lashermes et al. 1994a). About half the doubled haploids did not survive, suggesting a strong, negative effect of homozygosity. Inbreeding depression was particularly severe on general vigor and reproductive aspects. Considerable genetic variations were observed for several characters such as leaf shape, leaf rust resistance and bean weight within and between groups of doubled haploids produced from the same genotype. Fifty-five doubled haploids were then crossed with either heterozygous genotypes or doubled haploids in order to study their combining ability (Lashermes et al. 1994b). Marked hybrid vigor was observed for all characters analyzed including yield. Major differences were observed among top crosses involving different doubled haploids produced from the same genotype reflecting the high level of heterozygosity of genotypes. Factorial mating design analysis indicated that all genetic variances were attributable to additive effects in estimates of yield as well as plant height and leaf characteristics. The general combining ability variance component was also predominant for stem girth and susceptibility to leaf rust, although effects due to interaction were detected. Some hybrid combinations had yields comparable to standard varieties. The development of F_1 hybrid varieties should thus be envisaged.

Doubled haploids were used to construct genetic maps of *C. canephora*. A partial map was developed on the basis of a population of 85 doubled haploids (Paillard et al. 1996). The markers including 47 restriction fragment length polymorphism (RFLP) and 100 random amplified polymorphic DNA (RAPD) formed 15 linkage groups and the total length was 1,402 cM. Another map of *C. canephora* was then constructed using two complementary segregating populations produced from the same genotype (Lashermes et al. 2001). One doubled haploid (DH) population comprised 92 individuals derived from female gametes, while the other test cross (TC) population consisted of 44 individuals derived from male gametes. Based on the DH population, a genetic linkage map comprising 160 loci was constructed. Eleven linkage groups that putatively correspond to the 11 gametic chromosomes of *C. canephora* were identified. The mapped loci included more than 40 specific sequence-tagged site markers, either single-copy RFLP probes or microsatellites that could serve as standard landmarks in coffee-genome analyses. Furthermore, segregation distortion and recombination frequency were compared between the two populations. Although segregation distortions were observed in both populations, the frequency of loci exhibiting a very pronounced degree of distortion was especially high in the DH population. This observation is consistent with the hypothesis of strong zygotic selection among the DH population. The recombination frequencies in both populations were almost indistinguishable. These results offer evidence in favor of the lack of significant sex differences in recombination in *C. canephora*. Doubled haploids were also used to map the region containing the *S*-locus, which controls self-incompatibility system (Lashermes et al. 1996a). The availability of an RFLP marker associated with the *S*-locus should facilitate the genetic analysis of self-incompatibility in relation to coffee breeding programs.

3.5.2 *Haploids of* C. arabica

In *C. arabica*, interest in haploids remains limited because of the high homozygosity revealed in the species (Lashermes et al. 1999). Haploid plants were isolated from polyembryonic seeds in large sowings. Of 2,850 seeds sowed from a wild accession, only one haploid plant was found (Berthaud 1976). The proportion of viable pollen seeds ranged from 1 to 2%, which corresponds to almost total sterility.

3.6 Role in Classical and Molecular Genetic Studies

3.6.1 *Molecular Genetic Linkage Maps*

Several genetic maps have been constructed for coffee. The low polymorphism of *C. arabica* species has been a major drawback for developing linkage maps.

Hence, the works reported so far are often restricted to alien DNA fragments introgressed from *C. canephora* (Pearl et al. 2004) or *C. liberica* (Prakash et al. 2004) into *C. arabica* genome. To overcome the limitation of low polymorphism, efforts have been directed toward the development of genetic maps of *C. canephora* (see Sect. 3.5.1) or interspecific crosses.

Accessions of wild coffee belonging to four diploid species have been used to produce interspecific progenies. Estimates of the total map lengths were 1,144 cM, 1,360 cM, and 1,502 cM in the backcrosses [(*C. liberica* × *C. pseudozanguebariae*) × *C. liberica*] (Ky et al. 2000a), [(*C. canephora* × *C. heterocalyx*) × *C. canephora*] (Coulibaly et al. 2002) and [(*C. canephora* × *C. liberica*) × *C. liberica*] (N'Diaye et al. 2007), respectively. The differences observed in map lengths could be related to reduced recombination rate that were associated with variations in the genome size of the parental species since *C. pseudozanguebariae* has a smaller genome than *C. liberica*, *C. canephora*, and *C. heterocalyx* (Cros et al. 1995; Noirot et al. 2003). The relationship between parental chromosome distribution and nuclear DNA content was studied in progenies of the interspecific cross (*C. liberica* × *C. pseudozanguebariae*) (Barre et al. 1998). Progenies of this cross were also used to study inheritance of several characters such as bean sucrose content (Ky et al. 2000b), fructification time (Akaffou et al. 2003), and seed sensitivity to desiccation (Dussert et al. 2004). The genetic map allowed interspecific polymorphisms detected in the synthesis of the caffeoyl-coenzyme A 3-*O*-methyltransferase (Campa et al. 2003) and the phenylalanine ammonia-lyase (Mahesh et al. 2006) enzymes to be located.

3.6.2 Gene Mapping

Genetic mapping of the *C. arabica* genome was performed for genes controlling mainly pest and disease resistance. The *Mex*-1 gene from *C. canephora*, which confers resistance to the root-knot nematode *Meloidogyne exigua*, was mapped in a region of 8.2 cM using a segregating population (Noir et al. 2003). Similarly the region containing the S_H3 gene from *C. liberica*, which is one of the nine genes controlling coffee leaf rust, was mapped on a 6.3 cM interval (Prakash et al. 2004). Other mapping analysis of the S_H3 region led to the identification of two small linkage groups (5.6 and 5.9 cM), one being the introgressed fragment carrying the S_H3 gene (i.e., markers in coupling) and the other the corresponding fragment inherited from the non-introgressed parent (i.e., markers in repulsion) (Mahé et al. 2008). RAPD markers associated with the *T* gene of resistance to coffee berry disease from *C. canephora* were also identified (Gichuru et al. 2008). The markers linked to these resistance genes are suitable for marker-assisted selection and should facilitate pyramiding several resistance genes in a single genotype.

Regarding diploid species, self-compatibility segregation was assessed in backcross progenies originating from an interspecific cross between *C. canephora* (self-incompatible) and *C. heterocalyx* (self-compatible) (Coulibaly et al. 2002). Segregation ratios were consistent with monofactorial control of self-compatibility. Using molecular linkage analysis, the *S*-locus was mapped to a short linkage group. The study of sucrose biosynthesis genes using a bacterial artificial chromosome (BAC) library of *C. canephora* showed that sucrose synthase was encoded by at least two genes, one of which (*CcSUS1*) was cloned and sequenced (Leroy et al. 2005).

3.6.3 Comparative Genomics

The *Arabidopsis thaliana* genome sequence provides a catalog of reference genes that can be used for comparative analysis of other species thereby facilitating map-based cloning in economically important crops. A coffee BAC contig linked to the S_H3 leaf rust resistance gene was used to assess microsynteny between *C. arabica* and *A. thaliana* (Mahé et al. 2007). Microsynteny was revealed and the matching counterparts to *C. arabica* contigs were seen to be scattered throughout four different syntenic segments of *A. thaliana* on chromosomes (At) I, III, IV, and V. Coffee BAC filter hybridizations were performed using coffee putative conserved orthologous sequences to *A. thaliana*-predicted genes located on the different syntenic regions of *A. thaliana*. The coffee BAC contig related to the S_H3 region was successfully consolidated and subsequently validated by fingerprinting. Furthermore, with the exception of a single inversion on AtIII and AtIV chromosomes, the anchoring markers appeared in the same order on the

coffee BAC contigs and in all *A. thaliana* segments. However, the S_H3 coffee region appears to be closer to the ancestral genome segment (before the divergence of *A. thaliana* and coffee) than to any of the duplicated counterparts in the present-day *A. thaliana* genome. The genome duplication events at the origin of its *A. thaliana* counterparts most probably occurred after the separation (94 Ma) of Euasterid (coffee) and Eurosid (*Arabidopsis*).

3.7 Role in Crop Improvement Using Traditional and Advanced Tools

3.7.1 *Using Wild Accessions (*C. arabica*) as Cultivars*

In the diversity center of *C. arabica*, Ethiopia, where the species was domesticated, four major production systems are commonly distinguished (Woldetsadik and Kebede 2000; Labouisse et al. 2008):

- The forest coffee system, which includes simple coffee gathering and forest production where coffee trees are simply protected and tended for convenient picking.
- The semi-forest coffee system, where farmers slash weeds, lianas and competing shrubs, thin forest trees and fill open spaces with local seedlings. Both systems predominate in southwestern Ethiopia and in Bale.
- The garden coffee system, which is a further step in the cultivation process. Seedlings are taken from forest coffee plantations and transplanted closer to farmers' dwellings. In this system, coffee is generally grown on smallholdings and combined with other crops in open sunlight or under a few shade trees. The garden coffee system predominates in the south (Sidamo), in the west (Wellega), and in the east (Harerge and Arsi). Very small-scale coffee growing in the marginal zones of northern Ethiopia, e.g., in Gojam and Wello, can also be included in this category. Garden coffee accounts for 40–50% of national production.
- The plantation coffee system, where coffee is cultivated after land clearing with systematic soil preparation and planting, as well as tending and harvesting to maximize the volume of production and productivity. This sector includes a few large private and state farms located in the southwest, as well as smallholder plantations throughout the coffee growing areas. It accounts for 10–20% of national production.

Aside from Ethiopia, nowhere else in the world are these four systems found along with intermediate or mixed situations. In addition, the systems are not isolated from each other, as genes are exchanged between them. In forest coffee and semi-forest coffee systems, the germplasm, commonly called "wild coffee," is directly derived from spontaneous coffee trees in the forest. In the garden coffee system, the germplasm results from a complex process of transport, exchanges and selection by farmers, and adaptation to environments that are sometimes distant (in geographical and ecological terms) from its original habitat. The germplasm of these gardens is commonly referred to as "landraces." In the coffee plantation system, the germplasm may be coffee landraces but, in most cases, consists of a limited number of coffee lines selected by national research institutions. Around the beginning of the 1970s, the advent and dramatic spread of coffee berry disease caused by *Colletotrichum kahawae* resulted in a significant drop in Ethiopian coffee production. Consequently, an important breeding program based on the selection of naturally resistant genotypes from farmers' fields and forest or semi-forest was launched (Robinson 1974; Van der Graaff 1981; Bellachew et al. 2000). In 2006, 23 cultivars were available for distribution. A large number of promising landrace selections are currently being verified in Sidamo, Wellega, Limu, and Harerge (Bellachew and Labouisse 2007).

Average yield increases gradually from the forest coffee system to the plantation coffee system. Coffee yield never exceeds 200 kg ha^{-1} per year for forest coffee and 400 kg ha^{-1} per year for semi-forest coffee (Woldetsadik and Kebede 2000). In the area of Bonga, in the Keffa zone, very low yields were recorded of around 15 kg ha^{-1} per year for forest coffee and 35 kg ha^{-1} per year for semi-forest coffee (Schmitt 2006). For garden coffee, yield is very variable, ranging from 200 to 700 kg ha^{-1} per year depending on agricultural practices and the occurrence of coffee berry disease. For plantation coffee, yield varies

between 450 and 750 kg ha^{-1} per year although the average potential of selected cultivars is 1,700 kg ha^{-1} per year under optimum growing conditions.

In Latin America, production of coffee, especially by smallholders, is often associated with different forms of shade management. Shade can improve cup quality and limit disease incidence, but reduce productivity (Bertrand et al. 2006). To increase the profitability of the agroforestry system, some producers have adopted wild Ethiopian cultivars such as Java or Geisha. They produce an excellent quality coffee for specialty markets, but productivity is 20–50% lower than that of traditional cultivars.

In Brazil, three genotypes from an accession collected in Ethiopia produce beverage almost completely free of caffeine (Silvarolla et al. 2004). The mutation probably occurred in the caffeine synthase gene. The transfer of the mutation to highly productive cultivars has started using conventional breeding methods.

3.7.2 Using Wild Accessions (C. arabica) as Parents of F_1 Hybrids

In order to increase genetic diversity of *C. arabica* cultivars (Charrier 1978) and to exploit heterosis between genetic groups (Walyaro 1983; Van der Vossen 1985), breeders have proposed the production and selection of F_1 hybrids between traditional cultivars and wild accessions from the diversity center of the species. Wild accessions provide resistance to several root-knot nematode species (Anzueto et al. 2001; Anthony et al. 2003; Boisseau et al. 2009), leaf rust (Gil et al. 1990) and coffee berry disease (Bellachew 2001), and possibly high beverage quality. More than 150 F_1 hybrids have been created and evaluated in Central America (Bertrand et al. 2005a). By applying selection on yield and bean weight, the selected hybrids produced 11–47% higher yields than the best cultivar (Fig. 3.7), along with identical or significantly

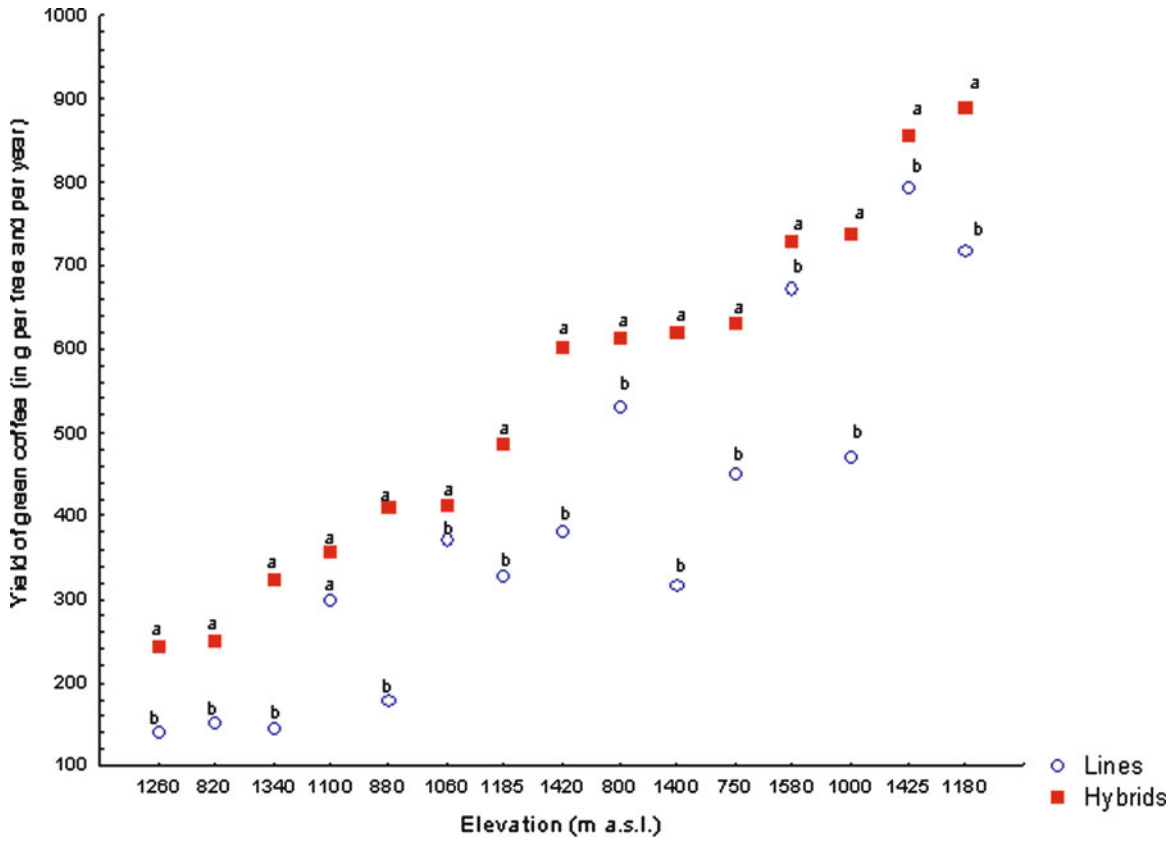

Fig. 3.7 Comparison of (cultivars × wild) F1 hybrids with cultivars in 15 trials for annual green coffee yields per tree (Ymean) in Central America (El Salvador, Costa Rica, Honduras) (from Bertrand et al. 2011)

higher bean weight, and identical fertility. The estimated heterosis (22–47%) was similar to the magnitude observed in previous studies (Carvalho and Monaco 1969; Walyaro 1983; Ameha 1990; Fazuoli et al. 1993; Netto et al. 1993).

Organoleptic evaluations under a range of soil-climatic conditions and elevations revealed no clear differences between F_1 hybrids and traditional cultivars for bean chemical contents and cup quality (Bertrand et al. 2006). For acidity and aroma, F_1 hybrids were variable but, regarding the overall standard, F_1 hybrids were equal to or better than traditional cultivars. In fact, the higher homeostasis of F_1 hybrids resulted in better nutrient supply to the fruits, whatever the environment. The use of F_1 hybrids should thus help reduce variations in the biochemical content of coffee beans, and at the same time favor stability of beverage quality. Moreover, introducing genetic progress provided by F_1 hybrids in agroforestry systems would help increase the productivity of the cropping system and make it more sustainable than other production systems.

The best F_1 hybrids were selected and propagated massively using somatic embryogenesis (Etienne-Barry et al. 1999; Etienne 2005). A large-scale multisite trial totaling, to date, 100,000 trees from 20 selected hybrids has been set up in Central America (Lashermes et al. 2009). The risk of somaclonal variation was estimated at 1–5% depending on the genotype (Etienne and Bertrand 2003).

3.7.3 *Using Wild Coffee (*Coffea *spp.) as Donors of Alien Genes*

Exploitation of coffee genetic resources has so far relied on conventional procedures in which a hybrid is produced between an outstanding variety and a donor genotype carrying the trait of interest, and the progeny is then backcrossed to the recurrent parent. Undesirable genes from the donor parent are gradually eliminated by selection. In so doing, conventional coffee breeding methodology faces considerable difficulties. In particular, major limitations are due to the long generation time of coffee-tree (5 years from a seed to a flowering plant), the high cost of field trials, and the lack of accuracy of the current strategy.

3.7.3.1 Introgression in *C. arabica* Genome

Sources of Introgression

The transfer of desirable genes, in particular for disease resistance, from diploid ($2n = 22$) species into tetraploid ($2n = 44$) cultivars of *C. arabica* without affecting quality traits has became the main objective of breeding programs (Carvalho 1988; Van der Vossen 2001). Genes can be transferred through production of triploid ($2n = 33$) or tetraploid interspecific hybrids. Triploid hybrids derive from a direct cross between the diploid progenitor and *C. arabica*, while tetraploid hybrids are obtained by crossing the two species after chromosome duplication of the diploid progenitor. The use of triploid hybrids is more difficult than that of tetraploid hybrids because of low fertility (Krug and Mendes 1940; Berthaud 1978a, b). However, productive seedlings have been selected as early as the first backcross to *C. arabica* or after the second generation by selfing (Orozco-Castillo 1989). *C. canephora* has been the main source of disease and pest resistance traits not found in *C. arabica*, including resistance to coffee leaf rust, coffee berry disease, and root-knot nematodes. Other coffee species are also of considerable interests in this respect. For instance, *C. liberica* has been used as a source of resistance to leaf rust (Srinivasan and Narasimhaswamy 1975), while *C. racemosa* is a promising source of resistance to the coffee leaf miner (Guerreiro Filho et al. 1999).

Introgression from Diploid Species

The presence of *C. canephora* DNA fragments was sought in accessions derived from a tetraploid interspecific hybrid known as the Timor Hybrid. This interspecific hybrid originated from a spontaneous cross between *C. arabica* and *C. canephora* on the island of Timor in 1927 (Bettencourt 1973). Following a backcross with a *C. arabica* cultivar, progenies were selfed and selected over three to five generations in several coffee-producing countries including Brazil, Colombia, and Kenya. The amount of alien genetic material was substantial, but varied between Timor Hybrid-derived genotypes (Lashermes et al. 2000). The introgressed fragments were estimated to represent from 8 to 27% of the *C. canephora* genome. The effect of *C. canephora* gene introgression on

biochemical composition of beans and cup quality in *C. arabica* breeding lines was investigated (Bertrand et al. 2001, 2003, 2005b). High amounts of introgression appeared frequently to be associated with lower quality factors. However, lines combining resistance to leaf rust and root-knot nematode (*M. exigua*), and good cup quality were successfully developed (Bertrand et al. 2003).

A similar study was undertaken to analyze accessions derived from a putative natural hybrid (*C. arabica* × *C. liberica*) (Prakash et al. 2002). The number of introgression markers was found to be similar in the *C. liberica* introgressed accessions and in the *C. canephora* introgressed accessions. Analysis of genetic relationships in the introgressed lines suggested that introgression was limited to a few fragments. Moreover, the alien genetic material appeared to be fixed and there was no elimination or counter-selection over generations, from introgressed parent to F_4.

Factors Controlling Gene Introgression

During the last few years, differences in the behavior of interspecific hybrids between *C. arabica* and the diploid species, *C. canephora* and *C. eugenioides*, has been investigated. Many plant populations resulting from the backcross of either triploid or tetraploid interspecific hybrids to *C. arabica* were analyzed (Herrera et al. 2002a, b, 2004). Flow cytometric analysis of the nuclear DNA content revealed that most of the backcross individuals were tetraploid or nearly tetraploid, suggesting that among the gametes produced by the interspecific hybrids, those representing 22 chromosomes were strongly favored. Molecular markers (i.e., RFLP, SSR, and AFLP) combined with the evaluation of morphological characteristics and resistance to leaf rust were applied to check gene transfer from the donor species into *C. arabica*, and to estimate the amount of introgression present in backcross individuals. While a high amount of introgression was observed in the progenies derived from the tetraploid interspecific hybrids, the backcross individuals generated from the triploid interspecific hybrids differed considerably. The mean proportion of introgressed markers per plant was significantly lower in populations derived from *C. eugenioides* than from *C. canephora*. Moreover, the comparison of reciprocal progenies between *C. arabica* and triploid interspecific hybrids (*C. arabica* × *C. canephora*) used as male or female parent revealed a very strong effect of the backcross direction. A major reduction in the frequency of *C. canephora* introgressed markers was observed when the triploid hybrids were used as the male parent. Breeding strategies based on gene introgression can now be designed according to selection objectives.

3.7.3.2 Introgression in *C. canephora* Genome

Interspecific hybrids have also been produced mainly to improve the bean size and cup quality of *C. canephora*. The species used for controlled or spontaneous hybridizations were *C. arabica* (Capot 1972; Charmetant et al. 1991), *C. congensis* (Cramer 1948; Yapo et al. 1989), and *C. liberica* (Louarn 1987; Yapo et al. 1991). However, the high expectations placed in the interspecific strategy were not fulfilled because interspecific progenies presented persistent problems of genetic instability and lower fertility than intraspecific progenies (Van der Vossen 2001).

3.8 Genomics Resources Developed

3.8.1 BAC Libraries

A BAC library of *C. arabica* was constructed to facilitate genome research on coffee trees (Noir et al. 2004). A cultivar derived from the Timor Hybrid that exhibited a fair level of resistance to several pathogens was chosen. The library contains 88,813 clones with an average insert size of 130 kb, and represents approximately eight *C. arabica* dihaploid genome equivalents. It was used to construct an integrated genetic and physical map of the coffee genome. The mapping approach combined hybridization with mapped markers and BAC fingerprinting. Hybridization with both low-copy RFLP markers distributed on the 11 chromosomes and probes corresponding to disease resistance gene analogs was successful (Noir et al. 2001). Positive BAC clones from subgenomes E^a and C^a were assembled into separate contigs, showing the efficiency of the combined approach for mapping purposes. The *C. arabica* BAC library was

also successfully used to assess microsynteny between *C. arabica* and *A. thaliana* (Mahé et al. 2007).

A *C. canephora* BAC library was developed using a genotype with relatively good cup quality (Leroy et al. 2005). The library contains 55,296 clones with an average insert size of 135 kb, representing almost nine haploid genome equivalents. The library was used to analyze the copy number of sucrose-metabolizing enzymes, mainly sucrose synthase and invertases (Leroy et al. 2005).

3.8.2 EST Resources

The expressed sequence tags (ESTs) available from coffee were reviewed recently (Lashermes et al. 2007). For *C. canephora*, a total of 55,692 ESTs is publicly available from developing seeds (Lin et al. 2005) and from leaves and fruits at different stages of development and maturation (Poncet et al. 2006). For *C. arabica*, only 1,226 ESTs are publicly available, comprising sequences expressed during infection by the rust fungus *H. vastatrix* (Fernandez et al. 2004) and in response to chemically induced systemic acquired resistance (De Nardi et al. 2006), and some others from leaves (Cristancho et al. 2006; Salmona et al. 2008). Other sequence sets that are not publicly available at the time of writing, have been developed by research groups involved in molecular genetics and genomics of coffee, mainly in Brazil (Vieira et al. 2006), Colombia (Cristancho et al. 2006), India (Aggarwal et al. 2007), and Italy (De Nardi et al. 2006). Around 250,000 good-quality ESTs from at least four different coffee species (i.e., *C. arabica*, *C. canephora*, *C. liberica*, *C. racemosa*) have already been produced worldwide (Lashermes et al. 2007).

3.9 Conclusions and Recommendations

The high number of coffee species (134) in the *Coffea* and *Psilanthus* genera is a consequence of high adaptive capacity displayed by this group of plants. Their evolutionary history is recent (likely in the Late Pleistocene) and low divergence has been detected in their genome. As a consequence, all coffee species are able to cross and produce relatively fertile interspecific and intergeneric hybrids. The possible transfer of agronomic traits from wild coffee into cultivars of either *C. arabica* or *C. canephora* means wild coffee has great potential value. Certain wild genotypes carrying traits of interest for breeding (e.g., resistance to pathogens) have been identified in several field genebanks. However, up to now, few genotypes have been used in breeding programs and genomic projects, mainly because of lack of genetic evaluation. The recent discovery of the first naturally caffeine-free plant (Silvarolla et al. 2004) indicates that evaluation in large genebanks has not been sufficiently intensive.

The largest use of wild coffee is undeniably in Central America for the creation and selection of F_1 hybrids of *C. arabica* (Bertrand et al. 2005a). F_1 hybrids exhibit particularly marked heterosis on yield, which could be exploited in agroforestry production systems. The gain in the production of F_1 hybrids could counterbalance the drop in yields observed in agroforestry systems, which is estimated to be between 20 and 40% (Vaast et al. 2006). Moreover, beverage produced by F_1 hybrids presents more stable quality than coffee from homozygous lines. Promoting F_1 hybrid cultivation in agroforest conditions would be a major step toward sustainable coffee production.

Despite the socio-economic importance of coffee, the conservation of genetic resources has received little attention. Natural habitats of wild coffee have been threatened by deforestation in Africa and Madagascar for decades. For instance, in Ethiopia, between 1971 and 1997 deforestation was responsible for the destruction of 10,000 ha per year, and this process always appeared to be linked to human activities (Woldemariam et al. 2002). In this context, new explorations in African and Madagascan forests are a priority in the short term, as only half known coffee species have been collected and placed in genebanks (Dulloo et al. 2009). Almost all genetic resources are currently conserved in field conditions and are therefore vulnerable. Moreover, considering the lifespan of coffee trees (30–50 years), there is now an urgent need to replace aging coffee trees (Dulloo et al. 2001). The conservation of coffee genetic resources should be managed by the coffee scientific community with the support of international institutions like the International Coffee Organization and Bioversity International.

In the future, the ability to capture and manipulate genetic diversity and effectively use germplasm in

breeding programs is vital for sustainable coffee production. In this way, the development of genomic tools and large-scale phenotyping technologies will help breeders to identify, characterize, and use diversity present in wild coffee. The recent development of high-capacity methods for analyzing genomes represents a new paradigm with broad implications. The advent of large-scale molecular genomics for the cultivated species *C. arabica* and *C. canephora* will provide access to previously inaccessible sources of genetic variation that could be exploited in breeding programs. Anticipated outcomes include (1) rapid characterization and management of genetic resources, (2) enhanced understanding of the genetic control of important traits, (3) identification of candidate genes or tightly linked genomic regions underlying traits of interest, and (4) identification of accessions in genetic collections with variants of genomic regions or alleles of candidate genes with a favorable impact on desirable traits. In this way, the recent constitution of an international commitment (ICGN http://www.coffeegenome.org) to unite research efforts for the development of common sets of genomic tools, plant populations, and concepts appears extremely useful. The sequencing of end sequences of 73,728 BACs is expected in 2010 thanks to French funds. This is a step toward sequencing the whole genome in 2011.

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Chapter 4
Cola

P.O. Adebola

4.1 Basic Botany of the Species

4.1.1 Areas of Origin and Present Distribution

The earliest written records on kola date back to the sixteenth and seventeenth centuries. According to Nzekwu (1961), Jahannus Leo Africanus was the first to refer to kolanut in 1556. Odoardo Lopez also recorded the occurrence of kola trees in Congo (Zaire) in 1591 followed by Andre Alvares, who saw them in Ghana and Guinea in 1594. The kola trees were thereafter recorded along all the West coast of Africa from Gambia through Benin to Angola. The distribution of *Cola* species in Africa lies approximately between 12°N and 12°S of the equator and ranges from Senegal to Angola in the West to Tanzania and Mozambique in the east.

The center of diversity of the genus *Cola* is West Africa (Bodard 1955). Large concentrations of the various species are represented in three major areas: Sierra Leone/Liberia, Nigeria/ Cameroon, and Gabon. *Cola acuminata*, the "Abata" kola, is native to lowland rainforest area of western Nigeria. Its original area of distribution stretched from Nigeria to Gabon. According to Conde Ficalho, as cited by Opeke (1984), *C. acuminata* also occurs naturally in the mountainous area of Angola and the Island of Principe and Sao Tome. This species has since been introduced into West Indies and various parts of South America. Areas of *C. acuminata* cultivation in Nigeria include Ogun, Osun, Ondo, Ekiti, Edo, Anambra, Abia, and Enugu States. *Cola nitida*, which is the main kola of commerce, occurred frequently in Sierra Leone and the forest areas of Cote d'Ivoire and Ghana, where it is considered to be indigenous (Nzekwu 1961). These areas, which have been the local sources of *C. nitida* some 800 years ago, must have depended to a large extent on naturally occurring trees, as plantations were not established until the beginning of this century (van Eijnatten 1969). *C. nitida* was also introduced into other areas from its original area of distribution. It can now be found in Australia, Trinidad, Jamaica, India, Madagascar, and South America. It was introduced into southwestern Nigeria shortly before 1900, where it replaced to a large extent the traditionally grown *C. acuminata*. It has also spread eastwards to Mauritius and Malaysia (Russel 1955; Purseglove 1968; FAO 1982). Kola seeds were distributed from Kew Royal Botanic Gardens for planting in Calcutta, Singapore, Sri Lanka, Java, West Indies, and the USA (Moloney 1887).

Less is known about the remaining edible *Cola* species that are rarely cultivated. These species include *Cola ballayi, Cola verticillata*, and *Cola anomala*. Others are *Cola heterophylla, Cola lepidota, Cola millenii, Cola gabonensis*, and *Cola simiarum*. The area of distribution of *C. verticillata* stretches from Cote d'Ivoire to Congo. It is as a rule, not cultivated, as its nuts are valued far less than those of *C. nitida* or *C. acuminata* because of its slimy nature. The seeds of *C. ballayi* are similar to those of *C. acuminata* and have the same properties when chewed. It occurs naturally in Central and East Africa from Cameroon eastwards. *C. anomala* is reported to be cultivated on the highlands of Cameroon, where it is locally referred to as "Bamenda kola." Wherever

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these trees occur, they were usually spared during land preparation for the establishment of annuals or permanent crops and their produce are usually utilized when the true kolanuts are scarce.

4.1.2 Taxonomy and Reproductive Biology

The family Sterculiaceae was recognized by Ventenat in 1804 within the order *Malvales*. Schott and Endlicher described the genus *Cola* in 1932. The family comprises about 125 species and about 50 different species of the genus have been recorded and identified in West Africa (Hutchinson and Dalziel 1958). Schumann, who recognized several subgenera, proposed a systematic grouping of the various species within the genus *Cola* in 1900. This was slightly altered by Chevalier and Perrot in 1911 and again by Bodard (1962), who listed five subgenera, viz. *Parrosemenecola* M. Bod; *Macrocola* (A Chev.) M. Bod; *Cola* (= *Eucola* A Chev.); *Haplocola* (K Schum M.Bod.); and *Cheirocola* K. Schum. They are mainly distinguished by the presence of one or two rings of anther on the connate androecium, the position of the inflorescence and fruit characteristics. The edible or true kola belongs to the subgenus *Cola*, which is characterized by an androecium with two rings of anthers; inflorescence placed in leaf axils and enclosed in bracts forming a hood with a circular line of dehiscence prior to emergence; ovoid follicles with many seeds which fill the carpel cavity and a fleshy seed coat. The main species in this group are *C. nitida* (Vent) Schott & Endl. and *C. acuminata* (P.Beauv) Schott & Endl. Other species of the subgenus with edible nuts but which are rarely cultivated include *C. verticillata* (Thonn.) Stapf ex Chevalier, *C. ballayi* Cornu, *C. anomala* Schum, *C. heterophylla* (P.Beauv.) Schott & Endl. *C. gabonesis* Mast, and *C. simiarum* Spraque ex Brenan & Keay.

Opeke (1984) described the accepted definition of the genus *Cola* as follows:

> Trees and shrubs with alternate leaves; stipule present although sooner or later dropping. Male and hermaphrodite flowers grouped into a panicle of cymes, or in fascicles on the branches or on the trunks. The anther loculi of the male flowers are placed laterally at the top of the androecium in one or two superimposed rings. The hermaphrodite flowers have five to ten carpels narrowly placed together in the center of the flower, short style, fleshy stigma that are more or less recurved and a vestigial androecium at the base of the gynoecium. The fruit have five to ten follicles placed perpendicularly on the peduncle. They are dehiscent at maturity and contain one to twelve seeds that are fleshy, without endosperm and with radicles directed towards the hilum. After germination of the seedling the cotyledons are subhypogynous.

Russel (1955) had earlier published a detailed description of the two main edible species of kola. He described *C. nitida* as a robust tree that usually grows to a height of between 9 and 12 m, sometimes up to 24 m. It has dense foliage with flat simple leaves. The leaves have long petiole and vary in shape from obovate to oblanceolate. The inflorescences are panicle-like cymes, determinate in growth with the terminal flowers opening first. In *C. nitida*, flowering occurs twice annually; a major flowering in August/September that extends into October or later and a minor flowering from January to March during which inflorescence are sporadic and few. *C. acuminata* on the other hand flowers in January and February; the fruits becoming available in April or May.

The kola plants are functionally monoecious possessing both male and hermaphrodite flowers. The male flowers have rudimentary gynoecium, which is non-functional. The hermaphrodite flowers have well-developed androecium and gynoecium. The pollen sacs in the hermaphrodite flowers are indehiscent and do not sporulate. Although pollen from hermaphrodite flowers is viable, they have been confirmed to be non-functional when used for either self- or cross-pollination (Opeke 1984). The hermaphrodite flowers are, therefore, considered as functionally female flowers. The male and female flowers are borne on separate inflorescence or mixed within the same inflorescence.

Cola is both self- and cross-pollinated although with varying degrees of cross and self-incompatibilities among individual trees. Due to the sticky nature and the size of *Cola* pollen, natural pollination is unlikely to be anemophilous. The flowers of kola have in addition a penetrating scent and both Russel (1955) and Bodard (1962) pointed out that this attracts different types of insects. *Torma colae, Crematogaster buchneri*, and *Oecophylla longinoda* are the major insects often found on kola inflorescence and on few occasions, sticky kola pollen has been found adhering to their bodies. The significance of the role of these insects as kola pollinators has, however, not been substantiated.

Anthesis occurs early in the morning from 4.00 a.m. to 8.00 a.m. When the male flowers are about to open, they are subspherical in shape and are about 12–20 mm in diameter while the female flowers at this stage are larger and oval in shape usually 30–40 mm in diameter. The flowers have five connate sepals, each with three parallel red lines, which may vary in width and length. Petals are absent. The flowers start drying up at the end of the third to fourth day, although some flowers may persist up to 10 days. The receptivity of the gynoecium decreases until the fifth day after anthesis when all pollinations are usually unsuccessful. The anthers of male flower sporulate at anthesis and some of the pollen grains are deposited on the adaxial side of the sepals just below the pollen sacs.

The five carpels contain 10–12 anatropous ovules implanted along the ventral adaxial side of the carpels. The carpels end in five receptive stigmatic lobes and distinct styles are absent. Complete fertilization in one flower result into the formation of five fruiting follicles. As the fruit develops the stalk thickens and curves downwards with the five follicles assuming a star shape. Fruits mature between 120 and 135 days after pollination. At maturity, the follicles usually dehisce along the ventral suture. Sometimes fruits may dehisce prior to maturity; this occurs irregularly and the split occurs at the point of highest tension independent of the ventral suture. Mature pods contain variable number of seeds usually from 1 to 11. Seeds can be white, pink or red usually with two cotyledons. Trees with yellow nuts have also been identified in Okuku, Osun State, Nigeria (Jacob 1973).

C. acuminata tree is very similar to *C. nitida*. The "Abata" kola tree (*C. acuminata*) as described by Russel (1955) is a slender tree usually 6–9 m tall with sparse foliage that were usually confined to the tips of the branches. The flowers are smaller than those of *C. nitida* and are produced during the dry season in January and February. The fruit also consists of five sessile follicles with rough surface and a straight tip. There are up to 14 seeds in each follicle. The nut may have three to six cotyledons, which are pink, red, and sometimes white in color. Three new species of *Cola, C. cecidiifolia* Cheek, *C. metallica* Cheek and *C. subopposititifolia* Cheek, all with simple leaves and short petioles have also been described from western Cameroon (Cheek 2002).

4.1.3 Cytology

Mitotic chromosome count of $2n = 40$ established for *C. lateritia, C. ballayi, C. verticllata*, and *C. gigantea* by Adebola and Morakinyo (2005) is the first report of chromosome counts in these species. Earlier counts of $2n = 40$ for *C. millenii, C. nitida, C. acuminate*, and *C. lepidota* have been reported by Morakinyo (1978) confirming that the two cultivated species of *Cola* (*C. nitida* and *C. acuminata*) and some of the wild relatives possess the same number of chromosomes ($2n = 40$). According to Purseglove (1968), the basic chromosomes number of *Cola* is $X = 10$ indicating that these species are probably polyploids of $2n = 4x = 40$ with each chromosome represented four times in the somatic complement. The chromosomes are mostly metacentric and submetacentric. Structurally, the chromosomes of *Cola* species were generally small in size making it difficult to see their morphological details by ordinary microscopy. The small chromosome size has also been reported for other species of the genus by Morakinyo (1995) confirming that this is a distinct karyotypic feature of the genus *Cola.*

The similarity in chromosome morphology of the species is very evident especially chromosome staining intensity, the range and gradation of chromosome length, and the centromeric positioning (Adebola 2003). It also partly explains the high degree of morphological similarity in the species indicating the commonness of their ancestry. The divergence of their genetic systems from this common or related ancestral parental stock might have led to parallel variation and speciation. The similarity in chromosome morphology suggests that the species are somehow related and gene exchange among these species is, therefore, expected barring cryptic structural differences in the chromosome and other pre- and post-zygotic cross-breeding barriers.

4.2 Conservation Initiatives

4.2.1 In Situ Conservation

Kola trees are traditionally found in large reservoirs of outlying farms in West and Central Africa. These trees arise from natural regeneration having been protected

during bush clearance in and around compound farms or in relatively nearby outlying farms (Tachie-Obeng and Brown 2004). The status of cultivation within forest or farm systems is related to kola's various uses and roles in providing food, revenue, and particularly, in the social life and religious customs of the local people living along the forest fringes (Russel 1955; Okafor 1980). Despite the multipurpose functions of planting kola trees, like providing shade and income, etc., few farmers protect regenerating kola seedlings, preferring to focus on annual crops, which bring more tangible and immediate benefits. The land tenure system also have an impact on other sustainable agriculture, agroforestry and community forestry programs, and many kola trees are destroyed by shifting cultivation and inappropriate management because of ownership problems. Furthermore, most kola trees, either in forest or farmlands, are considered an open-access resource and sustainable management is often lacking (Tachie-Obeng and Brown 2004).

4.2.2 Ex Situ Conservation

The seeds of the *Cola* spp., like many other tropical tree species, are recalcitrant because they are very sensitive to desiccation. The kolanut can, therefore, only be conserved for a short period (weeks to months), even under optimal moisture conditions, before losing viability. Facilities for long-term storage are not available in most kola-producing countries. This has resulted in the establishment of field genebanks in research and experimental stations across the West African subregion. In Nigeria, kola plantations as well as a few wild species were established at Cocoa Research Institute of Nigeria (CRIN) headquarters in Gambari and other suitable substations across the country, in the form of germplasm and experimental plots (Adebola et al. 2002).

The major problem facing the germplasm plots is labor shortages, as a result of which some of the plots have been taken over by forest trees. The plots are very expensive to maintain in order to prevent the loss of materials by the rampant annual bush fire. Moreover, most of the kola germplasm collections in Nigeria have been conserved in the headquarters at Gambari alone without being duplicated elsewhere. The localized conservation of these valuable materials, therefore, raises the concern of their genetic vulnerability.

Among the conserved materials at CRIN, Nigeria are five wild relatives of kola, which include *C. verticillata*, cultivated on a much smaller scale, but whose seeds are slightly slimy and are chewed only when the other preferred species are scarce. *C. millenii*, the "Monkey Kola," is another conserved wild relative. The edible part of this species is not the seed but the fleshy sweet-tasting mucilaginous seed coat (Williams 1986). The remaining three wild species, *C. gigantea*, *C. lateritia* and *C. ballayi*, are potential sources of useful genes for genetic improvement of the cultivated species. In Ghana, four accessions of *C. gigantea*, one each of *C. acuminata* and *C. caricifolia*, as well as five of *C. millenii* were reported to be conserved in Aburi Botanical Gardens (2002).

4.3 Role in Crop Improvement Through Traditional and Advanced Tools

4.3.1 Interspecific Hybridization *in* Cola

Interspecific hybrids (natural and synthetic) have long been known among plants (Stebbins 1950; Burham 1962). Extensive use has also been made of hybridization as a means of elucidating intergeneric and interspecific relationship. Interspecific crosses between *C. nitida* and *C. acuminata* have been widely reported and it has been shown that there are no physiological or structural barriers to interspecific pollination and fertilization between these two cultivated species (Town 1967; Jacob and Okoloko 1974; Adebola 1999). Artificial interspecific crosses between these two species are done possibly with an aim to incorporate the vigor and comparatively early-bearing habit of *C. nitida* into *C. acuminata*. The two species are related very closely and differ only in a few characters such as number of cotyledons, pod morphology, and fruit-bearing habit. F_1 hybrid progenies of these two species are, therefore, common in nature. The F_1 hybrids are viable but non-fruiting at maturity and are therefore suspected to be sterile (Jacob 1973). This is particularly so because pollen grains from several *C. nitida* genotypes have proved ineffective on some interspecific hybrids (van Eijnatten 1966).

Jacob (1973) also supported this assertion by both selfing and backcrossing the hybrid to the *C. nitida* parent. Adebola (1999), however, found out that the interspecific hybrids of *C. nitida* and *C. acuminata* were male-fertile and not completely sterile as earlier reported.

The first attempt of interspecific crosses between the cultivated *Cola* (*C. nitida*) and its wild relatives was reported by Morakinyo (1978). In reciprocal *C. nitida* × *C. millenii* crosses, 80% fruit set was recorded on *C. millenii* and 10% on *C. nitida*. All the seeds obtained from the crosses did not germinate due to lack of adequate food in the hybrid seed cotyledons to support the embryo. Although some of these crosses indicated some level of compatibility, true interspecific hybrid plants were not produced. Adebola (2003) also carried out experimental crosses involving the two cultivated species, *C. nitida* and *C. acuminata* and four wild relatives, *C. ballayi, C. millenii, C. lateritia* and *C. verticillata*. Flowering non-synchrony as well as the edaphoclimatic conditions at the time of pollination greatly limited the pollination success. The implication of flower non-synchrony is the restriction of hybridization to species of similar anthesis times. This might be a natural isolation mechanism that effectively prevents gene flow among these *Cola* species. Except for *C. verticillata*, all other wild *Cola* species do not have the same flowering period as *C. nitida*. This explains the very low number of pollinations that was possible during the 3-year study period. On the other hand, the flowering period of *C. acuminata* synchronizes with those of the other wild species but the dryness of the weather aids flower drop and abortion of most of the pollinations carried out. Although some of the crosses indicated a high degree of compatibility as evident in the percentage fruit sets obtained (Table 4.1), no clear-cut pattern of cross-compatibility was shown from the crossing program. Species, such as *C. nitida, C. millenii* and *C. ballayi*, seems to have a higher crossability than the others. The other extreme was *C. verticillata* that has a low crossability in all combinations showing that direct gene exchange between this species and the others will be difficult, if not impossible. The result of the study also indicated that a large number of fruits that were initiated do not reach maturity. The successful interspecific crosses are shown in Table 4.2.

In the *C. nitida* × *C. ballayi* cross, the F_1 seeds obtained possess multiple cotyledons (Fig. 4.1), a characteristic feature of *C. ballayi*, the pollen parent. According to Smatt (1979), inheritance of such pollen parental characteristics authenticates the establishment of hybridity. The possession of an additional seed coat that was hard and corky gave the F_1 nuts a characteristic wrinkled appearance. This phenomenon cannot be explained, as the feature was not found in

Table 4.1 Results of pollinations in full diallel combinations among six *Cola* species (Adebola 2003)

♀	♂						
		C. nitida	*C. acuminata*	*C. millenii*	*C. ballayi*	*C. lateritia*	*C. verticillata*
C. nitida	P	467	342	164	94	116	93
	S	170 (36.4)	87 (25.43)	28 (17.07)	31 (32.97)	18 (15.51)	12 (12.90)
	H	224 (11.99)	172 (12.57)	11 (1.67)	26 (6.91)	0	0
C. acuminata	P	302	542	206	316	98	85
	S	52 (17.21)	37 (6.80)	26 (12.62)	86 (27.21)	32 (32.65)	8 (9.41)
	H	0	31 (1.42)	0	0	0	0
C. millenii	P	155	81	131	64	82	32
	S	34 (21.97)	40 (49.38)	80 (58.00)	36 (57.00)	51 (62.50)	6 (18.75)
	H	84 (6.77)	38 (5.86)	244 (23.28)	43 (8.40)	0	0
C. ballayi	P	118	206	98	286	112	58
	S	48 (41.00)	94 (46.00)	12 (12.50)	102 (36.00)	21 (18.75)	7 (12.10)
	H	0	0	0	0	0	0
C. lateritia	P	59	63	74	48	88	31
	S	5 (8.47)	12 (19.04)	21 (28.30)	6 (12.50)	70 (80.00)	4 (12.90)
	H	4 (3.38)	0	0	0	79 (45.00)	0
C. verticillata	P	84	69	82	110	76	69
	S	13 (15.40)	8 (11.50)	14 (17.07)	6 (5.45)	16 (21.05)	13 (18.84)
	H	0	0	0	0	0	0

P: Number of flowers pollinated; S: Number of fruit set; H: Number of pods harvested. Percentages in parenthesis

Table 4.2 Successful interspecific crosses obtained in *Cola* species (Adebola 2003)

Interspecific crosses	Number of flowers pollinated	Number of fruit set (%)	Number of pods expected	Number of pods harvested (%)
C. millenii × *C. nitida*	155	34 (21.97)	1,240	84 (6.77)
C. millenii × *C. acuminata*	81	40 (49.38)	648	38 (5.86)
C. millenii × *C. ballayi*	64	36 (57.0)	512	43 (8.4)
C. nitida × *C. acuminata*	342	87 (25.43)	1,368	172 (12.57)
C. nitida × *C. ballayi*	94	31 (32.97)	376	26 (6.91)
C. nitida × *C. millenii*	164	28 (17.07)	656	11 (1.67)
C. lateritia × *C. nitida*	59	5 (8.47)	118	4 (3.38)

Fig. 4.1 Nuts obtained from C. *nitida* × *C. balayi* interspecific crosses 107 days after pollination

Fig. 4.2 Mature pods obtained from *C. lateritia* × *C. nitida* 135 days after pollination

both the parents. Results of the crossing program (Adebola 2003) corroborates to the qualitative and multivariate analyses and showed that *C. ballayi* is the closest wild relative of the two cultivated *Cola* species indicating its possible value for use as a bridge species. *C. lateritia, C. ballayi*, and *C. millenii* are wild species with characters of great breeding value. These plants fruits heavily, branch extensively, and are disease-resistant. *C. millenii* and *C. ballayi* are particularly outstanding among the wild *Cola* species because of their moderate height. The F_1 seeds of all successful interspecific crosses between these wild and cultivated species of *Cola* were not viable as they all had failed to germinate when planted. Genetic exchange among these plants is, therefore, far from being achieved. The results obtained in the study, however, point to the operation of post-zygotic barriers as the cause of the failure in most of the crosses. This was because a reasonable level of fruit set (Fig. 4.2) was obtained in all the pollination combinations. The formation of embryo in some of the hybrid seeds was also a proof of the occurrence of fertilization. The results obtained suggest that possibly many fertilized ovules abort at an early stage. According to Raghavan (1986) hybrid breakdown, hybrid weakness, and hybrid sterility are generally the main crossing barriers to interspecific hybridization. This may be caused by arrested embryo development, endosperm disintegration, abnormal development of ovular tissues or chromosomal or genetic instability (Singh et al. 1990). This barrier to gene exchange at the hybrid seed level can be circumvented by the application of

appropriate tissue culture technique to rescue the embryo. According to Pierik (1987) the technique had, indeed, determined the success of hundreds of different wide crosses. The use of somatic embryogenesis possibly from the immature zygotic embryos has also been suggested (Adebona 1992). Adebola and Morakinyo (2006) reported that the number of female flowers per inflorescence, number of stigmatic lobes per flower, fruit length, number of pods per hand, number of nuts per pod, and pod wall thickness are useful characters of agronomic interest with potentials for utilization in Kola breeding. The first step toward achieving this goal in *Cola* is the development of appropriate protocol for *Cola* embryo culture. Thus, the improvement of the cultivated species of *Cola* through hybridization with related wild species is by a necessity a long-term program because, even after obtaining viable F_1 hybrids, there are invariably problems of low fertility to contend with. Other undesirable characteristics might also have been transferred from the wild species. These characters could, however, be bred out, while desirable ones are fixed through systematic backcrossing.

4.4 Scope for Domestication and Commercialization

4.4.1 Scope for Domestication

According to Tachie-Obeng and Brown (2004), there is considerable potential for further development of *Cola* species as a commercial crop under sustainable management. The edible wild species also possess considerable potential for domestication. As a traditional crop in West and Central Africa, the cultivation, harvesting and processing of kola are widely known. Although prone to a number of pests and diseases, experience has shown both *C. nitida* and *C. acuminata* to be tolerant of a wide range of environmental conditions. The crop is easily cultivated and these species appear to be more resistant to pest and disease attack than other plantation crops of the region. *Cola* species are suitable for agroforestry combinations with both agricultural crops and timber yielding tree species/woodlots. Kolanuts have a high market value and there is considerable potential for expanding markets outside Africa.

4.4.2 Commercial and Industrial Values

Apart from a few species being used as a masticatory when the two commercial species are scarce, seeds of wild *Cola* species do not really have any commercial value. The trees are, however, used as excellent timber material. The seeds of *C. nitida* and *C. acuminata* are referred to as Kolanut and are consumed in large quantity in the Sudan Savannah areas of West Africa because of its stimulating and sustaining properties. The nuts contain two alkaloids, caffeine and theobromine, which are powerful stimulants that counteract fatigue, suppress thirst and hunger, and are believed to enhance intellectual activity (Sundstrom 1966; Nickalls 1986). The nuts when chewed dispel sleep and fatigue and therefore serve as a substitute for coffee drinking. On account of its sleep suppressing effect, kolanut chewing is now very popular among students in West Africa. Laborers and drivers on long distance journeys also frequently chew it. Due to their unique bitter taste, kola nuts are effective for refreshing the mouth, and the twigs are used as "chewing sticks" to clean the teeth and gums (Lewis and Elvin-Lewis 1985). Kolanut's active ingredients include caffeine (2–3%) and smaller amounts of theobromin and kolatin, a phenolic substance. The nuts are also nutritious and contain 10% protein, 1.35% fats, and 45% starch (van Eijnatten 1969). It is, therefore, useful in pharmaceutical industries and in the production of kola-type beverages, dyes, wines, and confectionery (Ogutuga 1975). Kolanut has been used as a base for a new brand of chocolate and wine (Famuyiwa 1987). Eka (1971) has also reported the possible use of pulverized kolanuts for the preparation of hot non-alcoholic beverages. It could also be used in jam and jelly production because of its high pectin content. Kolanut testa has high potassium content and has been suggested as a possible ingredient for making fertilizers (Olubamiwa et al. 2002). The pod husk has also been utilized for the production of liquid soap and for the replacement of up to 60% of the maize used in poultry feed formulations (Olubamiwa et al. 2002). The fresh pod testa has also been proven to be an excellent

material for feeding the African giant snails (Hamzat et al. 2002).

The demand for kolanut is increasing and efforts are still going on to diversify its uses. This indicates that kola will continue to play a significant role as an important economic crop for internal and regional trade and an increasingly important role in foreign trade.

4.4.3 Medicinal Uses

Cola species also plays significant roles in traditional herbal medicine. According to Mshana et al. (2000), the bark of *C. gigantea* is used to treat yaws while that of *C. lateritia* is very efficient in the treatment of wound. The fruit of *C. nitida* is also reported to be used for the treatment of excessive appetite and fracture. The cotyledon is used to treat herpes and the bark is also good in the treatment of dystocia. The leaves, twigs, flowers, fruits follicles, and the bark of both *C. nitida* and *C. acuminata* are used to prepare a tonic as a remedy for dysentery, coughs, diarrhea, vomiting (Ayensu 1978), and chest complaints (Irvine 1961). The nuts have considerable potential for the development of new pharmaceuticals and foods (Fereday et al. 1997). Extracts of *C. nitida* bark have been reported to show inhibitory activity against various pathogenic bacteria (Ebana et al. 1991). Benie et al. (1987) reported that stem bark extracts of *C. nitida* has potential to be used as a natural fertility regulator.

4.5 Recommendations for Future Actions

1. Information on *Cola* is still very scanty despite its economic importance in West and Central Africa. Greater research effort is needed in this crop especially on improving productivity, diseases and pest control, and reduction of post-harvest loses in the two commercial species. Speeding up the breeding cycle and circumventing the interspecific crossability barriers through the use of appropriate molecular tools should also be of priority.
2. In view of the various potential uses of *Cola* species, efforts should be made to create awareness and disseminate information on the potential food and medicinal products that can be derived from the plants.
3. Concerted conservation effort is needed for systematic documentation and conservation of all known *Cola* germplasm in the *Cola* belt. The current on-farm and plantation conservation effort is not enough and should be complemented by the use of modern conservation techniques such as cryopreservation. This will be an additional insurance against loss as a result of unexpected destruction by fire or human activities.

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Chapter 5
Digitalis

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5.1 Basic Botany of the Species

5.1.1 The Genus Digitalis

The genus *Digitalis*, commonly known as the "foxglove," is a member of the Plantaginaceae. The name *Digitalis* is Latin for "finger of a glove," which refers to the shape of the flowers. All *Digitalis* species are biennial or perennial herbs, rarely small shrubs with simple, alternate leaves, which are often crowded in basal rosettes. Flowers are zygomorphic and arranged in terminal, bracteate racemes, and vary in color with species, from purple to pink, white, and yellow. The calyx is equally five-lobed and shorter than the corolla tube. The corolla, with a cylindrical-tubular to globose tube, is often constricted at the base and the limb is more or less two-lipped. The upper lip is usually shorter than the lower, which is spotted or veined inside (Bräuchler et al. 2004). Several *Digitalis* species are used therapeutically, as they are the main source of cardiac glycosides and most of them are of great ornamental value.

Based on the morphological characterization of the genus *Digitalis* L. by Werner (1961, 1965), Luckner and Wichtl (2000) divided the genus into five sections: Frutescentes, Digitalis, Grandiflorae, Tubiflorae, and Globiflorae. A short characteristic of each sectio is outlined in Table 5.1.

Currently, the genus *Digitalis* comprises 23 species (Table 5.2) including the four species of the former genus *Isoplexis* (Bräuchler et al. 2004; Herl et al. 2008). A detailed discussion about the molecular phylogeny of the genera *Digitalis* was published by Bräuchler et al. (2004). As a result a different subsectio pattern was created: Digitalis (*D. minor* L. syn. *D. dubia* Rodr.; *D. purpurea* L.; *D. thapsi* L.; *D. purpurea* subsp. *toletana; D. mariana* Boiss. subsp. *mariana; D. mariana* subsp. *heywoodii* P. et M. Silva); expanded Macranthae (*D. ciliata* Trautv., *D. viridiflora* Lindl.; *D. grandiflora* Mill.; *D. davisiana* Heyw.; *D. atlantica* Pomel; *D. lutea* L. subsp. *australis* [Corsica]; *D. lutea* L. subsp. *lutea*); Isoplexis (*D. sceptrum* Loudon Masf.; *D. chalchantha* Svent. & O'Shan. Albach, Bräuchler & Heubl; *D. isabelliana* Loudon; *D. canariensis* Loudon: *D. canariensis* Loudon subsp. *trichomana*); Parviflorae (*D. parviflora* Jacq.), Frutescentes (*D. obscura* L. emend. Pau; *D. obscura* subsp. *laciniata*), Subalpinae/Tubiflorae (*D. lutea* L. subsp. *australis* [Tuscany]; *D. subalpina* Br.-Bl.); and Globiflorae (*D. ferruginea* L. subsp. *schischkinii; D. ferruginea* L. subsp. *ferruginea; D. laevigata* Waldst. subsp. *laevigata; D. levigata* subsp. *graeci; D. nervosa* Steud.; *D. cariensis* Boiss. subsp. *trojana; D. lanata* subsp. *leucophaea; D. lanata* subsp. *lanata*). Starting from morphological and biogeographical data several relationships of the genera in connection with the molecular aspects were found more recently (Carvalho and Culham 1997, 1998; Nebauer et al. 2000).

Only two species, viz. *Digitalis lanata* Ehrh. and *Digitalis purpurea* L. are of economic interest. The Grecian foxglove (*D. lanata*, Fig. 5.1) is preferred over *D. purpurea* (Fig. 5.2) as a source of glycosides for pharmaceutical industry (Bown 1995).

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Table 5.1 Sectio, subsectio, and species of the genus *Digitalis* (modified after Luckner and Wichtl 2000)

Section	Name	Features
I.	Frutescentes BENTH.	Small shrubs, plants non-hairy (except the flowers), leaves glossy, flowers in short clusters
II.	Digitalis	Biennial or short-lived perennial herbal plants, mostly thickly covered with hairs, bell-shaped, purple to white colored crowns, insight points or marks
III.	Grandiflorae BENTH. emend. WERNER	Short-lived perennial herbal plants, rosettes, differently covered with hairs, bell-shaped yellow flowers, upper site with dark veins
IV.	Tubiflorae BENTH.; subsection: *Acutisepalae*, *Obtusisepalae*	Short-lived perennial herbal plants, rosettes, differently covered with hairs
V.	Globiflorae BENTH.; subsection: *Hymenosepalae*, *Blepharosepalae*	Short-lived perennial herbal plants, rosettes, differently covered with hairs, smooth leaves sometimes leather-like surface

Table 5.2 Species of the genus *Digitalis*

Werner (1965)	Bräuchler et al. (2004)	Subspecies
D. atlantica Pomel	*D. atlantica*	
D. cariensis Boiss.	*D. cariensis*	*lamarckii* *trojana* *cariensis*
D. ciliata Trautv.	*D. ciliate*	
D. davisiana Heyw.	*D. davisiana*	
D. ferruginea L.	*D. ferruginea*	*ferruginea* *schischkinii*
D. grandiflora Mill.	*D. grandiflora*	
D. heywoodii P. et M. Silva	*D. heywoodii*	*mariana*
D. laevigata Waldst.	*D. laevigata*	*laevigata* *graeca*
D. lanata. Ehrh.	*D. lanata*	
D. lutea L.	*D. lutea*	*lutea* *australis*
D. mariana Boiss.	*D. mariana*	
D. dubia Rodr.	*D. minor*	
D. nervosa Steud.	*D. nervosa*	
D. obscura L.	*D. obscura*	*oscura* *laciniata*
D. parviflora Jacq.	*D. parviflora*	
D. purpurea L.	*D. purpurea*	Several ssp.
D. subalpina Br.-Bl.	*D. subalpina*	Several ssp.
D. thapsi L.	*D. thapsi*	
D. viridiflora Lindl.	*D. viridiflora*	
I. isabelliana (Webb & Berthel.) Morris	*D. isabelliana (Webb) Linding*	
I. canariensis (L.) Loudon	*D. canariensis* L.	Several ssp.
I. chalcantha Svent. & O'Shan.	*D. chalcantha* (Svent. & O'Shan.) Albach, Brüchler & Heubl	
I. sceptrum (L.) Loudon	*D. sceptrum* L.	

5.1.2 Geographical Distribution

The genus *Digitalis* is mainly distributed throughout two large geographical areas: the Iberian Peninsula, northwestern Africa; Macaronesia and Balkan Peninsula, Asia Minor. The areas in between do not have large numbers of species, for example, in Germany one can find only three species (*D. purpurea, D. grandiflora,* and *D. lutea*).

A similar situation is described for the Caucasian Mountains where *D. nervosa*, *D. ciliata*, and *D. ferruginea* (ssp. *schischkinii*) can be found. The geographical

Fig. 5.1 *Digitalis lanata* Ehrh. (reproduced with permission of Iris Voswinkel). (http://home.kpn.nl/wink0396/tuin/en/plants/DigitalisLanata.html)

Fig. 5.2 *Digitalis purpurea* L. (Prof. M. Costa. University of Valencia, Spain)

distribution of the species neatly coincides with their taxonomic relationship (Werner 1964). The distribution of some *Digitalis* species is restricted to small areas. Figure 5.3 shows the distribution of the genus *Digitalis*. Similar maps are also available for all other species of the genus (Luckner and Wichtl 2000). Some members of the genus are also familiar in the USA. *D. purpurea* is flowering in the northern states, whereas *D. lanata* and *D. lutea* can be found in the northeastern states. All members of the sectio Isoplexis represent species endemically growing in restricted areas in the Macaronesian region (excl. Azores; Sventenius 1968).

5.1.3 Taxonomy, Evolution, and Phylogenetics

Reviews on the botany of foxglove (*Digitalis*) date back to the 1950–1965 (Ivanina 1955; Werner 1960, 1965; Melchior 1964). Botanists at that time supported the well-known nomenclature and taxonomy of Werner (1965, 1966) who described 19 species of *Digitalis* and three species of *Isoplexis*. Subsequently, Sventenius (1968) described a new species, namely *Isoplexis chalcantha* Svent. et O'Shan. Later on, several authors tried to upgrade the taxonomy but their contributions are still under discussion (Bocquet and Zerbst 1974; Hinz et al. 1986; Hinz 1987b, 1989a, b, 1990a, b).

Werner (1960, 1961, 1964, 1966), summarizing the most prominent morphological characteristics, divided the genus into five sectios, viz. Frutescentes, Digitalis, Grandiflorae, Tubiflorae, and Globiflorae (Fig. 5.4). This classification was again very strongly based on morphological parameters.

The sectio Frutescentes represents only D. obscura. Five species form the sectio *Digitalis* including *D. thapsi, D. dubia, D. heywoodii, D. mariana,* and *D. purpurea*. Sectio Grandiflorae contains *D. grandiflora*, *D. atlantica*, *D. davisiana,* and *D. ciliata*. The sectio Tubiflorae includes *D. lutea*, *D. subalpina*, *D. viridiflora*, and *D. parviflora*. The sectio Globiflorae encompasses *D. laevigata*, *D. nervosa*, *D. ferruginea*, *D. lanata,* and *D. cariensis*. Finally, the sectio Isoplexis was introduced by Loudon (1829) and Bentham (1835). Both authors referred to the high similarity of several morphological parameters to the closely related *Digitalis* species, especially *D. obscura*. *Isoplexis* was first raised to generic rank by Loudon (1829). In 1968, *I. chalcantha* Svent. et O'Shan. was described for the first time. The reorganized sectio Isoplexis in the genus *Digitalis* now comprises four species, viz. *D. sceptrum, D. canariensis*, *D. isabelliana,* and *D. chalcantha* (Bräuchler et al. 2004).

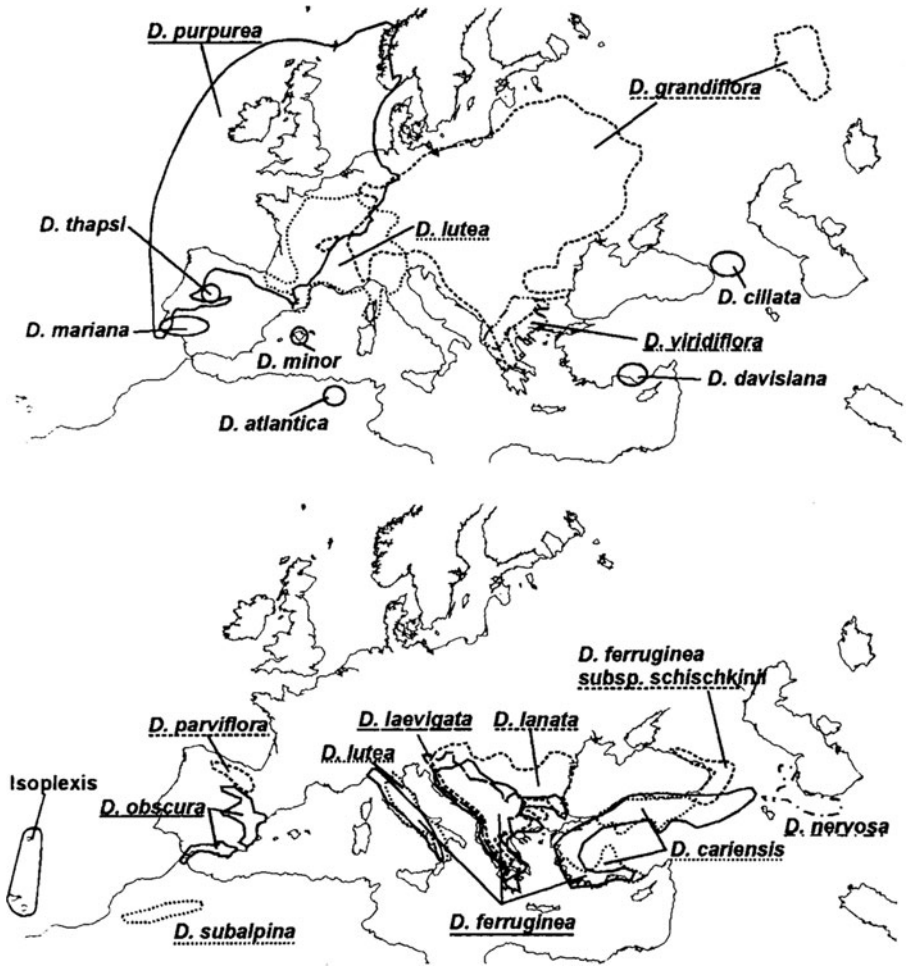


Fig. 5.3 Geographical distribution of the genus *Digitalis* (Bräuchler et al. 2004)

Interestingly, *Digitalis* and *Isoplexis* have common morphological features. Phytochemical analysis also revealed similar cardenolide patterns (see below). Both genera traditionally have been placed within the family Scrophulariaceae of the Lamiales. The analysis of the Scrophulariaceae by Olmsted et al. (2001) using three plastid genes (*rbc*L, *ndh*F, and *rps*2) revealed at least five distinct monophyletic groups. The tribus Digitaleae was placed into the Veronicaceae together with the tribes of Angelonieae, Antirrhineae, Gratioleae, Cheloneae, and the "traditional" families Callitrichaceae, Globulariaceae, Hippuridaceae, and Plantaginaceae. Albach and Chase (2004) demonstrated several incongruences in this newly formed Veronicaceae. Later on, Albach et al. (2005) analyzed this clade in a phylogenetic study of 47 members of Plantaginaceae and could arrange together the "new" Plantaginaceae. Oxelman et al. (2005) disintegrated further the Scrophulariaceae and finished the revision of this family. As a result of these considerations the newly circumscribed Plantaginaceae was established, in which *Digitalis* and many related genera form a clade with *Plantago*, which is well separated from *Scrophularia*.

Based on a molecular phylogenetic investigation of the genera *Digitalis* and *Isoplexis* using internal transcribed spacer (ITS)- and *trn*L-F sequences, it was shown that *Isoplexis* is nested within the genus *Digitalis* (Bräuchler et al. 2004). However, the use of nrDNA and plastid DNA regions has sometimes been insufficient to fully resolve species level relationships. Kelly and Culham (2008) used *MAX4*-like genes as a phylogenetic utility to resolve the *Isoplexis*/*Digitalis* issue. The analysis of the *MAX4*-like dataset revealed

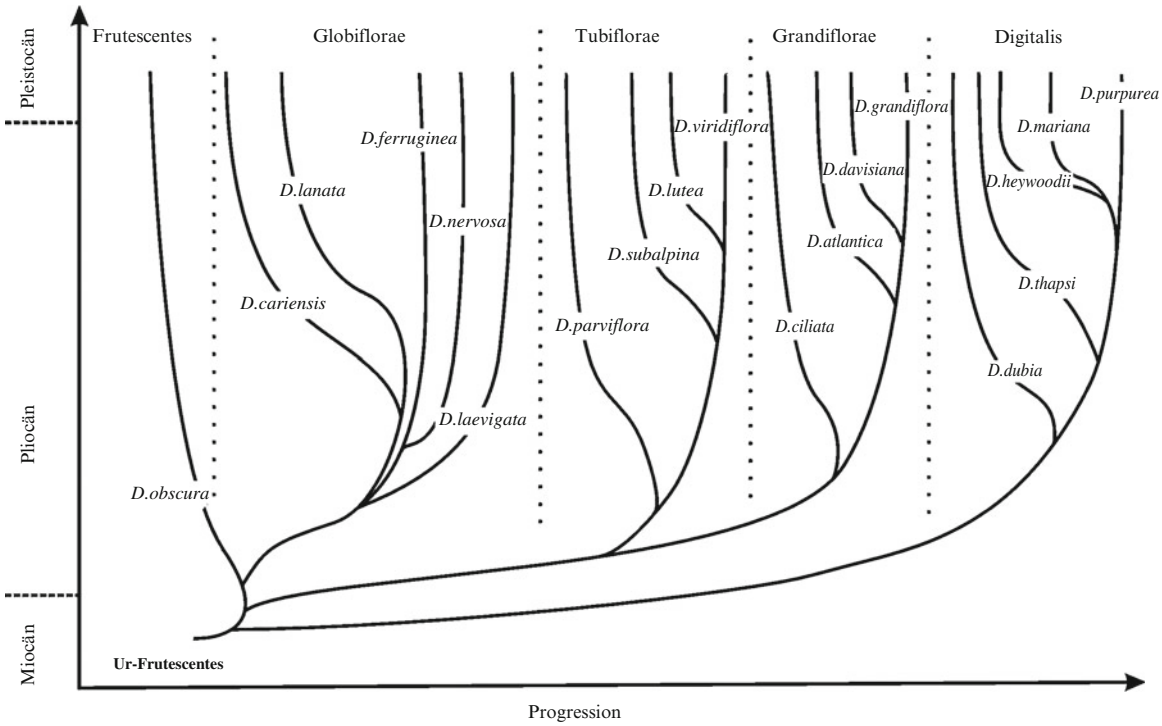


Fig. 5.4 Phylogenetic origin and relationship of the *Digitalis* species as proposed by Luckner and Wichtl (2000). The sectio Isoplexis was not included here

a high degree of incongruence with the molecular phylogeny deduced by Bräuchler et al. (2004). It was admitted, however, that the incidence of paralogy restricts the use of *MAX4*-like genes. Herl et al. (2008) sequenced the progesterone 5β-reductase genes (*P5βR*) of more than 20 species of *Digitalis* and *Isoplexis* to infer phylogenetic relationships. This gene was chosen as a marker for plant secondary metabolism and compared to the previously used nuclear ITS- and plastid *trn*L-F sequences (Bräuchler et al. 2004). The results show high congruence within the genus *Digitalis* and support the conclusion that all species of *Isoplexis* have a common origin and should be embedded in the genus *Digitalis*. Hence, the *Isoplexis* species should be reintegrated into the genus *Digitalis* as *D. isabelliana* (Webb.) Linding, *D. canariensis* L., *D. chalchantha* (Svent. & O'Shan.) Albach, Bräuchler & Heubl and *D. sceptrum* L.

5.1.4 Hybrids

The number of naturally occurring hybrids is limited, which is not surprising because of the geographical distribution of the individual species. In 1777, Koelreuter first performed backcrossing experiments on *Digitalis* to generate hybrids. Years later, hybrids were analyzed more thoroughly (von Gärtner 1849; Focke 1881; Wilson 1906; Jones 1912; Hill 1929). In the meanwhile, the number of *Digitalis* hybrids produced artificially has been very high. They differ very much in morphology and cardenolide content (Michaelis 1929). Some of the hybrids are allopolyploids and completely fertile, e.g., *D. grandiflora* × *D. lutea* or *D. purpurea* × *D. lutea* (Stein 1963). Detailed analysis does exist for a number of hybrids, such as *D. cariensis* ssp. *lamarckii* × *D. ciliate*; *D. cariensis* ssp. *lamarckii* × *D. lanata*; *D. ferruginea* × *D. ciliata*; *D. ferruginea* × *D. lutea*. *D. sibirica* seems to be a hybrid derived from *D. grandiflora* and *D. laevigata* or *D. grandiflora* and another *Globiflorae* species. For details, see Luckner and Wichtl (2000).

5.1.5 Agricultural Status

Heeger (1956) reported about the possibilities for cultivation of several *Digitalis* species. Nevertheless,

plants of *D. lanata* as raw material are of most use in big scale for cardenolide production in Europe. Less plant material to be used for extraction is produced with *D. purpurea* mostly in small farms or agricultural companies. *D. purpurea* is also cultivated for the production of homeopathic stocks from fresh leaves.

5.2 Basic Phytochemistry of *Digitalis*

Many secondary metabolites of several biosynthetic groups of compounds have been identified in the various members of the genus *Digitalis* (incl. *Isoplexis*) the most important among them being the cardioactive glycosides of the cardenolide type. These compounds will be reviewed in more detail here than phenolic (anthranoids, phenylpropanoic acids, flavonoids) or other steroidal (steroidal saponins, sterols) compounds (see Luckner and Wichtl 2000 and the references therein).

5.2.1 Cardiac Glycosides

The therapeutic action of cardiac glycosides depends on the structure of the aglycone and on the type of sugar (or sugar chain) attached to it. Two types of aglycones are known, namely the cardenolides, bearing an α,β-unsaturated five-membered lactone ring (butenolide) at C-17β, and the bufadienolides, with a six-membered lactone ring (cumaline). Members of the genus *Digitalis* contain cardenolides only. The stereochemistry of these compounds is important for their biological activity. The typical *Digitalis* cardenolides (e.g., digoxin, digitoxin, methyldigoxin) are characterized by a steroid nucleus with its rings connected *cis–trans–cis*, possessing a 14β-hydroxyl group, at position 3β a sugar side chain with up to five carbohydrate units is attached containing glucose and various rare 6-deoxy, 2,6-dideoxy, and 6-deoxy-3-methoxy sugars, such as D-fucose, D-digitoxose, or D-digitalose (Fig. 5.5). More than 100 different cardenolides have been isolated from *Digitalis* species of which all have been analyzed for the occurrence of this important group of plant secondary metabolites.

The members of the Sectio Digitalis are usually rich in derivatives of gitoxigenin and lack cardenolides of the digoxin type. The relative content in tetrasaccharides is usually high.

The most important species in the Digitalis sectio is the purple foxglove (*D. purpurea*), which is common in Europe and northern America. *D. purpurea* is potentially very toxic due to the cardenolide content (about 40 different structures, including derivatives of digitoxin, gitoxin, and gitaloxin) of up to 0.5% in the leaves.

Digitalis minor is a species endemic to the eastern Balearic Islands (Mallorca, Menorca, and Cabrera) that occurs in two morphologically varieties: *D. minor* var. *minor* (pubescent) and *D. minor* var. *palaui* (glabrous). *D. minor* is believed to be a schizoendemic vicariant of *D. purpurea* ssp. *purpurea* (Contandriopoulos and Cardona 1984).

Digitalis thapsi, an endemic of the west of the Iberian Peninsula, is commercialized as a perennial outdoor ornamental.

Digitalis mariana is a drought tolerant, perennial species with evergreen foliage and a succession of compact stems with deep rose red flowers all summer. *D. mariana* subsp. *heywoodii* is native to a small area in Portugal and is sometimes also referred to as a subspecies of *D. purpurea* L. Differences and similarities between the members of the Sectio Digitalis have been investigated from botanical and phytochemical perspectives (see Luckner and Wichtl 2000).

The members of the Extended Macranthae contain gitoxigenin derivatives but no digoxigenin derivatives (exception: *D. viridiflora*). Tetrasaccharides are not very dominant or even absent in several species.

Digitalis ciliata is native to the Caucasus and bears its epitheton because of the very fine hairs covering the stalks and even the yellow flowers.

Digitalis viridiflora is a perennial species native to woods in the Balkans. It resembles *D. lutea* with pale greenish yellow flowers with a brownish tinge.

Digitalis lutea (Central Europe) bears small yellow flowers and is a rather short-lived perennial plant. This species is often grown in gardens.

Other species in the sectio Extended Macranthae are *D. grandiflora* (Central Europe), *D. davisiana* (Turkey), and *D. atlantica* (northwestern Africa).

The sectio Isoplexis is represented by four species. Three species are endemic to the Canary Islands and one (*D. sceptrum*) to Madeira. All species but *I. sceptrum* contain cardiac glycosides of the cardenolide type. *D. chalcantha* may be regarded as the most

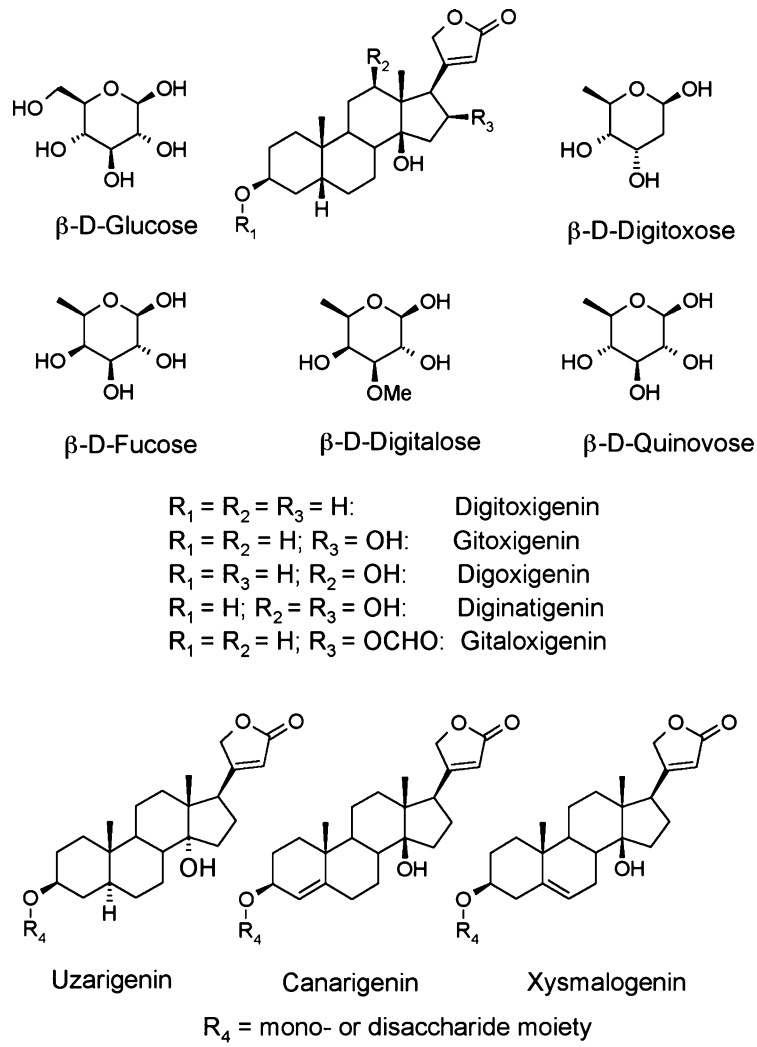


Fig. 5.5 Cardenolide genins and sugars forming the glycosidic part of the cardenolides found in *Digitalis lanata*. Cardenolides with uzarigenin, canarigenin, and xysmalogenin structure are only found in three members of the sectio Isoplexis

endangered species among them since it is native to a few places on Gran Canaria. The members of this section are regarded as more primitive than the other *Digitalis* species thereby, their cardenolides may also be regarded as "more primitive" because (a) only mono- and diglycosides are produced and (b) that 5β-configured cardenolides (uzarigenin type) as well as Δ4 and Δ5 unsaturated cardenolides of the xysmalogenin and canarigenin type occur side by side with the "typical" 5β-cardenolides (Freitag et al. 1967; Gonzales et al. 1985; Gavidia et al. 2002; Schaller and Kreis 2006).

Digitalis parviflora (sectio Parviflorae), which is native to mainland Spain, is a hardy perennial with tall spikes of densely packed, brown flowers and evergreen leaves. Cardenolides of the digoxigenin type are absent.

Digitalis obscura (sectio Frutescens) comes from the mountains of Spain and is an attractive foxglove with narrow evergreen foliage and pendulous flowers in burnt orange and beige with red veins. It is a close relative to the members of the sectio Isoplexis. It does not contain cardenolides of the digoxigenin type.

Digitalis subalpina (sectio Subalpinae) is native in the Atlas Mountain. No remarkable differences in the cardenolide pattern of the four varieties could be found.

Subalpinoside (oleandrigenin glucodigitoxoside) is the main cardiac glycoside in all varieties (Lichius et al. 1992). It does not contain cardenolide tetrasaccharides or digoxigenin derivatives.

Most of the members of the Globiflorae are rich in lanatosides, i.e., tetrasaccharides bearing an acetyldigitoxose and a terminal glucose. They contain cardenolides of the digoxigenin series (exception: *D. nervosa*, *D. lavigata*). Actually, digoxigenin derivatives seem only to be present in this section (exception: *D. viridiflora*, see above).

The section Globiflorae contains the commercially most important *Digitalis* species, namely *D. lanata* (Grecian foxglove). The species, native to Italy, the Balkans, Hungary, and Turkey, is preferred over *D. purpurea* as a source of glycosides for the pharmaceutical industry (Bown 1995).

Digitalis laevigata is a rare perennial foxglove from southern Europe, with distinctive red stems and purple-veined orange-yellow flowers with a white lower lip.

Digitalis ferruginea (Rusty foxglove) is a native of the northern Mediterranean. The brownish flowers have red to dark brown veins.

Other species in this sectio are *D. nervosa* (rare plant from northern Turkey) and *D. cariensis*.

5.2.2 Digitanols

Digitanols are C_5–C_6 unsaturated C_{21}-pregnanes (e.g., Tschesche and Buschauer 1957; Satoh et al. 1962; Tschesche 1966; Liedke and Wichtl 1997). Interestingly, some of them possess the 14β-hydroxyl function typical for cardenolides. Digitanols and cardenolides may, therefore, share part of their respective biosynthetic pathways. Thus, digitanols may also be regarded as degradation products of cardenolides. Usually they bear oxygen functions at C_{15}, have a sugar side chain attached at C_3, and may occur as tetracyclic or pentacyclic compounds. In the latter case, C_{12} and C_{20} are bridged with an oxygen to form a tetrahydrofurane. Known digitanol genins are digiprogenin, digipurpurogenin, purpnigenin, purprogenin, digacetigenin, digifoligenin, and diginigenin (Fig. 5.6).

5.2.3 Steroidal Saponins

Saponins are classified in triterpenoid and steroidal saponins. They all have surfactant and soap-like properties and cause hemolysis. Steroidal saponins, though occurring in the genus *Digitalis*, are rare in dicotyledonous plants and quite abundant in monocotyledons. Steroidal saponins are derived from C_{27} sterols in which the side chain of cholesterol has undergone structural modifications to form a spiroketal. Interestingly, cholesterol was detected in several *Digitalis* species contributing significantly to the total sterol pool (e.g., Jacobsohn and Frey 1968; Helmbold et al. 1978). The steroidal saponins found in *Digitalis* are neutral compounds with a weak saponin character only. They may occur as furostanol-based bisdesmosides or spirostanol-based monodesmosides. Typical sapogenins found are digitonin, tigogenin, and gitogenin (e.g., Tschesche et al. 1972, 1974). Members of the tribus *Isoplexis* are characterized by steroidal glycosides derived from crestagenin, sceptrumgenin, funchaligenin, and the isoplexigenins A–D (e.g., Delgado Benitez et al. 1969; Freire et al. 1970) (Fig. 5.7).

5.2.4 Anthranoids

About 40 different anthraquinones have been identified in the genus *Digitalis* (e.g., Imre et al. 1971, 1976; Luckner and Wichtl 2000), such as digitolutein and other compounds derived via the so-called alizarin pathway, i.e., a synthetic route for the formation of an anthraquinone skeleton by cyclizing a dimethylallyl substituent on to a naphthaquinone system. Digitolutein seems to be a typical compound of all *Digitalis* species but not the members of the sectio Isoplexis (Imre et al. 1976; Fig. 5.8).

5.2.5 Other Secondary Metabolites

Phenols. Phenolic glycosides were isolated from the leaves of *D. purpurea* as well as from other species (Matsumoto et al. 1987; Lichius et al. 1995). In a chemosystematic investigation of Digitalideae, the water-soluble part of extracts of *D. thapsi*, *D. purpurea*, *D. chalcantha,* and *D. sceptrum*, as well as *Erinus alpinus* and *Lafuentea rotundifolia* were studied with regard to their content of main carbohydrates, iridoids, and caffeoyl phenylethanoid glycosides (Taskova et al. 2005). The *Digitalis* species contained sorbitol, cornoside, and a number of other

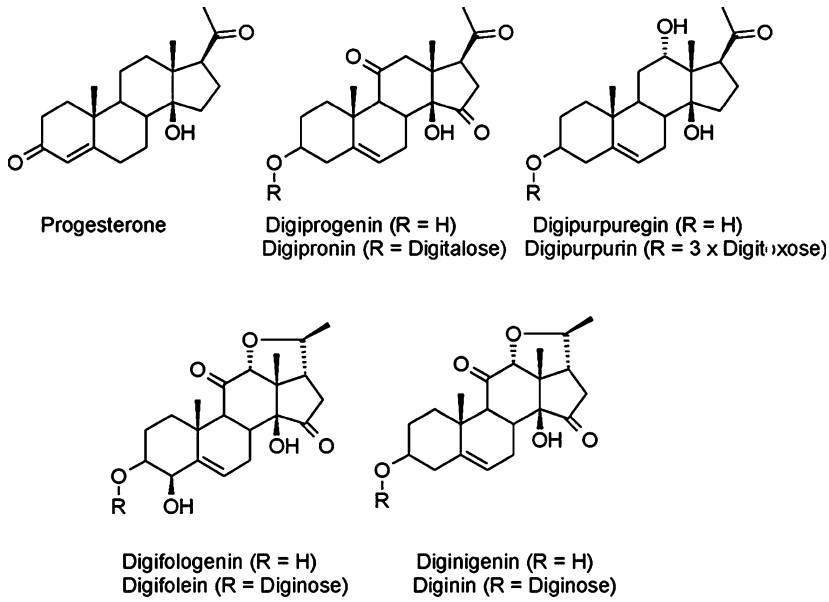


Fig. 5.6 Tetracyclic and pentacyclic digitanols and digitanol glycosides found in *Digitalis* species

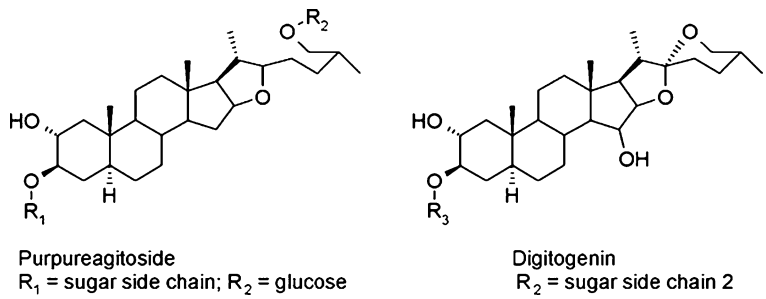
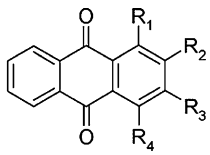


Fig. 5.7 Furostanols and spirostanols isolated from various *Digitalis* species (examples)



Digiferruginol (R_1 = OH; R_2 = CH_2OH; R_3 = R_4 = H)
Digiferrol (R_1 = OH; R_2 = H; R_3 = CH_2OH; R_4 = OH)
2-Methylchinizarin (R_1 = OH; R_2 = CH_3; R_3 = H; R_4 = OH)
Pachybasin (R_1 = OH; R_2 = H; R_3 = CH_3; R_4 = H)
Digitolutein (R_1 = OCH_3; R_2 = OH; R_3 = CH_3; R_4 = H)
Madeirin (R_1 = OCH_3; R_2 = H; R_3 = CH_3; R_4 = OH)
3-Methylpurpurin (R_1 = OH; R_2 = OH; R_3 = CH_3; R_4 = OH)

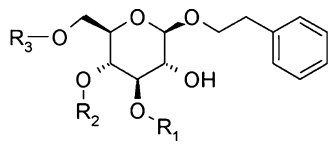
Fig. 5.8 Anthranoids isolated from various *Digitalis* species (examples)

phenylethanoid glycosides including the new tyrosol β-D-mannopyranoside, sceptroside but were found to lack iridoid glucosides (Fig. 5.9).

Flavonoids. Most of the *Digitalis* species have also been investigated with respect to their flavonoid content and pattern (see Luckner and Wichtl 2000 for review). About 40 different flavonoids mainly of the flavone and 3-methoxyflavone group have been described, among them digicitrin, the most highly oxygenated naturally occurring flavonoid substance (Meier and Fürst 1962).

5.2.6 Other Metabolites of Pharmaceutical Relevance

Sterols. Besides the common phytosterols, such as sitosterol or stigmasterol, *Digitalis* species also contain rare sterols. Steryl esters and steryl glycosides have been described as well (Evans 1973; Jacobsohn and Frey 1968; Jacobsohn and Jacobsohn 1976).



Verbascoside (R_1 = rha; R_2 = caff; R_3 = H)
Calceolarioside A (R_1 = H; R_2 = Caff; R_3 = H)
Calceolareoside B (R_1 = H; R_2 = H; R_3 = caff)
Purpureaside A (R_1 = gluc; R_2 = caff; R_3 = H)
Purpureaside B (R_1 = gluc; R_2 = rha; R_3 = H)
Purpureasside C (R_1 = rha; R_2 = caff; R_3 = gluc)
Maxoside (R_1 = gluc; R_2 = caff; R_3 = gluc)

Fig. 5.9 Phenolic glycosides isolated from various *Digitalis* species (examples)

Polysaccharides. Polysaccharides were isolated in yields of up to 4 mg/mL from the culture media of suspension-cultured cells from *D. lanata*. Methylation analysis indicated the neutral polysaccharide fractions to contain xyloglucans besides minor amounts of highly branched arabinogalactans. In addition, an acidic arabinogalactan was isolated. Four main crude polysaccharide fractions, which represented the complete polymeric carbohydrate pool, were isolated by sequential extraction from *D. purpurea* leaves. The water soluble reserve polysaccharides were mainly composed of neutral and acidic arabinogalactans, neutral and acidic glucomannans, and starch. Pectic material was identified as rhamnogalacturonan. Hemi-cellulosic cell wall polysaccharides consisted of a neutral, low substituted arabinoxyloglucan and several acidic xylans. Hemi-cellulosic polymers associated with cellulose were shown to be highly branched xyloglucans. Interestingly, 2,6-dideoxysugars, the typical carbohydrate components of cardiac glycosides of several *Digitalis* species, were not detected in these polysaccharides (Hensel et al. 1997, 1998).

5.2.7 Species Used for the Technical Production of Cardenolides

The main sources for the cardenolides used in therapy are *D. lanata* and *D. purpurea*. Both species are cultivated for this purpose. The cardenolides of interest are digoxin, lanatoside C, digitoxin, acetyldigoxin (Fig. 5.10), and the semi-synthetic methyldigoxin.

5.2.7.1 *Digitalis lanata*

Cardenolides. The total cardenolide content is about 0.9–1.5% dry weight.

Major constituents: lanatoside C, lanatoside A, glucolanadoxin, digitalinum verum, glucogitoroside, glucoverodoxin, glucoevatromonoside.

Minor constituents: digitoxigenin glucosidoglucomethyloside, *neo*-glucodigifucoside, glucodigifucoside, lanatoside B, digoxigenin glucosidobisdigitoxoside, lanatoside E, glucogitofucoside, desacetyllanatoside C, *neo*-digitalinum verum, digitoxigenin-glucosidoallomethyloside, acetyldigitoxin, acetyldigoxin, digoxin, lanadoxin, strospeside, gitoroside.

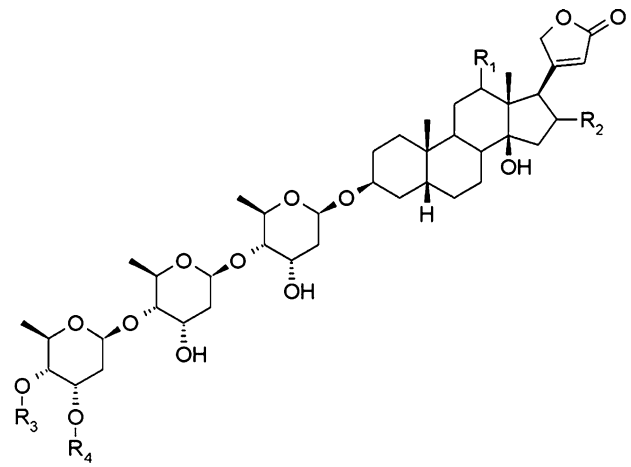
Trace constituents: verodoxin, lanatoside D, purpureaglycoside A, *neo*-lanatoside C, digitoxigenin glucosidobisdigitoxoside, purpureaglycoside B, *neo*-odorobioside G, digitoxin, glucogitaloxin, acetylgitoxin, digiproside, digitoxigenin glucomethyloside, digitoxigenin glucosido acetylglucomethyloside, neodesacetyl lanatoside C, acetylgitaloxin, gitoxin, evatromonoside, odoroside H, digoxigenin bisdigitoxoside, gitaloxin, digitoxigenin glucoside, acetyldiginatin, gitoxigenin fucoside, digitoxigenin allomethyloside (Kaiser 1966; Imre et al. 1981; Wiegrebe and Wichtl 1993).

Digitanols. Progesterone, digipronin, digifolein, lanafolein, glucodigifolein, glucolanafolein, 14β-hydroxydigitalonin (Tschesche and Buschauer 1957; Satoh et al. 1962; Tschesche and Brügmann 1964; Wurst et al. 1983; Liedke and Wichtl 1997).

Saponins. Lanagitoside, lanatigoside, digalogenin, neodigalogenin, lanadigalonin I, neogitogenin, neogitogenin, tigogenin, neotigogenin, tigonin, lanatigonin I (Tschesche and Balle 1963; Tschesche et al. 1972).

Anthranoids. 2-methyl-chinizarin, 3-methyl-purpurine, digitolutein, 4-hydroxydigitolutein, 3-methylalizarin, 1-methoxy-2-methyl anthraquinone, 2-methoxy-3-methyl anthraquinone (Burnett and Thomson 1968; Imre et al. 1976).

In their natural environment, *D. lanata* plants may contain levels of cardenolides lower than 0.5% since high-yielding varieties have been selected for cultivation with a view to producing cardenolides. The glycoside pattern also may vary. For example, the content of the main cardenolides in *D. lanata* cultivated in Brazil was determined in two different stages of growth. The analyzed plants presented great variation in the contents of lanatoside, digoxin, lanatoside A,



$R_1 = R_2 = R_3 = R_4 = H$:	Digitoxin
$R_1 = R_2 = R_4 = H$; R_3 = glucosyl:	Purpureaglycoside A
$R_1 = R_2 = R_3 = H$; R_4 = acetyl:	β-Acetyldigitoxin
$R_1 = R_2 = H$; R_3 = glucosyl; R_4 = acetyl:	Lanatosid A
R_1 = OH; $R_2 = R_3 = R_4 = H$:	Digoxin
R_1 =OH; $R_2 = R_3 = H$; R_4 = acetyl:	β-Acetyldigoxin
R_1 = OH; $R_2 = H$; R_3 = glucosyl; R_4 = acetyl:	Lanatoside C
$R_1 = R_3 = R_4 = H$; R_2 = OH:	Gitoxin
$R_1 = R_4 = H$; R_2 = OH; R_3 = glucosyl:	Purpureaglycoside B

Fig. 5.10 Important primary and secondary glycosides isolated from *D. lanata* and *D. purpurea*

lanatoside B, glucoevatromonoside, odorobioside G, glucogitoroside, glucoverodoxine, glucodigifucoside, and digitalinum verum. The sum of the analyzed cardenolides in the 12-month-old plants was higher than the determined concentrations in plants collected 6 months later. Lanatoside A content decreased in the older plants, whereas lanatoside C showed the opposite trend (Castro Braga et al. 1997).

The main compounds seen in the fresh rosette leaves are the lanatosides, which constitute about 50–70% of the total cardenolides. Lanatosides resemble the purpureaglycosides (see below) but contain an acetyl ester function on the third (i.e., terminal) digitoxose. Drying of the leaf material is accompanied by partial hydrolysis of the "primary glycosides" and both the terminal glucose and the acetyl group can be released so that "secondary glycosides" will be produced during the technical process. This process is termed fermentation and is included as a defined step in the isolation process. *D. lanata* cardenolides are based on six aglycones, namely digitoxigenin, gitoxigenin, gitaloxigenin, digoxigenin, diginatigenin, oleandrigenin. Lanatoside A and C constitute the major components in the fresh leaf (about 50–70%) and are based on the aglycones digitoxigenin and digoxigenin, respectively. Lanatosides B, D, and E (gitoxigenin, diginatigenin, and gitaloxigenin derivatives, respectively, see Fig. 5.5) are minor components derived from gitoxigenin, diginatigenin, and gitaloxigenin, respectively. Enzymatic hydrolysis of lanatosides generally involves loss of the terminal glucose prior to removal of the acetyl function, so that compounds like acetyldigitoxin and acetyldigoxin as well as digitoxin and digoxin are present in the dried leaf as decomposition products from lanatosides A and C, respectively (Fig. 5.10).

5.2.7.2 *Digitalis purpurea*

Cardenolides. About 30 different cardenolides. Total content: rosette leaves, 1 year, approximately 0.4–1.0%; in leaves of flowering plants 0.3–0.8% dry weight.

Major constituents: purpureaglycoside A, glucogitaloxin, purpureaglycoside B.

Minor constituents: digitalinum verum, glucoverodoxin, digitoxin, gitaloxin, gitoxin, glucoevatromonoside,

glucolanadoxin, glucogitoroside, stropeside, verodoxin, glucogitaloxigenin bisdigitoxoside.

Trace constituents: glucodigitoxigenin bisdigitoxoside, odorobioside G, evatromonoside, glucodigifucoside, odoroside H, gitoroside, digiproside, digitoxigenin glucosidoglucomethyloside, digitoxigenin glucomethyloside (Kaiser 1966).

Anthranoids. Digitolutein, 3-methylalizarin, phomarin, phomarin 6-methylether, digitopurpon, 1-methoxy-2-methyl, 3-methoxy-2-methyl- and 1-methoxy-2-methyl-anthraquinone, 4-hydroxydigitolutein, pachybasine methyl ester (Burnett and Thomson 1968; Imre et al. 1976).

Digitanols. Digipronin, purpnin, purpronin digipurpurin, digacetihin glucdigfolein, diginin, glucodiginindigitalonin (Satoh et al. 1956, 1962; Tschesche 1966; Liedke and Wichtl 1997) (Fig. 5.6).

Saponins. Purpureagitoside, digalogenin, neodigalogenin, digalonin, digitogenin, beodigitogenin, digitonin, deglucodigitonin, gitogenin, neogitogenin, gitonin, tigogenin, neotigogenin, tigonin, degalactotigonin (Tschesche et al. 1962, 1972, 1974; Tschesche and Balle 1963; Tschesche and Wulff 1961; Fig. 5.7).

Other secondary compounds. Desrhamnosylacteoside, forsythiaside, purpureaside A and B, 3,4-dihydroxyphenethylalcohol-6-*O*-caffeoyl-β-D-glucoside.
Four phenolic glycosides were isolated from the callus tissue: purpureaside A, B, and C, acteoside (Matsumoto et al. 1987)

Though potentially toxic, *D. purpurea* is unlikely to be ingested by humans erroneously, mainly because of its bitter taste. Like *D. lanata* (see above) *D. purpurea* is cultivated for drug production. Rosette leaves are harvested in the first year and then dried at 60°C. This procedure inactivates but does not destroy glucosidases capable of hydrolyzing cardioactive glycosides, giving rise to various artifacts. Excess heat may cause dehydration in the aglycone to inactive Δ^{14}-anhydro compounds. It is interesting to note in this context that the hydrolyzed enzymes are more temperature tolerant than the cardiac glycosides (May and Kreis 1997). Because of the pronounced cardiac effects of *Digitalis* cardenolides, the variability in the cardiac glycoside content, the crude leaf drug is usually assayed biologically (e.g., isolated frog heart, anesthetized guinea pig). However, the crude drug is hardly ever used now, only few herbal or homeopathic preparations use the plant extract as a starting material.

The major components are based on the aglycones digitoxigenin, gitoxigenin, and gitaloxigenin. The glycosides comprise two series of compounds, those with a tetrasaccharide unit (primary glycosides) and those with a trisaccharide unit (secondary glycosides). As described for *D. lanata* (see above) the secondary glycosides are produced (at least in a great part) by hydrolysis from the respective primary glycosides during processing. Therefore, the principal genuine glycosides, viz. purpureaglycosides A and B, are almost completely converted into digitoxin and gitoxin, respectively. In the fresh leaf, purpureaglycoside A can constitute about 50% of the glycoside mixture whereas it is nearly absent in old or fermented drug. Digitoxin, released from purpureaglycoside A during controlled fermentation is the only compound used as a drug.

5.3 Biosynthesis of Compounds of Economic Value

5.3.1 Cardenolides

The most prominent compounds formed throughout the genus *Digitalis* (except for *D. sceptrum*) are the cardenolides. Only their biosynthesis will be considered here. Possible biosynthetic routes leading to the cardenolides are shown in Fig. 5.11. It shows, besides the "classical" pregnane pathway, the optional "norcholanic acid" pathway (see Kreis and Müller-Uri 2010 for a review).

5.3.1.1 Early Biosynthetic Studies

During the 1970s and 1980s, studies on the ability of cultured *Digitalis* cells to modify exogenous cardenolides were conducted (e.g., Reinhardt and Alfermann 1980; Rao and Ravishankar 2002). These studies will not be reviewed here although some of them lead to the identification of some steps in cardenolide-specific biosynthetic pathways. The isolation and characterization of enzymes and genes involved in pregnane and cardenolide metabolism have now allowed new insights into the secondary metabolite pathway that leads to cardenolides and may open the route for manipulating cardenolide biosynthesis in these plants.

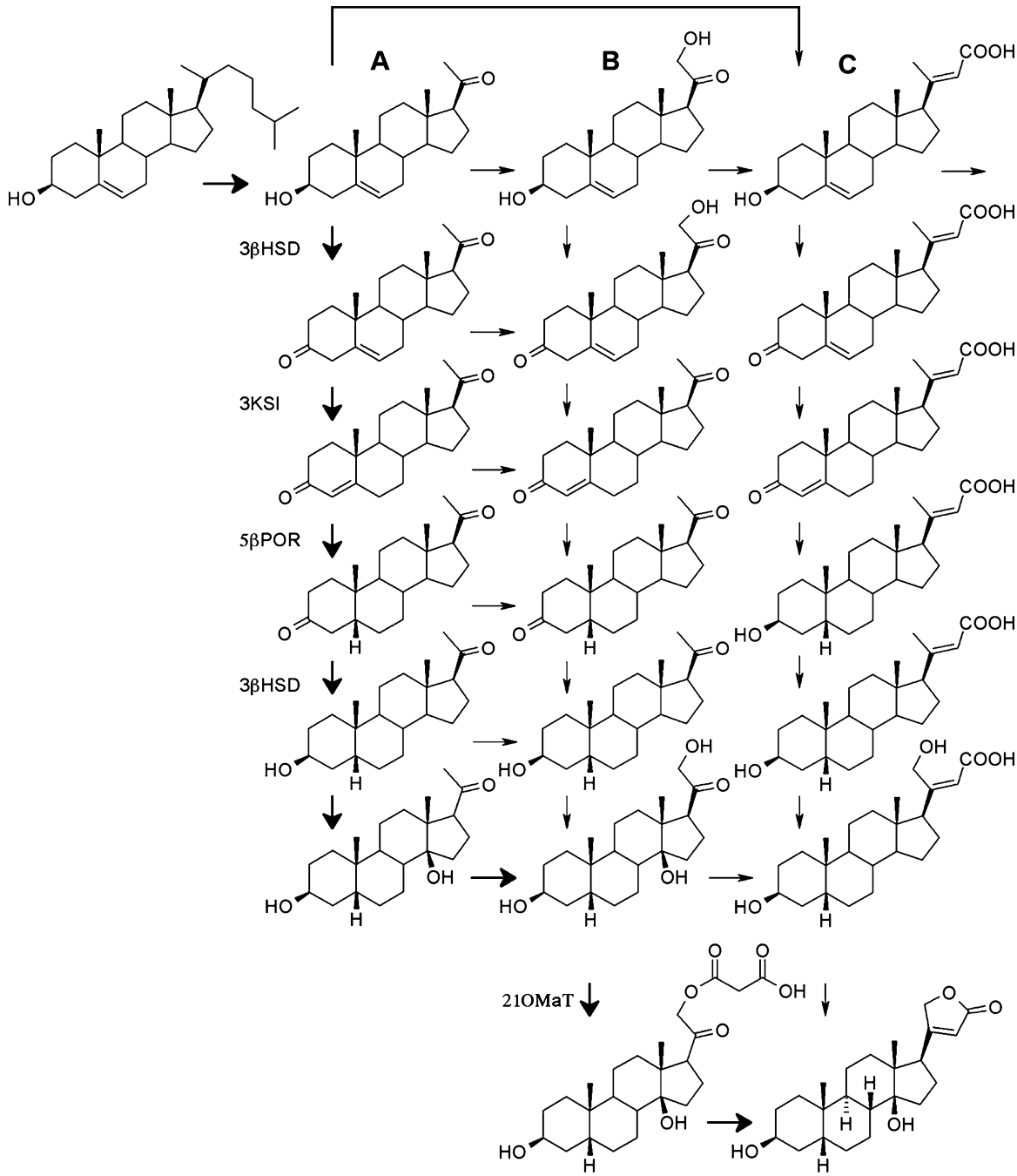


Fig. 5.11 Possible biochemical pathways leading to cardenolides. Starting from cholesterol, the actual textbook pathway (*Lane A*) is highlighted by *thick arrows*. Alternative pathways such as a "complete" norcholanic pathway (*Lane C*) may be operative. Steps generally assumed to be late steps in the pathway, such as 21-hydroxylation (*Lane B*) may also be much earlier events in the pathway. *3βHSD* 3β-hydroxysteroid dehydrogenase, *3KSI* 3-ketosteroid isomerase, *P5βR* progesterone 5β-reductase

Cardenolides are steroids and thus supposed to be derived from mevalonic acid via triterpenoid and phytosterol intermediates. Radiolabeled mevalonic acid is incorporated into the steroid part of digitoxin (Ramstad and Beal 1960) and chemical degradation revealed that the label of 2-^{14}C-mevalonic acid

appeared in C-1, C-7, and C-15 of the cardenolide genin (Gros and Leete 1965), which is consistent with a biosynthetic route via the mevalonic acid pathway. Later on, it was found that the carbon atoms C-22 and C-23 of the butenolide ring of the cardenolides are not derived from mevalonic acid (Gregory and Leete 1969). It was thus concluded that a pregnane has to be condensed with a C_2 donor, such as acetyl CoA or malonyl CoA, to yield the cardenolide genin. The early tracer studies leading to the proposed pathway of cardenolide formation in plants have been summarized, e.g., by Kreis et al. (1998).

More recently, feeding experiments with labeled C_{23} steroids revealed that 23-norcholanic acids can serve as cardenolide precursors. It has been shown that the radioactivity of side chain labeled appears in the butenolide ring thus indicating the incorporation of the C_{23} steroid without degradation (Maier et al. 1986). When 21-[^{3}H]-2β,20ξ-dihydroxy-23-nor-5β-cholanic acid was administered together with 21-[^{14}C]-3β-hydroxy-5β-pregnane-20-one the so-called norcholanic acid pathway was even the preferred route for cardenolide formation (Deluca et al. 1989). However, the carbon atoms C-22 and C-23 of the butenolide ring of the cardenolides are not derived from mevalonic acid, which should be the case in a "full" norcholanic acid pathway, where the respective intermediates directly derived from a sterol precursor.

5.3.1.2 Enzymes Involved in the Formation of Cardenolide Aglycones

Cholesterol is supposed to be a precursor of cardenolides during the formation of which the side chain of cholesterol has to be cleaved between C-20 and C-22. However, indirect evidence for a favored route not involving cholesterol was provided by studies with a specific inhibitor of 24-alkyl sterol biosynthesis. Feeding of 25-azacycloartanol led to an increase of endogenous cholesterol in *D. lanata* shoot cultures. On the other hand, the content of 24-alkyl sterols was dramatically reduced as was the content of cardenolides (Milek et al. 1997), indicating a route via typical phytosterols, such as campesterol or β-sitosterol.

In analogy to the formation of steroids in animals, this reaction is thought to be catalyzed by P_{450}scc ("cholesterol side-chain cleaving enzyme") which, however, has never been characterized in details in plants. The enzyme was associated with mitochondria and microsomal fractions of proembryogenic masses, somatic embryos, and leaves of *D. lanata* (Lindemann and Luckner 1997).

The conversion of pregnenolone into progesterone is composed of two steps: the first reaction is the NAD-dependent oxidation of the 3-hydroxy group yielding Δ^5-pregnen-3-one catalyzed by the Δ^5-3β-hydroxysteroid dehydrogenase; subsequently the double-bond shifts from position 5 to position 4. The animal enzyme (Pollack 2004) is involved in the conversion of progesterone to androgens and catalyzes two steps, namely the oxidation of the steroid substrate (3βHSD activity) and the subsequent isomerization of the intermediate (KSI activity). The protein is active as a dimer and the monomer has a molecular mass of about 42 kDa.

A plant 3βHSD was isolated from *D. lanata* cell suspension cultures as well as from shoot cultures and leaves of *D. lanata* plants (Seidel et al. 1990). NAD is the preferred proton acceptor of the enzyme that was purified having a molecular mass of 80–90 kDa as determined by size exclusion chromatography (Finsterbusch et al. 1999). First attempts to isolate the plant *3βHSD* gene were reported by Lindemann et al. (2000). Deduced oligonucleotide primers from peptide fragments obtained from the digestion of the 3βHSD isolated from *D. lanata* leaves (Finsterbusch et al. 1999) were used for the amplification of *3βHSD* gene fragments. Subsequently, Lindemann et al. (2000) amplified and sequenced a 700-nucleotide cDNA fragment for a putative *3βHSD*. Based on these reports, Herl et al. (2006b) generated primers for PCR amplification of the *D. lanata 3βHSD* gene. The isolated cDNA clone was nearly identical with the *3βHSD* gene sequence reported (Lindemann et al. 2000). PCR amplification of the fragments was performed with DNA templates from several *Digitalis* species. All genes were found to be of similar size and they did not differ much from each other or from their genomic fragments.

As already stated by Lindemann et al. (2000) the *3βHSD* shows some sequence similarities with microbial hydroxysteroid dehydrogenases and contains a conserved putative short chain dehydrogenase (SDR) domain. The *Digitalis 3βHSD* genes also share some similarities with putative alcohol dehydrogenase genes of *Arabidopsis thaliana*, *Lycopersicon esculentum*, *Oryza sativa*, *Nicotiana tabacum*, *Forsythia* × *intermedia*, *Solanum tuberosum,* and

Mentha × *piperita* (BLAST analysis). No obvious similarities with the animal 3βHSD/KSI were seen.

Molecular cloning and heterologous expression of the Δ^5-3β-hydroxysteroid dehydrogenase (*3βHSD*) from *D. lanata* was reported by Herl et al. (2007). In *Digitalis*, 3βHSD is a soluble enzyme and shares this property with other members of the SDR family (Janknecht et al. 1991; Oppermann and Maser 1996). In the presence of NAD, r*Dl*3βHSD converts pregnenolone to isoprogesterone. Besides pregnenolone, several other pregnanes were accepted as substrates. Testosterone, a C_{17} steroid with a 3-carbonyl group and a 17β-hydroxy group, was converted to 4-androstene-3, 17-dione indicating that rD/3βHSD possesses 3β- as well as 17β-dehydrogenase activity. The r*Dl*3βHSD was also able to catalyze the reduction of 3-keto-steroids when NADH was used as cosubstrate.

In many aspects, r*Dl*3βHSD behaves like the hydroxysteroid oxidoreductases supposed to be involved in cardenolide metabolism (Warneck and Seitz 1990; Seitz and Gärtner 1994). It was presumed (Finsterbusch et al. 1999; Herl et al. 2007) that 3βHSD catalyzes at least two steps in cardenolide biosynthesis, namely the dehydrogenation of pregnenolone and the reduction of 5β-pregnane-3,20-dione (Fig. 5.11). Occurrence of 3βHSD in other species not accumulating cardenolides indicates that the enzyme may also be involved in other metabolic pathways (Herl et al. 2008).

Dehydrogenase activity could clearly be separated from a ketosteroid isomerase (KSI, see below), indicating that r*Dl*3βHSD is related to microbial HSDs of the short-chain dehydrogenase/reductase (SDR) family but not with mammalian HSDs. Δ^5-3-Ketosteroid isomerase (KSI) catalyzes the allylic isomerization of the 5,6 double bond of Δ^5-3-ketosteroids to the 4,5 position by stereospecific intramolecular transfer of a proton.

It was shown that KSI activity was present in crude protein extracts prepared from *D. lanata* cell suspension cultures and leaves. From the latter source it was partially purified and it was found that KSI did not copurify with 3βHSD. The molecular mass of the enzyme is about 15 kDa as determined by SDS-PAGE (N Meitinger et al. unpublished). However, it is not yet finally clear whether KSI activity is also associated with the 3βHSD although circumstantial evidence implies that this is not the case. The spontaneous isomerization of 4-pregnene-3,20-dione represents a crucial problem and this may explain why 5-pregnene-3,20-dione was also found when 5-pregnene-3β-ol,20-one was used as substrate for the *D. lanata* or recombinant HSD (Finsterbusch et al. 1999; Herl et al. 2006b).

The progesterone 5β-reductase (P5βR) catalyzes the transformation of progesterone into 5β-pregnane-3, 20-dione (Gärtner et al. 1990, 1994). The P5βR requires NADPH as the cosubstrate, which cannot be replaced by NADH. The 43-kDa enzyme was purified to apparent homogeneity from the cytosolic fraction of shoot cultures of *D. purpurea*. The gene for P5βR of *D. obscura* (*Dop5βr;* AJ555127) was first identified by Roca-Pérez et al. (2004a). Herl et al. (2006b) reported the cloning and heterologous functional expression of P5βR from leaves of *D. lanata* Ehrh. The P5βR was amplified by RT-PCR from cDNA prepared from *D. lanata*, *D. purpurea,* and *D. obscura*. DNA fragments of nearly identical length were also obtained when genomic DNA of *D. purpurea* was used as template. The DNA fragments and the nucleotide sequences obtained from *D. lanata*, *D. purpurea,* and *D. obscura* did not differ in size. The sequence of the genomic clone contained a small intron.

It seems as if the *P5βR* genes are highly conserved within the genus *Digitalis* (Herl et al. 2008). The deduced P5βR protein sequences were found similar to those of *Oryza sativa* (about 58%) and *Populus tremuloides* (about 64%). Interestingly, no obvious similarities were found with the pulegone reductase of *Mentha piperita*, described as a medium-chain dehydrogenase/reductase (Ringer et al. 2003), or animal Δ^4-3-ketosteroid-5β-reductase, described as an aldo-keto-reductase (Kondo et al. 1994), implying very different evolutionary origins in spite of the similarity of the reactions catalyzed or even substrates used (Herl et al. 2008).

The r*Dl*P5βR did not only accept progesterone but also testosterone, 4-androstene-3,17-dione, cortisol, and cortisone. NADPH is the only cosubstrate and cannot be replaced by NADH. Essential structural elements for substrates of r*Dl*P5βR are the carbonyl group at C-3 and the double bond in conjugation to it (Herl et al. 2006a). Egerer-Sieber et al. (2006) and Thorn et al. (2008) reported on the purification and crystallization of recombinant P5βR from *D. lanata*.

Progesterone 5α-reductase, which catalyzes the reduction of progesterone to 5α-pregnane-3,20-dione, probably in a competition situation to the progesterone

5β-reductase, was isolated and characterized from cell cultures of *D. lanata* (Wendroth and Seitz 1990). The enzyme, which requires NADPH as cosubstrate, was found to be located in the endoplasmic reticulum. Recently, it was found that the addition of finasteride, an inhibitor of animal and human testosterone-5α-reductase, at 180 μM inhibited 5α-POR of *D. lanata* completely but left P5βR of the same source unaffected (Grigat 2005).

The 3β-hydroxysteroid 5α-oxidoreductase activity in *D. lanata* cell cultures was first reported by Warneck and Seitz (1990). The enzyme catalyses the conversion of 5α-pregnane-3,20-dione to 5α-pregnan-3β-ol-20-one. 3β-hydroxysteroid 5β-oxidoreductase catalyses the conversion of 5β-pregnane-3,20-dione to 5β-pregnan-3β-ol-20-one (Gärtner and Seitz 1993).

The 3α-hydroxysteroid 5β-oxidoreductase catalyses the conversion of 5β-pregnane-3,20-dione to 5β-pregnan-3α-ol-20-one. 3α-cardenolides have never been described in the genus *Digitalis* and the final products of a putative 3α-pregnane pathway are not yet known (Stuhlemmer et al. 1993). These conditions were inhibitory for the formation of 3β-hydroxy-5β-pregnan-20-one. The enzyme activity was found in microsomal preparations.

Finsterbusch et al. (1999) already discussed that these reactions may also be catalyzed by 3βHSD although they were previously assigned to the rather putative enzymes 3β-hydroxysteroid 5α-oxidoreductase, 3β-hydroxysteroid 5β-oxidoreductase, and 3α-hydroxysteroid 5β-oxidoreductase. Thus, this issue has to be examined further before clear conclusions concerning the role of the mentioned enzymes in the cardenolide pathway can be drawn.

The enzymes involved in pregnane 21-hydroxylation and pregnane 14β-hydroxylation in the course of cardenolide formation have not been described as yet. However, 14β-hydroxyprogesterone was incorporated into cardenolides, and it was supposed that 14β-hydroxylation must occur prior to the formation of the butenolide ring (e.g., Haussmann et al. 1997). It still remains unclear whether pregnane 14β-hydroxylation precedes 21-hydroxylation or vice versa.

Intra- or intermolecular nucleophilic attack at the C-20 carbonyl of an appropriately activated acetate or malonate is proposed as a possible mechanism of attaching C-22 and C-23 to the pregnane skeleton. The formation of the butenolide ring system can then be accomplished by formal elimination of water and lactonization. Experimental evidence for these steps is still lacking and recently a different mechanism of butenolide ring formation has been suggested, involving the formation of a pregnane 21-*O*-malonyl hemi-ester with subsequent intramolecular condensation under decarboxylation and dehydratization (Stuhlemmer and Kreis 1996; Pádua et al. 2008). When 5β-pregnane-14β,21-diol-20-one 3-β-*O*-acetate was incubated together with malonyl-coenzyme A in a cell-free extract of *D. lanata* leaves, a product was formed, which was identified as the malonyl hemi-ester of the substrate (Stuhlemmer and Kreis 1996). Recently, Kuate et al. (2008) reported the purification and characterization of malonyl-coenzymeA: 21-hydroxypregnane 21-*O*-malonyltransferase (*Dp*21MaT) from leaves of *D. purpurea*. Gel filtration and native SDS-PAGE analysis showed that Dp21MaT exists as a monomer with a molecular mass of 27 kDa.

Steroid 12β-hydroxylation and 16β-hydroxylation can occur at the pregnane, the cardenolide genin, and the glycoside level (Furuya et al. 1970; Tschesche 1971; Reinhardt 1974). A microsomal cytochrome P_{450}-dependent monooxygenase is capable of converting digitoxigenin-type cardenolides to their corresponding digoxin-type ones (Petersen and Seitz 1985). This enzyme, termed digitoxin 12β-hydroxylase, was first isolated from cell suspension cultures of *D. lanata*, where the enzyme was found to be located in the endoplasmic reticulum (Petersen et al. 1988).

5.3.1.3 Enzymes Involved in the Formation and Modification of the Sugar Side Chain of Cardenolides

So far, only few investigations have focused on the formation of the sugar side chain of cardenolides, especially the point of time when the characteristic 2,6-dideoxysugars are attached to the cardenolide genin. The hypothetical cardenolide pathways, both the pregnane and the optional norcholanic acid pathway, imply that the various sugars are attached at the cardenolide aglycone stage, although it cannot be ruled out that pregnane glycosides are obligate intermediates in cardenolide formation. Cardenolide genin glycosylation was discussed in more depth and detail in a previous review (Kreis et al. 1998) but has not been studied much since then. The putative

cardenolide pathway implies that the various sugars are attached at the cardenolide aglycone stage, although it cannot be ruled out that pregnane glycosides are obligate intermediates in cardenolide formation (e.g., Haussmann et al. 1997). Some results indicate that digitoxose is formed from glucose without rearrangement of the carbon skeleton (Franz and Hassid 1967) and that nucleotide-bound deoxysugars are present in cardenolide-producing plants (Bauer et al. 1984). Digitoxigenin was fed to light-grown and dark-grown *D. lanata* shoot cultures, as well as to suspension-cultured cells (Theurer et al. 1998). In either system, the substrate was converted to digoxigenin (Fig. 5.5), digitoxigen-3-one, 3-epidigitoxigenin, digitoxigenin 3-*O*-β-D-glucoside, 3-epidigitoxigenin 3-*O*-β-D-glucoside, glucodigifucoside, and additional cardenolides. Interestingly, digitoxosylation was not observed in these studies. Administration of cardenolide mono- and bisdigitoxosides or cardenolide fucosides did not lead to the formation of cardenolide tridigitoxosides. These results support the hypothesis that cardenolide fucosides and digitoxosides may be formed via different biosynthetic routes and that glycosylation may be an earlier event in cardenolide biosynthesis than previously assumed. Luta et al. (1998) synthesized a set of pregnane and cardenolide fucosides and they have shown that feeding of the 3-*O*-β-D-fucoside of 21-hydroxypregnenolone to *D. lanata* shoot cultures leads to a 25-fold increase in the formation of glucodigifucoside, when compared to a control where the respective aglycone was fed (unpublished observations).

UDP-glucose: digitoxin 16′-*O*-glucosyltransferase (DGT) catalyzes the glucosylation of secondary glycosides to their respective primary glycosides as was first demonstrated by Franz and Meier (1969) in particulate preparations from *D. purpurea* leaves. It was investigated in more detail in cell cultures of *D. lanata* (Kreis et al. 1986). Cardenolide monodigitoxosides, such as evatromonoside, and cardenolide tridigitoxosides, such as digitoxin, were substrates accepted very well, whereas cardenolide genins or bisdigitoxosides were glucosylated at a much slower rate. Faust et al. (1994) concluded from their studies that DGT accepts only substrates with an equatorial OH group in the 4′-position.

UDP-fucose: digitoxigenin 3-*O*-fucosyltransferase (DFT) is a soluble enzyme in *D. lanata* leaves and catalyzes the transfer of the sugar moiety of UDP-α-D-fucose to cardenolide genins (Faust et al. 1994).

UDP-glucose: digiproside 4′-*O*-glucosyltransferase (DPGT) has not yet been characterized in detail but seems not to be identical with the glucosyltransferase described earlier. Glucodigifucoside was formed by a soluble enzyme from young leaves of *D. lanata* in the presence of UDP-α-D-glucose and digiproside (Faust et al. 1994).

Acetyl coenzyme A: digitoxin 15′-*O*-acetyltransferase (DAT) is a soluble enzyme that catalyzes the 15′-*O*-acetylation of cardenolide tri- and tetrasaccharides. Using acetyl coenzyme A as the acetyl donor, DAT activity was detected in partially purified protein extracts from *D. lanata* and *D. grandiflora*, both known to contain lanatosides (Sutor et al. 1993).

Lanatoside 15′-*O*-acetylesterase (LAE) is able to convert acetyldigitoxose-containing cardenolides to their corresponding non-acetylated derivatives as was demonstrated in *D. lanata* cell suspension cultures and leaves (Sutor et al. 1990). This enzyme was found to be bound to the cell wall. LAE seems to be a specific cardenolide acetylesterase capable of removing the 15′-acetyl group of lanatosides and their deglucosylated derivatives. LAE was isolated, purified, and partially sequenced (Sutor and Kreis 1996; Kandzia et al. 1998). A fragment obtained by Lys-C digestion showed partial homology to other hydrolases and apoplasmic proteins. It included the probable location of an active-site histidine (Kandzia et al. 1998).

Cardenolide 16′-*O*-glucohydrolase (CGH I) was found to be associated with plastids (Bühl 1984) and could be solubilized from leaves of various *Digitalis* species using buffers containing Triton X-100 or other detergents (Kreis and May 1990). CGH I was purified from young leaves (May and Kreis 1997; Schöninger et al. 1998). Purified CGH 1 was digested and the resulting fragments were sequenced. One fragment had the typical amino acid sequence of the catalytic center of family 1 of glycosyl hydrolases (Schöninger et al. 1998). A clone of cardenolide 16′-*O*-glucohydrolase cDNA (CGH I) was obtained from *D. lanata*. The amino acid sequence derived from CGH I showed high homology to a widely distributed family of β-glucohydrolases (glycosyl hydrolases family 1). The recombinant CGH I protein produced in *E. coli* had CGH I activity. CGH I mRNA was detected in leaves, flowers, stems, and fruits of *D. lanata* (Framm et al. 2000). The coding sequence for the *D. lanata* CGH I was inserted downstream of the 35S promoter in the binary vector pBI121 resulting in the plant

expression vector pBI121cgh (Shi and Lindemann 2006). Explants excised from seedlings of *Cucumis sativus* were transformed using *Agrobacterium rhizogenes* harboring pBI121cgh. Hairy roots were obtained from infected explants. Glycolytic activity of the recombinant CGH I was demonstrated by HPLC using lanatosides as the substrates.

Cardenolide glucohydrolase II (CGH II) was isolated from *D. lanata* and *D. heywoodii* leaves and cell cultures. This soluble enzyme hydrolyzes cardenolide disaccharides with a terminal glucose and appears to be quite specific for glucoevatromonoside, which is supposed to be an intermediate in the formation of the cardenolide tetrasaccharides. The tetrasaccharides, deacetyllanatoside C and purpureaglycoside A, which are rapidly hydrolyzed by CGH I, were only poor substrates for CGH II (Hornberger et al. 2000).

Cardenolide β-D-fucohydrolase (CFH) was isolated from young *D. lanata* leaves. This soluble enzyme catalyzes the cleavage of digiproside and synthetic pregnane 3β-*O*-D-fucosides to D-fucose (6-deoxygalactose) and the respective genin. Digitoxigenin 3β-*O*-D-galactoside was not hydrolyzed by the enzyme. It seems not to be identical with the cardenolide glucohydrolases described earlier, which do not accept β-D-fucosides as substrates (Luta et al. 1997).

5.3.1.4 Regulation of Cardenolide Formation

Lindemann and Luckner (1997) speculated that cardenolide formation is regulated mainly by the availability of cholesterol and its transport into mitochondria, where the P_{450}-scc is assumed to be located. However, direct evidence has not been presented yet.

Cell suspension cultures established from different plants producing cardiac glycosides did not produce cardenolides (Luckner and Diettrich 1985; Seidel and Reinhardt 1987; Stuhlemmer et al. 1993). However, somatic embryos, green shoot cultures, as well as plants obtained by organogenesis or somatic embryogenesis were found to produce cardenolides (Diettrich et al. 1991). Several studies have reported a positive correlation between light, chlorophyll content and cardenolide production (e.g., Hagimori et al. 1982a). It seems as if chloroplast development is not sufficient for expression of the cardenolide pathway, since photomixotrophic cell cultures were shown to be incapable of producing cardenolides (Reinhardt et al. 1975). *Digitalis* roots cultivated in vitro are not capable of producing cardenolides, although they do contain these compounds in situ, indicating that roots are a sink organ for cardenolides (Christmann et al. 1993).

Suspension-cultured *Digitalis* cells, which do not synthesize cardenolides de novo (Reinhardt et al. 1975; Kreis et al. 1993), as well as roots or shoots cultivated in vitro (Theurer et al. 1998), are able to take up exogenous cardenolides and modify them. Cardenolides may enter and leave the cells by simple diffusion. Only the primary cardenolides, i.e., those containing a terminal glucose, are actively transported across the tonoplast and stored in the vacuole (Kreis et al. 1993). Cardiac glycoside transport was also investigated at organ and whole plant level. The long-distance transport of primary cardenolides from the leaves to the roots or to etiolated leaves was demonstrated and it was established that cardenolides are transported in the phloem (Christmann et al. 1993). Primary cardenolides may thus serve as the forms of both transport and storage of cardenolides.

It is important to note that the comparison of the sequences for low copy genes like *P5βR* provides useful new information for the phylogenetic reconstruction of the organismic evolution (Herl et al. 2008). Interestingly, morphology-based taxonomy as thoroughly performed by Werner (1965) is highly consistent with the molecular findings, in this way corroborating classical taxonomy.

5.4 Conservation Initiatives

Digitalis species are commonly propagated by seeds. According to the EURISCO catalog (http://eurisco.ecpgr.org), 250 entries of *Digitalis* spp. are preserved, most of them as seed collections, in European National Germplasm Banks in Germany, Hungary, Bulgaria, Romania, and Poland, among others. The Leibniz Institute of Plant Genetics and Crop Plant Research in Germany maintains the highest number of accessions, including selected landraces of *D. lanata* and *D. purpurea*. Many of them are from Germany but others are from Austria, Bulgaria, Italy, Greece, or Spain. The National Plant Germplasm System of the US Department of Agriculture stores some entries of *D. grandiflora* and *D. purpurea*. Recently, Probert et al. (2007) reviewed some of the threats to

seed-collection quality that arise during the period between collection, processing, and storage under ideal conditions, and presented data on *D. purpurea* that reveal the beneficial effect on seed quality of post-harvest treatments that result in delayed seed drying. The review also deals with the effects of environmental conditions and the condition of seeds themselves at the time of harvest on the potential rate of deterioration during the immediate post-harvest period. In this way, Butler et al. (2009a) demonstrated that the rate of germination and the longevity of immature *D. purpurea* seeds were improved by holding seeds at a wide range of humidity after harvest. A treatment with a solution of −1 MPa PEG 600 for 48h (priming) improved the longevity of the seeds dried immediately after harvest, but not of those first held at 95% RH for 8 days prior drying. Butler et al. (2009b) also demonstrated that the extent of prior deterioration and the post-priming desiccation environment affected the benefits of priming to the subsequent survival of mature *D. purpurea* seeds, suggesting that rehydration–dehydration treatments may have potential as an adjunct or alternative to the regeneration of seed accessions maintained in gene banks for plant biodiversity conservation or plant breeding.

Successful conservation of *Digitalis* requires a better understanding of levels and apportionment of genetic diversity within and between populations (Hayward and Sackville-Hamilton 1997). To the best of our knowledge, reports pertaining to this subject have been applied to *D. obscura* (Nebauer et al. 1999a, 2000), *D. minor* (Sales et al. 2001a), and *D. grandiflora* (Boronnikova et al. 2007).

In recent years, a series of molecular markers, based on either proteins or DNA polymorphisms, have significantly facilitated research aimed at improving medicinal plant species. These markers, particularly those based on differences at the DNA sequence level, can be used for the characterization of the population structure (the distribution of variability within and between populations), authenticating plant material used for drugs, and for marker-assisted breeding (Joshi et al. 2004; Canter et al. 2005; Sucher and Carles 2008). Although both random genomic and functional markers have been utilized in several medicinal and aromatic plant species (Kumar and Gupta 2008) there have been few studies of this type in *Digitalis* species, all of which are described in the next paragraphs.

Nebauer et al. (2000) assessed the genetic relationships within the genus *Digitalis* based on random amplified polymorphic DNA (RAPD) markers. RAPDs were efficient in detecting interspecific variation among six species of the genus *Digitalis: D. obscura*, *D. lanata*, *D. grandiflora*, *D. purpurea*, *D. thapsi*, and *D. dubia*, synonym *D. minor*, and the hybrid *D. excelsior* (*D. purpurea* × *D. grandiflora*). In fact, individuals from the different sections gave rise to characteristic RAPD profiles, which were so obviously different as to allow identification at the section level by eye (Fig. 5.12). The species relationships revealed by RAPD were fully consistent with those previously obtained using morphological affinities, corroborating previous taxonomic data of Werner (1965). Then, RAPDs may be important for strain identification and cultivar characterization, and can be used to detect instances of natural interspecific gene introgression. Nevertheless, further analysis with more species and primers will be required to fully establish the specificity of loci to particular taxa and subsequent interspecific gene flow in *Digitalis*. Also, efforts should be made on *Digitalis* for identification of molecular markers associated with quality and quantity of cardiac glycosides and other secondary metabolites. To date, only a study focussed on chemotaxonomic markers has been published (Taskova et al. 2005).

RAPD markers also proved to be a powerful tool for the detection of spatial genetic variation in Spanish wild populations of *D. obscura* and *D. minor* and for the identification of individuals from the different populations (Nebauer et al. 1999a; Sales et al. 2001a). The most relevant conclusions of these two investigations are summarized below.

D. minor is an endemism from the eastern Balearic Islands (Mallorca, Menorca, and Cabrera) that shows a high level of morphologic variation. Two infraspecific taxa have been described, *D. minor* var. *minor* and *D. minor* var. *palaui,* according to the presence or absence of leaf pubescence, respectively (Hinz 1987a). The presence of leaf trichomes in var. *minor* has been shown to be an efficient mechanism in preserving the photochemistry apparatus, when compared to the glabrous var. *palaui*, although such pubescence was not related to a lower leaf water loss (Galmés et al. 2007). The RAPD survey of the two infraspecific taxa of *D. minor* did not find a significant partitioning of genetic diversity among islands, probably as a result of a relative recent gene flow when the three islands

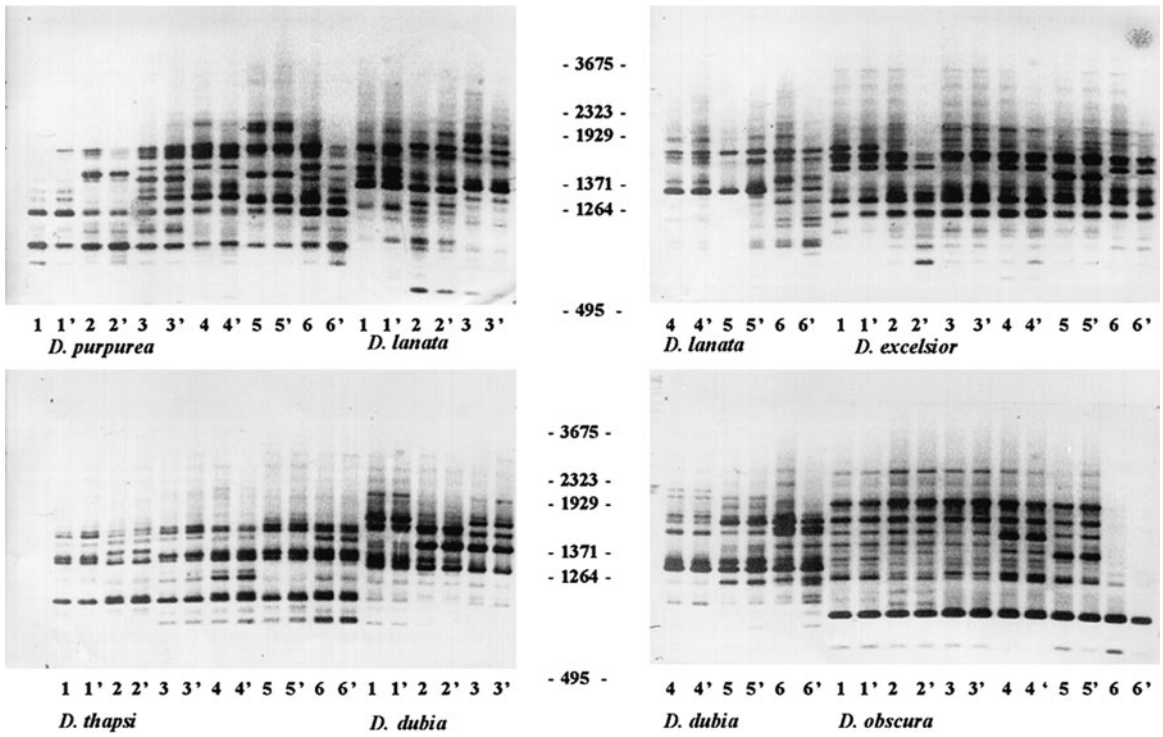


Fig. 5.12 Examples of RAPD profiles in six individuals from *Digitalis* species using the primer OPA13. Each individual appears in duplicate in *two lines*. From Nebauer et al. (2000)

constituted a single landmass; furthermore, the RAPD data did not support the taxonomic differentiation of *D. minor* (Sales et al. 2001a).

Distance-based clustered methods separated by their area location *D. obscura* and *D. minor* individuals; consequently, RAPDs seem to be highly effective in distinguishing *Digitalis* genotypes from geographically distinctive areas, suggesting that this technique can be useful in population fingerprinting and germplasm assessment.

The RAPD-based analyses of molecular variance (AMOVA) revealed that most genetic variation in *D. obscura* and *D. minor* was recorded within populations (84.8% and 92.9%, respectively), a result consistent with those from most other outcrossing plant species (Hamrick and Godt 1996). The value of RAPD for detecting this intrapopulation variation is endorsed by the study on cardenolide content in wild *D. obscura* plants cited below, in which the proportion of phytochemical variation attributable to individuals was significantly higher than that attributable to population differences (Nebauer et al. 1999b). Nevertheless, AMOVAs also indicated a significant population differentiation (fixation indices of 0.152 and 0.071 for *D. obscura* and *D. minor* populations, respectively). These estimates were close to those given in the literature for analysis of population structure in mixed and outcrossing species: 0.1–0.24 (Loveless and Hamrick 1984); 0.099–0.216 (Hamrick and Godt 1990); and 0.03–0.31 (Heywood 1991).

More recently, *D. grandiflora* populations growing in Russia were also analyzed by RAPD and ISSR (intersimple sequence repeats) markers (Boronnikova et al. 2007); results from both markers revealed a weak population structure, with most of the genetic variation accounting for within population variability. In contrast, a study of the consequences of crossing distance on lifetime progeny fitness in a population of *D. purpurea* demonstrated the existence of within-population outbreeding depression, suggesting substantial genetic structuring at moderate distances in the species (Grindeland 2008).

The above-mentioned results have a number of implications in the development of conservation

strategies for *Digitalis* species. The detection of population differentiation may assist in the definition of adequate units for conservation, thus providing an appropriate focus for conservation management or monitoring. The definition of such management units will also be a valuable tool when sampling germplasm for ex situ conservation and for restoring degraded populations of the species. These strategies would be also of interest for the conservation of high yielding cardenolide plants or populations where it would be possible to select parental strains for new crosses. Several micropropagation methods, as described below should be also of interest for rapid multiplication of adult *Digitalis* plant species thereby facilitating the propagation and conservation of the selected high-yielding plants.

5.4.1 In Vitro Culture of the Genus Digitalis

Generally, plant cell and tissue cultures can be established from any living plant cell. These cells then "despecialize" or "dedifferentiate." Plant hormones or growth regulators trigger growth, organ formation, and regeneration. First work on in vitro culture of *Digitalis* species was reported by Staba (1962) using *D. lanata* and *D. purpurea* cells. In the following years, cell and tissue cultures from almost all parts of the plant could be initiated and cultivated in vitro over long periods of time (Diettrich 1986; Schöner and Reinhard 1986; Luckner and Diettrich 1987a, b, 1988; Rücker 1988).

Plants can be regenerated in vitro by the following methods (a) enhancing axillary bud-breaking, (b) differentiation of adventitious buds, and (c) somatic embryogenesis. The first two approaches lead to plant regeneration through the production of unipolar shoots, which must then be rooted in a multistaged process. In contrast, somatic embryogenesis leads to the formation of embryos having shoot and root apices (bipolarity). All these regeneration methods have been successfully applied to *Digitalis* species and have been reviewed in depth by Rücker (1988), Luckner and Diettrich (1988), and Segura and Perez-Bermudez (1992). In vitro production of *Digitalis* haploids is summarized in Sect. 5.5. The mentioned reviews summarize work done on *D. ambigua, D. cariensis, D. ferruginea, D. grandiflora*, *D. heywoodii*, *D. laevigata*, *D. lanata*, *D. lutea, D. mertonensis, D. obscura,* and *D. purpurea*, and describe explant source, basal medium and growth regulators, environmental conditions tested, and the morphogenic responses obtained. In vitro regeneration protocols have also been described for *D. thapsi* (Herrera et al. 1990; Cacho et al. 1991), *D. minor* (Sales et al. 2002), and *D. trojana* (Çördük and Aki 2010), endemic species of the central/western part of the Iberian Peninsula, of the Balearic archipelago and of the Ida Mountain, Canakkale, Turkey, respectively. Table 5.3 includes the methodology used for in vitro propagation of those *Digitalis* species that were not included in the mentioned reviews.

From Table 5.3 and the above-mentioned literature (see also Gavidia et al. 1993; Lapeña and Brisa 1995), it can be concluded that almost every explant of most of the *Digitalis* species studied, including cells, protoplasts, and anthers, have the potential to regenerate plants through direct or indirect organogenesis or embryogenesis. In general, in vitro organogenesis in *Digitalis* species was the result of a specific auxin–cytokinin interaction, although other growth regulators, specially ethylene and gibberellins may affect the caulogenic action of auxins and cytokinins. Shoot regeneration was also obtained after infection of *D. minor* with the wild type *Agrobacterium tumefaciens* strain 82.139, which induced shooty tumors. This shoots were not transgenic, as revealed by nopaline assays and the use of a C58pMP90/T139GUSINT strain harboring the intron inactivated *gus*A gene (Sales et al. 2002). In another study, Palazón et al. (1995) evaluated the effect of phenobarbital on organogenesis from *D. purpurea* callus; the interaction of this xenobiotic with IAA not only reduced the production of biomass but also increased the volume of mitochondria per cell and the formation of shoot buds in callus tissues. Somatic embryogenesis can be also easily achieved from primary explants or callus cultured on media with auxin alone or with several auxin–cytokinin combinations; at least in *D. obscura*, gibberellic acid favored both differentiation and normal embryo development. It is worth noting that a cell strain line of *D. lanata* was used to characterize somatic embryogenesis of the species by means of two-dimensional gel electrophoresis of in vivo and in vitro synthesized polypeptides (Reinbothe et al. 1992a). The study demonstrated that processes

Table 5.3 Explants and media used for the micropropagation of *D. minor*, *D. thapsi*, and *D. trojana* (here described as *D. cariensis* ssp *trojana*)

Species	Explant	Basal medium	Growth regulators	Goal	Reference
D. minor	Shoot tips from 30-day-old seedlings Shoot tips from 30-day-old shoot cultures	MS* with ½ NH_4, 3% sucrose, 0.7% agar	1 μM BA	Axillary shoots proliferation	Sales et al. (2002)
	Leaves from 30-day-old seedlings Leaves from 30-day-old shoot cultures	MS*, 3% sucrose, 0.7% agar	8.9 μM BA + 0.6 μM IAA	Shoot organogenesis	
D. thapsi	Shoot tips from 30-day-old seedlings	MS*, 3% sucrose, 1% agar	0.5 or 2.3 μM KIN + 2.7 μM NAA	Axillary shoots proliferation	Herrera et al. (1990)
	Leaves from 30-day-old seedlings		23.2 μM BA alone or + 5.4 μM NAA		Cacho et al. (1991)
	Hypocotyls from 30-day-old seedlings		23.2 μM BA or 4.4 μM BA + 2.9 μM IAA	Shoot organogenesis	
	Roots from 30-day-old seedlings		13.3 μM BA or 4.4 μM BA + 2.9 μM IAA		
D. trojana	Leaves from 90-day-old seedlings	MS*, 3% sucrose, 0.8% agar	13.3 μMBAμMNAA	Shoot organogenesis	Çördük and Aki (2010)

**MS* Murashige and Skoog medium (1962)

normally occurring during zygotic embryogenesis need not necessarily take place in a similar way during somatic embryogenesis. Note, however, that this embryogenic line of *D. lanata* showed a very similar expression pattern of LEA transcripts and of certain in vitro translatable mRNAs found for *Nicotiana plumbaginifolia* somatic embryos regenerated from leaf mesophyll protoplasts, suggesting that common embryogenesis-related gene expression programs were realized in both plant species (Reinbothe et al. 1992b).

As previously mentioned (see Sect. 5.1.3), the genus *Digitalis* and *Isoplexis* have a common origin, and the latter should be reduced to sectional rank and embedded in *Digitalis*, close to *D. obscura*. Because of this, we also include in this revision work done on micropropagation of *I. canariensis*, *I. chalcantha* and *I. isabelliana*. Papers published by Schaller and Kreis (1996), Arrebola et al. (1997), Pérez-Bermudez et al. (2002), and Arrebola and Verpoorte (2003) describe methods for the micropropagation of these *Isoplexis* species through axillary bud proliferation, using apical and or nodal explants cultured on either liquid solidified Murashige and Skoog medium with cytokinin alone or in combination with auxins. Rooting was easily achieved in hormone-free medium or supplemented with auxin. In general, cultural requirements and propagation pattern for *Isoplexis* are quite similar to those of *D. obscura* (Segura and Perez-Bermudez 1992), which is in agreement with the close relationships of the species.

The protocol described by Vela et al. (1991) for the micropropagation of adult *D. obscura* was used for the in vitro establishment, regeneration, and conservation of high-yielding genotypes. In a first study (Gavidia et al. 1996), wild-growing plants of this species were characterized according to their capacities to biosynthesize cardenolides and to regenerate in vitro, founding high genotype dependence in both parameters; one of the high yielding genotypes was maintained in vitro, through serial shoot tip subculture, for 2 years. RAPD analysis suggested that the micropropagation method used (axillary bud-breaking) preserved the genetic stability of long-term cultures of the species. In contrast, Sales et al. (2001a) found RAPD variation in long-term cultures of a different high-yielding genotype of the species. Although differences between both results could be attributable to the use of a different set of primers, we emphasize that the absence of RAPD polymorphism alone does not guarantee the genetic stability of the regenerants (Renau-Morata et al. 2005 and references therein). Thus, an appropriate selection of the primers as well as other corroborating approaches should be used to ensure an accurate interpretation of RAPD results.

Long-term conservation by freezing plant cells or tissues in liquid nitrogen have been developed for cell cultures of *D. lanata* (Diettrich et al. 1986b) and *D. thapsi* (Morán et al. 1999) as well as shoot tips from *D. lanata* (Diettrich et al. 1986a) and *D. obscura* (Sales et al. 2001b). The protocols employed are summarized in Table 5.4. In this last study, explants were taken from in vitro cultures established with *Digitalis obscura* elite genotypes previously selected by its higher cardenolide contents (Nebauer et al. 1999b). The cryopreservation method used a simple procedure of encapsulation-dehydration for shoot tips (Fig. 5.13; Table 5.4); survival and shoot regeneration was dependent upon genotype, reaching percentages of 93% and 86%, respectively, when shoot cultures were cold-hardened. A RAPD marker survey demonstrated that cryopreservation of *D. obscura* shoot tips was more effective than repetitive subcultures in order to maintain the genetic fidelity (Table 5.5; Fig. 5.14).

5.5 Role in Development of Cytogenetic Stocks and Their Utility

The establishment of callus and cell cultures from *Digitalis* facilitates investigations on metabolism and development independently from the complex organism. Starting from callus it was possible to generate permanent suspension cultures submerged in liquid media (see Luckner and Wichtl 2000). Phytohormone-autotrophic cell lines were established from *D. lanata* (Kreis and Reinhard 1985; Kreis 1987) and *D. purpurea* (Hirotani and Furuya 1975). Photo-autotrophic lines were first described by Hagimori et al. (1984a, b).

Cell, tissue, and organ cultures showed morphogenetic capacity for regeneration but this capacity decreased in long-term cultures. Somatic embryo cultures derived from embryogenic cell lines of *D. lanata* were reported (Garve et al. 1980; Tewes et al. 1982;

Table 5.4 Cryopreservation methods applied to *Digitalis* species

Species	Donor plants pre-treatment	Plant material	Alginate beads	Pre-freezing conditioning	Reference
D. lanata	–	Cell suspension	No	Pre-culture with 0.15 M mannitol + addition of cryoprotectors (sucrose and glycerol or DMSO) + slow cooling	Diettrich et al. (1986b)
	Shoot cultures at 4°C × 8 weeks	Shoot tips	No	Pre-culture for 2 h with DMSO 2 M + slow cooling	Diettrich et al. (1987)
D. obscura	Shoot cultures at 4°C × 15 days	Shoot tips	Yes	Pre-culture for 24 h in 0.5 M sucrose + 2.5 h dehydration in laminar flow	Sales et al. (2001b)
D. thapsi	–	Cell suspension	No	Pre-culture with 0.15 M mannitol for 3 days + addition of cryoprotectors (sucrose + glycerol + DMSO) + slow cooling	Morán et al. (1999)

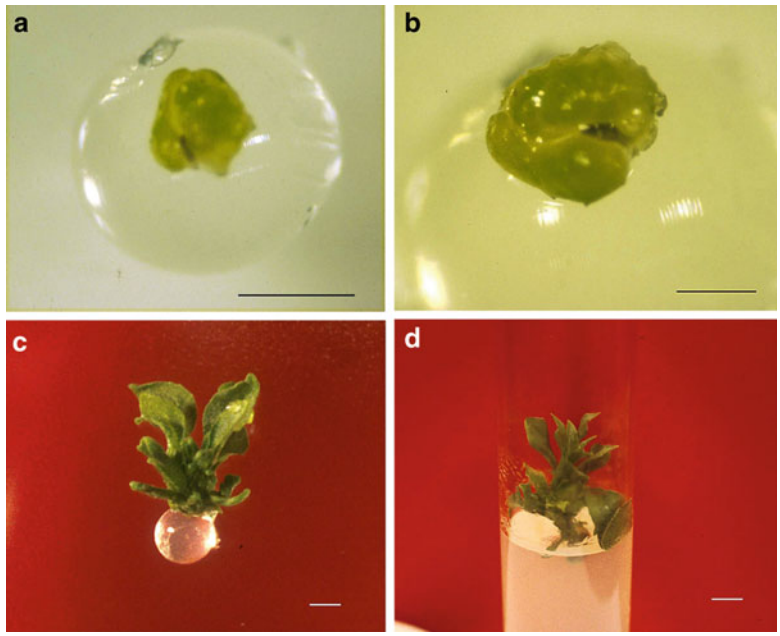


Fig. 5.13 Recovery of cryopreserved *D. obscura* shoot tips 7 (**a**), 15 (**b**), and 30 (**c**) days after thawing. Developed shoot 75 days after thawing (**d**). Scale bars represent approximately 1 mm (**a**, **b**, **c**) or 5 mm (**d**). From Sales et al. (2001b)

Diettrich et al. 1986a, b, 1991). Embryogenic cell lines were also established from other species, e.g., *D. obscura* (Arrillaga et al. 1986, 1987). Root cultures are another system in which several aspects of growth and development can be studied (Pérez-Bermúdez et al. 1987). Haploid cell cultures from anthers, pollen, non-fertilized egg cells, and pistils have also been generated (Ernst et al. 1990; Pérez-Bermudez et al. 1990; Diettrich et al. 2000).

Meristematic stem cells were the source for stem cultures formation (Lui and Staba 1979). A number of stem cultures were generated in several groups (Hagimori et al. 1982a, b; Luckner and Diettrich 1985; Gärtner and Seitz 1993; Stuhlemmer et al. 1993). The growth and cultivation conditions differ from culture to culture and have been optimized over the years. These cultures were used to investigate several developmental and environmental processes as well as the

Table 5.5 Variation found in RAPD profiles generated for in vitro cultures of two *D. obscura* genotypes (AY3, HU3). From Sales et al. (2001b)

	AY3 (2nd subculture)				HU3 (16th subculture)			
	A	B			A	B		
		Control	Frozen (+LN)			Control	Frozen (+LN)	
		(−LN)	1 h	2 days		(−LN)	1 h	3 months
Total number of bands	47	47	47	47	54	54	54	54
MNPB[y]	0.4	0.3	0.3	0.3	1.5	0.5	0.8	0.8
Match percentage	93.4	99.5	99.3	99.5	84.9	99.1	98.6	98.6

MNPB[y], mean number of polymorphic bands

A, comparisons, for each genotype, between wild-growing parent plant and subcultured shoots

B, comparisons, for each genotype, between subcultured shoots and their respective control and frozen progenies

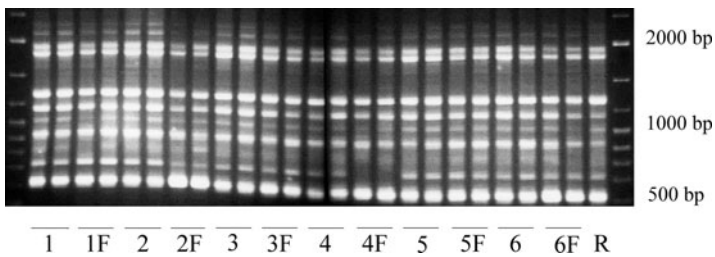


Fig. 5.14 Examples of RAPD profiles generated with primer OPC-8 for *D. obscura* genotype HU3. Duplicated lanes show band patterns of six subcultured shoots (1–6) and their corresponding cryopreserved (1F–6F) progenies. Mother plant profile was used as reference (R). From Sales et al. (2001b)

influence of different exogenous factors on their development. At the same time first attempts have been applied to cultivate roots or root meristematic cells (Rücker et al. 1981; Pérez-Bermúdez et al. 1987).

Stable cardenolide formation in cell cultures was found only in cultures of stems and somatic embryos (PEMs) as described by Nover et al. (1980), Ohlsson et al. (1983), Kuberski et al. (1984), and Scheibner et al. (1987, 1989). Most non-differentiated or specialized tissue or cell cultures do not produce cardenolides or do so in negligible amounts only, rarely detectable.

Apart from being a short way for the production of homozygous lines, haploids provide an important tool for crop improvement. Gametic cell cultures also offer the scope to detect spontaneous or induced genetic variants at a higher frequency than in somatic cultures. The application of these techniques to breeding programs is limited because the frequency of haploid production is seldom sufficient to evaluate genetic properties of the regenerants. Cultured anthers, microspores, and ovaries of a high number of plant species, including *Digitalis*, have been used to regenerate haploid plants via organogenesis or embryogenesis (Bajaj 1990; Pérez-Bermudez et al. 1990; Don Palmer and Keller 2005; Ferrie 2007).

Plants from anther cultures of different *Digitalis* species have been generated (Table 5.6). The degree of ploidy was different and intensively investigated by Ernst et al. (1990) and Diettrich et al. (2000). In *D. lanata,* androgenic callus was obtained from cold-treated anthers and pollen. The callus obtained was mixoploid and contained haploid, diploid, and tetraploid cells as shown by impulse cytophotometry. Haploid cell lines were selected by single colony cloning. They were unstable and selection had to be repeated every 1–2 months. Mixoploid shoot cultures were derived from embryogenic haploid cell lines via somatic embryogenesis. Haploid shoots were analyzed that showed a wide variability with regard to cardenolide content and profile. Rooting of the haploid shoots resulted in haploid plants that were transferred into soil. The regenerated plants were smaller in size than diploid plants. Flowers, if developing at all, were morphologically abnormal and showed male sterility due to crippled anthers (Diettrich et al. 2000). Finally,

Table 5.6 Anther cultures from *Digitalis* species

Digitalis	Developmental stage	Ploidy level	References
Digitalis spec.	Callus	Haploid, non-stabile	Schröder (1985)
	Callus	Haploid, non-stabile	Scheibner et al. (1987)
	Regenerated plants	Diploid (dihaploid)	
	Callus	Haploid	Diettrich et al. (2000)
	Stems	Mixoploid	
	Regenerated plants	Haploid, diploid	
D. obscura	Callus, embryos	–	Pérez-Bermúdez et al. (1985b)
	Regenerated plants	Haploid (50%)	
D. purpurea	Callus	Haploid	Corduan and Spix (1975)
	Regenerated plants	Haploid (most)	

Arnalte et al. (1991) reported the isolation of protoplasts from immature pollen of *D. obscura* that provides a tool for further gene transfer studies.

5.6 Role in Crop Improvement Through Traditional and Advanced Tools

Since the medical superiority of series C cardenolides was demonstrated, *Digitalis lanata* became the industrial source of these compounds. The species is a biennial plant cultivated as annual for cardiac glycosides production. Crops are established from seeds and harvested mechanically at the end of the first growing season to obtain the leaves that are immediately dried and processed to produce three valuable therapeutic agents for the treatment of cardiovascular diseases: a primary glycoside, the lanatoside C, and two secondary glycosides, digoxin and digitoxin (Fonin and Khorlin 2003).

As other *Digitalis* species (Nazir et al. 2008, and references therein), *D. lanata* is an outbreeding but self-fertile plant that shows high variability among individuals for cardenolide production-related traits, as plant size and digoxin content. Genetic variation for plant size has been found to be mainly additive; therefore conventional breeding techniques can be effective in producing varieties with high biomass yield (Kennedy 1978). Mass selection programs conducted in the Netherlands (Mastenbroek 1985) and France (Brugidou and Jacques 1987) resulted in varieties with increased leaf yield, and also with improvement in other traits such as upright habit of leaves and resistance to diseases and bolting.

Initial essays on obtaining *Digitalis* plants containing high digoxin levels involved interspecific crosses with *D. purpurea, D. lutea,* and *D. grandiflora* (Calcandi et al. 1961; Kennedy 1978 and references therein). Since these *Digitalis* species produce digoxin at a very low, if any, level, interspecific hybrids showed lower digoxin content than *D. lanata*. However, more recently Ikeda and Fujii (2003) reported that the level of lanatoside C in the hybrid *D. ambigua* × *D. lanata* was higher than in *D. lanata*. Other phytogenetic resources, as wild populations of *D. obscura* and *D. purpurea,* have also been prospected in Spain and Sardinia, respectively, looking for high digitoxin-yielding donor plants (Nebauer et al. 1999b; Usai et al. 2007). A high degree of diversity was found in both studies, since digitoxigenin content of 1-year-old greenhouse-grown *Digitalis obscura* plants ranged between 202.6 and 1,166.8 mg/kg DW, while 2-year-old plants of *D. purpurea* showed digitoxigenin contents ranging between 11.3 and 240.6 mg/kg FW.

Cardenolide production in *Digitalis* plants has proved to be affected by genotype, developmental stage, as well as environmental factors. Thus, Nebauer et al. (1999b) estimated cardenolide productivity in six different wild populations of *D. obscura* in Spain, by quantification of series A and B genins (digitoxigenin and gitoxigenin, respectively), in 49 individual plants. Digitoxigenin and gitoxigenin content differed widely among the six *D. obscura* populations, and showed a remarkable diversity in individual plants from a given location (Fig. 5.15). Corroborating this, results from a hierarchic analysis of variance showed significant variations in cardenolide content (digitoxigenin plus gitoxigenin) due to differences among and within

populations. Changes in the environmental conditions generally alter the production of plant secondary metabolites (Hartmann 1996), and *D. obscura* was not an exception; nevertheless, the proportion of variation attributable to single plant differences was higher than that attributable to population differences (50.6 vs. 38.9%), which could be related to the similar bioclimatic conditions of the sampled populations.

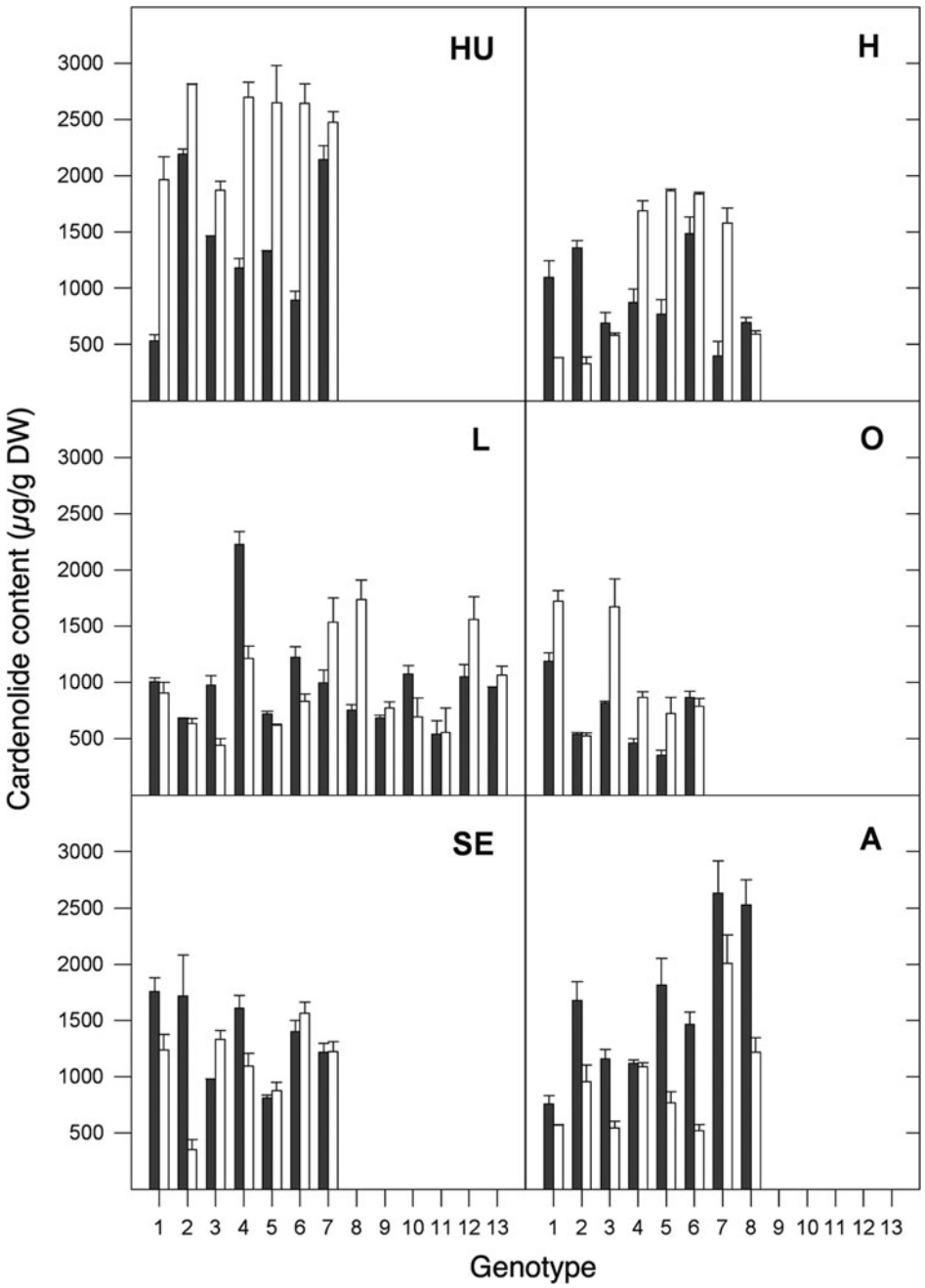


Fig. 5.15 Gitoxigenin (*gray bars*) and digitoxigenin (*white bars*) content in HCl-hydrolyzed extracts from the six populations of *D. obscura*: Huesa (HU); Huesa (H); Llanorell (L); Oliete (O); Segart (SE); and Aiora (A). Each value (µg/g DW) represents the mean of three determinations (±SE). From Nebauer et al. (1999b)

This high dependence on the genotype for the content of lanatoside C and digoxin has also been observed in native or selected cultivars of *D. lanata* (Castro Braga et al. 1997, and references therein).

Several studies in *D. lanata* demonstrated that variations in the relative profile and percentual composition of cardenolide series depended on environmental conditions (Stuhlfauth et al. 1987), development (Castro Braga et al. 1997), and method of propagation (Schöner and Reinhard 1986). This also holds true for *D. obscura*, where the proportion of series A and B genins varied among the studied populations (Nebauer et al. 1999b). In contrast, Roca-Pérez et al. (2004a) found that pre-dominance of the series A cardenolides over those of the series B had been independent of the natural *D. obscura* population studied. This apparent contradiction could be attributed to the methodological approach for cardenolide determination (genins in Nebauer et al. 1999b and glycosides in Roca-Pérez et al. 2004a). Although most of the cardenolide glycosides are accurately quantified by HPLC analysis, genin determinations should be the preferred method when screening of high-yielding plants is pursued (hydrolysis of cardiac glycosides avoids problems related to the quantification of highly hydrophilic glycosides and facilitates comparisons among natural populations by reducing the number of scored data). In any case, further research is required to establish whether the variation found in the proportion of series A and B genins in *D. obscura* populations is genetically determined or whether it can be due to climatic or other environmental factors.

Several studies have related the nutrient status of *D. obscura* leaves to soil characteristics, plant nutrients, and cardenolide production. Thus, cardenolide content exhibited negative correlation with plant P levels and with Cu content in soils (Roca-Pérez et al. 2002). On the other hand, cardenolide content in leaves was negatively correlated with Zn level in young leaves and with Mn level in old leaves, but positively correlated with Fe content in young leaves (Roca-Pérez et al. 2004b). Finally, cardenolide contents were negatively correlated with the N, P, and K contents in young leaves but highly significant and positive for Mg (Roca-Pérez et al. 2005).

Roca-Pérez et al. (2004a) also studied seasonal fluctuations of cardenolides in natural populations of *D. obscura* and found that cardenolide contents changed in the time course of the four seasons as a multiple response to distinct plant and/or environmental factors. The lowest production was recorded in May, followed by a fast cardenolide accumulation in summer, a decreasing phase in autumn, and a stationary phase in winter. In the same way, Brugidou et al. (1988) reported that digoxin contents in *D. lanata* grown in both controlled and natural conditions was related to the seasonal variations of light intensity, photoperiod, and thermoperiod.

Besides mineral soil characteristics, rhizosphere communities can also affect cardenolide production. Thus, Gutierrez et al. (2003) isolated rhizobacteria from wild populations of *D. thapsi* and *D. parviflora*, and demonstrated that some *Bacillus* strains were able to provoke systemic induction of the terpenic pathway in *Digitalis lanata*.

Interspecific hybrids have also been obtained in studies designed to elucidate genetic regulation of cardenolide production in *D. lanata* and *D. purpurea*. Low heritability rates have been estimated for this trait (Lichius et al. 1992; Ardelean et al. 2006) as non-genetic factors affecting cardenolide accumulation in leaves were determined in previous studies (Balbaa et al. 1971; Schaffer and Stein 1971; Rajukkanu et al. 1981). However, Mastenbroek (1985) reported a 50% increase in digoxin rates after mass selection in a *D. lanata* cultivated variety. Weiler and Westenkemper (1979) also reported the selection of *D. lanata* strains with high digoxin content. There is also a reference on the use of mutagenesis to induce genetic variation on cardenolide accumulation, which was found among plantlets derived from irradiated shoot tips of a *D. obscura* genotype (Gavidia and Pérez-Bermudez 1999).

In contrast to these few references about traditional breeding of *D. lanata,* biotechnological approaches to improve cardenolide production have widely been reported. Some of these studies deal with the in vitro production of cardiac glycosides. Since the production of these secondary metabolites from in vitro cultures of *Digitalis* spp. is dependent on morphological differentiation, it is necessary to establish shoot-proliferating cultures. This has been successfully reported for *D. purpurea* (Hagimori et al. 1982a, b), *D. obscura* (Pérez-Bermudez et al. 1984; Vela et al. 1991), *D. lanata* (Luckner and Diettrich 1988; Rücker 1988), *D. thapsi* (Cacho et al. 1991), and *D. minor* (Sales et al. 2001a). However, this approach is limited

by the low cardenolide accumulation rates determined in the *Digitalis* in vitro grown shoots. For example, *D. purpurea* shoots (Hagimori et al. 1984a, b) and *D. lanata* embryos (Greidziak et al. 1990) were successfully grown in fermenters, although the productivity was small and should be improved by a factor of 100–1,000 before cardenolide production can be economically viable (Kreis and Reinhard 1989). More recently, Sales et al. (2002) reported for shoot cultures of *D. minor* the highest cardenolide content of 226 mg/kg DW, while greenhouse-grown plants accumulated 800–1,000 mg/kg DW. Furthermore, cardenolide content of *D. minor* leaves was significantly reduced (14 μg/g DW) when shoots were cultured in liquid medium, which is the usual condition for large-scale cultures performed in bioreactors. This negative effect of the liquid medium, also evident in shoot cultures of *D. obscura*, is related to the appearance of hyperhydricity, a physiological disorder that reduces cardenolide accumulation in the in vitro grown plants (Lapeña et al. 1992). Several strategies have been used for the improvement of secondary metabolite production in plant cell cultures, including medium optimizations in undifferentiated and/or morphogenic systems (Kreis and Reinhard 1989; Stuhlemmer et al. 1993; Palazón et al. 1995; Gavidia and Pérez-Bermudez 1997; Cacho et al. 1991), addition of biosynthetic precursors (Haussmann et al. 1997), elicitors (Bonfill et al. 1996; Paranhos et al. 1999), and the use of temporary immersion system (Pérez-Alonso et al. 2009). Unfortunately, none of these strategies led to a significant enhancement in cardenolide production, therefore the improvement of this process rests on the genetic engineering of *Digitalis* spp.

Metabolic engineering of plant secondary compounds has become an area of great biotechnological interest and several strategies have been applied (for a review, see Verpoorte and Alfermann 2000). Abovementioned organogenesis protocols opened up possibilities of biotechnological strategies for the genetic improvement of *Digitalis* species, but reports on this matter showed only limited results for *D. lanata* and *D. purpurea*. The first transgenic *Digitalis* plants were obtained by Lehmann et al. (1995), who reported an *A. tumefaciens*-mediated transformation of *D. lanata* by using protoplasts derived from an embryogenic line. Pradel et al. (1997) also reported the regeneration of *D. lanata* plants from hairy root cultures established after infection with several wild type strains of *Agrobacterium rhizogenes*.

A more efficient transformation protocol was developed by Sales et al. (2003) for *D. minor*. In these experiments, up to an 8.4% of the leaf explants infected with *A. tumefaciens* formed at least one transformed plant (Fig. 5.16). This transformation efficiency allowed the genetic engineering of this plant species using an *Agrobacterium* strain containing a Ti plasmid with the catalytic domain of the 3-hydroxy-3-methyl-glutaryl coA reductase gene (*HMG1*) isolated from *A. thaliana* (Sales et al. 2007). Constitutive expression of this transgene resulted in an increased sterol and cardenolide production in both in vitro and greenhouse-grown plants. Although

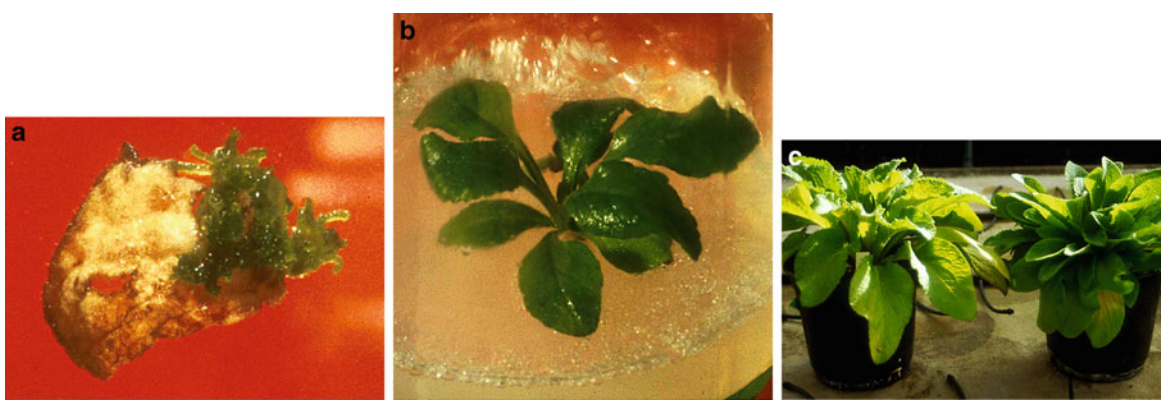


Fig. 5.16 Generation of transgenic *D. minor* plants from leaf explants cultivated with *A. tumefaciens* EHA105 harboring the plasmid p35SGUSINT. (**a**) Adventitious bud differentiation on selection medium; (**b**) elongation of a KAN + shoot; (**c**) plants growing in the greenhouse. From Sales et al. (2003)

a clear correlation between *HMG1* expression and cardenolide accumulation in transgenic plants could not be established, since HMGR up-regulation in *D. minor* seems to be more effective in modifying sterol than cardenolide metabolism, lines with the higher *HMG1* expressing level also showed higher cardenolide contents. These results suggest that additional critical steps exist in the cardenolide biosynthesis pathway. However, further progress in this field is limited due to the lack of sufficient information concerning the genes involved in the biosynthesis of these secondary metabolites.

About 20 enzymes probably involved in the formation of cardenolides have been identified and characterized in *Digitalis*, but only some of the genes coding these enzymes have been cloned, as the progesterone 5β-reductase isolated in *D. obscura* (Roca-Pérez et al. 2004a), *D. lanata* (Herl et al. 2006a), and in *D. purpurea* (Gavidia et al. 2007); the cardenolide-16′-O-glucohydrolase (Framm et al. 2000), the lanatoside-15′-O-acetylesterase (Kandzia et al. 1998), and the Δ^5-3β-hydroxyesteroid dehydrogenase (Lindemann et al. 2000; Herl et al. 2007) of *D. lanata*. Promising results obtained in the genetic engineering of *D. minor* could be improved with further studies that combine both an increase in carbon flux and committing cardenolide biosynthesis regulating genes. In addition, cardiac glycosides of *D. minor* may be improved by the introduction of additional hydroxyl functions, which generate digoxigenin (series C) derivatives, the most commonly used for clinical purposes. Transgenic *D. minor* plants are then a valuable system to study and achieve metabolic engineering of the cardenolide pathway and in consequence for the genetic improvement of *Digitalis* species.

Protoplast fusion produces cells containing a mixture of the DNA from both parents, then provides an ideal system for genetic modification and for use in plant breeding. To date, the only studies dealing with this subject in *Digitalis* are those reported by the team of Prof. Carmen Brisa at the University of Valencia, Spain. In these studies (see Vela 1996), factors influencing electrofusion of protoplasts from callus of *D. obscura* with mesophyll protoplasts of *D. lanata* were investigated. Protoplasts previously aggregated with polyethylene glycol were fused with variable direct current pulses and hybrid cells that underwent sustained mitotic division producing small colonies were obtained.

Many plant cell cultures fail to synthesize secondary metabolites, but they are still important due to their ability to perform specific reactions, a process known as biotransformation. Results of cardenolide biotransformation with *Digitalis* cell cultures (for review, see Kreis and Reinhard 1989, 1990) offer the advantage of transforming cardenolides seldom used in pharmacy into compounds of medicinal importance.

Table 5.7 summarizes biotechnological approaches applied to the genetic improvement of *Digitalis* species.

5.7 Scope for Domestication and Commercialization

Some *Digitalis* species have economical relevance because of their ornamental use: many varieties of *D. ferruginea*, *D. grandiflora*, *D. lutea,* and *D. thapsi* are cultivated worldwide as garden herbs due to their appreciated inflorescences. However, the main interest of the *Digitalis* genus comes from other two species, *D. purpurea* and *D. lanata* (Duke 2002), which besides their ornamental value, have been traditionally used as medicinal plants.

There are references of the medicinal uses of *D. purpurea* leaves of wild growing plants from as early as in the seventeenth century. This species is a source of digitalin, digitoxin, and gitalin, and is cultivated in several countries of Europe, Asia, and America. *D. lanata* is the source of acetyldigoxin, deslanoside, digoxin, and lanatosides A, B, and C. Digitoxin rapidly strengthens the heartbeat but is excreted very slowly. Digoxin is therefore preferred as a long-term medication (Chevallier 1996) and *D. lanata* the main crop for the commercial production of this cardiac glycoside (Hill 1952; Uphof 1959; Launert 1981; Grieve 1984; see also Newman et al. 2008). The species is widely cultivated in Europe, India, Nepal, and Brazil.

D. lanata was used in herbal medicine with a recognized stimulatory effect upon the heart. In allopathic medicine, *D. lanata* leaves serve as the main source for the isolation of those cardiac glycosides used in the treatment of heart complaints (Bown 1995; Chevallier 1996). *Digitalis* glycosides have a profound tonic effect upon a diseased heart, enabling

Table 5.7 Biotechnological tools for the genetic improvement of *Digitalis* species

Cell culture	*D. lanata*	Nickel and Staba (1997)
	D. purpurea	Pilgrim (1977)
Shoot cultures	*D. lanata*	Schöner and Reinhard (1982)
		Diettrich et al. (1990)
	D. minor	Sales et al. (2002)
	D. obscura	Vela et al. (1991)
	D. purpurea	Hagimori et al. (1982a, b)
	D. thapsi	Herrera et al. (1990)
Shoot organogenesis	*D. lanata*	Luckner and Diettrich (1988)
		Rücker (1988)
	D. minor	Sales et al. (2002)
	D. obscura	Pérez-Bermúdez et al. (1985a, b)
	D. purpurea	Rücker et al. (1981)
		Hagimori et al. (1982a, b)
	D. thapsi	Cacho et al. (1991)
	D. trojana	Çördük and Aki (2010)
Somatic embryogenesis	*D. lanata*	Kuberski et al. (1984)
		Reinbothe et al. (1990)
	D. obscura	Arrillaga et al. (1987)
Protoplast-derived plants	*D. lanata*	Diettrich et al. (1982)
		Schneider (1988)
	D. obscura	Brisa and Segura (1987)
	D. purpurea	Diettrich et al. (1980)
Haploid plants	*D. lanata*	Diettrich et al. (2000)
	D. obscura	Pérez-Bermúdez et al. (1985a, b)
	D. purpurea	Corduan and Spix (1975)
Cryopreservation	*D. lanata*	Diettrich et al. (1986a, b, 1987)
	D. obscura	Sales et al. (2001b)
	D. thapsi	Morán et al. (1999)
Hairy root cultures	*D. lanata*	Pradel et al. (1997)
	D. purpurea	Saito et al. (1990)
Transgenic plants	*D. lanata*	Lehmann et al. (1995)
	D. minor	Sales et al. (2003)

the heart to beat more slowly, powerfully, and regularly without requiring more oxygen. At the same time, it stimulates the flow of urine, which lowers the volume of the blood and lessens the load on the heart (Chevallier 1996). The leaves should only be harvested from plants in their second year of growth, picked when the flowering spike has grown and about two-thirds of the flowers have opened. Harvested at other times, the content in the medically active glycosides is lower. The seed has been used traditionally. The leaves are strongly diuretic and were used with benefit in the treatment of dropsy (Grieve 1984). Great care should be taken when using leaves or extracts prepared thereof since the therapeutic dose is very close to the lethal dose (Foster and Duke 1990). Cardiac glycoside may cause nausea, vomiting, slow pulse, visual disturbance, anorexia, and fainting (Bown 1995). Therefore, their use is obsolete and only a homeopathic remedy, used in the treatment of cardiac disorders, is still prepared from the leaves (Launert 1981).

Heralded as the oldest known cardiovascular drug, digoxigenin remains widely used today in the face of increasing rates of heart failure and atrial fibrillation despite the emergence of new medications (Vivo et al. 2008). Recent findings suggested for a regulatory role of cardiac glycosides in several cellular processes, thus highlighting new therapeutic applications for these compounds, especially as anticancer drugs (Nesher et al. 2007; Prassas and Diamandis 2008). Digitoxin can inhibit the growth and induce apoptosis in cancer cells, probably by inhibition of glycolysis, malignant cells being more susceptible to this natural compound (López-Lázaro 2007). This

implies that production of both digoxin and digitoxin is of increasing interest.

Despite advancements in synthetic chemistry, we still depend upon biological sources for a number of secondary metabolites including pharmaceuticals. One of these is digoxin, the chemical synthesis of which is not economically viable and is therefore produced from dried leaves of *D. lanata*, reaching a price of $3,000 per kg (Rao and Ravishankar 2002).

Although the international scale of the medicinal plants trade is difficult to assess, mainly due to the companies' secrecy, the economical importance of this market can be inferred from studies that estimated that 25% of the prescriptions of pharmaceuticals in the developed countries contain plant-derived chemicals, while in the developing countries about 75% of the population relies on plants for traditional medicine (see references in Canter et al. 2005). The European cardiovascular drugs market is expected to reach more than $36 billion in 2012 according to a study by Frost & Sullivan consultants. There is an urgent need for domestication, production, and biotechnological studies and genetic improvement of medicinal plants, instead of the use of plants harvested in the wild (Calixto 2000).

5.8 Recommendations for Future Actions

Cardiac glycosides or cardenolides are natural products contained in several unrelated angiosperm families, although leaves from *Digitalis* species are the most important source of these compounds. Due to their effectiveness in the treatment of heart insufficiency, cardenolides from *Digitalis*, especially *D. lanata*, are still used very extensively worldwide (Wasserstrom and Aistrup 2005; Vivo et al. 2008). Recent findings suggest for a regulatory role of cardiac glycosides in several cellular processes (Prassas and Diamandis 2008), thus highlighting new antitumoral applications for these compounds (Nesher et al. 2007). Given the high therapeutic and commercial value of cardenolides, biotechnological approaches in *Digitalis*-breeding programs should attain a special significance. Paradoxically, the actual impact of biotechnological tools on the genetic improvement of *Digitalis* species to date has been minimal. In fact, with the exception of a paper demonstrating that transgenic *D. minor* plants are a valuable system to achieve metabolic engineering of the cardenolide pathway (Sales et al. 2007), there is no work reporting engineering of other *Digitalis* species with genes of potential economic importance. Among potentially valuable genes are primarily those for key enzymatic or regulatory steps of the cardenolide biosynthetic pathway. The two main biotechnological approaches that will have a high impact for the genetic improvement of *Digitalis* in the near future are as below.

1. Development of reliable genetic transformation systems for those *Digitalis* species of high economic value, viz. *D. lanata* and *D. purpurea*; this would facilitate the metabolic engineering of cardenolides and the engineering of agronomic traits in these species; for the former molecular approach to succeed, a better understanding of the cardenolide biosynthetic pathway and their genetic control is still needed.
2. A major use of molecular marker techniques that should be of help to assess genetic diversity in natural or managed *Digitalis* populations and to authenticate *Digitalis* material employed for cardenolide isolation and breeding.

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Chapter 6
Elaeis

Maizura Ithnin, Rajinder Singh, and Ahmad Kushairi Din

6.1 Introduction

6.1.1 Economic Importance of Oil Palm

Oil palm is the most productive oil-bearing plant species. In good growing conditions, a hectare of oil palm produces on average 4.5 tons crude palm oil (CPO) per year, 0.50 tons palm kernel oil (PKO), and 0.45 tons palm kernel cake. Oil yield of the oil palm is almost three times that of coconut and about ten times that of soybean (Table 6.1). Oil palm has the potential to produce up to 40 tons of fresh fruit bunch (FFB) or 10 tons of CPO per hectare per year.

In 2008, the total oil palm area in Malaysia was 4.48 million ha. The CPO production for that year was 17.73 million tons, an increase of 12.1% compared to previous year. The crude PKO production rose from 1.91 million tons in 2007 to 2.13 million tons in 2008. The 2008 Malaysian average production for FFB yield increased by 6% to 20.18 tons/ha and CPO production was 4.08 tons/ha, the highest recorded since 1987. This was attributed to recovery of FFB from biological stress and larger matured areas (MPOB Statistics 2009).

The Malaysian palm oil exports expanded to 15.41 million tons or 12.1%, mainly to China, the European Union (EU), Pakistan, United States, India, Japan, Bangladesh, and Egypt. China was the largest importer, accounting for 24.6% of the total exports. There was a significant (31.8%) increase in palm oil import by the United States due to the *trans*-fatty acid labeling, which was enforced in the country since 2006. Reduced import duties for palm oil and Government's efforts to distribute vegetable oil to low-income households had stretched palm oil exports to India by almost 90% in 2008. Exports of oleochemical products, however, showed a decrease of 3.2% or 2.07 million tons. Exports of biodiesel increased to 0.18 million tons (91.7%), primarily to United States (39.2%) and EU (38.6%) (MPOB Statistics 2009).

World production of oils and fats stood at 160 million tons for year 2008. Palm and PKOs jointly remain the largest contributor accounting for 30% of the total production. Malaysia contributed about 11% of the global oils and fats yield, and maintained dominance in palm oil trading (GOFB 2009).

6.1.2 Distribution of Elaeis Species

Oil palm belongs to the genus *Elaeis* under the family Palmae, which is an important member of the monocotyledonous group. It is included in the *Cocoineae* tribe together with the genus *Cocos* (Uhl and Dransfield 1987; Latiff 2000). Within the genus *Elaeis*, two species are distinguished: the economically important oil palm species *E. guineensis* and the species of American origin, *E. oleifera*. These two species hybridize readily suggesting a close relationship despite their geographical separation to two different continents (Hardon and Tan 1969).

The two species, *E. guineensis* and *E. oleifera*, are distinctly isolated in Africa and Latin America, respectively. The commercial oil palm, *E. guineensis*, is endemic to West Africa stretching from Senegal through Angola. The Latin American *E. oleifera* is

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Table 6.1 Oil yield in various crops (Robbelen 1990)

Crop	Oil (kg/ha/year)
Oil palm	2,500–4,000
Coconut	600–1,500
Olive	500–1,000
Rapeseed	600–1,000
Sunflower	280–700
Groundnut	340–440
Soybean	300–450

distributed in Honduras, Nicaragua, Costa Rica, Panama, Colombia, Venezuela, Surinam, Ecuador, Peru, and Brazil.

The main oil palm (*E. guineensis*) belt runs through the southern latitudes of Sierra Leone, Liberia, Ivory Coast, Ghana, Togo, Nigeria and Cameroon and into the equatorial region of the Congo and Angola between 10°N and S (Zeven 1967). Tribal migration or intergroup exchanges has spread oil palm across Africa (Smith et al. 1992). Oil palm was taken to Congo and East Africa before the arrival of Europeans. It was introduced to Sudan about 5,000 years ago (Clark 1976). The sporadic occurrence on the East African coast in Uganda, Kenya, Tanzania, Rwanda, and Burundi was probably due to slave traders. The Africans probably brought oil palm to Madagascar in the tenth century (Pursglove 1972). *E. guineensis* was grown in European conservatories in 1793. It was in Calcutta in 1836 and must have reached Mauritius at an earlier date.

In 1848, the Dutch imported oil palm seeds from West Africa resulting in the four oil palm seedlings planted at Buitenzorg (now Bogor) Botanical Garden, Indonesia. Two of the seedlings came from the Amsterdam Botanic Garden and two from Bourbon (now Reunion) (Pursglove 1972; Hartley 1988). This introduction laid the foundation for the oil palm industry in Southeast Asia. Development of the oil palm industry came about when demand for vegetable oils escalated sharply as Europe was experiencing industrial revolution at the turn of the twentieth century.

The first commercial oil palm estate in Malaysia was established in 1917 at Tenammaran estate near Kuala Selangor. Up to the 1950s, it was common for estates to plant large commercial oil palm areas with seeds, which were either of virtually unknown origin or which had been collected from the best-looking palms in an avenue or high yielding field. With the discovery of the single gene inheritance for shell thickness (Beirnaert and Vanderweyen 1941), the potential value of pedigree seed from appropriately selected palms was much appreciated and there was a sudden surge for *dura* × *pisifera* (DxP) hybrid planting materials. The switch to DxP was however gradual due to the limited supply of planting materials. The impetus for planting large areas with DxP seedlings came about in early 1960s when the Malaysian Federal Land Development Authority (FELDA) developed large areas for resettlement programs.

6.2 Basic Biology of *Elaeis* Species

6.2.1 Morphology

Both *E. guineensis* and *E. oleifera* are large-feathered palm. At maturity, a solitary columnar stem with persistent leaf bases is developed, which carries a crown of feathery leaves. The orientation of the *E. oleifera* leaflets on the rachis is in one plane, instead of two ranks in *E. guineensis*. *E. oleifera* is relatively much shorter than *E. guineensis*, with annual height increment between 5 and 10 cm, which is less than 20% of that observed in *E. guineensis*. The length of the leaf is much shorter and as such has smaller leaf area than *E. guineensis* (Hartley 1988). The *E. guineensis* stem remains erect while *E. oleifera* has a procumbent trunk when a height of 3 m is reached. They are monoecious plants, i.e., producing separate male and female flowers on inflorescences of the same palm. Although monoecious, oil palm is functionally cross-pollinated due to the alternating cycle of male and female inflorescences (Pursglove 1972; Hartley 1988). The flowers are bisexual in origin, but in the males the stigmas are suppressed, whereas in the females the stamens are undeveloped. Beirnaert (1953) has shown that the oil palm flower primodium has both male and female organs. In a potentially female flower primodium, the two accompanying male flowers are suppressed and remain rudimentary. In a potentially male flower primodium, the female organ is suppressed.

The male inflorescences of both species are similar. At maturity, a male inflorescence has 100–200 spikelets. For *E. guineensis*, one male inflorescence yields

on average 30–50 g of fresh pollen. A female inflorescence on a matured *E. guineensis* may have more than 100 spikelets with over 4,000 floral buds. Similar number of spikelets emerge in *E. oleifera* with floral buds between 2,000 and 5,000 (Tan 1983).

The female inflorescences of both species are covered by spathe. On *E. oleifera* palms, the female inflorescences are covered by a double sheath making it more recalsitrant to pollination. These sheaths remain even when the bunch is ripe. Although the anthesis period is longer; 3–4 weeks compared to 3–4 days for *E. guineensis*; the flowers are hidden in the spikelet, hence, also affecting pollination efficiency. This is one of the reasons that point to the high bunch failure among *E. oleifera*, as well as the OxG (*oleifera* × *guineensis*) hybrids.

The oil palm fruit is a drupe. The fruits are borne on spikelets, which are spirally arranged to form a compact bunch. After fertilization, fruit development is more rapid on the upper than the lower spikelets and similarly, fruits on the upper spikelets ripen earlier than the lower ones. The individual *E. oleifera* fruits are smaller than those of *E. guineensis*. The *E. oleifera* bunches are usually smaller because about 90% of the fruits are parthenocarpic and abortive. The color of the *guineensis* fruit varies considerably. The commonest type is deep violet, blakish color before ripening known as nigrescens. An uncommon type is green and is called virescens. The virescens is dominant over nigrescens (Pursglove 1972; Hartley 1988). In general, the color of unripe *oleifera* fruits is pale yellow and gradually change to orange when matured. The fruit color of this species also varies from immaturity to maturity; mainly from yellowish green to ivory at the base of the bunch. However, there are some reports that *E. oleifera* bunches from Colombia undergo change in fruit color from bright green to pale yellow, whereas for *oleifera* in Suriname, unripe bunches are green but turn red when ripe (Tan 1983).

E. guineensis has three fruit forms – *dura*, *tenera*, and *pisifera* – categorized by thickness of the shell, which is controlled by a single gene (Beirnaert and Vanderweyen 1941). The *dura* is homozygous dominant (Sh^+Sh^+) for thick shell and the *pisifera* is homozygous recessive (Sh^-Sh^-) for shell-less fruit. The *tenera* is thin shell and is the hybrid (Sh^+Sh^-) between the *dura* and *pisifera*. Palm oil is extracted from the mesocarp and PKO from the kernel. Because of differences in shell thickness, the *tenera* has higher oil-bearing mesocarp (60–90%) than the *dura* (20–65%). Kernel contents of both fruit forms are similar (5–10%). The *pisifera* is usually female sterile, hence, not used as planting materials. Instead, it is used as the pollen source in DxP crosses for the production of *tenera* planting materials. There is no evidence that these fruit structure differences exist in the *E. oleifera* palms. The *oleifera* fruits have rather thin mesocarp with extremely low oil yield (about 0.5 tons/ha per year), far below the average of Malaysian commercial materials (4.08 tons/ha per year in 2008).

6.2.2 Cytogenetics

Both *E. guineensis* and *E. oleifera* are diploids with chromosome number $2n = 32$ (Hardon 1979; Maria et al. 1995). Differences between paired homologous chromosomes of these species were not significant. Although these two species differ in several morphological characteristics, normal pairing of chromosomes of both the species occurs during hybridization (Maria et al. 1998).

Maria et al. (1999) carried out an investigation on the introgressed parental genomes of OxG hybrids using the genomic in situ hybridization (GISH) technique. There was a clear differentiation between the *E. oleifera* and *E. guineensis* genome. There are 16 *E. oleifera* and 16 *E. guineensis* chromosomes in the OxG hybrids. However, in backcross progenies, due to exchange of genetic materials and independent assortment during meiosis, variable number of parental chromosomes are inherited. This information is very useful to oil palm breeders to access the genomic composition of the backcross progenies and select only hybrids with a high proportion of the *guineensis* parents for further backcrossing.

6.2.3 Genome Size

The haploid genome size of oil palm has been estimated to be 1.7 Gbp (Rival et al. 1997). Using flow cytometry, the genome sizes of *E. guineensis*, *E. oleifera*, and their OxG hybrids have also been estimated (Maria et al. 2008). Within *E. guineensis*, the genome sizes of the *dura* and *pisifera* appear to be significantly

different. Maria et al. (2008) also found that the OxG hybrids had larger genome size than their parents.

6.3 Conservation Initiatives

Initially, oil palm improvement in Malaysia made use of progenies from the four Bogor seedlings as genetic foundation in breeding programs. With these materials, significant progress in improving oil palm yield was obtained over the last few decades. However, the limited gene pool restricts the progress that can be achieved through breeding for the various traits of interest such as high iodine content, high kernel content, slow height increment, and minor components (carotene and vitamin E). The development of oil palm planting materials with such traits requires the availability of genetic variability. This realization provided the initiatives for Malaysian Palm Oil Board (MPOB) to search for new genes in the oil palm center of origin. The first comprehensive and systematic effort in *E. guineensis* germplasm collection was made in Nigeria in 1973 (Rajanaidu 1985), an effort jointly undertaken by the Malaysian Agricultural Research and Development Institute (MARDI) and the Nigerian Institute for Oil Palm Research (NIFOR). The collections were later extended to other countries within the African oil palm belt including Cameroon, Congo DR, Tanzania, Madagascar, Angola, Senegal, Gambia, Sierra Leone, Guinea (Rajanaidu 1994), and Ghana. The germplasm materials are currently being maintained by MPOB. The materials are being utilized to raise the yield potential, improve nutritional qualities, and address future challenges faced by the oil palm industry. The available genetic variability within the germplasm has paved the way for the introduction of new genes into the existing breeding stocks, enhancing the prospects of the oil palm industry.

E. oleifera genetic materials were collected in 1981–1982 in six countries, namely Colombia, Panama, Costa Rica, Honduras, Brazil, and Surinam (Rajanaidu 1986). The American oil palm is attractive to breeders because it possesses a number of desirable traits such as slow height increment, high iodine value, and possible tolerance to disease such as Fusarium wilt. The fatty acid composition (FAC), iodine value, and carotene content of the *E. oleifera* are more attractive compared to *E. guineensis*.

The germplasm collection at MPOB has made it possible to introduce novel genes into the current oil palm breeding materials. Creation of new oil palm breeding populations is possible based on the new introductions. The germplasm collection has also made it possible to conserve a cross-section of oil palm's wild relatives, since natural palm grooves in its center of origin are affected by development and human intervention.

The wild materials cannot be exploited directly for commercial purposes, and have to be introgressed into advanced breeding materials. As such, the wild collections have to be evaluated extensively and screened for interesting traits, for incorporation into the existing commercial materials. In this respect the wild materials from Nigeria have proven to have interesting, as about 3% of the *teneras* had oil yield comparable or close to commercial materials at the time of evaluation, while a third of the collection exhibited slower height increment compared to the commercial material (Rajanaidu et al. 1992). The Nigerian materials as such were quickly exploited and several *dura* families, which exhibited high bunch number and the dwarfness trait, were used as mother palms to produce MPOB's Planting Series 1 (PS1), which exhibits slower height increment and are higher yielding compared to commercial material (Kushairi et al. 1999a). The dwarf Nigerian *pisiferas* have also been used as pollen source to produce MPOB's Planting Series 1.1 (PS 1.1). The dwarfness trait is attractive for oil palm, as it facilitates easy harvesting and helps lengthen the replanting cycle of the palm.

Many palms in the Nigerian collection also showed higher oil unsaturation (measured in terms of iodine value, IV). A higher IV value indicates that the oil is more unsaturated. Many individual palms had IV in excess of 60, which is higher than the commercial palm oil, which has IV ranging from 50 to 53 (Rajanaidu et al. 2008). The exploitation of the Nigerian palms to increase the level of unsaturation can enable palm oil to better penetrate into the liquid oil market. The development of Planting Series 2 (PS2) (Kushairi et al. 1999b) that produces oil that is more unsaturated and liquid compared to the standard *Deli dura* × *AVROS pisifera* is a result of exploiting the high IV Nigerian palms.

The *E. oleifera* collections from South America also showed interesting attributes. Slow height increment (Hardon and Tan 1969; Meunier and Boutin

1975) and resistance to certain diseases (Meunier 1987; Tan 1987; Guen et al. 1991; Amblard et al. 1995) are of course traits of interest in *E. oleifera*. In addition, the palms were also actively screened for FAC. It was found that the oil characteristic of *E. oleifera* was quite close to olive oil (Rajanaidu et al. 1985). Screening of MPOB *E. oleifera* germplasm collection for FAC indicated that the species has relatively high levels of oleic (C18:1) and linoleic (C18:2) acids but low levels of palmitic (C16:0) acid resulting in oil with higher unsaturation compared to oil from the commercial *E. guineensis*. Higher IV was also observed among the *E. oleifera* populations. Palms collected from Colombia, Panama, and Costa Rica germplasm collection had iodine value of more than 90, compared to IV of about 50 for the current *E. guineensis* planting materials. Their C18:1 level ranged from 53 to 68%, whereas for C18:2 the level varied between 11 and 26% (Mohd Din et al. 2000). Obviously, the *oleifera* species offers great opportunities for oil palm improvement, in terms of oil quality.

Apart from oil higher in unsaturation, the *E. oleifera* collections also have a higher content of carotene (Mohd Din et al. 2002, 2004). Some palms of the *E. oleifera* collections have shown carotene content in excess of 3,000 ppm, compared to the current commercial material, which has carotene content of only between 500 and 700 ppm (Mohd Din et al. 2002, 2004). High carotene palm oil can be an important source of alpha- and beta-carotene, which are important for human health. Selected high carotene *oleifera* palms have been included in breeding programs and sampled for cloning (Mohd Din et al. 2004).

Breeders have traditionally focused on having higher level of mesocarp oil in the breeding populations. However PKO is a rich source of lauric acid (C12:0), which is important for the oleochemical industry. The availability of high kernel *dura* palms within Nigerian wild materials (10% kernel/bunch, compared to 5% for commercial material) (Rajanaidu et al. 2008), has made it possible to develop planting materials with high kernel content.

The wild collections housed at MPOB were also evaluated for vitamin E content, which is naturally present in small amounts in vegetable oils and acts as antioxidant. Palm oil contains an average of about 800 ppm vitamin E (Choo and Yusof 1996). Among the germplasm, palms with vitamin E ranging from 1,300 to 2,500 ppm were identified (Kushairi et al. 2004). Within the germplasm collection at MPOB, the *teneras* had a higher level of vitamin E compared to *duras* (Kushairi et al. 2004). The high vitamin E palms are currently being subjected to progeny testing.

Harvesting of oil palm bunches is mostly carried out using a chisel attached to aluminum or bamboo pole for tall palms (Noh et al. 2005), although mechanization is slowly being introduced. Harvesting remains a tedious and time-consuming process. Several palms from the wild collections were found to have long stalk (measuring 20–36 cm) compared to current commercial materials, which have short stalk length (10–15 cm) (Noh et al. 2005). The introgression of the long stalk trait into current commercial material is on-going with the aim of helping to ease harvesting of bunches and improve worker productivity.

The wild *E. guineensis* palms from Madagascar deserve a special mention. In Madagascar, the distribution of the oil palm is very sparse (Rajanaidu et al. 1992). The palms are generally much shorter than *E. guineensis* palms from the other collections in Africa. The palms growth, bunch, and fruit traits were also poor when compared to materials collected from elsewhere in Africa (Rajanaidu et al. 1992). The palms nevertheless can be exploited to introgress the slow height increment character into the current commercial materials.

Apart from the wild *E. guineensis* and *E. oleifera* collections, MPOB also has a collection of peach palms (*Bacrtis gasipaes*), which belong to same Palmae family as oil palm. Peach palms are an important food crop in the humid lowlands of central and South America (Rajanaidu et al. 2004). The peach palm which gives two edible products – starchy fruits and palm heart – is one of the good candidates to diversify the current plantation crops in Malaysia dominated by oil palm, rubber, and cocoa.

In the near future, the wild *guineensis* and *oleifera* collections look the most promising to help the industry move forward. With respect to *E. oleifera* although the oil composition of *E. oleifera* palms is interesting, the oil yield of pure *oleiferas* is very low (oil to bunch ratio of 5%), compared to *E. guineensis* (oil to bunch is >25%) (Rajanaidu et al. 1992). As such the pure *oleiferas* cannot be directly utilized as commercial planting materials. However, the interesting traits from *E. oleifera* can be introgressed into *E. guineenis* through interspecific hybridizations.

6.4 Interspecific Hybridization

Oil yield is of primary interest in oil palm cultivation. Kernel yield is a secondary product. The selection of these traits involves evaluation of the FFB yield and bunch components. In improvement programs of *E. guineensis*, oil palm breeders adopted the reciprocal recurrent selection (RRS) or its modification (modified reciprocal recurrent selection – MRS) as the breeding scheme. Under these schemes, the parents (*dura* and *pisifera* populations) are kept as distinctly separate base populations. Selection for oil yield is emphasized in both parental populations.

However, these schemes are not applicable in exploiting interesting traits detected in the *E. oleifera* palms due to its extremely low oil yield. It is known that *E. guineensis* can be crossbred with *E. oleifera* giving rise to an OxG hybrid. Backcross method seems to be most suitable for transferring specific traits from *E. oleifera* to the palm of commerce, *E. guineensis*. In this scheme, selected OxG hybrids will be repeatedly backcrossed with the guineensis parent (Fig. 6.1). Theoretically, by the sixth generation of backcrossing, the entire genotype of the *guineensis* parent would be recaptured, together with the desired traits.

Yap and Tan (1988) proposed a modified backcross method (Fig. 6.2). The scheme recommended that backcrossing to the recipient is carried out alternately, with selfing of selected hybrids, to obtain *dura* and *tenera* populations for eventual DxP seed production. Once the cycles are completed, the final progenies are subjected to field evaluation to select short *guineensis* with traits derived from the *oleifera*.

Yong and Chan (1993) suggested the single seed descent scheme, beginning with selfing of the selected backcross progenies to develop recombinant inbred lines having high unsaturation and oil yield.

6.5 Classical Genetic Studies Involving Interspecific Hybrids

The OxG hybrid programs have been initiated worldwide by oil palm breeders to exploit the economically important genes of *E. oleifera*. Apart from the long breeding and selection cycle, i.e. 10 years, difficulties in estimating the actual value of the OxG hybrid were highlighted. The low yields among the hybrids were

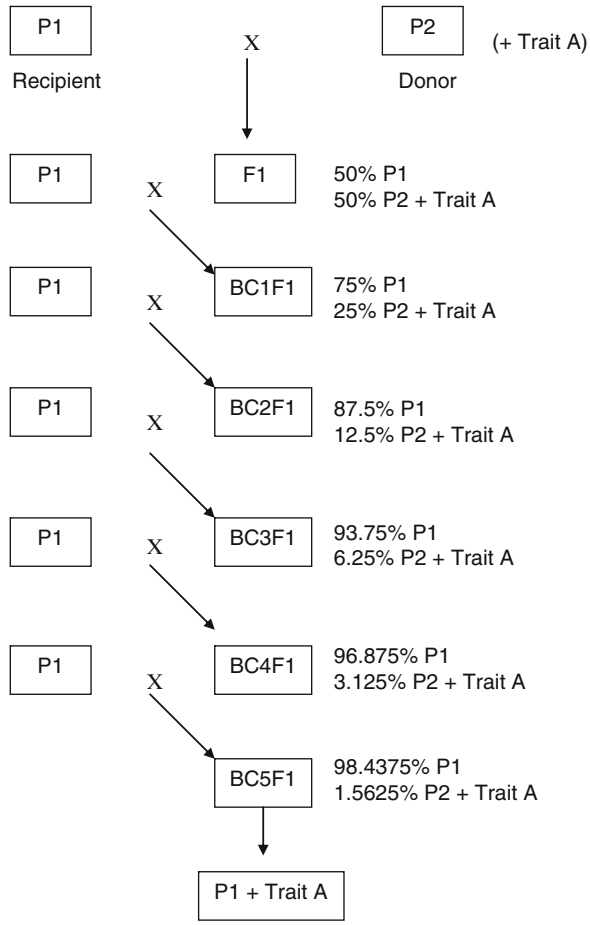

Fig. 6.1 Backcrossing showing increment of P1 (usually *guineensis*) genome and the transfer of trait A from P2 (usually *oleifera*) into backcross progenies

believed to be due to the faulty gamete functioning after hybridization (Schwendiman et al. 1982), which caused abortion of female flowers and low pollen viability. In addition, the persistent spathe covering the female inflorescences during anthesis affects pollination rate. The large number of parthenocarpic fruits influences the measurement of some components such as fruit-to-bunch and mesocarp-to-bunch ratios (Meunier 1987). Amblard et al. (1995) suggested that planting some *E. guineensis* palms surrounding the OxG experimental plots might possibly provide good pollen source for pollination. Assisted pollination may be a possible solution to improve fruit set however, this has been found to be impractical at commercial scale. Due to the high frequency of parthenocarpic fruits and low yield, direct commercial use of the hybrids may not be viable.

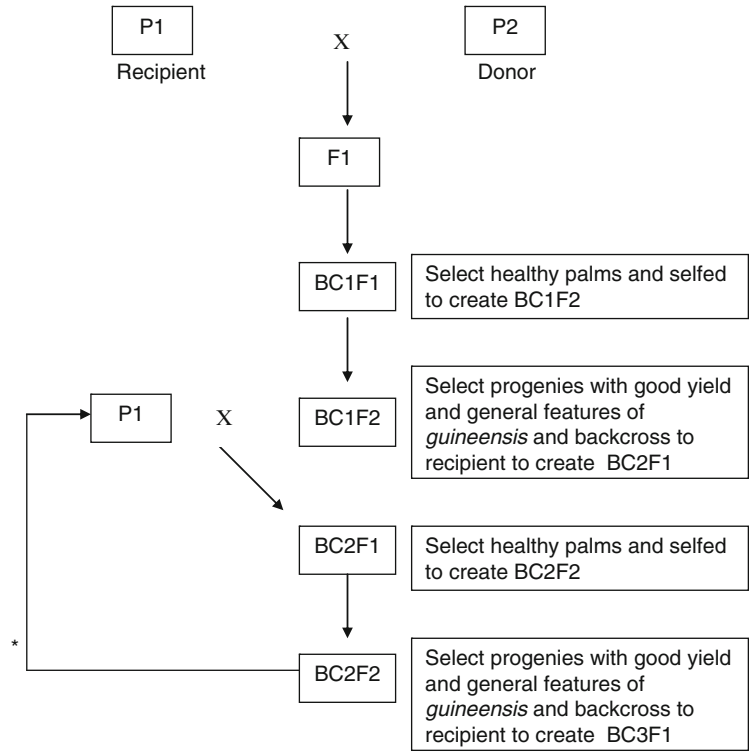

Fig. 6.2 Modified backcross method (adapted from Yap and Tan 1988)

Nevertheless, several researchers have reported encouraging yield performance among the hybrids. Samples of some first backcross (BC1) and second backcross (BC2) progenies showed 10–26% more oil yield, respectively, compared to commercial materials (Chin et al. 2003). Similarly, Amblard et al. (1995) highlighted FFB yield of their best hybrids achieving 85–90% of the commercial *E. guineensis*. However, Pamin et al. (1995) reported lower FFB yields in their backcross progenies as compared to the commercial oil palm. Positively, there is a 5% increase in the oil extraction rate among the backcross progenies over their F_1 hybrids. Due to genetic segregation, the progenies showed high variation in bunch characteristics and oil quality, which are advantageous for further selection. It was evident that most of the bunch characteristics of the OxG hybrids were inherited from the *E. oleifera* parents (Hardon 1969; Sterling et al. 1995). As such, choosing *oleifera* parents with reasonably good bunch characteristics would be useful to increase the productivity of the OxG and backcross progenies.

An increase in unsaturation is desirable due to consumer demands for monounsaturated and polyunsaturated dietary oils and fats. A higher unsaturated palm oil will enable better penetration into the liquid oil market. The FAC of palm oil from current planting materials limits its share of the liquid and salad oil markets in temperate countries. High iodine value palm oil is more liquid and tolerable to lower temperatures. Notably, the OxG hybrids produced oil richer in IV and unsaturated fatty acids (oleic (C18:1) and linoleic (C18:2) acids) than that of *E. guineensis* (Rajanaidu et al. 1985; Amblard et al. 1995; Pamin et al. 1995). Among the backcross progenies, the unsaturated fatty acids and IV among backcrossed progenies were significantly higher than DxP commercial, but unfortunately lower than the OxG hybrids (Yong and Chan 1993; Chin 1991). These observations highlighted the possibility of developing more superior palm oil.

In plantation practices, short palms are favored over tall ones for the ease in harvesting and to lengthen the economic life of the palm, which is

around 20–25 years. The current oil palm planting materials grow too tall, making harvesting cumbersome after 20 years of field planting. In addition, slow-growing palms are environment-friendly as they lengthen the replanting cycle of the plantation. Lower growth rate and compactness were observed among the OxG hybrids compared to the commercial materials (Yong and Chan 1993; Amblard et al. 1995; Pamin et al. 1995; Rajanaidu et al. 1995; Sterling et al. 1995). It is important to note that the origin of the *pisifera* parent used in the backcrossing program has significant impact on the height of the hybrids. OxG hybrids descended from AVROS *pisifera* parents were taller and had longer frond than those from other *pisiferas* (Sterling et al. 1995). While the OxG hybrids showed intermediate height between the parents, the height is further reduced among backcross progenies (Yong and Chan 1993; Pamin et al. 1995).

Among the OxG hybrids, undesirable excessive vegetative growth in the fronds and leaves gives rise to problems with harvesting and also results in inter-palm competition (Yong and Chan 1993). The subsequent backcross progenies however showed significant reduction in plant canopy, but were still above that seen in the commercial materials. In contrast, Lubis et al. (1987) and Pamin et al. (1995) reported more compact backcross progenies over their F_1 hybrids, presumably due to different *E. oleifera* sources used.

In the succeeding second backcross progenies (BC2), Chin et al. (2003) reported *E. guineensis*-like features. These progenies exhibited high variability in growth, from short and compact to typical *E. guineensis* stature. Distinct *dura*, *tenera*, and *pisifera* fruit types can be identified and selected for further improvement. High-density planting materials can then be developed, which will increase yield per hectare basis.

Some OxG hybrids, although not systematically proven, showed certain level of tolerance to some diseases and pests such as Ganoderma, bagworm (Tan 1987), bud rot (Amblard et al. 1995), vascular wilt, spear rot, and sudden wither (Meunier et al. 1976). The hybrids were shown to have survived in areas heavily infected with bud rot (Amblard et al. 1995). However, their tolerance to vascular wilt varies greatly even though Renard et al. (1980) reported a high tolerance among their parental lines.

At this juncture, high variability in oil yield, height, compactness, and oil quality still persist among the hybrids and backcross progenies. Commercialization of the best progenies through conventional seed production method is restricted due to limited source of parental lines. Cloning technology offers a rapid and short-term method to exploit the best individuals among the best-performing progenies (Meunier 1987; Tan 1987; Guen et al. 1991; Yong and Chan 1993; Amblard et al. 1995; Pamin et al. 1995). Selection of elite ortets from interspecific hybrids and their backcrosses were carried out (Chin et al. 2003; Escobar et al. 2005). The compact clones, mainly developed from selected backcross progenies, have been field-evaluated and low abnormality rate was reported (Escobar et al. 2005).

6.6 Molecular Genetic Studies Involving Interspecific Hybrids

Breeding efforts to develop oil palm with higher unsaturated oil is complicated because the genes controlling the traits are polygenic. Scientific evidence proved that the oil fatty acids are inherited in an additive or codominance manner (Ong et al. 1981; Rajanaidu et al. 1985; Yong and Chan 1993). Although higher unsaturated oil can also be achieved through breeding for high IV, the crop's long life cycle is a factor that hinders rapid production of newly, high-valued planting materials.

Biotechnology holds great potential for plant breeding as it promises to expedite the time taken to produce crop varieties with desirable traits as well as increase selection efficiency. Identification of quantitative trait loci (QTLs) using the current conventional phenotypic characterization is impossible. Molecular markers, with their ability to utilize information at the genetic level, offers a practical solution to plant breeding. Polygenic characters, which were previously very difficult to analyze using traditional breeding methods, can now be tagged using molecular markers. Establishment of such markers allows development of marker-assisted selection (MAS), a method whereby a phenotype is selected based on the genotype predicted by some markers, which can improve the efficiency of conventional breeding selection.

Tagging of molecular markers to specific traits of interest is unachievable without a saturated genetic linkage map. MPOB has adopted the capacity for construction of a linkage map and using it in MAS. An interspecific (OxG) population that segregates for fatty acid contents and iodine value (IV) was chosen in an effort to search for markers linked to QTLs associated with FAC in oil palm. Controlled pollination was done in crossing *E. oleifera* palm UP1026 and *E. guineensis* tenera palm T128 to generate a mapping population. A total of 118 palms from this interspecific cross were evaluated in the field. The oil was extracted from the palms and analyzed for FAC and iodine value.

DNA samples from the interspecific progenies were screened with 71 informative restriction fragment length polymorphism (RFLP) probes, 19 informative simple sequence repeat (SSR) primers, and 67 amplified fragment length polymorphism (AFLP) primer pairs. Using the pseudo-test cross strategy, a map was constructed for the male *E. guineensis* parent. In total, 252 markers (199 AFLPs, 38 RFLPs, and 15 SSRs) mapped in 21 linkage groups (Singh et al. 2009). The total genetic distance covered by the markers was 1,815 cM, with an average interval of 7 cM between adjacent markers. The average number of markers per linkage group was 12 (Singh et al 2009).

Interestingly, Singh et al. (2009) reported that at a genome wide significant threshold of $P < 0.01$ and $P < 0.05$, significant QTLs were detected for IV, C16:0, and C18:1 using interval mapping (Table 6.2). These QTLs were mapped on the same group. The QTLs showed similar-shaped likelihood profiles, suggesting that the same QTL may be influencing the three traits. Furthermore, these traits have been reported to be significantly correlated (Rajanaidu et al. 1985). IV is a measure of unsaturation of oils and fats. C18:1 is the most abundant unsaturated fatty acid, whereas C16:0 is the most abundant saturated fatty acid in palm oil. As such, it is not surprising that the same locus could be influencing the three traits.

Table 6.2 QTLs for IV and fatty acid composition found to be significant at the empirical genome wide mapping threshold (interval mapping) (adapted from Singh et al. 2009)

Trait	Linkage group	Linked markers	LOD at peak
IV	1	EAGG/MCAT-198 CB75A	8.90
C16:0	1	EAGG/MCAT-198 CB75A	8.06
C18:1	1	EAGG/MCAT-198 CB75A	5.69

The work reported shows the promise in developing markers to help improve oil quality in backcross program. If the marker or QTL linkage holds true across genotypes, a strategy for improving oil quality through MAS can be developed. With MAS, selection of palms and the subsequent crossing can be started soon after flowering (about 2–3 years) without having to wait for the fruits to be formed and analyzed (normally can take about 5 years) (Singh et al. 2009).

6.7 Genomic Resources Developed for *Elaeis* Species

One of the most important genomic resources developed for oil palm is the collection of expressed sequence tags (ESTs). ESTs were generated for the primary purpose of establishing a database for oil palm gene sequences. Toward this end, several groups have reported the generation of ESTs from oil palm tissues. Initially, Jouannic et al. (2005) reported about 2,000 ESTs from inflorescences, shoot apices, and zygotic embryos. This was followed by Ho et al. (2007) who reported the generation of 14,000 ESTs from various tissues of oil palm. More recently, Low et al. (2008) reported over 17,000 ESTs from different developmental stages in oil palm tissue culture. Although the number of ESTs available for oil palm are minimal compared to other more extensively studied annual crops, it does represent an important start to the identification of the genes in oil palm. Although these sequences are available in GenBank, MPOB has also established an additional database known as *PalmGenes* (http://palmoilis.mpob.gov.my/palmgenes.html). The database has additional information on selected gene clones, such as position on genetic maps or information obtained on expression profile in selected tissues. *PalmGenes* is a resource that is intended to expedite research in oil palm biotechnology.

Although EST sequencing is useful, studies in other crops have revealed that EST sequencing generates minimal additional data after the first few thousand sequences have been obtained. Furthermore, the regulatory sequences that govern gene expression are not

sampled via EST sequencing. As such, alternative methods such as genomic sequencing may add value. In this respect, MPOB also exploited the GeneThresher™ technology (Rabinowicz et al. 1999) to expedite the sequencing of oil palm genes. The GeneThresher™ technology allows the selective sequencing of hypomethylated regions, which most likely contain genes and their regulatory regions. The use of this technology has allowed MPOB to generate over 360,000 sequences from three individual palms (Budiman et al. 2005), and it is estimated that 90% of the genes in oil palm have been touched by a sequence. More importantly, the gene sequences have made available a large set of molecular markers such as SSRs and single nucleotide polymorphisms (SNPs) for exploitation by breeders in crop improvement programs.

The existing sequences (mainly ESTs) have also led to the design, validation of a DNA chip for oil palm (Low et al. 2006). The Chip containing over 3,000 oil palm genes is the first step toward developing a comprehensive microarray platform for expression profiling in oil palm. The current oil palm DNA chip has proved useful in the identification of candidate genes associated with tissue culture amenity (Low et al. 2006).

6.8 Future Direction

Current trend suggests the need to breed for elite oil palm planting materials to increase productivity. New planting materials with special features are being developed by extensively exploiting MPOB oil palm germplasm collection from Africa and South America (Kushairi et al. 2003). The introduction of commercial materials selected for high carotene, high iodine value, and dwarfness provides an alternative choice of planting materials to entrepreneurs. This will help promote the plantation sector and enhance the growth of the oil palm industry.

Suitable genotypes within the *E. oleifera* and the *E. guineensis* germplasm have shown prospects for the development of high-grade palm oil. Although the oil extraction rates of such planting materials are lower than those of the current materials, the high valued carotene can be encapsulated as health products that fetch premium prices compared to merely using palm oil for edible purpose.

Through systematic backcross breeding, there is a great possibility to transfer the dwarf genes from the *E. oleifera* to the current oil palm commercial variety. Furthermore, the *E. oleifera* palms are the only known source for resistance to some lethal diseases. Improvement of hybrids for this purpose seems to be also promising.

A great amount of breeding and improvement work must be done before the hybrids can be exploited at commercial scale. The infertility problem may be overcome if hybridizations are confined to parents that have lower incompatibility. The suggestion made by Meunier (1987) on the study of combining ability among the parents should be taken into consideration for improvement of the OxG hybrids.

The availability of an increased number of genes for oil palm, as well as high-density genetic map, will aid in the development of markers linked to important agronomic traits. This will directly allow the selection of improved planting materials via MAS.

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Chapter 7
Euphorbia

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7.1 Basic Botany

Euphorbia is one of the largest and morphologically the most diverse genus among the flowering plants. The genus comprises about 2,160 recognized species and is classified in the family Euphorbiaceae and order Malpighiales. Euphorbs are primarily distributed across the tropical and subtropical regions of Africa and the Americas, but also extend into temperate zones worldwide. The distinctive succulent species originate mostly from Africa, the Americas, and Madagascar.

7.1.1 General Description

The plants are of diverse habit and include annual, biennial, or perennial herbs, xerophytes, and woody shrubs or trees. The xerophytes are well-adapted in dry environments: they may be spiny or unarmed and include leaf succulents, stem succulents resembling cacti, caudiciform species, and geophytes. *Euphorbia* is the only plant genus that utilizes all the three photosynthetic systems (C3, C4, and CAM) for CO_2 fixation; the succulent species use CAM, section *Chamaesyce* are C4 with typical Krantz leaf anatomy and the remainder are C3 (Webster et al. 1975; Reddy et al. 2003). Euphorbs commonly contain abundant milky sap (latex), which is the cytoplasm of specialized multi-nucleate cells called laticifers. The latex contains metabolites such as bioactive natural products and rubber, and unusually shaped starch grains (see Sect. 7.1.3). The roots are fibrous or thick, fleshy, and sometimes tuberous. Some species such as leafy spurge (*Euphorbia esula*) produce vegetative buds on their root systems. The simple, undivided (rarely lobed) leaves are persistent or deciduous and are arranged in alternate, opposite, or in whorl phyllotaxy. In succulent species the leaves are mostly small and short-lived. The stipules are mostly small, but can be fused, missing, or modified into spines ("spine shield" species) or glands.

Like all members of the Euphorbiaceae, *Euphorbia* have unisexual flowers often surrounded by brightly colored leaves as with *Poinsettia* or modified bracts with nectar glands to attract insect pollinators (Fig. 7.1). Their flowers are greatly reduced and grouped into a unique aggregated inflorescence (pseudanthium) called a cyathium. This specialized type of pseudanthium resembles a bisexual flower with a cup-like structure (involucre) of fused bracts, often with nectar glands and petaloid appendages around its rim that enclose a single central female flower surrounded by 4 or 5 groups of stamens arranged as cymes (Prenner and Rudall 2007). These groups of 1–10 stamens are interpreted as partial inflorescences with each stamen representing a highly reduced male flower. The majority of species are monoecious (bearing male and female flowers on the same plant), although some are dioecious with male and female flowers occurring on different plants. It is not unusual for the central cyathia of a cyme to be purely male, and for lateral cyathia to carry both sexes. Sex expression can also be environmentally influenced such that young plants or those growing under unfavorable conditions exhibit only male cyathia, and then produce female flowers at maturity or as growing conditions

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Fig. 7.1 Blooms of two spurges showing flowers borne in cyathia, three-parted central fruits and brightly colored pink or yellow bracts

Fig. 7.2 Growth of underground shoot buds of *E. esula* following loss of dormancy induced by excision of the above-ground portion of the plant

improve. The fruits are three (rarely two) compartment capsules usually explosively splitting at maturity; they are rarely modified as fleshy drupes. The seeds are angled, oval, or spherical, and in some species are arillate with a caruncle at one end. The brown or gray seed coat can be smooth or rough, pitted and warty; some species (especially sect. *Chamaesyce*) become mucilagenous on wetting. Some perennial varieties such as *E. esula* are capable of vigorous vegetative reproduction through development and controlled growth of underground adventitious buds on their often extensive root systems (Fig. 7.2). Cytogenetic studies indicate the species form an aneuploid series of 6, 7, 8, 9, and 10 with geographical cytotypes and polyploidy also occurring in some species (Hans 1973).

7.1.2 *Potential Uses and Valuable Products of the* Euphorbia

Spurges have long been used for their medicinal properties (Turner 1995; Appendino and Szallasi 1997). The common name "spurge" derives from the Middle English/Old French *espurge* ("to purge"), due to the use of the plant's latex as a purgative. Latex was also used as an exfoliant. The botanical name *Euphorbia*

derives from Euphorbus, the Greek physician of King Juba II of Numidia (52–50 BC–23 AD) who was noted to have used the latex to produce a medicine (Mozaffarian 1996). Resin spurge (*E. resinifera*) is noted as a potential herbal remedy for herpes (Yarnell and Abascal 2005). *Ixbut* (*E. lancifolia*) was known to the Mayan Indians of Guatemala to increase milk production, which has been verified experimentally in both cattle and postpartum women (Schultes 1987). *E. pekinensis* is used in traditional Chinese medicine, where it is regarded as one of the 50 fundamental herbs (Wong 1976). Australian asthma herb, *E. hirta*, has long been used as a medicinal to treat hypertension and edema. Studies on this weed have been shown it to increase urinary output and electrolytes in rats (Johnson et al. 1999). A compound from *E. hyberna* was shown to be a very effective anti-HIV agent (Bedoya et al. 2008). Indeed, at the time of this writing, nearly 900 references mentioning *Euphorbia* ssp. were noted in the Biomedical Literature citations and abstracts database (http://www.ncbi.nlm.nih.gov/pubmed/), indicating that work on medicinal compounds from this genus is a highly active area of research.

There are uses for various *Euphorbia* ssp. beyond potential medicinal preparations. Several spurges are grown as ornamentals, notably including the poinsettia (*E. pulcherrima*), which is the best selling potted flowering plant sold in the US. *Euphorbia* extracts have proven to be effective in controlling sting nematode in soil (Cox et al. 2006). Several species, particularly *E. esula*, *E. lathyris*, *E. lactiflua*, and *E. tirucalli* have been investigated as oilseed sources with potential as biofuel crops. *Euphorbia* spp. yield candilla wax in Mexico from epicuticular secretions, rubber from their latex, and fish and arrow poisons (Maxwell et al. 1985; Calvin 1987; Gnecco et al. 1996; Siler et al. 1997; Solymosi 2000).

7.1.3 Toxicity

The latex of *Euphorbia* is often caustic and poisonous and acts as a deterrent for herbivores as well as a wound healer. Usually it is white, but in rare cases (e.g., *E. abdelkuri*) it can be yellow or in other Euphorbiaceae it also can be watery, red, or pink. As the latex is under pressure, it exudes from the slightest wound and congeals within a few minutes of contact with the air. Among the chemical components in the latex are many di- or triterpenes, and different species vary in their chemical profiles (Rizk 1987). The terpene composition determines how caustic and irritating to the skin it is. The latex can produce extremely painful inflammation on contact with mucous membranes (eyes, nose, and mouth). In experiments with animals, it was found that the terpene ester resiniferatoxin had an irritating effect 10,000–100,000 times stronger than capsaicin, the "hot" substance found in chili peppers (Szallasi and Blumberg 1999). Several irritant diterpene esters also known as "phorbol esters" are cocarcinogenic and tumor promoters, although they are not directly carcinogenic (Adolf and Hecker 1975; Seip and Hecker 1982). Leafy spurge (*E. esula*) is toxic to some range animals causing irritation and weakness or death on ingestion of large quantities; its impact on cattle causes large economic losses in rangelands in North America where it is endemic.

7.1.4 Systematics, Taxonomy, and Evolution

Given the large number of *Euphorbia* species, it is not surprising that their taxonomy and sytematics are complex and historically have been much debated. Over 10,000 scientific names have been published but only a fraction of these are presently considered "good" taxa. *Euphorbia* and closely related "satellite genera" (i.e., *Cubanthus*, *Elaeophorbia*, *Endadenium*, *Monadenium*, *Pedilanthus*, and *Synadenium*) are all united by the cyathium and traditionally have been classified as subtribe Euphorbiinae, within tribe Euphorbieae and subfamily Euphorbioideae (Webster 1994; Radcliffe-Smith 2001). These satellite genera are primarily recognized based on specialized modifications to their cyathial form. For example, *Pedilanthus* is unique in bearing elongate, bilaterally symmetrical cyathia that are adapted for hummingbird pollination (Steinmann 2003). Recent molecular phylogenetic studies have indicated that the satellite genera associated with *Euphorbia* are nested deep within the latter (Steinmann and Porter 2002; Wurdack et al. 2005; Bruyns et al. 2006). The taxonomic implications of these findings are that *Euphorbia* must be broadly defined (*sensu lato*; *s.l.*) to include all these

satellite genera; an alternative classification to even further split up this group into many poorly defined smaller genera has not gained broad acceptance.

The closest relatives to *Euphorbia* are four small genera (*Anthostema*, *Calycopeplus, Dichostemma*, and *Neoguillauminia*) from Africa, Madagascar, New Caledonia, and Australia that have pseudanthial inflorescences that are not quite as reduced as a cyathium. This suggests an Old World origin for *Euphorbia* and provides some insights into the inflorescence evolution of the cyathium from a determinate thyrse. The origin of the cyathium has been frequently implicated as a "key innovation" that enabled the adaptive radiation of the genus, but recent phylogenetic results indicate that the evolution of varied, specialized growth forms is an important additional factor in their diversification. For example, the evolution of succulence and weediness are each associated with species radiations. Species radiations have also occurred on islands (i.e., Hawaiian and Canary Islands and Madagascar).

Within *Euphorbia*, the phylogenetic studies indicate four main natural groups, which have been recognized as newly defined subgenera: *Esula*, *Rhizanthium*, *Euphorbia*, and *Chamaesyce* (Bruyns et al. 2006). Further reclassification into sections has begun, but requires additional detailed molecular phylogenetic studies, which are underway. Subgenus *Esula* is "basal" to the other three groups and includes many weedy leafy taxa (e.g., *E. esula*) but few succulents. Subgenus *Rhizanthium* is rich in African succulent species. Subgenus *Euphorbia* contains most of the segregate genera (except *Chamaesyce*), a diverse array of growth and floral forms, and is distributed around the world. Finally, subgenus *Chamaesyce* contains few succulents and is mostly confined to the New World except for the "basal" species and some derived weedy *Chamaesyce*. It contains the C4 segregate genus *Chamaescyce,* which is now classified as a distinct section of the subgenus.

7.2 Conservation Initiatives

Many species of *Euphorbia* have limited distributions and are of conservation concern. Additionally pressure from horticultural trade has resulted in CITES II (Convention on International Trade in Endangered Species of Wild Flora and Fauna) status for all the succulent species, which are in particular demand. For example, the widely grown *E. obesa* has been over-collected to the point of extinction in the wild, but fortunately is easy to propagate in cultivation. *E. clivicola* is endangered by habitat destruction and fractionation in S. Africa (Pfab and Witkowski 2000). *E. conzattii*, a variety of slipper spurge, was thought to be extinct, but was rediscovered in Oaxaca, Mexico in 2002 (SEMARNAT 2002; Olson et al. 2005). Few studies have examined the population genetics of the many rare taxa. Endangered taxa of sect. *Chamaesyce* from the Hawaiian Islands were found to have unusually high levels of genetic diversity (Morden and Gregoritza 2005) but ex situ conservation measures were considered a priority due to habitat loss. Efforts to collect seeds and DNA from threatened populations and species should be undertaken.

The perennial taxa, especially the succulents, are long-lived plants in cultivation under appropriate growing conditions. Propagation of many species is easy by seed or vegetative means. Seeds can be produced via artificial self- or cross-pollination followed by care to contain the explosive capsules, which would otherwise scatter them. Seeds of many species, including xerophytes reportedly have a short shelf-life. Vegetative reproduction, which is seasonal, can be done by cuttings or grafting. Grafting is usually applied to succulent taxa with advantages for rare or difficult species of faster growth and easy to care for stocks (i.e., stocks that are less prone to root rot). Compatibility appears high, at least among related species; it remains to be closely examined in a phylogenetic context to see in particular if it extends between subgenera. Tissue culture via somatic embryogenesis has been successful in poinsettia (Jasrai et al. 2003) and in vitro micropropagation of shoot explants has been achieved in others.

7.3 Role in Elucidation of Origin and Evolution of Allied Crop Plants

Major crops including cassava (*Manihot esculenta*), rubber (*Hevea brasiliensis*), castor bean (*Ricinus communis*), tung oil tree (*Vernicia fordii*) are among other members of Euphorbiaceae. Cassava and rubber are closely related, but the other species along with *Euphorbia* are widely spread across phylogenetic trees of the family and relatively distant from each

other (Wurdack et al. 2005). These species have had varying time periods for domestication. No equivalent domestication has occurred in *Euphorbia*. The domestication of cassava is ancient and its wild relatives and geographical origins were finally determined through non-coding DNA sequence and microsatellite variation (Olsen and Schaal 2001). Relationships among accessions of rubber were reconstructed with a hypervariable non-coding mitochondrial sequence (Luo and Boutry 1995).

7.4 Genomics Resources

With two notable exceptions, relatively few genomic resources are available for *Euphorbia* species. At the time of this writing, only 865 protein sequences were available, many of these were partial plastid *NADH DEHYDROGENASE* (*ndh*F) used in phylogenetic analyses of *Euphorbia*. The internal transcribed spacer region of rDNA, including 5.8S, is an additional widely sequenced locus for phylogenetic purposes. Partial sequences from nearly 60,000 cDNAs (DNA copies of the messenger RNAs from transcribed genes – often referred to as expressed sequence tags, ESTs) are publicly available for *Euphorbia* ssp. The vast majority of these (47,915) are for *E. esula*. However, 9,302 are from *E. tirucalli*, 303 from *E. lagascae*, with a few from various other *Euphorbia* of interest such as *E. pulcherrima*.

E. esula has been well developed as a model herbaceous perennial (Chao et al. 2005). As such, the EST database developed for *E. esula* was developed from all plant tissues including dormant, after-ripened and germinating seeds, plant material from roots, stem, leaves buds, flowers, and developing seed pods, as well as from leaf and meristem material from plants that were drought and cold stressed as well as plant infected with gall midges and flea beetles (Anderson et al. 2007). The EST database has been annotated and found to contain 23,000 unique sequences representing about 19,000 unigenes. The cDNAs representing these 19,000 unigenes along with about 4,000 additional unigenes from cassava that did not match those from *E. esula* have been used to develop cDNA microarrays for gene expression studies. Preliminary data demonstrated that these microarrays could be used to follow gene expression from all members of the Euphorbiaceae that were tested (cassava, poinsettia, and castor bean). Preliminary analysis indicated that relative hybridization intensity corresponds to overall sequence similarity of the hybridizing species and thus the microarrays might be useful for determining the relationship between family members. Thus, these microarrays could readily be used to study gene expression in any member of the *Euphorbia* and therefore could serve as a powerful tool for answering questions concerning developmental and ecological responses of euphorbs to various environments or treatments.

These microarrays have been used to follow changes in gene expression in underground shoot buds of *E. esula* as it transitions through various dormancy states (Horvath et al. 2008). They have also been used to follow changes in gene expression in *E. esula* and cassava following infection with various pathogenic bacteria (Santana and Horvath, In preparation unpublished). The response of various euphorbs to drought stress has also been studied using these arrays (James Anderson personal communication). These studies have helped identify a specific MADS-box transcription factor that appears to be a conserved key regulator of dormancy, provided clues as to the mechanisms of pathogen resistance, and identified common signaling pathways regulating their response to drought stress. Additional studies on seed and flower development (Foley et al. 2009, 2010) are likely to provide information that may be useful in further developing various *Euphorbia* ssp. as potential biofuel crops. Likewise, planned studies on the response of *E. esula* in its native and invaded ranges will provide information on how invasive euphorbs such as *E. esula* evolved invasive and weedy characteristics.

In addition to the microarrays, several genomic and cDNA libraries have been developed to facilitate study of *E. esula*. A bacterial artificial chromosome (BAC) library providing 5× coverage of the *E. esula* genome is now available from the Arizona Genomics Institute. Also, the normalized cDNA library used to produce the EST database is available from James Anderson at the USDA-ARS in Fargo, ND. A cDNA library produced from growing and dormant buds suitable for two-hybrid analysis to identify possible protein–protein or protein–DNA interactions is available from Dr. Horvath at the USDA-ARS in Fargo, ND. Finally, a genomic library built in a ZAP-Expression vector with average insert size of about 5,000 bp is also

available from Dr. Horvath. A cDNA library produced from callus tissue of *E. tirucalli* has been made by Kanji Ohyama, Research Institute of Agricultural Resources, Ishikawa Agricultural College, Ishikawa, Japan (Kajikawa et al. 2004).

Genomic resources for related Euphorbiaceae are considerably developed. Whole genome sequencing is complete for castor bean and cassava (sequencing by DOE-JGI) and is underway for two biofuel species of *Jatropha* (*J. curcas* and *J. tanjorensis*) with high seed-oil content (sequencing by Nandan Biomatrix Ltd). The chloroplast genome is completed for cassava (Daniell et al. 2008). Large EST libraries have been generated for castor bean, cassava, and rubber. The tung oil tree is being developed as a model system for unusual seed oils (Shockey et al. 2006). Poplar (*Populus* spp.) also belongs to Malpighiales, the same order as Euphorbiaceae, and has served as a model organism for trees; its completed and well-annotated genome provides a firm basis for broad comparative genomics.

7.5 Role in Crop Improvement Through Traditional and Advanced Tools

Euphorbia ssp. are not currently cultivated as crop plants with the exception of poinsettia as an ornamental crop. However, as mentioned above, there is potential use as biofuel sources. The major Euphorbiaceae crop species could benefit from advanced genetic resources developed for *Euphorbia*, such as improved disease resistance, yield and post-harvest physiological deterioration in cassava, reducing toxic ricin in castor bean, and resistance to fungal blight and tapping panel dryness in rubber. Genomic resources and tools developed from *E. esula* could be also used to identify regulatory genes or biochemical/physiological processes that be used to improve drought stress in these crop species. With over 23,000 gene sequences from leafy spurge, and numerous transcriptome studies to identify potentially important regulatory genes involved in many biochemical processes, it is possible that genes from leafy spurge could be used to modify developmental and physiological processes in poinsettia or other Euphorbiaceae crops. Transformation of cassava, poinsettia, and leafy spurge via *Agrobacterium tumefaciens* have been reported (Li et al. 1996; Clarke et al. 2008; WS Chao personal communication). Likewise, these tools can be used to identify genes and pathways that could improve biofuel production and/or biomass yield in these potentially new crop species or improve desirable traits in ornamental species of *Euphorbia*. For example, early versions of the *E. esula* microarrays helped identify growth-related genes from poinsettia that were responding to the endemic phytoplasma responsible for the desirable dwarf phenotype of the horticultural varieties (Nicolaisen and Horvath 2008). Numerous *Euphorbia* ssp. live in arid or semi-arid environments and genes involved in xerophyte adaptations could be useful in crop improvement for marginal land.

7.6 Scope for Domestication and Commercialization and Potential Problems

As noted above, wild *Euphorbia* ssp. have been cultivated and collected for millennia for their medicinal value. Due to the great phytochemical diversity of *Euphorbia* with the potential of identifying antiviral and anticancer drugs, their ability to grow in extremely xeric environments, and their potential as novel biofuel sources, some members of this genus may be recruited as crops. However, as with many ornamentals and emerging crops, there is potential for escapes that may become invasive weeds. Many *Euphorbia* ssp. are noxious and/or invasive weeds, including 11 species on Federal and state noxious weed lists in the US. Among the most notable is *E. esula* for which an invasive variety was brought to the US in the late 1800s most likely with seed stocks by German immigrants from the Ukraine (Dunn 1985). *E. esula* is now listed as one of the 12 most problematic weeds in the Northern Great Plains of the US and Canada, causing more than $100 million annually in damage, lost land use, and expense of control efforts (Leitch et al. 1994). Leafy spurge has been only marginally controlled with current integrated pest management systems using mechanical, herbicidal, and biological agents. Biological control with host-specific herbivorous flea beetles has shown promise but remains to be widely used. Host fidelity has been one particular concern, especially if rare native species of *Euphorbia* become

alternative host-plants. *E. hirta* is noted as an invasive species in Australia (Cowie and Werner 1993). Wild poinsettia (*E. heterophylla*) has become herbicide resistant (Trezzi et al. 2005) and is highly problematic in soybean fields in Brazil (Lorenzi 2000). *E. maculata* and *E. prostrata* are listed as invasive species in Croatia (Boršić et al. 2008). Phylogenetic relationships indicate that the weedy taxa fall into three groups, each from different subgenera of *Euphorbia*. It should be possible to use phylogenetic evidence to predict which closely related species are potential noxious weeds and accorded special precautions.

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Chapter 8
Gladiolus

Maria Cantor and Janakiram Tolety

8.1 Introduction

Gladiolus, *Gladiolus hybridus* L., is a perennial, geophyte, semi-rustice herb, and belongs to the Iridaceae family. Other important genera in this family are *Crocus*, *Freesia*, and *Iris*.

The genus name, *Gladiolus*, comes from the Latin word *gladius*, meaning sword, and refers to the shape of the leaves of all members of the genus, actually a characteristic feature of the entire *Iris* family and the genus was so known by the Romans. The ancient Greek name for the wild gladiolus was *xiphium*, from the Greek word *xiphos*, also meaning sword.

The gladiolus has a global history. Countless species grow throughout the world, from South Africa to the Mediterranean to West Asia.

Gladiolus were recognized over 2,000 years ago growing in the field of Asia Minor and were called "corn lilies." The European species were cultivated for more than 500 years and known for their striking, colorful flowers. Gladiolus was so popular that it led to the creation of the American Gladiolus Society in Boston in 1910. But it was known that these garden plants were grown from hybrids of wild gladioli native to South Africa.

Today gladiolus is one of the world's most important bulbous ornamental, valued both as an ornamental garden plant and as a cut flower crop used for bouquets and arrangements. The modern gladiolus cultivars offer a diversity of colors, shapes, and sizes available in few other flowering plants. It is cultivated in almost all countries of the world where spring and summer conditions are favorable.

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8.2 Basic Botany

8.2.1 Origin and Distribution

Very few flowers match the complex ancestry of *Gladiolus* and a revision of the South African species (Lewis et al. 1972) has further complicated the understanding of its development. The total number of species of *Gladiolus* recognized by Lewis et al. in southern Africa was 103. Thorough research on systematics of the genus have been methodically investigated, first by Peter Goldblatt in Madagascar (Goldblatt 1989), tropical Africa (Goldblatt 1993, 1996), and recently in southern Africa (Goldblatt et al. 1998) that led to a conclusion that Africa is a major center of diversity and speciation for *Gladiolus* (163 species are recognized in southern Africa).

The current number of species in the genus *Gladiolus* appears to be more than 255 centered in southern Africa extending throughout tropical Africa and Madagascar and into the Arabian Peninsula, the Mediterranean basin, Europe and Asia as far east as Iran and Afghanistan (Goldblatt and Manning 1998).

On the basis of the total number of species, it has been observed that England, France, Albania, Yugoslavia, Italy and Sardinia, Corsica, Sicily, Cyprus, former USSR, Iraq, Israel, Lebanon, Ghana, Ivory Coast, Mali, Sierra Leone, Reunion Island, Uganda, each contains one species of *Gladiolus*. However, *G. communis* is common in Italy, Spain, Greece, Corsica, Sicily, Sardinia (Italian island in the west in the

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Mediterranean), Portugal, Mediterranean area, and North Africa; while *G. illyricus* is common in Lebanon, Israel, Turkey, and England; *G. palustris* in Central Europe, Albania, and Yugoslavia; and *G. atroviolaceus* in Greece, Iran, and Turkey. *G. natalensis* (Eckl.) Reinw. is common in western Arabia, Ethiopia, Botswana, Namibia, Nigeria, Zimbabwe, and South Africa. Countries like Spain, Greece, Portugal, Mediterranean areas, tropical Africa, Guinea, Botswana, Namibia, and Somali contain two species each. Romania, Turkey, East Africa, Cameroon, and Madagascar contain three species each; Iran, Ethiopia, Kenya, and Zambia contain four species each. Nigeria and Zimbabwe contain five species each; whereas Mozambique contain 10 species; Zaire 11 species; Tanzania 13 species; Malawi 14 species; and Angola contains 16 species. About 20 species have been described with uncertain origin. South Africa is the only country from where more than 114 species have been recorded, out of which 62 are said to be winter flowering and 35 as scented ones: *G. acuminatus, G. alatus, G. arcuatus, G. brevifolius, G. brevitubus, G. carinatus, G. caryophyllaceus, G. ceresianus, G. engysiphon, G. equitans, G. esxilis, G. floribundus, G. gracilis Jacq., G. guthriei, G. jonquilliodorus, G. lewisiae, G. liliaceus* (night scented), *G. longicollis* (night scented), *G. maculates, G. marlothii, G. mutabilis, G. odoratus, G. orchidiflorus, G. permeabilis, pillansii. G. pritzelii var. pritzelii, G. recurvus, G. rebertsoniae, G. tenellus, G. tristis* (night scented), *G. uysiae, G. vaginatus, G. viridifflorus* and *G. watermeyerl*; however, *G. callianthus* Marais is a scented species from Ethiopia (Misra 1995).

Species of *Gladiolus* in southern Africa fall into two broad geographic categories: one from the summer rainfall and another winter rainfall zones of the subcontinent. Although there is no clear geographic separation between the two areas only a few species occur in both. Most of these species crosses from one zone to the other for only a short distance, and even then respond to the rainfall pattern of their main range. It is thus useful to clearly indicate about the species from summer rainfall or winter rainfall areas when discussing different aspects of *Gladiolus* (Goldblatt and Manning 1998).

This climatic distinction is significant in an evolutionary as well as ecological point of view, for the whole sections appear to have spread largely in one or the other rainfall zones and a few species of any section extend outside their main centers of diversity. Sections *Densiflorus* and *Ophiolyza* occur exclusively in the summer rainfall zone, with species extending only to the north, into tropical Africa where the same rainfall regime prevails. Likewise sections *Blandus, Hebea*, and *Homoglossum* have largely radiated in the winter rainfall zone. Just a few species of the later two sections occur outside the winter rainfall zone and a scant handful occurs exclusively in summer rainfall zone of southern Africa. The section *Heterocolon* is unusual in having three of the nine species in southern Africa occurring in the winter rainfall zone and six species in tropical Africa and the section *Linearifolius* stands out in having a more or less even distribution in summer and winter rainfall zones of southern Africa and in tropical Africa.

In UK, Toone (2005) reported that *Gladiolus illyricus* was found for the first time in New Forest in 1855 by Alexander More when he found a single plant of "Wild gladiolus" (then the plant had been found in more numbers in the New Forest in 1856) and attracted the attention of botanists when Alexander More published the record (More 1862).

The original sites were New Forest enclosures and plantations and many of the first records from them were also the last. There is no obvious reason for the plant's disappearance in these areas. There is also no evidence so far, apart from the first UK record, of the species being known locally before these early records, which is highly surprising.

Report of Townsend (1904), perhaps significantly, sits on the fence but he noted opinions from the literature and mentioned the lone dissenting voice against H.C. Watson, and his suggestion that every site might be associated with planted trees or shrubs, adding *G. illyricus* might also have come from there.

While in 1987, Jonathan Stokes comments – "Whatever the species" origin, AP Hamilton (personal communication 1987) is of the opinion that *G. illyricus* in Britain is sufficiently different from its European counterparts to warrant its designation as the separate subspecies *britannicus*. This split is based upon the genetic variation found in various populations, and the significant differences in floral morphology, e.g., lip shape, size, etc. Chromosome number of *G. illyricus* in the British population was reported to be $2n = 90$, while in Europe to be $2n = 60$ (Tutin 1980). The only population that resembled the English colonies was found on Belle Isle in Brittany, but these were

recently destroyed during the construction of a dam, although specimens are held at Kew (S Everett personal communication 1987).

A.P. Hamilton speculates about the origin of *G. byzantinus* Mill. – ". . .two main possibilities as to its origin are likely (1) from tetraploid *G. illyricus* via an unreduced gamete (pollen or egg) and (2) from a hybrid between *G. illyricus* and *G. byzantinus*" (Hamilton 1976). He points out the high sterility of the British plant as opposed to fertile populations in North Africa and that *G. byzantinus* from UK is hexaploid ($2n = 90$) and that from Africa octoploid. Surprisingly Hamilton (1967) thought *G. illyricus* was collected prior to extinction at the earliest sites (in which case what happened to the material? This does not seem to be justified by herbarium holdings) (Hamilton 1968).

In UK *G. illyricus* was cultivated at the Cambridge Botanic Gardens in the 1980s from French material, but is apparently no longer present. It is worthwhile to mention that it was frost hardy (P. Atkinson personal communication 2003).

Grazers in the New Forest (UK) avoid the capsules though they will eat the leaves early in the year before the Bracken closes off their view. It is not known whether birds will eat the seeds and there no information about small mammals or insects. It is possible that the New Forest populations survive only in bracken because of the grazing of plants in more open areas; first records date from 5 years after a major cull of deer authorized by an Act of Parliament.

The practice of bracken cutting was common in the New Forest until the mid-twentieth century; scythed patches provided winter bedding for animals and it was also used as fuel. As a means of spread this is suggestive, though inconclusive without more data. Vera Scott, who has gathered many of the best records with the New Forest Study Group says that British species have diminished since the cessation of bracken cutting in about the 1960s. She also comments that hard frosts help to select the resistant plant. *G. illyricus* can, however, persist in very dense bracken (>2 m) and it is doubtful that bracken cutting can claim continuity over the last 8,000 years (Toone 2005).

It is yet not known about the means of dispersal apart from gradual site migration, but consider Stokes's comment – "Human interest also extended to planting Gladioli; and one site (C), originally considered to be natural, was subsequently found to have been planted from seed in 1944 by the owner of the neighboring house" (R. Grove-White personal communication 1987). It is doubtful that the example is unique.

Cytological and morphological differences between *G. illyricus* in UK and in the Europe continent remain inexplicable without further genetic investigation. With Mediterranean/Atlantic floristic it appears to hybridize with *G. communis* ssp. *byzantinus* within its core range in South Spain producing an evenly graded range of intermediates (A Lockton personal communication 2004).

Switzerland's various bio-geographical zones harbor different numbers of species facing global extinction. Particularly a large number of endangered plant species occur in the western-central Alps. The only change within the past 15 years has been observed in the eastern-central Alps, where the marsh gladiolus (*G. palustris*) has been deemed regionally extinct for several years. However, it still occurs in a few other Swiss localities.

The flora of the Carpathians has been studied by many generations of Austrian, Czech, Hungarian, Polish, Romanian, Russian, Slovakian, and Ukrainian botanists. Species distribution is presented in the Carpathian countries and includes the wild *Gladiolus* species *Gladiolus felicis* Mirek in Poland and *G. palustris* Gaudin in Czech Republic, Slovakia, and Ukraine. These species are strictly protected or partially protected (Tasenkevich 2003).

In Romania, Ciocârlan (2009) mentioned in "The Romanian Illustrated Flora. Pteridophyta et Spermatophyta" that in flora Transylvania grew three wild *Gladiolus* species: *Gladiolus imbricatus* L., which has wonderful 4–10 magenta decorative flower, full bloom in the spring months throughout the meadows of *Fagus sylvatica* (beech tree); *G. palustris* L. found in areas with oak tree and humid location; and *G. illyricus* Koch. found also in areas with oak tree.

In Greece, island of Crete grows *Gladiolus italicus,* flowering from March through June, and filling the meadows with their deep pink color. About 8–16 deep pink flowers grow on a single tall spike-like stem and sway gently in the spring breeze.

Gladiolus saundersii is amongst the Drakensberg endemic species (Huntley 1993).

Gladiolus communis ssp. *byzantinus* is the example of a wild European species with 18 spikes of magenta flowers blooming in June and is very different from the

South African cultivars. This is the species, the "eastern Gladiolus" that has long appeared as a weed in cultivation in Sweden as well as being used in gardens for centuries.

The *Gladiolus* taxa was categorized by Selimov (2008) according to the phyto-geographic regions, and mainly listed as follows: 17.79% Iranio-Turanian, 12% Hirkan, and 8.59% Mediterranean. *Gladiolus atroviolaceus* Boiss. was identified in the Iranio-Turanian region. Its distribution is in the Nakhchivan and Lerik regions, 0–650 m altitude (Cabbarov 2000); habitats are stony steppe and rocky places, and phytogeographical element is Iranio-Turanian.

No *Gladiolus* species has been recorded yet from India and South Korea.

8.2.2 Brief History of the Crop

The gladiolus has quite a long history, with many old species coming from Africa and Asia. Many consider the mention of lilies of the field in the Bible as the earliest mentioned reference to the gladiolus.

There are about 200 species scattered throughout Tropical and South Africa; Zambezi river near Victoria Falls, Rhodesia; Mt. Kilimanjaro, Tanganyika; Natal and Cape of Good Hope, Republic of South Africa; Madagascar and Abyssinia and Caucasus in erstwhile USSR, Syria, Persia, etc., but a large number of them belong to South Africa in the Cape regions, and about 15 species grow only in the north of Sahara (Misra 1977).

Since the days of ancient Greece, the gladioli are said to be cultivated. History reveals that it is known since 1578, as evidenced by a record in Lyte's *Nievve Herball*. Gladioli were first introduced into France and soon after they spread to England, Germany, Holland and North America. In 1596, John Gerard, gardener to Lord Buleigh, who also owned his own "Physic Garden" in Holborn, had two European species, *G. communis* from Mediterranean region and *G. segetum* from South Europe, the canaries and Mediterranean region; as is evidenced by the catalog of some 1,030 plantlets he grew during that period. In 1597, Gerard published his famous *Herball*, *Geeral Histrie of Planets* describing these two species.

Prior to 1730, the major garden species in England were *G. communis, G. segetum*, and *G. byzantius*, the latter being introduced in 1629 from Constantinople. With the establishment of trade routes from England to India via the Cape of Good Hope, several South African species were sent to England starting in 1737.

The expedition of the Siomon van der Stel, Governor of the Dutch colony at the Cape in 1685 (De Wet and Pheiffer 1979) conducted a botanical exploration and an artist Claudius, who accompanied the expedition, painted two species of *Gladiolus* (*G. esculentus* syn. *G. speciosus* and *G. aquilegia* syn. *G. caryophyllaceaus*).

In 1753, the Swedish scientist Carl Linnaeus published six species of *Gladiolus* in *Species Plantarum,* the modern system of naming plants: two Eurasian and four from the Cape of Good Hope. Just one of these later four, *Gladiolus angustus*, still belongs to the genus as it is understood today. The other three have long since been shifted to different other genera.

By 1753 however, several more species of *Gladiolus* from southern Africa were already known in Europe, although their generic affiliation was not always clear. These species included *G. alatus, G. blandus, G. recurvus*, and *G. tristis*. These species were formally named according to the binomial system in the later years. Phillip Miller had the honor to find the first flower, a South African *Gladiolus*, *G. tristis,* in England in 1745.

During 1772–1775, Carl Peter Thunberg in South Africa collected hundreds of new species and sent thousands of specimens to Europe. Linnaeus's son and many more botanists described some. The results were remarkable. Where handful of species of Cape *Gladiolus* were known in 1770, the *Flora Capensis*, published in various editions from 1807 to 1823 (Thunberg 1823), included some 50 species, almost one-third of the total recognized today.

Gladiolus aethiopian, described first by Cornutus, is from Cape region bearing white red-scarlet flowers. It was used in Cape region about the middle of the eighteenth century until the first South African species were introduced. *Gladiolus tristis*, a sweet-scented species closely related to *Gladiolus grandis*, was first introduced in Chelsea Physic Garden, US, in 1745 from Cape region and Natal. In 1772, Kew Gardens sent overseas the first of its numerous plant collectors, the Aoerdonian, Francis Masson who traveled principally in Cape region and collected many wild growing species. *Gladiolus citricus is a native* to Mediterranean region, which later on was found growing in

England as the area of diversity. *G. grandis*, in 1749, was naturalized in Spain from south Africa. In 1784, C.P. Thunberg published *Dissertatio de Gladiolo*, which may be taken as the starting point of post-Linnaean era. In 1789 W. Aiton published his *Hortus Kewensis* describing two *Gladiolus* species brought by Masson during 1773–1775.

8.2.3 Taxonomy and Classification

The genus *Gladiolus* of the petaloid monocot plants belongs to the family Iridaceae Juss. Iridaceae is among the largest families of the order Asparagales (Goldblatt 2001). The taxonomical hierarchy of *G. hybridus* is as follows (NCBI Taxonomy 2008):

Domain: Eukaryota
Kingdom: Plantae
Subkingdom: Viridaeplantae
Phylum: Tracheophyta
Class: Liliopsida
Order: Iridales
Family: Iridaceae
Subfamily: Ixioideae
Genus: *Gladiolus* L.
Botanical name: *Gladiolus* × *hybridus* Hort.

Bentham and Hooker (1883) placed the family in the series Epigynae whereas Engler (1908) placed it under Liliflorae, and Hutchinson (1932) put it in a separate order Iridales through his treatise, *The Families of Flowering Plants II*: *Monocotyledons*. In 1959, when this book was revised, the genus *Gladiolus* was placed in tribe 10 – Gladioleae. Goldblatt (1971a, b) placed the genus *Gladiolus* under subtribe Gladiolineae of the tribe 3 – Ixieae.

The current intrafamilial taxonomy of the Iridaceae recognizes four subfamilies: Isophysidoideae; Nivenioideae, Iridoideae, and Crocoideae (Goldblatt et al. 2008) (Fig. 8.1). The first description of the subfamily Crocoideae included 29 genera and contained 1,032 species (Burnett 1835). The tribe Gladioleae included one of the largest genus *Gladiolus* L., which comprises ca 262 species (Dumortier 1822).

Pax, in 1888, classified Iridaceae (Egler and Prantl) into Crocoideae, Iridoideae, and Ixioideae, further dividing the latter Iridoideae into Ixieae, Watosonieae, and Gladiolus based on the forms of the flowers, i.e., xygomorphic or actinomorphic and styles complete or divided into two, mono-or-bi-carpellary ovary. This latter subdivision comprises eight genera with *Gladiolus* L. as one of them. The *Gladiolus* genus has further been divided into four sections:

Section I: *Eugladiolus*, which includes 100 species of Europe, western Asia, Tropical and south Africa.
Section II: *Habea,* which includes 12 species of south Africa (Cape Peninsula) and the Madagascar Island.
Section III: *Schweiggeria,* which includes only two species, i.e., *Gladiolus montana G. arenarius* from Cape.
Section IV: *Homoglossum*, which includes five or six species of South Africa.

Baker, in 1892, through his treatise *Handbook of Irideae*, also adopted the same placing: 109 species under *Eugladiolus*, 15 under *Habea*, two under *Schweiggeria* and 51 species under *Homoglossum*. Lewis et al. (1972) divided the genus *Gladiolus* into four groups. The species belonging to different groups along with their places of origin in parenthesis and flower colors are given below.

8.2.3.1 *Plurifoliati*

Twenty-two species having 5–8 or more well-developed leaves, distichously arranged to form fan shape.

Gladiolus cardinalis (Cape), *G. sempervirens* (Cape), *G. cruentus* (Natal and Lesotho), *G. saundersii* (Natal and Lesotho) – all of red or scarlet color; *G. oppsibiflorus* (Cape) – flowers white or pink; *G. eeliotii* (Natalk Botswaa and Rhodesia) – white, speckled with purple; *G. sericeo-villosus* (Cape, Natal, Swaziland and Transvaal) – pink and/or yellow flowers, arranged in opposite directions; *G. virus* (Transvaal) – pink*: G. ochroleucas* (Cape) – cream to yellow; *G. ecklonii* (Cape) – white red and mauve; *G. buckerweldii* syn. *Antholyza buckerveldii, Petamenses buckerveldii* (Cape) – yellow*; G. matelensis* (Cape and Natal) – red, orange, yellow and or greenish*; G. papilio* syn. *G. purpuireoauarntus* (Transkei to Transvaal) – yellow; all have one side facing spike; *G. hollandii (*Transvaal) – white, speckled with pink and mauve*; G. densiflorus* (Transvaal) – white, with redspots*; G. invenustus* (Swaziland and Natal) – white to mauve, with blotches*; G. calcaratus* (Transvaal) – white*; G. appendiculatus* (Transvaal, Natal and

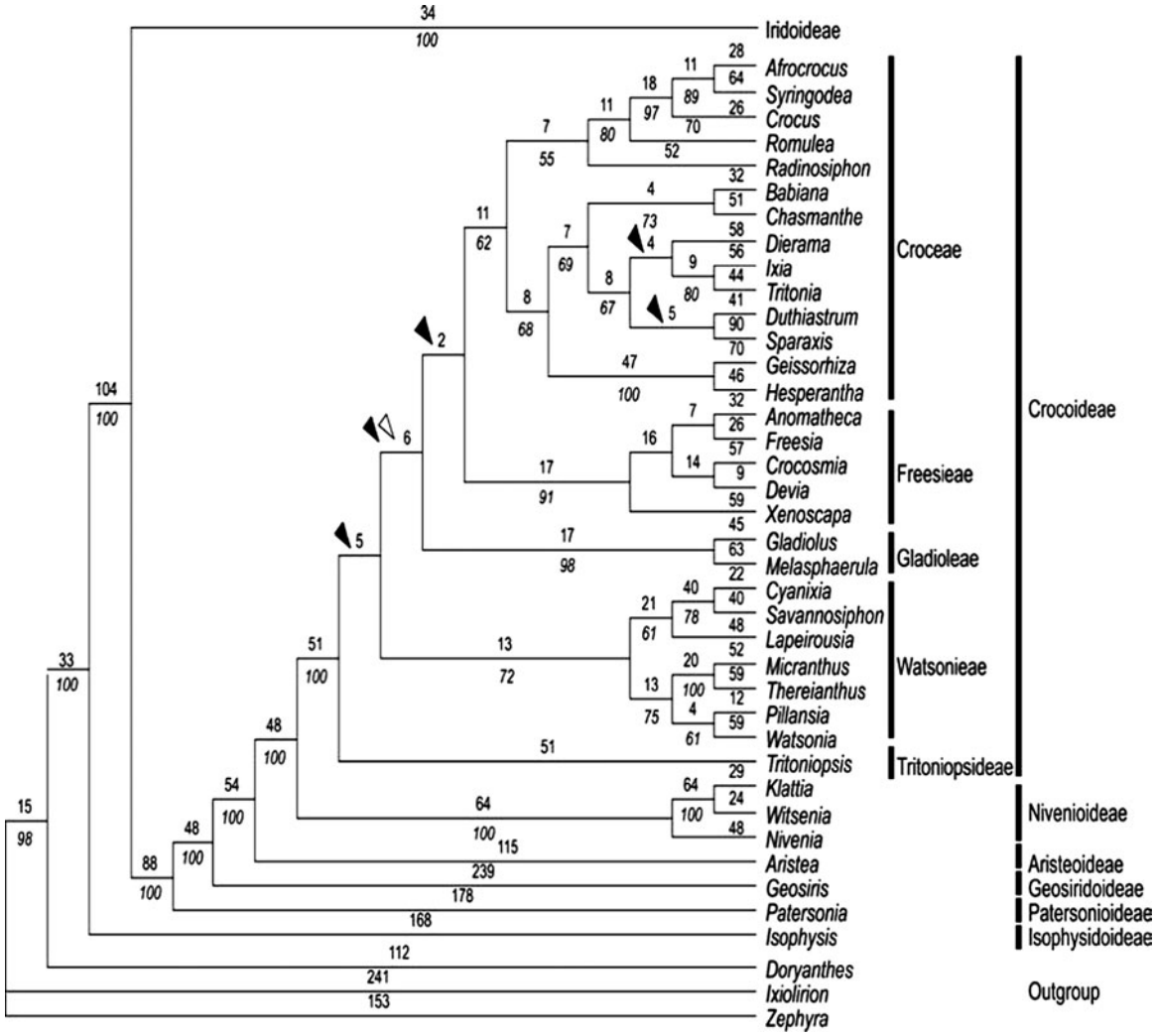

Fig. 8.1 One of three most parsimonious phylogenetic trees of the genera of Iridaceae obtained after successive weighting. The *number* of steps after optimization of equally weighted characters is shown above the branches (ACCTRAN optimization) and bootstrap percentages above 50% are shown in *bold* below the branches. *Black arrows* indicate the group that collapses in the strict consensus of the equally weighted trees; *unfilled arrows* indicate additional nodes collapsing in the strict consensus of the equally weighted tree. Subfamilial and tribal classification is shown on the *right*. A. Outgroups and *Isophysis* to subfamily Crocoideae (reprinted from Goldblatt et al. 2008 with permission of Systematic Botany)

Swaziland) – white, with mauve marking and *G. poleevansii* (Transvaal) – pinkish lilac, hairy on all vegetable parts.

8.2.3.2 *Paucifoliati*

Thirty-four species, having 2–5 well-developed leaves, distichously or spirally arranged.

Gladiolus stellantus (Cape) – fragrant, whitish to pale mauve; *G. gueinzii* (Crape and Natal) – pink, marked with purple; *G. acuminatus* (Cape) – fragrant dull greenish yellow; *G. lapeirousiodes* (Cape) – white, with the lower lobes each bearing two red blotches; *G. leptosiphon* (Crape) – cream to cream-brown; *G. vigilans* (Cape) – pale rose-pink; *G. carneus* (Cape) – white, cream, pink or mauve; *G. macneilii* (Transvaal) – salmon-pink; *G. microcarpus* (Natal) – whitish, pink or mauve; *G. buckerveldii* (also descried under Plurifoliati); *G. angustus* (Cape) – white to stream-yellow; *G. floribundus* (Cape) – fragrant, with white, cream pink or mauve, closing at night; *G. udulantus* (Cape) – greenish to cream-white or pink; *G. carlyophyllacleus* (Cape) strongly scented,

flowers pale to mauve; *G. lewisiae* (Cape)- fragrant, creamy white, flushed with pink; *G. involutus* (Cape) – pale pink or white; *G. sclullyi* (*Cape*) fragrant, cream, yellow, lime-green, pink, mauve or maroon; *G. arcuatus* (Cape) – fragrant, mauve or purple; *G. salteri* (Cape) – pink; *G. kamiesbergensi* (Cape) – fragrant, mauve or purple; *G. permeabillis* (Cape) – fragrant, white to cream, mauve pink or brown; *G. vernus* (Transvaal) – pale magenta, pink; *G. pretoriensis* (Transvaal) – dark purple to pink; *G. mostertiae* (Cape) – pale pink. *G. rufoarginatus* (Transvaal) – white or cream-yellow, densely flecked with red or purple; *G. orchidiflorus* (Cape) – fragrant, grey-green, densely flecked with red or purple; *G. watermeyeri* (Cape) – fragrant, cream or greenish cream, flushed with dull purple; *G. irescenss* (Cape) – fragrant, cream or greenish cream, flushed with dull purple; *G. virescens* (Cape) –fragrant, cream or greenish cream, flushed with dull purple; *G. seresianus* (Cape) – fragrant dull or greenish yellow; *G. uysiae* (Cape) – fragrant, cream or greenish; *G. alatus* (Cape) – scented, red, orange or salmon but lower leaves greenish; and *G. equitans* (Cape) – fragrant, orange, red or vermillion, with yellow-green patches on laterals and falls.

8.2.3.3 *Unifoliati*

Twenty-nine species, having one well-developed basal leaf, rest of the leaves, bract-like with short blades spirally arranged on the scapes.

Gladiolus brevitubus (Cape) – pale pink or red; *G. quadrangulus* (Cape) – fragrant, white pale, blue, pinkish, mauve; *G. citrinus* (Cape) – bright yellow; *G. tenellus* (Cape) – fragrant, yellow, cream or white tinged purple: *G. oreocharis* (Cape) – white to mauve; *G. inflatus* (Cape) – pale, pink or mauve; *G. robersoniae* (Transvaal) – fragrant, mauve or white tinged pale mauve; *G. cylindraceus* (Cape) – pale pink or creamy pink, flushed with salmon-pink; *G. debilis* (Cape) – white to pale pink, with red dots in throat; *G. longicollis* (Cape, Natal, Lesotho, Orange Free State, Swaziland and Transvaal) – evening-fragrant, white-cream or yellow; *G. tristis* (Cape) – night-fragrant, white to buff-cream or pale yellow tinged green; *G. liliaceus* (Cape) – night-fragrant, yellowish to dull yellow, flecked with brown, pink, red or purple; *G. hyalinus* (Cape) – slightly fragrant towards evening, yellow-brown, yellowish cream or greenish brown, spotted with brown and purple; *G. recurvgus* (Cape) – fragrant, pale greenish grey or greenish mauve to pale yellow or cream; *G. symonsii* (Natal) – pink; *G. punctulatus* (Cape) – pink to mauve; *G. carnatus* (Cape) – fragrant, pale grayish blue to pale pink; *G. viridiflorus* (Cape) fragrant, pale green; *G. blommesteinii* (Cape) – pale pink or mauve: *G. ornatus* (Cape) – pale to deep pink: *G. gracilis* (Cape) – fragrant, pale mauve or pink, sometimes white or yellow; *G. exilis* (Cape) – slightly fragrant, pale blue, mauve or white tinged pale mauve; *G. mutabilis* (Cape) – fragrant, greyish mauve to cream, brown or yellow; *G. violaceolineatus* (Cape) – mauve; *G. comptonii* (Cape) – bright yellow with reddish streaks; *G. rogersii* (Cape) – purple; *G. bullatus* (Cape) – deep mauve; and *G. pritzelli* (Cape) – fragrant, yellow sometimes tinged red or grey outside.

8.2.3.4 *Exfoliati*

Twenty-four species, having well-developed leaves (or leaf) absent at the time of flowering, the scape bearing 1–3 (or 4) sheathing bracts without or with small free blades.

Gladiolus stefaniae (Cape) – red; *G. stokoei* (Cape) – scarlet: *G. nerineoides* (Cape) – pale salmon-pink to deep golden red; *G. guthriei* (Cape) – fragrant, deep pink or reddish; *G. carmineus* (Cape) – deep rose-pink or carmine, with a white or cream median stripe; *G. monticola* (Cape) – cream colour tinged pink; *G. maculatus* (Cape) – fragrant, yellow, pink or white, streaked and spotted with brown, purple or red; *G. engysiphon* (Cape) – strongly and sweetly scented, white with a crimson median line on three lower and upper lateral lobes: *G. bilineatus* (Cape) – white or cream, faintly flushed with pink; *G. emiliae* (Cape) – fragrant, yellow, densely speckled with red, brown or purple; *G. odoratus* (Cape) – fragrant, dull or brownish yellow, speckled and striped with purple, red or maroon; *G. vaginatus* (Cape) – fragrant, mauve to grey-mauve, rarely white; *G. brevifolius* (Cape) – scented, pale to pink, rarely white, mauve, brownish green or brown; *G. pillansii* (Cape) – fragrant, mauve or pink; *G. martleyi* (Cape) – white, suffused with rose-pink; *G. subcaeruleus* (Cape) – mauve or pinkish; *G. jonquilliodorus* (Cape) – fragrant, cream-coloured or pale yellow, flushed with pink or mauve; *G. aurantiacus* (Natal,

Swaziland and Transvaal) – golden yellow or orange and yellow, streaked with red; *G. brachyphyllus* (Transvaal, Swaziland and Mozambique) – pink to mauve, with red spots; *G. unguiculantus* (Sierra Leone and Transvaal) – mauve, with purplish blotches on lower lobes; *G. woodii* (Natal, Transvaal and Swaziland) – dark brown; and *G. parvulus* (Cape, Natal and Transvaal) – mauve, pink or white.

The current number of species in the genus is 255 (Goldblatt and Manning 1998). The majority of *Gladiolus* species are native of Africa (South, Eastern, and Western), Madagascar, Europe, and Middle East. Approximately ten of the 255 species in the genus occur north of the Sahara in Eurasia as far east as Afghanistan, and another eight occur in Madagascar. There are some 84 species in tropical Africa, where the genus is well understood as a result of research of Goldblatt (1996). In the South Africa, the area south of the Cunene-Okaavango-Limpopo River axis – now it was recognized 163 species. Only about 12 are originating from Mediterranean areas, western Asia, and Europe. South Africa, and particularly the Cape of Good Hope, is considered to be the center of diversity of the genus (Lewis et al. 1972; Delpierre and Du Plessis 1973; Ohri and Khoshoo 1985a).

Goldblatt and Manning (1998) in the book *Gladiolus in southern Africa* presented a classification of 163 species from southern Africa. These are arranged in seven sections and 27 series, as following:

1. Section *DENSIFLORUS*
   - Series *Paludosus* (*G. paludosus, G. papilio*)
   - Series *Densiflorus* (*G. crassifolius, G. hollandii, G. serpenticola, G. exiguous, G. densiflorus, G. ferrugineus, G. lithicola, G. varius*)
   - Series *Calcaratus* (*G. appendiculatus, G. calcaratus, G. macneilii*)
   - Series *Scabridus* (*G. ochroleucus, G. mortonius, G. microcarpus, G. scabridus, G. caractarum, G. pavonia, G. brachyphyllus*)
2. Section *OPHIOLYZA*
   - Series *Oppositiflorus* (*G. dolomiticus, G. poleevansii, G. oppositiflorus, G. sericeovillosus, G. elliotii*)
   - Series *Ecklonii* (*G. ecklonii, G. vinosomaculatus, G. rehmannii*)
   - Series *Ophiolyza* (*G. antholyzoides, G. aurantiacus, G. dalenii, G. magnificus, G. flanaganii, G. saundersii, G. cruentus*)
3. Section *BLANDUS*
   - Series *Phoenix* (*G. oreocharis, G. crispulatus, G. phoenix*)
   - Series *Sabulosus* (*G.* gueinzii)
   - Series *Blandus* (*G. carneus, G. pappei, G. geardii, G. aquamontanus, G. undulatus, G. augustus, G. buckerveldii, G. bilineatus, G. insolens, G. cardinalis, G. sempervirens, G. stefaniae, G. carmineus*)
   - Series *Floribundus* (*G. rudis, G. grandiflorus, G. floribundus, G. miniatus*)
4. Section *LINEARIFOLIUS*
   - Series *Pubigerus* (*G. woodii, G. malvinus, G. pardalinus, G. pubigerus, G. parvulus*)
   - Series *Linearifolius* (*G. hirsutus, G. caryophyllaceus, G. guthriei, G. emiliae, G. overbergensis, G. bonaspei, G. aureus, G. bervifolius, G. monticola, G. nerineoides, G. stokoei*)
5. Section *HETEROCOLON*
   - Series *Unguiculatus* (*G. oatesii*)
   - Series *Heterocolon* (*G. rubellus, G. pretoriensis, G. filiformis*)
   - Series *Vernus* (*G. rufomarginatus, G. vernus, G. kamiesbergensis, G. marlothii, G. mostertiae*)
6. Section *HEBEA*
   - Series *Involutus* (*G. leptosiphon, G. loteniensis, G. involutus, G. vandermerwei, G. cunonius, G. splendens, G. saccatus*)
   - Series *Permeabilis* (*G. permeabilis, G. uitenhagensis, G. acuminatus, G. stellatus, G. wilsonii, G. inandensis, G. robertsoniae*)
   - Series *Deserticola* (*G. arcuatus, G. viridiflorus, G. deserticola, G. scullyi, G. venustus, G. salteri, G. lapeirousioides*)
   - Series *Hebea* (*G. orchidiflorus, G. watermeyeri, G. virescens, G. ceresianus, G. uysiae, G. equitans, G. speciosus, G. pulcherrimus, G. alatus, G. meliusculus*)
7. Section *HOMOGLOSSUM*
   - Series *Carinatus* (*G. atropictus, G. violaceolineatus, G. comptonii, G. roseovenosus, G. carinatus, G. griseus, G. quadrangulus*)
   - Series *Mutabilis* (*G. mutabilis, G. exilis, G. vaginatus, G. maculates, G. albens, G. meridionalis, G. priorii*)
   - Series *Brevitubus* (*G. brevitubus*)
   - Series *Gracilis* (*G. rogersii, G. bullatus, G. blommesteinii, G. virgatus, G. debilis, G. variegates, G. vigilans, G. ornatus,*

G. inflexus, G. taubertianus, G. gracilis, G. caeruleus, G. reculvus)
- Series *Teretifolius* (*G. inflatus, G. cylindraceus, G. nigromontanus, G. engysiphon, G. patersoniae, G. subcaeruleus, G. martleyi, G. jonquilliodorus, G. trichonemfolius, G. sufflavus, G. pritzelii, G. delpierrei*)
- Series *tristis* (*G. hyalinus, G. liliaceus, G. tristis, G. longicollis, G. symonsii*)
- Series *Homoglossum* (*G. watsonius, G. teretifolius, G. quadrangularis, G. huttonii, G. fourcadei, G. abbreviatus*)

Species outside southern Africa include ten in Eurasia (assigned to a section *Gladiolus*), nine in Madagascar, one of these shared with Africa; and 83 in tropical Africa plus Arabia.

8.2.4 Morphology

Gladiolus is an herbaceous plant, having deciduous leaves with overlapping bases and a flowering stem bearing a terminal spike (Fig. 8.2). *Leaves* usually contemporary with the flowers borne on the same shoot, sometimes borne earlier or later than the flowers and on separate shoots, two to several, basal, or some inserted above ground level, with sheathing base and isobilateral, unifacial blade, sometimes the blade reduced or lacking thus the entire leaf partly to entirely sheathing, blades linear to lanceolate, either plane and the margins, midrib and sometimes other veins not or only lightly thickened and hyaline, or the margins or midrib strongly raised, sometimes even winged (thus H-or X-shaped in section), or the midribs and margins much thickened and the blade evidently terete but with four narrow longitudinal grooves.

Flowering stem aerial, terete, simple or branched, erect or flexed downward above, often above the sheath of the uppermost leaf.

Inflorescence a spike (rarely reduced to a single flower), the flowers second or distichous in a few species; *floral bracts* two usually green, sometimes dry above or entirely, usually relatively large, the inner (adaxial) usually slightly smaller than the outer, sometimes much smaller, or rarely slightly longer, usually notched apically for 1–2 mm or entire.

Fig. 8.2 *Gladiolus hybridus* Hort. cultivated plant (photo Maria Cantor)

Flowers bilaterally zygomorphic (actinomorphic in a few tropical African, Madagascar, and South African species), tepals united below in a tube, the lower tepals usually with contrasting markings constituting a nectar guide, the stamens arcuate and unilateral, ascending to horizontal, the style arched over them frequently, closing at night; *perianth tube* well developed, usually obliquely funnel-shaped, shorter than or to about as long as the bracts, or sometimes much longer than the bracts; *tepals* usually unequal, the upper most (dorsal) broader and arched to hooded over the stamens, the lower three narrower, in subgenus *Gladiolus* usually clawed below and united for a short distance, as long as, shorter than, or exceeding the upper tepals. Florets number up to 30 or more and are either bilateral or radially symmetric. Flower size also varied from 2 to 20 cm in diameter. *Filaments* filiform inserted at the base of the upper part of the perianth tube, usually exerted but occasionally extending only to the mouth of the tube; *anthers* symmetrically disposed around the style in species with actinomorphic flowers,

usually unilateral, parallel ascending to horizontal lying below the dorsal tepal, dehiscing longitudinally, subbasifixed to centrifixed, rarely with sterile tails, the connective obtusely mucronate above or prolonged into a prominent acute to apiculate appendage (some tropical African species).

Individual flowers enclosed in two bracts are more or less tubular with a six-membered perianth, three stamens, an inferior ovary, and three-chambered capsule. *Ovary* ovoid to oblong; *style* filiform, dividing opposite to or beyond the anthers, the branches simple, filiform below, expanded gradually to abruptly above and channeled to bilobed. *Capsules* large, usually slightly inflated, ovoid to ellipsoid or globose, rarely elongate and nearly cylindric.

The capsule contains between 50 and 100 ovules that mature within 30 days after fertilization. *Seeds* usually many per locule, discoid with a broad membranous, circumferential wing or, rarely, few per locule and the seeds wingless and more or less globose to angled by pressure.

The *Gladiolus* corm grown from a flowering one is formed by the swelling of 5–8 shortened basal internodes of the flower stalk. A bud is present at each alternate node and usually one or two apical buds develop into a shoot. The leaves overlap at base and may number from 1 to 12. The corm is surrounded by husks (tunics) that are the dry bases of foliage leaves. A new corm is produced each new growing season over the mother corm. At the same time cormels are formed at the tip of branched stolons that develop from buds located at the base of the new corm. The wild species produce one or rarely two corms and a few cormels while modern cultivars can produce 2–4 corms and between 30 and over 400 cormels.

8.2.5 *Chromosome Numbers in* Gladiolus *Species*

Chromosomes of *Gladiolus* are relatively small, 0.7–2.9 μm, and few details of the karyotype apart from the diploid numbers are known. Karyotypes of most species comprise metacentric to submetacentric chromosomes of fairly uniform size. All chromosomes have a large heterochromatic segment that stains darkly at early prophase. Counts are available for just 60 of the 163 species in southern Africa of which some ten counts are new (Goldblatt and Takei 1997). The pattern is surprising. All species have numbers based on $x = 15$ and most are diploid, $2n = 30$. An identical base number and a similar chromosomal configuration in most species of *Gladiolus* explain why it is relatively easy to hybridize species, even from different sections, and why hybrids are so often fertile. Chromosome number is clearly of virtually no value in understanding the systematics and phylogeny of the genus in southern Africa.

Both polyploidy and dysploidy are rare in the subcontinent, $2n = 45$ or 60, and the only count for the southern Cape species, *G. leptosiphon* is also polyploid, $2n = 60$. Polyploidy has evidently played a minimal role in the evolution of *Gladiolus* except in Eurasia and North Africa. Polyploidy elsewhere is rare. The reports of triploidy in the southern African *G. scullyi* and *G. orchidiflorus* hardly suggest a need for additional study. Excluding these examples, only two southern African species have been reported as polyploid, *G. papilio* and *G. saundersii* and both the counts are for so-called hybrids (Bamford 1935). The significance of polyploidy in *G. dalenii* is difficult to gauge. It is one of the most successful species of the genus and certainly the one with the widest geographical distribution (Goldblatt 1989, 1996; Goldblatt et al. 1998).

The majority of the African species of *Gladiolus* are primarily diploids ($2n = 30$) whereas the European species are polyploids ($2n = 60$–130), indicating a southern origin of the genus. Modern hybrids, designated as *G. grandiflorus*, are a complex of at least 11 species, several of which are represented by different color, forms or botanical varieties.

Some of these diploid species have been used in crosses and constitute the genetic base for the spring flowering cultivars, including *G. colvillei, G. nanus, G. ramosus, G. tunbergenii* (Ohri and Khoshoo 1985b; Anderton and Park 1989).

In the northern areas of South Africa and in subtropical Africa the vigorous species flower from summer to autumn and rest during the cool winters. They have various levels of ploidy:

- *G. natalensis* var. *psittacinus*: $2n = 75$–90
- *G. oppositiflorus:* $2n = 30$
- *G. saunderssi:* $2n = 30$–45
- *G. papilio:* $2n = 75$
- *G. natalensis* var *primulinus:* $2n = 60$

After their introduction into Europe, many breeders used these species. All modern cultivars of summer flowering types (*Gladiolus grandiflorus* Hort.) originated from complex crosses made since the middle of the last century (Buch 1972; Ohri and Khoshoo 1985b; Anderton and Park 1989).

Species found in countries bordering the Mediterranean Sea, in western Asia and Europe are all polyploids:

- *G. communis:* $2n = 90–180$
- *G. italicus:* $2n = 120$
- *G. illyricus:* $2n = 60–90$
- *G. atroviolaceus:* $2n = 90$

Their growth resumes in autumn and they flower in late spring. The flower color is limited to pink, magenta, and reddish purple tones. Breeders have not used these species. However they can be valuable because of their relative hardiness and their low sensitivity to *Stromatinia* (Van Eijk et al. 1986).

The Romanian *G. imbricatus* and *G. palustris* are tetraploid ($2n = 60$ chromosomes) and *G. illyricus* has $2n = 60–90$ chromosomes.

8.3 Conservation Initiatives

8.3.1 Conservation and Bulbous Plants

During the last few years there has been a growing awareness of the possibility of over-collecting of wild bulbous plants for the horticultural trade. This has resulted in the placement of some Turkish bulbous plants on the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). Besides the fact that collecting for commercial use has seriously impacted some wild populations, the major threat remains the conversion of wild bulb fields to agriculture. This is particularly true on the Iberian Peninsula, as well as other regions (Koopowitz 2008).

In Koopowitz opinion much of the hype about the collection and sale of wild flower bulbs was built more on ignorance rather than fact. When flower species on sale in the bulb market were first tallied, it was clear that no distinction was made between domesticated and propagated species and wild species. It seemed much more dramatic to lump the two together, despite the fact that many of the tulip species in the trade were selections that had been painstakingly built up and propagated for decades. Rumors still occur that pictures purported to show piles of bulbs of *wild stocks of calanthus* were in fact pictures taken from farmed stock. Was there really a misrepresentation of the true situation by idealistic conservationists or an honest mistake?

Gladiolus aureus was collected for the first time by C.B. Fair in the southern Cape Peninsula in 1894, and was described by the Kew botanist J.G. Baker in 1896. Although known from several populations in the past, it has always been a rare species, restricted to the southern Cape Peninsula.

The variety *Gladiolus alatus* var. *algoensis* is classified as endangered but there is no information available indicating what specific threats this plant may face. However, as it is endemic to the Cape Floristic Region, it is likely to be affected by a number of threats that are impacting the natural habitat of this botanically unique area. These threats include urban development, the encroachment of agriculture, and the invasion of alien plant species (Rouget et al. 2003).

Like other plant communities within the Cape Floristic Region, such as fynbos, only a small proportion of reserve is protected (Kemper et al. 1999). Through researching its ecology, the Cape Action for People and the Environment partnership is working to determine how best to manage and restore and conserve its biodiversity. These management strategies can then be employed by willing landowners, thereby ensuring that beautiful species such as the *G. gracilis* (blue pipe) are preserved (Cape Action for People and the Environment 2008).

Within the Cape Floristic Region, there are a number of protected areas and a number of conservation organizations working to conserve this botanically rich habitat (UNEP-WCMC 2008). Conservation actions include purchasing land to protect it from the threats of encroaching agriculture and urban development (Fauna and Flora International 2008), the removal of alien plants, and the establishment of new protected areas; measures of which should benefit the endangered variety of this beautifully unusual flower.

During 1976, 1,100 *G. aureus* seeds collected in the wild were deposited at the Wakehurst Place Seed Bank (now the Millennium Seed Bank) in the UK, to determine whether cold storage of seed as a measure of

long-term conservation was possible. This proved successful and tests carried out several years later at the seed bank showed a germination of 99% at 11°C. *G. aureus* has been successfully cultivated at Kirstenbosch Botanic Garden for many years and is also being grown by several specialist bulb growers in many countries. Ideally its natural habitat should be formally protected, but should this not be possible, ex situ material could possibly be used to re-establish this species elsewhere, in suitable sites (Duncan 1981, 2002). The Kirstenbosch Botanical Garden in the Cape Town area of South Africa, one of the leading botanical gardens in the world, has one of the largest collections of *Gladiolus* species and related corms. It has a seed bank and a major source for species seeds.

At the present time, the University of California Arboretum in Irvine, California, has one of the largest collections of gladiolus and other related corm plants.

In Romania, the wild *Gladiolus* species are protected in some national reservations in Transylvania. Corms in a germplasm collection at the University of Agricultural Sciences, Cluj-Napoca, and Fruit Research Station Cluj preserve the modern cultivars.

8.3.2 Modes of Preservation and Maintenance

8.3.2.1 Sexual Reproduction

Despite the capacity for vegetative reproduction in *Gladiolus*, conventional sexual reproduction and the recruitment of new plants by seed production and dispersal remain vital for long-term survival and the maintenance of populations, as well as long-distance dispersal and the establishment of new colonies. Sexual reproduction is also essential for the maintenance of genetic variability and the production of novel genotypes through recombination. This may be stating the obvious, but it seems necessary to emphasize on the importance of sexual reproduction and seed dispersal in geophytic plants such as *Gladiolus*. The conservation of these plants does not simply require that they may be left undisturbed in their habitat. This is certainly a recipe for their ultimate downfall and loss. Production of seeds also depends on successful pollination. Conservation thus also implies conservation of pollinators and the maintenance of the environment to the extent that pollinators are available and predator populations are limited. In the absence of large mammal predators over much of the range of *Gladiolus* today, damage by herbivores may be disastrous to plant populations. Likewise, human activities, although they may be distant from plant populations, may affect them through disturbance or loss of habitat for specialist pollinators.

8.3.2.2 Vegetative Reproduction

Although a few species of *Gladiolus* have much reduced corms, they all have the capacity for annual regeneration from the corm. Many species also have the ability to increase their numbers by vegetative reproduction through the production of cormlets in various ways (Goldblatt and Manning 1998). It may thus be thought that annual regeneration and vegetative reproduction are sufficient for ensuring the continuing survival of the species. This is, however, unlikely that corms may in theory live to a great age, but in fact they are highly nutritious sources of food for a variety of animals adapted in special ways to locate and consume them. Major predators are baboons, porcupines, molerats and other rodents, as well as some birds, notably guinea fowl, and they account annually for huge reductions in population numbers of mature plants. Therefore, in the absence of predators, species of *Gladiolus* and other corm-bearing Iridaceae may form dense stands and even become noxious weeds. The same species in southern Africa are seldom conspicuous and are often declining in numbers due to impaired reproductive capacity for a variety of reasons, not least an increase in natural predation. Reproduction through the more conventional means of cross-pollination and seed production is thus vitally important for geophytic plants such as *Gladiolus*.

In the face of predation pressures, population numbers are maintained in short term by individuals surviving and even increasing clonally through their cormels, which are often too small or too inconspicuous to interest a predator. Species may even make a virtue of the inevitable loss of mature individuals by exploiting the behavior of predators to disperse plants to new habitats. Molerats are particularly important dispersal agents for many species. These animals harvest corms and bulbs in huge numbers and maintain stores of food

in their underground burrows. Undamaged mature corms can in this way be carried to large distances. Uneaten corms will grow in the following season in a new site. The dispersal of plants of some species may depend to a large extent on molerat activity.

8.3.2.3 Cryopreservation of Gladiolus

Modern cultivars have been bred by introgression of genetic traits derived from several species and hence it becomes difficult to distinctly assign cultivars to different species (Misra and Singh 1989). Prolonging pollen viability through pollen storage is reported in many crop species as a means for overcoming asynchrony in flowering, scheduling hybrid seed production and haploid gene pool conservation. However, published reports on this aspect are restricted to a few ornamental crops. Among bulbous ornamentals, long-term pollen storage of Narcissus in liquid nitrogen is reported by Bowes (1990). In *Gladiolus*, pollen storage for duration of 730 days at −40°C has been reported by Koopwitz et al. (1984) for five species. This demonstrates the feasibility of cryogenic storage of gladiolus pollen and establishment of cryo banks for conservation of species.

Many plant lines that are genetically different are generated while genetically engineering plants. These plants must be screened to determine which lines have the same phenotype as the non-transformed plant and are most resistant to the pathogen of interest. Cryopreservation of *Gladiolus* is a way to maintain these plant lines in a minimal amount of space and to eliminate the labor needed to routinely transfer plants growing in culture or in the greenhouse. Only four bulb crops have been cryopreserved by vitrification include lilies (Bouman et al. 2003), taro (Takagi et al. 1997), garlic (Baek et al. 2003), and gladiolus (Joung et al. 2007).

8.4 Role in Genetic Improvement of Domesticated Gladiolus

8.4.1 History of Breeding

Expert breeders and amateurs have devoted more than a century to hybridization and selection to modify plants to the colorful, blossom laden gladiolus varieties those are in demand today.

A commercial cultivar must possess numerous horticultural qualities (Manley 1968, 1969; Wilfred 1971). All individuals from the bulb grower to the ultimate consumer must be satisfied with the cultivars. The cut flower and bulb growers require the following objectives:

1. Ability to produce flowers of high commercial quality under a wide range of temperature and light conditions.
2. Cut flowers those are easy to handle, pack, and store.
3. The ability to open from tight buds following 2–3 days of dry storage.
4. A high rate of propagation, a large number of cormels with a high germination rate and a solid husk.
5. Good corm enlargement under various soil and climatic conditions.
6. A low sensitivity to major pests and diseases and adverse climatic conditions.
7. Adaptation to mechanization of all operations, planting, lifting, and cleaning.

A consumer desires an inflorescence with a good ornamental value, pure color, excellent stem strength, and good disposition and attachment of the flowers. Judgment of the aesthetic characteristics is complex and difficult. It is more or less subjective and somewhat dependent on fashion.

The principles of gladiolus breeding are simple. The breeder wants to identify exceptional genotypes that may be present among progenies of crosses between good commercial cultivars possessing complementary qualities. Breeders' constraints are often related to the biological characteristics of the genus. Due to vegetative multiplication by corms and cormels, all materials must be planted, lifted, cleaned, and stored every year. These operations are time consuming and special equipment is required. Also if not done carefully, fungal and viral contaminations can easily be transmitted to the following generation. Modern cultivars are the result of complex interspecific crosses. They are heterozygous and tetraploid and the knowledge of the hereditary transmission of numerous characteristics is poor. As a result, rational bases for the choice of parents to be crossed are generally not available. Quantitative characteristics such as

earliness of flowering, plant height, spike length, number of floret/spike have a moderate to high heritability (Cohat 1988).

Qualitative traits such as inflorescence's aesthetic value and overall value have a very low heritability. Excellent parents can give poor progenies, since the phonotypic value of parents does not permit a prediction of the value of offspring. The number of favorable characteristics that must be combined is high and single defect is sufficient to reject a plant that is otherwise acceptable. Probability of success is largely linked to the number of plants per progeny subjected to selection.

The assessment of some traits such as propagation rate and ability to flower out of season is laborious, as it can only be evaluated after several growth cycles.

Due to a high genotype × environment interaction, plurilocal and pluriannual experiments are required, especially if the plants have to be grown under conditions different from those of the breeding area. Corms used for experiments must be grown and stored under the same conditions. In fact, an efficient breeding program is based upon a good knowledge of the plant's physiology in order to determine the required experimental conditions.

The large diversity available among species has not been adequately exploited. Improvement of some traits such as winter flowering capability, flower fragrance and resistance to major pathogens is desirable. Diseases are the main problem encountered in gladiolus culture. With the probable future limitation of pesticide utilization, breeding for resistance or at least low sensitivity to major pest and diseases is critical. Uses of biotechnology must be considered in the future (Cohat 1993).

Producing new gladiolus by self-pollination or natural cross-pollination (by insects or wind), give useful and interesting variations of gladiolus characteristics. Many people around the world are now engaged in growing glads from seeds obtained by this method. New gladiolus cultivars arise also by mutations (sports.) The sports arise from structural changes in the genetic make up of the gladiolus, such as changes in the chromosome structure. Mutations to produce sports of glads are relatively rare and may occur in two ways, such as to produce a new strain or to back form similar to a parent. A few very important glad cultivars have arisen as mutations (Hartline 1996).

The species *G. communis*, *G. careneus* (*G. bladus*), and *G. cardinalis* were the prominent types grown prior to 1880 and, since they are sexually compatible, many natural hybrids were cultivated (Buch 1972).

William Herbert, in England, was one of the first to start (1806) hybridization in the *Gladiolus* species, and while he did produce many hybrids all proved sterile and never got into commerce. They were probably what we now call triploids. Knowledge of chromosomes and genes was nil at that time. Many plant breeders have since found that these Cape species are incompatible for blending into other cultivars. Interest in the wild gladiolus was growing and plant collectors began to fan out east of Cape Town searching for other species. The first hybrids to enter commerce were the work of James Colville, in England, who used *G. tristis* var. *concolor* and *G. cardinalis* to create his Colvillei hybrids in 1823. While these hybrids did well in England, it was soon found that they required fall planting for spring bloom. *Gladiolus carneus* (*G. blandus*) was then crossed with *G. cardinalis* to produce *G. isignis* – a small, early-flowering type.

G. psittacinus (*G. natalensis*) came to Europe from Africa in 1825, *G. oppositiflorus* and *G. papilio* in 1830. These three species were found to differ from those previously known in that they bloomed in the summer of the northern hemisphere. Interest intensified when it was found that they could store the corms to overwinter for spring planting. *G. blandus* is one of the most variable of the South African species, due to the fact that it bloomed in the summer.

In 1833, Schneevogt in Holland pollinated *G. isignis* with *G. oppositiflorus* (*G. floribundus*) to produce the Ramosus hybrids.

In 1816, France, Holland, and Belgium started raising hybrids, the first being scent gladiolus (Gand in Belgium), *Gladiolus garndavensis* in 1841, by crossing *G. grandalis* and *G. psittacinus* or vice-versa. William Herbert, a British botanist has been evolved by crossing *G. appositiflorus* with *G. psittacinus*. He also records a hybrid, *Gladiolus brenchlelyensais*, obtained by crossing between *G. cardinalis* and *G. psttacinus*. It has light red flowers, and another race *G. citrinus* with yellow flowers was also developed from the same parent. In 1923, *Gladiolus colviellei*, *G. tristis concolour*, and *G.* cardinalis were developed by Colville at his nursery in Chelsea, England.

Hermman Josef Bedinghaus, gardener to Duc de Arenberg at Engheim, Belgium, made the successful

cross leading to the development of our present garden gladiolus. In 1837 he had crossed *G. psittacinus* (*G. natalensis*) and *G. cardinalis* to produce what was later called as Gandiensis (Gandavensis) hybrids.

Many nurserymen became hybridizers and hundreds of gladioli were developed between 1840 and 1850. Birders like Rykfogel and Souchet in France, Krelage in Germany, Schneevogt in Holland, and Standish and William Hooker in England were hybridizing and developing new cultivars each year. By the 1850s botanists were exploring in Africa for more species to use in their hybridization programs.

Eugene Souchet, royal gardener at Fontainbleu, in 1852 crossed Gandavensis and Ramosus hybrids with *G. blandus* (*G. floribundus*) thereby starting a long line of somewhat novel strains. These were the first hybrids to be imported into the United States. E.S. Rand, Jr. in the book *Corms* published in 1866 said, "all the world are raising seedling gladiolus."

In the mid-1860s the scarlet-flowering *G. cruentus* was introduced from Natal and used by J. Standish of England in crosses with the Brenchleyensis hybrids to produce over 100 named cultivars. In 1877, M. Leichtlin crossed *G. cruentus* (*G. saundersii*) with *Gandavensis* hybrids; the resultant progeny he called *Leichtlin* hybrids. These were then sold to J. Childs of Long Island in 1891 and became known as *Childsi* hybrids, which are the basis of many of our modern cultivars.

In the 1870s, a yellow-flowered form of *G. papilio* (*G. purpureo-auratus*) was introduced into France and it was not only hardy but also produced multiple daughter corms and numerous cormels from a single corm. V. Lemoine of France crossed *G. papilio* with the Gandavensis hybrids and named 152 Lemoine hybrids. In 1885, he crossed the Lemoine hybrids with the Childsi hybrids to produce the Nancianus hybrids, of which 75 cultivars were available. One of these was "Emile Aubrun," considered to be the grandmother of the "grandiflorus" gladiolus. The Nancianus hybrids were crossed with Souchets hybrids to produce many outstanding cultivars, one of which was "Golden Measure," which is the ancestor of many of the modern yellow gladiolus available today. Lemoine also crossed the dull-purple flowered form of *G. papilio* to the lemoine hybrids to produce the small flowered blue "Butterfly" gladiolus. At about the same time M. Froebel of Zurich crossed Gandavensis hybrids with *G. saundersii*, and J. Sander of England crossed Gandavensis with the maroon and green *G. dracocephalus*. J. Kelway of England made numerous hybrids of the Gandavensis and Lemoine hybrids and called the progeny Kelwayi hybrids, which where known for their size and hardiness. In 1900, Lemoine added *G. aurantiacus* to the gene pool for crosses with the Leichtlin hybrids. The progeny, which he called Precoces, were early flowering.

A whole new race of garden gladiolus originated when a yellow form of *G. natalensis* (*Gladiolus primulinus*) was collected at Victoria Falls, Zimbabwe and introduced in 1902 in Europe. It was crossed with larger hybrids and the progeny became the so-called Primulinus hybrids, which are important ancestors of modern cultivars, and the basis of many of the small flowered "prims." Use of these hybrids in breeding programs continued in Europe following World War II. Most of the European gladiolus breeding has continued in Holland and England. Gladiolus as cut flower in North America was developed from Souchet's hybrids and in 1870 up to 10,000 spikes per day were shipped to New York from local fields. Luther Burbank soon developed cultivars that had greater scent compounds and could withstand the bright sun and dry atmosphere of California. After the introduction of the Childsi hybrids in 1893, H. Groff of Canada incorporated the best qualities of Burbank's, Souchet's, and Child's hybrids to produce the Groff hybrids, known for their strong stems and good cut-flower characteristics. These could be grown in fields and then cut with the basal floret open and shipped to local markets. A. Kunderd of Indiana soon developed the ruffled and laciniatus types of gladiolus, which were unlike the plain-petaled European types. When Dr. E. F. Palmer of Canada had introduced "Picardy" in 1932, the gladiolus cut-flower industry of the South and West was born. This cultivar was a blend of the Gandavensis and Primulinus types and was the first that could be cut in the bud stage and shipped to distant markets where the florets would open. Field production of "Picardy" and its progeny soon eliminated greenhouse production of gladiolus. Soon after World War II, L. Butt of Canada introduced his "Rufmins," which were small-flowered non-prim types, the most famous of which was "Crinklette." These are now commercially known as "Pixiola" types. A few of the modern hybridizers of gladiolus cut flowers in the United States are C. Fischer

of Minnesota, H. Turk of Oregon, E. Frazee of California, and G. Wilfret of Florida.

Entire new strains of gladiolus were developed in the last century, with many hybridizers keeping their crosses secret, since selling, trading, profit, and pride were at stake.

Before moving onto the modern gladiolus of today, it is important to say that the African species were really responsible for the wide diversity of color, form, and unique variations in modern gladiolus. Thousands of hybridizers made millions of crosses that although people have long forgotten over the past 200 years but those brought us to where we are today. Many species were not necessarily beautiful, most having small florets and low bud count. Many opened only one or two florets at a time, with a bud count of four or five. Who knows, may be a species still undiscovered in Africa may hold the secret to a healthier gladiolus or a color still not achieved. The elusive highly scented gladiolus might be growing right in the mountains behind a rock, still to be discovered. The gene to make our modern cultivars hardy still is yet to be found.

Many of the lines of today can probably trace their roots to central and southern Africa, where the summer flowering species were discovered. Victor Lemoine of France achieved more colors and variations around 1880. He produced the race *Gladiolus lemoinei* from crossing of *G. gandavensis* or *G. bechelyensis* with *G. purpureoaurtus*. The hybrids extended the color range into browns, greens, and deep reds. Many of these descendants are where we get our color range today.

William Pfitzer and his son Paul Pfitzer were hybridizers during 1840 to 1930, covering almost 100 years. William created the first giant red-flowered cultivars and was able to tackle many problems of culture and breeding in the 1880s. From 1890 to 1900, the Pfitzer Strains formed the backbone of the German creations and were known for large flowers widely opened and unicolored gladiolus in bright shade. Other characteristics were well blending of colors and harmonious rows of florets, not crowded, but forming a strong and fine, spike. Their work raised the number of open florets on a single spike from one-quarter to a half of all buds blooming at one time.

G. primulinus is known as "Maid of the Misty" because its native haunts are the banks of the Zambezi river. This species grows on the spray of the Victoria Faith and so to protect the sex organs, especially pollen, upper central petals became hooded. It was introduced into England in 1879 from Tanganyika and the name *Gladiolus nubicola* was proposed for it. Again in 1902, it was introduced into England and in 1904 the flowers were exhibited at the Royal Horticultural Society, and since then it was extensively been used in hybridization for its elegant primrose-yellow flowers (Garrity 1975).

British further extended the color range in the late 1890s, including most veining, yellow and orange. By the early 1900s, red, orange, salmon, cream, and ivory were more common. In 1920, the large flower gladioli were being grown in the United States in large numbers. A. F. Kunderd did much of the hybridization in the United States during the 1920s.

Not much new progress was attained in the 1930s, except for the introduction of "Picardy" cultivar introduced in 1931 by Professor E. F. Palmer. "Picardy" was a large, outstanding shrimp pink cultivar that is still being grown today. This was a big step in improving American modern gladiolus, and was considered a world champion top show gladiolus for years.

By 1940s and early 1950s gladioli were at their glory with thousands of members in societies. Some of the important stepping stones during this era where the cultivars "Elizabeth the Queen" (560) introduced by D. W. White in 1941; "Burma" (556) introduced by Palmer in 1943; "Spic and Span" (446) introduced by Carlson in 1946 followed by Friendship (442) introduced in 1949 by Carl Fischer. In the 1950s, several hybridizers were introducing three quarters of the new cultivars of that decade in America. They were: Ralph E. Baerman, Dr. Robert A. Griesbach, Carl Fischer, Edwin Frazee, J. R. and C. T. Larus, Winston Robersts, Henry Turk, and Don Walker. There were a few introductions in the United States in the 1950s that were also stepping stones. "King David" (556) introduced by Anton Carlson in 1951 and "Peter Pears" (423) by Konynenburg and Mark in 1958 were two notable cultivars. "Peter Pears," imported from Holland is still sold in chain stores today. In the 1960s, through the present time, more energy was spent to achieve perfect show gladiolus. "Parade" (534)*, introduced in 1970 by Larus, leads the way and is still a very important show gladiolus today. Crossing existing cultivars has been strong, with little reintroduction of species being done and hybridizing for commercial cultivars. Karl Fischer of Minnesota, during his 67 years of growing gladiolus had 88

American selections from the 1950s through the 1990s have published 51 consecutive gladiolus catalogs.

Two more important introductions in the 1980s are the last notable stepping stones. "Ice cap" (400) introduced in 1981 by A.G. Carnefix and "White ice" (300) in 1987 by Dr. Jack Jones, were important for hybridization, which helped in the improvement of present-day gladiolus. There are a few show men and catalogs that have brought the glad to its caliber of today. Dr. Earl Hamilton brought the showing of glads to affine art and catalogers like Alex Summerville and Ed Squires were responsible for introducing many show quality glads, while many other notable catalogers are credited for introducing decorative and commercial cultivars (Hartline 1996).

Some other races were also produced by crossing *Gladiolus alatus* with *G. vittane* (Syn. *G. vinulus*); *G. Cardinalis* with *G. brachyandrus, G. cuspidatus, G. floribunda, G. irsutus, G. oppositiflorus,* and *G. tristis; G. gandavensis* with *G. floribunda* resistant race *G. massiliensis; G. psittacinus* with *G. floribundus, G. pseudo-aurens* and *G. saundersii* var. *Cooperi*; *G. recurvus* with *G. blandus* and G. tristis; *G. tristis* with *G. blandus* and *G. primulinus,* etc. Thousands of present-day cultivars of *G. hortulanus* and/or *G. hybridus* are the contributions of 8–12 species of the genus (Laurie et al. 1958).

Homoglossum watsonianum and *Acidanthera bicolor murielae* (first introduced from Abyssinia in 1893 and named by Perry, an English nurseryman in 1930 as scented species) are the last used donors in hybridization programs. Collingwood Ingram, in 1931, used the former genus in crosses with *Gladiolus tristis* and produced *Homoglad* hybrids, whereas Mrs. Joan Wright of New Zealand, in 1967, developed *Gladanthern* "Lucky star" by crossing gladiolus cv. Filigree with the latter genus, which is fragrant (Wright 1971). Tamberg (1972) has given an account of 188 *Gladiolus* species with their certain characters, and Hamilton (1976) of England collected and described European *Gladious* species i.e., *illyricus, communis, palustris, imbricatus, italicus,* and *atraviolaceaus* (Garrity 1975).

Of these only a small number of species have been used in the development of the cultivated *Gladiolus*. The present days' *Gladiolus* cultivars are complex hybrids and include the following species: *G. cardinalis* Curtis., *G. dalenii* van Geel., *G. oppositiflorus* Herb., *G. papilio* Hook. f., *G. carneus* Delaroche, *G. cruentus* Moore., *G. tristis* L., and *G. saunddersii* Hook. f. (Bailey and Bailey 1976; Huxley et al. 1992; Cohat 1993).

Gladiolus cultivars suitable for cultivation under the subtropical conditions as of the middle and southern areas of Korea have been developed by the National Horticultural Research Institute (NHRI), Rural Development Administration from 1995 to 2008. In South Korea, breeding work of gladiolus started in 1991 and the first cultivar released was "Hongkwang" in 1995. A total of 33 new gladiolus cultivars were released by NHRI in Korea from 1995 to 2008. These cultivars have broad spectrum of colors and desirable characters such as vigor; multiple flowering; resistance to virus, Fusarium, and neck rot; and high rate of multiplication. The promising gladiolus cultivars are "Adlib Salmon," "Adlib Scarlet," "Arirang," "Bel Canto," "Blue Bird," "Cardinal," "Carmen," "Lavender Bell," "May Queen," "Pink Fantasy," "Pink Pearl," "Pink Veloce," "Salmon Classic," "Spring Time," "White and Cool," "White Lace," "White Nova," and "Yellow Candy." "Adlib Salmon" is early flowering and resistant to *Pseudomonas and Fusarium*. "Bel Canto" has thick petals and is resistant to *Pseudomonas* and thrips (Cho 2007).

According to the changes of the South Korean domestic cut flower market during 2003 to 2008, the share of the domestic cultivars went up to 7.2% during the period, because of its advantages such as higher disease resistance and better adaptability to temperature change than foreign cultivars growing in Korea. So the number of commercialized cultivars was 18 in 2008 and cultivated area under domestic cultivar was 2.7 ha (Cantor et al. 2008a, b).

E.K. Kundred of Indiana, USA, grew the first ruffled gladiolus in 1907 together with a species *Gladiolus quarantinianu.* During the period from 1920 to 1940, the American Gladiolus Society facilitated the popularization of gladiolus. In Post war era, i.e., 1945–1955, the New England Gladiolus Society and the North American Gladiolus Society contributed towards improvement and popularity of gladiolus. "Palmer gave era," i.e., 1940–1950, contributed to world the novelties in which the special contributions were Carine, Shelley, Grazee, Snuder, Fisher, Arenias Gove, Milo, Palmer, Baerman, Rich, Woods, Roberts, to name a few. However, during the "Post War era," i.e., 1945–1950, cut-flower trade of Europe with USA began to flourish (Bose and Yadav 1989).

Nowadays in USA, many hybridizers work for creating new gladiolus varieties (Hartline, Euer, Madeson, Carl Fischer, etc.). The only other way new cultivars of gladiolus are developed is by spontaneous mutation. Such a mutation, for instance in the gene for color, leads to deletion, or change of position and when it is deleted, an albino results. Happening only once in thousands or so cases (ca. 10^{-6}), these mutations or variations are the incidents that a careful plant breeder looks for. They most often call these off-types "sports" of a given cultivar. These sports are of commercial value only when they outclass their parents in color, stature or health and can be propagated easily.

Use of irradiation or chemicals can also produce mutations, which cause point or chromosomal mutation. Such induced or artificial mutations have hardly been used in gladiolus since most glad fanciers focus their attention on sports produced by natural or spontaneous mutation.

Hybridization will probably continue as the most reliable method of developing new cultivars. The goal of hybridization or cross-fertilization is to create superior cultivars by bringing new combinations of genes together through crossing. The hybridization of gladiolus dates long back practiced in Europe and Africa. Species of *Gladiolus* native to Africa were mostly short budded and had small florets. Literally, there must have been millions of crosses made to bring the gladiolus to its present beautiful state. Over time chromosome numbers have changed and most modern gladiolus can no longer be crossed with the species found in Africa. There are millions of gladiolus cultivars created over the world.

The culture and selection work of gladiolus in Romania began in 1953 at the Research Station at Cluj-Napoca by Rudolf Palocsay (Neagu et al. 1976), Research Station Cluj, Floral Institute Vidra and University of Agricultural Sciences and Veterinary Medicine at Cluj-Napoca and Bucharest released a total of 41 new gladiolus cultivars during 1953 to 2008. Today the most popular varieties are: "Speranta," "Cipriana," "Candida Ali," "Excelsa," "Clujana," "Ramona" and "Amethyst" (Cantor et al. 2008a, b).

For a long period, a laborious breeding work was conducted in Slovakia by I. Admovic, who was a great hybridizer of glad. He created cultivars with a typical heavily ruffled and many types of fragrance (Adamovici 2005).

In Russia, many hybridizers worked for obtaining new cultivars. Vladimir Ivanovich Trifonov founded the St. Petersburg Gladiolus Club in 1993 and is its president since 2005. He started growing gladiolus in 1980 and almost immediately began hybridization. His aim from the beginning was to create new, uncommon sorts. From 1993 onwards, many of his cultivars won championships at countless Russian exhibitions. Since 1997, he had won awards from the Russian Gardeners Alliance, the Moscow Alliance Conservancy, and both the Moscow and St. Petersburg glad clubs. Some of his most popular cultivars in Russia are "Singing Fountains," "Fire of Desires," "Neva Exotic," and "Emerald Necklaces." In the last few years, he had been working on truer blues, greens, and blacks (Savchenko 2008).

In Canada, Peter Enterprises Gladiolus devoted a sustainable work for improving the gladiolus assortment by breeding, in order to obtain new varieties with superior characteristics and more adaptive ability to climatical conditions of this area. Peter Enterprises Gladiolus is working collaboratively with outstanding hybridizers such as Croteau, Euer, Everson, Frederick, Hartline, Madeson, Sander, and Selinger from North America and Mimranek from Europe (McDougall 2008).

Gladiolus × *hybridus* is a popular floricultural crop in India grown mainly for cut-flowers. Because of the variation in flower color and suitability for open cultivation, gladiolus is one of the important floricultural crops and is used for vase decoration and making bouquets. There are about 2,000 ha in the northern states (Uttar Pradesh, Uttarakhand, Himachal Pradesh and parts of Punjab and Haryana) and the popular cultivars grown are: "Friendship Pink," "Novalux," "Oscar," "Red Beauty," "Sylvia," "Tropic Sea," and "White Prosperity." Many cultivars have been developed in India by the National Botanical Research Institute (NBRI), Lucknow; Indian Agricultural Research Institute (IARI), New Delhi; Indian Institute of Horticultural Research (IIHR), Bangalore and many agricultural universities. The domestic market is the main source of consumption although export has been initiated recently into the Gulf countries. Under the National Horticultural Mission, gladiolus is one of the crops being emphasized for increased planting (Roy 2008).

8.4.2 Potential Breeding Objectives in Current Classical Breeding

8.4.2.1 Fragrance

Gladiolus cultivars have beautiful florets, but they are without fragrance. Currently no modern gladiolus cultivar has floral scent. It is believed that gladiolus cultivars with floral scent would attract consumers in flower markets. This conviction prompted to find suitable genetic resources in order to breed scented gladiolus cultivars. One particular trait found in wild species is floral scent. Previously studied wild species and their major scent compounds are: *G. liliaceus* – eugenol; *G. alatus*, *G. maculates*, *G. recurvus* and *G. tristis* – linalool; *G. jonquilliodorus*, *G. orchidiflorus*, *G. patersoniae* and *G. scullyi* – geraniol or geraniol acetate, nerol and citronellol; *G. carinatus* and *G. virescens* – B-ionone (Goldblatt et al. 1998; Goldblatt and Manning 2002). While these studies were performed to determine their attractiveness to pollinators, the data suggested large qualitative variations in floral scents including bengenoids and terpenoids in wild *Gladiolus* species.

Fragrance is an important feature of the pollination strategy in many species of *Gladiolus*. All moth-pollinated species of the genus produce a strong, sweet scent, usually with an undertone of cloves. The scent may be present throughout the day and night, as in *G. emiliae* (section *Linearifolius*), *G. robertsoniae* (section *Hebeae*), and *G. recurcvus* (section *Homoglossum*). In the moth-pollinated species, *G. liliaceus*, G. *longicollis,* and *G. tristis* of section *Homoglossum*, scent is produced only in the evenings. Scent production is accompanied by a shift in perianth color from dull brown or red to pale mauve in *G. liliaceus*, a particularly unusual phenomenon.

Bee-pollinated species do not depend on fragrance to attract visitors to the same extent as do species pollinated by moths. Nevertheless most bee-pollinated species of the predominantly winter–rainfall section *Hebea* and *Homoglossum* produce strong sweet odors, whereas bee-pollinated species of section in the summer-rainfall region do not produce scent. Species of section *Linearifolius*, which are pollinated by bees, produce scent inconsistently. The winter rainfall *Gladiolus brevifolius* and summer rainfall *G. pubigerus* produce scent in some populations. Fragrance in section *Hebea* is characteristically very strong and is a combination of violet and freesia. In the field, flowers can often be located by scent before they are seen. Particularly strongly scented species are the cryptically colored *G. orchidiflorus* and its allies – *G. ceresianus*, *G. uysiae,* and *G. watermeyeri*. *Gladiolus permeabilis* subsp. *permeabilis* is usually scented and subsp. *edulis* frequently so, with most populations north of the Wall River being unscented, whereas strongly scented populations occur in the Karoo, Free State, and Namibia. In section *Homoglossum* the southwestern Cape species, *G. carinatus* and *G. gracilis*, are well known for their highly fragrant flowers, but perhaps the finest fragrance is produced by *G. trichonemifolius*. In the nineteenth and early twentieth centuries the flowers of these species were treasured for the vase and a few stems would fill a house with their delightful fragrance.

Currently no modern gladiolus cultivars have floral scent. Recent studies were targeted to select suitable genetic resources for scent breeding and these have qualitatively and quantitatively analyzed floral scent emitted from nine wild *Gladiolus* species. The analyzed species were divided into four groups: Linalool/Benzenoid, Nnerol, Ionone, and Ocimene/Caryophyllene. Based on the kind and amount of scent compounds, *G. orchidiflorus*, *G. recurvus*, *G. tristis,* and *G. watermeyeri* were selected as potential genetic resources for fragrance (Suzuki et al. 2008). The differential release of scent compounds during the day is an important character to select for, in addition to the quality and quantity of scent compounds, in breeding program for gladiolus (Suzuki et al. 2008).

Attempts were made to incorporate gene(s) for fragrance from *G. callianthus* (*Acidanthera bicolor* var. *murieliae*) (Anonymous 1974, 1980). *Gladiolus dalenii* L. is tetraploid ($2n = 60$), while *G. callianthus* is diploid ($2n = 30$). It was observed that $4x \times 2x$ was successful only when tetraploid was used as female parent (Ohri and Khoshoo 1983). Rao and Janakiram (1992) reported to production of interspecific hybrids between *G. grandiflorus* Andr. cultivars and *G. callianthus* that have mild fragrance.

8.4.2.2 Stress Resistance

There is a lot of scope for breeding gladiolus using wild species to develop useful genotypes for biotic and abiotic stresses.

There is record that 23 wild species have so far been used in the development of the modern garden cultivars of gladioli (Misra and Kaicker 1986). These species are *G. alatus* L., *G. angustus* L., *G. aurantiacus* Klatt., *G. blandus* Ait., *G. communis* L. ssp. *byzantinus* (Mill.) Hamilton, *G. callianthus* Marais, *G. cardinalis* Curt., *G. cruentus* Moore, *G. cuspidatus* Hook. f., *G. dracocephalus* Hook., *G. floribundus* Jacq., *G. hirsutus* Jacq., *G. oppositiflorus* Herb., *G. papilio* Hook. f., *G. natalensis* cv. *primulinus* Bak., *G. natalensis* cv. *psittacinus* Hook., *G. pulchellus* Klatt, *G. purpoureo-auratus* Hook. f., *G. quartinianus* Rich., *G. recurvus* L., *G. saundersii* Hook. f., and *G. tristis* L. Apart from these, *Homoglossum watsonium* (Thumb.) N.E. Br. has also been used in gladiolus breeding and the × *Homoglad* hybrids were evolved in 1931.

It is thought that modern cultivars of G. × *grandiflora* originated from a small number of wild species, viz. *G. cruentus*, *G. natalensis*, *G. oppositiflorus*, *G. papilio,* and *G. saundersii* (Imanishi 1989a, b); thus, most *Gladiolus* species were not involved in the development of the modern cultivars. These species may possess useful characters for plant breeders.

8.4.3 Employment of Molecular Markers in Breeding

Molecular markers have widespread application in plant breeding. These markers can be used, for example, to characterize diversity within gene pools and to identify sources of genetic diversity that complement existing breeding populations (Mazur and Tingey 1995). Molecular markers are the basis of the marker-assisted selection (MAS) strategy. The precision of selection offered by DNA markers could make MAS more economic and the method of choice for breeding programs in the future. To date, marker-assisted breeding in gladiolus has been limited to screening germplasm.

The molecular information on the *Gladiolus* genome has been accumulated in the last period on the studies on some wild species of southern Africa. Methodologies for isozyme and random amplified polymorphic DNA (RAPD) markers have been devised and practically used.

Modern gladiolus cultivars are considered to have been bred originally from only 6–12 species (Barnard 1972; Anderton and Park 1989; Imanishi 1989a, b) and considerable genetic potential exist to develop new cultivars of gladiolus using wild species. Especially some species having a good scent should be useful material for breeding of a scented modern cultivar. However, there is little information of genetic relationships in wild *Gladiolus* species for interspecific hybridization. Recently, Goldblatt and Manning (1998) proposed a phylogenetic tree for 163 southern African gladioli using morphological, geographic, and ecological data. Although their study was unique and useful, we have not been able to obtain sufficient information, such as cross-compatibility for interspecific hybridization involving wild species.

Other studies made by Takatsu et al. (2001) used RAPD markers to characterize relationships among 33 *Gladiolus* species (32 wild *Gladiolus* species of southern Africa and a modern cultivar "Traveller") to estimate relationship between wild species and modern cultivars. They generated a dendrogram based on RAPD markers. However, the utility of this dendrogram in a breeding program of gladiolus has not been investigated. As a consequence, in 2005 Takatsu and coworkers have tried compatibility and hybridity of F_1 plants obtained by crossing *G. tristis* and *G. gracilis*. In addition, a segregation analysis of the floral scent trait was carried out using F_2 population derived from crosses between *G. tristis* and *G. gracilis*.

In a similar study, Wang et al. (2006) while studying classification and genetic relationship of 12 cultivars of *G. hybridus* Hort. applied RADP analysis. Twenty-six arbitrary primers screened from 80 primers were used for further PCR and diversity analysis. A total of 164 RAPD sites were detected with a mean of 6.31 fragments amplified for each primer. A total of 151 polymorphic DNA fragments were detected among all the 164 amplified fragments, which accounted for a high level (92.8%) of polymorphism and could be used for identification of different cultivars. The result revealed that the germplasm resource of *G. hybridus* Hort. cultivar had a narrow genetic base at the molecular level.

More recently in the Republic of South Korea, Cho and Lee (2008) examined the relationships among wild *Gladiolus* species, related species and commercial cultivars to know about polyploidy level. It was found that the DNA level of lines resulting from crosses between cultivars and wild species were distributed in the intermediate class of the wild species

and the commercial five species of *Gladiolus* had thrip resistance trait, especially resistance level of *G. carneus* was the highest among the *Gladiolus* species. On the basis of leaf injury index (LII), six cultivars and five lines were found to be highly resistant to thrips. The variety "BelCanto" had the highest level of resistance. However, another six cultivars and three lines were highly susceptible to thrips. The variety "Arirang" was the most susceptible. From cross-combination of highly resistant parents, more resistant descendants could be obtained (Cho and Lee 2008).

An affinity relation among 40 commercially available cultivars, 43 *Gladiolus* wild species, and three interspecific hybrids were analyzed using RAPD markers. Also, RAPD markers tightly linked to the locus controlling resistance to thrips in gladiolus were analyzed (Cho 2007). Among the 86 genotypes, the similarity coefficient was 0.61–0.99. Similarity coefficient between the commercial cultivar "Charming Beauty" and *G. carneus*, resistant to thrips, was 0.74. As a result of analyzing RAPD markers using bulked segregant analysis (BSA), eight markers including B09-700 were initially detected. It was assumed that they were tightly linked to the locus controlling resistance to thrips in gladiolus (Suzuki et al. 2005). Therefore, these results will contribute to give genetic information to improve the efficiency of crossing and selection for breeding for thrip resistance in gladiolus.

Consequently, few molecular resources relevant to gladiolus improvement were developed earlier. But important resources have been developed in recent years and there would be substantial opportunity for gladiolus improvement through the application of molecular markers in the development of molecular genetic maps, marker-aided breeding, and isolation of important genes.

8.4.4 Genetic Transformation

An initial report on stably transformed bulb scales of Eastern lily resulting from particle gun bombardment of bulb scales was verified by PCR analysis (Van Eck et al. 1994). There has been one report of infection of *Gladiolus* using *Agrobacterium*, but Southern hybridization was not presented (Graves and Goldman 1987). Kamo et al. (1995a, b) have developed transgenic Gladiolus plants by particle bombardment of suspension cell and callus culture. The success of this procedure depends on the ability to regenerate plants from suspension cells and callus and varies greatly depending on the cultivars. In another study conducted by Kamo (1995), callus or suspensions rapidly decreased the regeneration capacity with increased time in culture.

The first stable transformants of gladiolus that utilized the bulb tissue for transformation was reported by Kamo et al. (1995a, b). Initial characterization of β-glucuronidase (GUS) gene expression under the actin promoter was presented for this monocotyledonous bulb crop.

Transgenic plants of gladiolus were produced following particle bombardment of cormel slices. Plant cells were cotransformed with the gene for phospinothricin acetylttransferase under the control of cauliflower mosaic virus 35S promoter and *uid*A gene coding for GUS under the control of the actin promoter isolated from rice. Particle bombardment has been applied directly to cormels of *Gladiolus* cultivars. A concentration of 8 mg/l phospinothricin resulted in 14% of bombardment cormel slices regenerating plants that were transformed and confirmed by PCR amplification. Most transgenic plants resulting from the bombardment of plant tissues, rather than suspension cells, have been found to be chimeric. Transgenic plants of gladiolus have grown vigorously in vitro and formed cormels. Following 3-month period of dormancy, the cormels continued to express GUS. After dormancy the cormels were sliced transversely and multiple plants regenerated from each cormel slice.

Particle bombardment and selection using phosphinothricin (PPT) and bialaphos can now be successfully applied to develop transgenic plants of all floral crops that multiply by cormels.

Research on using transgenic approaches for developing disease-resistant ornamental plants has recently been reviewed (Hammond et al. 2006). Viruses are a major problem for gladiolus and other bulbous crops because the plants are propagated each year by bulbs that may harbor a bean yellow mosaic virus (Stein 1995).

Development of plants that are resistant to the virus is a more effective means of controlling virus infections in plants. Virus-resistant cultivars or germplasm of *Gladiolus* are unavailable for breeding, making genetic engineering for virus resistance an attractive alternative.

Almost all *Gladiolus* plants are infected with bean yellow mosaic virus (BYMV) (Stein 1995). *Gladiolus* plants were transformed with the BYMV coat-protein gene in either the sense or antisense orientation (Kamo et al. 2005). All the 11 plant lines were challenged with BYMV using controlled aphid transmission. One month following aphid transmission, several transgenic lines containing antiviral transgene showed a lower incidence of infection as compared to the non-transformed plants. Most of the plants that did not contain the virus one month after challenge were found to contain the virus the following season when the plants were grown outside. Apparently BYMV infection was delayed in the coat protein and antisense transgenic plants, but there was eventual infection with the virus. *Nicotiana benthamiana* and *N. benthamiana* plants transformed with the BYMV coat protein gene showed either delayed viral symptoms or recovered from initial infection following challenge with BYMV (Hammond and Kamo 1995a). One out of ten *N. benthamiana* lines transformed with the antisense gene was highly resistant (apparently immune) to the virus (Hammond and Kamo 1995b). The reason for this discrepancy in virus protection in gladiolus in contrast to *Nicotiana* is unknown.

Gladiolus plants were transformed to contain either the cucumber mosaic virus (CMV) coat protein serotype I gene, the CMV coat protein serotype II gene, the CMV replicase gene, an antibody gene to CMV serotype I or an antibody gene to CMV serotype II. Transgenic plants are currently being challenged with purified CMV using the hand-held gene gun to introduce the virus. Most of the transgenic plant lines with the CMV coat protein or replicase genes are susceptible to 2 μg of CMV, but one line with the replicase gene has been consistently found to be resistant to 10 μg (Aebig et al. 2005).

Several promoters (*Arabidopsis UBQ3*, rice actin, *rol*D, mannopine synthase, translation elongation factor 1 subunit α, CaMV 35S, duplicated CaMV 35S, potato *Ubi3* and *Ubi7*, and phosphenolpyruvate carboxylase) have been examined for levels of stable expression in transgenic plants of gladiolus (Kamo and Blowers 1999; Kamo et al. 2000).

The highest level of GUS expression was from the CaMV 35S promoter even though gladiolus is a monocot. The lowest levels of transient GUS expression in gladiolus were with promoters such as rice *Act1* and maize *Ubi1* that were isolated from cereal monocots (Kamo et al. 1995a, b). The maize *Ubi1* promoter has been used most frequently for developing transgenic plants of cereal monocots that show high constitutive levels of expression, but this promoter is not useful for high levels of expression in gladiolus.

It is often desirable to have more than one promoter for expressing both the disease resistance gene and a selectable marker gene. Three polyubiquitin promoters were isolated from gladiolus, and the *GUBQ1* promoter showed higher levels of transient gene expression than the CaMV 35S promoter in gladiolus (Joung and Kamo 2006).

8.5 Scope for Domestication and Commercialization

Many *Gladiolus* species are traditionally used as herbal medicines and deserve domestication.

Ancient Greeks used the roasted corms of *G. italicus* as food. C-vitamins that are obtained from *Gladiolus atroviolaceaeus* leaves at the end of the blossom can be used as a medicine for treatment of toothaches and kidney-stones (Ismailov 1985).

Jacot Guillarmod (1971) described the various uses of *Gladiolus* in Lesotho (South Africa) and the people residing at the high altitude mountains relish the flowers of certain species. The flowers of *G. saundersii, G. ecklonii, G. papilio, G. cruentus,* and *G. natalensis* are used as uncooked salad by nipping of the anthers. According to de Meyer Jr (1982), the culinary virtues of many flowers, including gladiolus can be found by dipping them in butter and fry until crispy, or by stuffing them with a savory hamburger and vegetables fillings and fry, and also by preparations like "Gladiolus Hors D'oeuvre" or "Gladiolus Cake" with pixiola flower and recommended recipes.

It has been found that the corms of Psittacinus hybrids contain high amount of carbohydrate mostly starch (65.4–78.615%) and protein (12.6–18.5%). Through an analysis, pentosan (2–91%), fat (0.58%), saponin (present only before alcoholic extraction), and ash (Sulphated, 3–48%) and after through extraction of glucose, xylose and arabnose were found whereas amino acid analysis revealed the presence of lysine, glycine, threonine, glutamic acid, alanine, pralines, tyrosine, valine, isoluecine, and some unidentified

ones (Khan et al. 1980). *Gladiolus crasifolius* is used for headache and lumbago (the whole plant is crushed, heated, and applied to the affected part). *G. saundersii* (Cooked corms) mixed with food is effective against diarrhea. Decoction prepared from the crushed corms of *Gladiolus ecklonii* is used as drink to get relief from rheumatism and pains.

Gladiolic acid is produced by the corms affected with *Pencillium gladioli* (Brian et al. 1948). The leaves of this genus are also rich in vitamin C, ranging from 1 to 7% depending on the species and cultivars (Maximov et al. 1945). Inhibitors are also found in sheath leaves and leaf bases but not on the tips of the leaves (Konoshima 1980). Raja Rao et al. (1983) isolated ten cytokinin-like substances, termed as X_1, X_2, X_3, X_4a, X_4b, X_5a, X_5b, X_6, X_7, and X_8, from the corms of the cv. Friendship. The factors X_4a, X_5a, and X_6 were tentatively identified as zeatin (Z), isopentenyl adenosine (iPA), and isopentenyl ademine (iP), respectively; factor X_3 behaved like zeatin ribsoide (ZR) or dihydrozeatin riboside (DHZR); X_1 and X_2 may be cytokinin glucosides and X_8 a cytokinin nlucleotide or a cytokinin conjugate similar to lupinic acid. Except X_2 and X_2, others were higher in non-dormant corms.

8.6 Some Dark Sides and Their Addressing

The "sword lily" is common name of gladiolus because of the sword shaped foliage. "Corn flag" is its other common name in Europe because *G. illyricus* is found wild as weed in the cornfields. *G. primulinus* is also known as "waterfall gladiolus" as it was found growing near the Victoria Falls in the tropical forests of Africa.

Only a small percentage of wild *Gladiolus* species are invasive. *G. angustus* native to South Africa has become invasive elsewhere and naturalized in many places, especially Australia. *Gladiolus undulates* is a plant on the list which are environmentally damaging and present a risk in Waitakere, New Zealand. It is distributed in this area but have not spread widely yet. Exclusion, early detection, and rapid response are by far the most cost-effective way of dealing with undesirable invaders. The popular step is to facilitate efforts to keep invasive species out of the state, find invasions before they establish permanent footholds and do whatever it takes to eradicate incipient populations of undesirable species. Education and cooperation are key components of an effective strategy. Much more education is needed to raise the general level of awareness of invasive species by the public and elected officials. More emphasis needs to be put on risk assessment, exclusion, early detection, and eradication. A systematic process needs to be put in place to address before any economic damage becomes apparent.

8.7 Recommendations for Future Actions

1. Establishment of a "Pollen Cryobank" through which pollen of desired species could be consolidated and obtained for breeding without any seasonal or geographic barrier.
2. Molecular genetics related research to provide a sound basis for future advances in the species.
3. International efforts to sequence the *Gladiolus* genome.
4. Future genetic mapping in *Gladiolus* will rely on integration of sequence from other bulbous plants.
5. Development of an interspecific map will facilitate the introgression of genes from wild germplasm into cultivated *Gladiolus* species, which will help in studying of gene flow from genetically engineered plants into the wild, and allow the processes of domestication to be elucidated if the progenitor species is included.
6. Development of genomic resources will be important for future breeding work. Improvement of gladiolus cultivars will be directed towards ornamental characteristics and disease resistance through traditional and molecular-aided breeding methods. Some of these traits may be improved by genes from wild *Gladiolus* species that can be used as a reservoir for identifying and screening closely related species for resistance factors.
7. Genetic transformation is potentially a valuable tool for improving gladiolus. Genetic transformation of selected gladiolus cultivars, with genes conferring specific traits of interest may create new phenotypes useful for different purposes of the grower and the consumer.

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Chapter 9
Lilium

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9.1 Some Basic Facts About the Genus *Lilium*

Genus *Lilium* has about 100 species distributed in the northern hemisphere extending up to Asian tropics (Latitude 10–60°). These perennial herbs have unsurpassed beauty and great commercial significance. *Lilium candidum* (Madonna lily) was used already as a cut flower in the middle Minoan IIIA–B period (ca. 1750–1675 BC) known to be of biblical importance. *Lilium longiflorum* is often used in Easter season as pot plant and known as "Easter lily" in the United States. In China, lilies have been cultivated for food and medicine for at least 2,000 years (Haw 1986). Today, lily hybrids are one of the most important cut flowers and pot plants of the worldwide horticultural bulb trade. Lily hybrids and species are also used as garden plants. In most cases, "lily species" are susceptible to various diseases and cultural problems that render them difficult to maintain in gardens. But, many species are attractive for gardeners in Europe and North America. More than 300 cultivars are registered per year and the accumulated number of registered lily cultivars is more than 9,400 (http://www.lilyregister.org). The important aim of lily breeding is to combine the delicate features of their wild relatives with disease resistance, hardiness, and year-round forcing ability in the cultivars. Lily cultivars can be divided into three classes based on uses: one for cut flowers in the greenhouse, another as pot plant, and further for garden cultivation. Eastern Asia and North America are centers of high diversity with about 60 and 21 species, respectively. The closest relatives to lilies are found in eastern Asia, where the genus *Lilium* originated, along with the genera *Fritillaria*, *Nomocharis* Franchet, *Notholirion* Wallich ex Boissier, and *Cardiocrinum* (Endicher) Lindley (Woodcock and Stearn 1950). Lighty (1968) reported that *Lilium* and *Nomocharis* are very closely related and some recent molecular classifications (Fay and Chase 2000) include *Nomocharis* in genus *Lilium*. The 21 species of *Lilium* that are native to North America are derived from Asian stock (Lighty 1968). *Lilium philadelphicum* and *L. catesbaei* are the only two North American lilies with erect flowers. These two species almost certainly represent a single introduction from Asia (Nishikawa et al. 1999; Hayashi and Kawano 2000). Most of the *Lilium* species possess $2n = 2x = 24$ chromosomes with the exception of *Lilium tigrinum*. Natural triploids in *L. tigrinum* are common in the habitat of Korea, where diploid plants predominate in the coastal area and the triploid plants predominate in the mountainous area. Most lilies are largely self-incompatible, and cross-pollination is required for seed set, however some species are confirmed as self-compatible.

Lily bulbs were used as a food and equally versatile as medicine, and the mashed bulbs were variously employed in the treatment of spider bites, cuts and bruises, fever, coughs, consumption, stomach ailments, and rheumatism. Contemporary medical use seems to be largely limited to *L. tigrinum*, which bulbs are used to treat a variety of internal discomforts including those associated with menstruation and menopause (Zhao et al. 1996; Zhang 2007).

Although there were previous attempts to classify *Lilium* species, the taxonomic work of Comber (1949) is by far the most comprehensive. Comber classified

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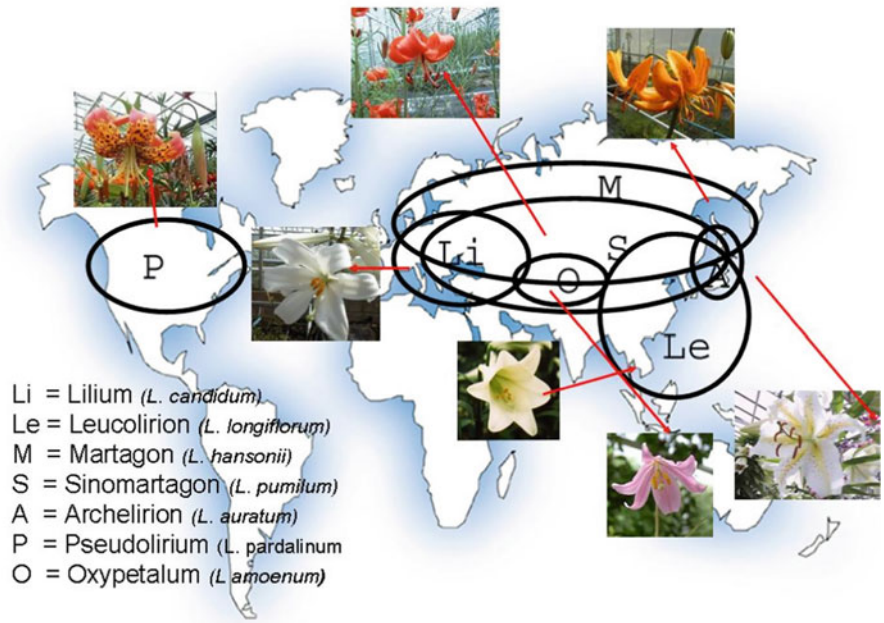

Fig. 9.1 The natural distribution of sections of the genus *Lilium* around the northern hemisphere with each section of a representative species

the genus into seven taxonomic sections, viz., *Martagon*, *Pseudolirium*, *Liriotypus*, *Archelirion*, *Sinomartagon Leucolirion*, and *Daurolirion*. This classification was based on 15 phenotypic characteristics. De Jong (1974) revised this classification: *Martagon*, *Pseudolirium*, *Lilium*, *Archelirion*, *Sinomartagon*, *Leucolirion*, and *Oxypetalum*. Besides this, information that is relevant to phylogenetic considerations has also emerged from the crossability data of species within and among different taxonomic sections (Lim et al. 2008) as well as meiotic studies (Sect. 1.1.8). In addition to the above approaches, molecular phylogeny of the genus *Lilium* has also been carried out on a limited scale (Nishikawa 2007). On basis of these data, a new classification should be proposed.

9.1.1 Characteristics of Classified Species

9.1.1.1 Section *Martagon*

Involved species: *L. tsingtauense*, *L. miquelianum*, *L. distichum*, *L. hansonii*, *L. martagon*, *L. medeloides*.

The species of the section *Martagon* are primitive in the genus *Lilium*, especially *L. hansonii*. Most *Martagon* species are native in Korea, Japan, China, Manchuria and Russia, and just have a restricted distribution in the countries and islands along the East Sea of Korea and Japan. Only *L. martagon* is widespread with its distribution area from East Russia to West Spain. Based on Lighty (1960), Korea and the adjacent area of Manchuria are very likely near the center of origin of the genus *Lilium*. China and Korea host *L. distichum* and *L. tsingtauence*. The Russian area around the Vladisvostok and up along the Amur River is also the home of *L. distichum*. *L. hansonii* habitats in the Ullung-Do Island of Korea and that is the only known area of native growth of this species. There, one finds the ancestral colonies of the wheeled lily of Japan, *L. medeoloides*. They all show a whorl of leaves near the middle of the stem plus a few scattered leaves above or below. Flowers of this section are nodding to upright, horizontal, and pendant with a range of 6–12 cm in diameter. They possess a range of perfume-like fragrances as in *L. distichum*, sweet-dung smelling in *L. hansonii*, and *L. tsingtauense* possess no smell. There is no clear criterion for discriminating the four highly related species: *L. distichum* and *L. medeoloides*, and *L. tsingtauense* and *L. miquelianum*.

Crosses with *Martagon* species within the *Martagon* section and with other sections produce quite different results. It is easy to cross and get seed set between *L. hansonii* as a female parent and *L. tsingtauense* (Martagon), and *L. cernuum* (Sino-martagon) as male parent. However, the reciprocal crossings showed almost no seed set and embryo formation. This may be highly related to the plant vigor, which is important for the fertilization and embryo growth as a female parent. Plant vigor of *L. cernuum* and *L. tsingtauense* is very weak and the flower size is also small. Many hybrids are made by crossing between *L. martagon* and other species, such as *L. hansonii*. Bridge-crossing can be made among three species: *L. martagon*, *L. hansonii*, and *L. tsingtauense*.

L. hansonii is very peculiar to have its only habitat in Ullung-Do Island, Korea. These plants are growing in a humus alkaline soil at the level of 200–300 m above sea level. A number of plants are seen in the habitats, which are well protected because digging of these is prohibited by Korean law. There are few plants that produce flowers with pure yellow colour without any spots. They possess strong odor, like sweet mixed with other unpleasant smells. All flowers showed pendant thick tepals with slightly unbalanced tepals like *L. distichum* found in other Korean region. Plants are vigorous and tall to reach about 1 m.

L. distichum, called Chosen lily (Chosen is one of the Korea dynasty), is native in China, Russia, and Korea. Lighty (1968) reported that *L. distichum* was found in northern Korea, but recently the author has found this in southern Korea around Mt. Dukyusan as well. This species has a side-facing dull orange-colored flower with one tepal pendant and rest of the five tepals ascending, showing asymmetrical shape of flowering. It has also fragrance but, mostly not so attractive, however, very few flowers posses a strong perfume-like fragrance unknown in other lilies.

L. medeoloides shows side- to down-facing orange-colored flowers with long flowering stems. It is mainly native to Japan above Shikoko through Hokkaido, Kamchatka, Sakhalin islands, and partly to the main land of Russia above Vladivostok. Baker (1871) stated in his synopsis that *L. medeoloides* are found in both Koreas.

9.1.1.2 Section *Pseudolirium*

Involved species:

2a. *L. bolanderi*, *L. columbianum*, *L. kelloggii*, *L. humboldii*, *L. rubescens*, *L. washingtonianum*
2b. *L. maritimum*, *L. nevadense*, *L. occidentale*, *L. pardalinum*, *L. parryi*, *L. parvum*, *L. roezlii*
2c. *L. canadense*, *L. grayi*, *L. iridollae*, *L. machuxii*, *L. michiganense*, *L. superbum*
2d. *L. catesbaei*, *L. philadelphicum*

North America is one of the centers of worldwide diversity of lily with about 21 species. Most of the species are distributed along the American West Coast (*L. bolanderi*, *L. columbianum*, *L. kelloggii*, *L. humboldii*, *L. rubescens*, *L. washingtonianum*, *L. maritimum*, *L. kalleyanum*, *L. occidentale*, *L. pardalinum*, *L. parryi*, *L. parvum*, *L. wigginsii*). A few species are native to eastern North America such as: *L. canadense, L. grayi*, *L. superbum*, *L. catesbaei*, *L. michiganense*, *L. michauxii*, *L. iridollae*. In natural condition, the species can be found from sea level (*L. columbianum*) to areas at high elevation of around 3,000 m (*L. parvum* and *L. parryi*). West Coast lilies can be divided into two main types – the "dry growers" and "wet growers" (Robinett and Robinett 1991). "Dry growers" usually can be located in areas of high seasonal rainfall, deep in soil that retains moisture. "Dry growers" can be found, at the edge of woodlands or scrubs. The following species can be included to this group: *L. bolanderi*, *L. columbianum*, *L. kelloggii*, *L. rubescens*, *L. washingtonianum.* "Wet growers" can be found in areas rich in water around the year: along the streams, in seeps, or in bog condition. This group comprises, *L. kelleyanum*, *L. maritimum*, *L. occidentale*, *L. pardalinum*, *L. parryii*, *L. parvum*, *L. wigginsii*.

Species of North American lily have a wide variation in their flower tepals' color (from white to peach, orange, yellow, pink, red, purple, scarlet), flower shape (Turk's cap, trumpet, bowl, bell-shaped) with or without spotting. Most of the species are characterized by nodding flowers with the exception of two species with erected flowers *L. philadelphicum* and *L. catesbaei*. Plants can reach a height from 30 cm (*L. catesbaei*) to 3 m (*L. superbum*). Leaves can be arranged in whorls or scattered on the stem. The bulbs of the North American species are radically asymmetrical, slowly growing, and scaly rhizomes.

A number of the successful hybridizations that have been done among species of *Pseudolirium* section created the group of American species hybrids. Successful crossing in this respect has been between: *L. pardalinum* and *L. humboldtii*, *L. kelloggii* and *L. parryi*, *L. pardalinum* and *L. bolanderi* (Fox 1974), as well as between *L. canadense* and *L. michiganense* (Wadekamper 1988). Hybrids were also obtained from crosses between *L. pardalinum* and oriental hybrids from the section *Archelirion*, while *L. canadense* has been successfully crossed with *L. longiflorum* (Fig. 9.2).

9.1.1.3 Section *Lilium*

Involved species: *L. candidum*, *L. carniolicum*, *L. chalcedonicum*, *L. monadelpum*, *L. polyphyllum*, *L. pomponium*, *L. pyrenaicum*, *L. albanium*, *L. ledebourii*, *L. kesselringianum*, *L. jankae*, *L. ponticum*, *L. ciliatum*, *L. akkusianum*, *L. bosniacum*, *L. szovitisanum*.

Species of the *Candidum* subsection are found in Europe and the Middle East. The species are important from the hybridizer's point of view. As *L. candidum* crosses readily, with *L. chalcedonicum* and *L. monadelphum*. *L. polyphyllum* in most of its characteristics is very close to *L. monadelphum* (Anurag 2007) *L. candidum* holds pleasant odor, whereas *L. pyrenaicum* produces an unpleasant one. Flowers of the species bloom during June–July. Interspecific crosses were made between *L. henryi* and *L. longiflorum* with *L. candidum* (Fig. 9.2).

9.1.1.4 Section *Archelirion*

Involved species: *L. auratum*, *L. brownii*, *L. japonicum*, *L. nobilissimum*, *L. rubellum*, *L. speciosum*.

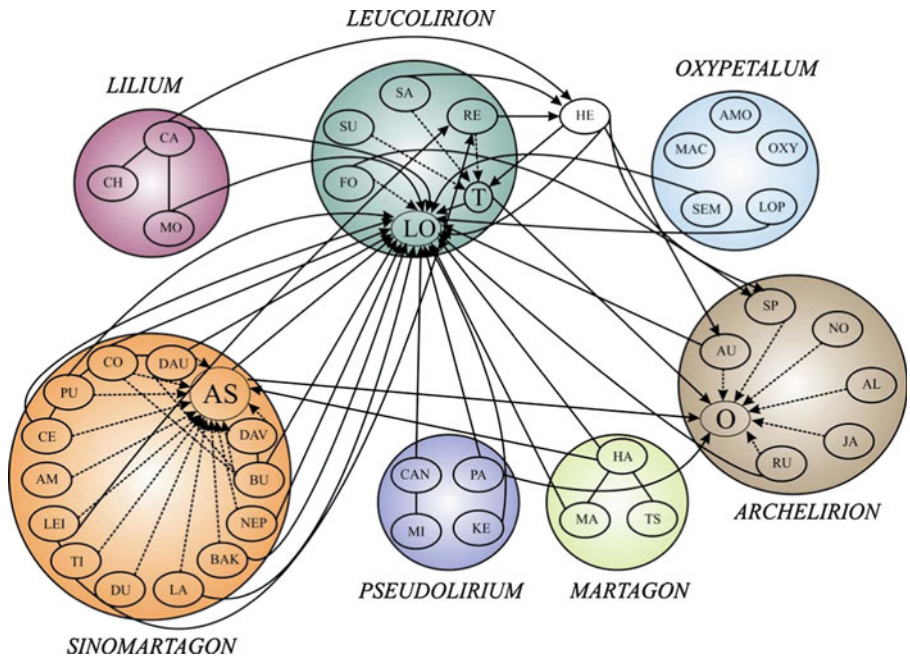

Fig. 9.2 *Crossing polygon* of the genus *Lilium* including all the successful crosses of species between different sections of the genus *Lilium*. In this figure, the connections between the Asiatic, Trumpet, and Oriental hybrid groups (*large ellipses*) are shown by *dotted lines*. In successful crosses between species (*small circles*) of different sections (*large circles*), the *arrows* point toward the female parent. Abbreviations: AL: *L. alexandrae*; AM: *L. amabile*; AMO: *L. amoenum*; AS: Asiatic hybrids; AU: *L. auratum*; BAK: *L. bakerianum*; BU: *L. bulbiferum;* CA: *L. candidum*; CAN: *L. canadense*; CE: *L. cernuum*; CH: *L. chalcedonicum*; CO: *L. concolor*; DAU: *L. dauricum;* DAV: *L. davidii*; DU: *L. duchartrei*; FO: *L. formosanum*; HA: *L. hansonii*; HE: *L. henryi*; JA: *L. japonicum*; KE: *L. kelloggii*, LA: *L. lankongense*; LEI: *L. leichtlinii*; LO: *L. longiflorum*; LOP: *L. lophophorum* MA: *L. martagon*; MAC: *L. macklinae* MI: *L. michiganense*; MO: *L. monadelphum*; NEP: *L. nepalense*; NO: *L. nobilissimum*; OXY: *L. oxypetalum*; O: Oriental hybrids; PA: *L. pardalinum*; PU: *L. pumilum*; RE: *L. regale*; RU: *L. rubellum*; SA: *L. sargentiae;* SEM: *L. sempervivoideum*; SP: *L. speciosum*; SU: *L. sulphureum*; T: trumpet hybrids, TI: *L. tigrinum*; TS: *L. tsingtauense*

The species of the section *Archelirion* are native to Japan with the exception of *L. speciosum,* which is also found in Taiwan and southeastern China (McRae 1998). In natural condition the species can be found on the margins of hillside woods and well-drained slopes (*L. auratum*), on the steep cliffs of the coast (*L. nobilissimum*) and in shaded and moist places in forest, grassy slopes (*L. speciosum*) in high mountain meadows (*L. rubellum*) and moist places (*L. japonicum).* *L. rubellum* and *L. nobilissimum* are threatened with extinction and are under special protection in Japan nowadays.

Species from the *Archelirion* Section range from early (*L. rubellum*, *L. auratum*) to late flowering (*L. nobilissimum*, *L. speciosum*), plants can reach a height from 30 cm (*L. rubellum*) and 50 cm (*L. nobilissimum*), to up to 2.5 m (*L auratum*). Strongly scented flowers are of white (*L. nobilissimum*, *L. japonicum*, *L. auratum*) or pink color (*L. rubellum*). The flowers of *L. speciosum* are white to pink in color with carmine spots and papillae; similarly flowers of *L. auratum* have gold radial markings, and orange spots. Most species of the section *Archelirion* are resistant to *Botrytis elliptica,* a pathogenic fungus that affects most of the lilies from other sections, but susceptible to *Fusarium* (Barba-Gonzalez et al. 2005a, b). In Japan, *L. auratum, L. speciosum, L. rubellum* and *L. nobilissimum* are cultivated for their edible bulbs, which are rich in starch.

A successful hybridization has been made between *L. rubellum* and *L. longiflorum* (section *Leucolirion*) where the former was used as a pollen donor. Hybrids were also obtained from reciprocal crosses between *L. nobilissimum* and *L. regale* (Section *Leucolirion*) (Obata et al. 2000). *L. auratum* has been successfully crossed with *L. henryi* and *L. longiflorum* and many modern oriental cultivars are derived in part from this species. *L. speciosum* has also been widely used for breeding, successful crosses have been made with other species of the Oriental section as well as with *L. henryi* and *L. alexandrae* (McRae 1998).

The hybridization among the species of the *Archelirion* section resulted in the Oriental hybrids (O-genome; Beattie and White 1993; McRae 1998). These hybrids nowadays form the most important group of cultivated lily hybrids, in spite of the fact that their forcing time is a few weeks longer than most of the Asiatic hybrids. Since 1990, around 2,000 cultivars have been registered (Leslie 1982–2005). Oriental hybrids have massive strongly scented flowers of white and pink color where the dark pink is dominant over white (Lim and Van Tuyl 2006). Flowers are side-wards or up-facing, and open flat while some of the petals may be bent back.

Oriental hybrids have been successfully crossed with *L. pardalinum* (Section *Pseudolirium*) and Asiatic hybrids (Section *Sinomartagon*) when used as female parent. One of special interest at present is to combine the resistance to *Fusarium oxysporum* and viral diseases from Asiatic hybrids with the resistance to *B. elliptica* from the Oriental hybrids into a new group of interspecific hybrids (Schenk 1990; Lim et al. 2000a). Crosses are also made between Oriental hybrids and *L. longiflorum*, and Trumpet hybrids.

9.1.1.5 Section *Sinomartagon*

Involved species:

5a. *L. dauricum*, *L. maculatum*, *L. wilsonii*, *L. bulbiferum*, *L. davidii*, *L. duchartrei*, *L. henryi*, *L. tigrinum*, *L. lankongense*, *L. leichtlinii*, *L. papilliferum*, *L. rosthornii*

5b. *L. amabile*, *L. callosum*, *L. cernuum*, *L. concolor*, *L. pumilum*, *L. fargesii*

5c. *L. aboricola*, *L. bakerianum*, *L. euxanthum*, *L. majoense*, *L. nepalense*, *L. orchraceum*, *L. paradoxum*, *L. poilanei*, *L. primulinum*, *L. sherifiae*, *L. souliei*, *L. stewartianum*, *L. taliense*, *L. wardii*

Many species involved in this section possess diverse phenotypic and physiological traits. Current Asiatic lily cultivars are derived from this section by anonymous crossings since 1890s. *L. bulbiferum*, *L. dauricum*, *L. tigrinum*, *L. amabile*, *L. cernuum*, *L. concolor*, among others have been widely used in hybridization being ancestors of the modern Asiatic hybrids. The tremendous diversity in phenotypical and physiological aspects in this section is, for example, shown in the flowers which are orange, pink, red, purple, yellow, white and green, and up-facing to downward-facing. The flower shape is bowl, flat, and recurved. Interspecific hybridizations among species and their hybrids made possible to release commercial hybrid cultivars. It encompasses large numbers of cultivar registration so far, but the number of new hybrids registered recently is decreased due to a decreasing economic importance of Asiatic hybrids.

China is the largest center of origin of this section, where more than 33 species are native. Seeds of this section show instant epigeal germination. *L. tigrinum* and *L. davidii* are edible lilies cultivated for cooking in China. *L. tigrinum* shows wide range of habitats in most areas of East Asia, distributed at 400–2,500 m elevation. This species shows diploid and triploid plants in the natural habitat of which diploids are mainly found on the seaside and triploids are mainly found in the mountainous area of Korea (Kim et al. 2006). It is very tolerant to the different environmental conditions in China, Korea, and Japan. *Lilium nepalense* is medium hardy and needs good protection against frost. It shows an affinity to *L. nobilissimum*, since an wonderful hybrid between these two species has been reported (McRae 1998). Nishikawa et al. (1999) compared nuclear ribosomal DNA (internal transcribed spacer region) of 55 species including *Nomocharis saluensis*. They found that *L. nepalense* together with *N. saluensis* showed great affinity to the subsection *Lilium*. *L. primulinum, L. nepalense*, *L. majoense*, and *L. poilanei* seem to be closely related species. *L. amoenum* is a rare species, which is considered to be on the borderline to the genus *Nomocharis* and closely related to *L. sempervivoideum* (which has more narrow leaves, in section *Oxypetalum*). *L. arboricola* seems to be related to *L. primulinum*, *L. lijiangense*, and *L. wardii*; however, the flowers are pure green without any other coloration. *L. bakerianum* shows diverse flower colors such as white, green, pink, yellow, or purple. Flower shapes are also very diverse in this section as recurved (*L. rosthornii*, *L. lankongense*, *L. leichtlinii* var *maximowiczii*, *L. amabile*, *L. pumilum*, *L. nepalense*, *L. taliense*), bell-shaped (*L. mackliniae*), lantern-shaped (*L. lophophorum*), Turk's cap-shaped (*L. arboricola*, *taliense*, *wardii*), and funnel-shaped (*L. majoense*, *L. nepalense*) flowers can be found. Some species are very rare and are classified by de Jong (1974) in *Oxypetalum* (*L. sempervivoideum*, *L. amoenum*, *L. henrici*, *L. lophophorum*, *L. mackliniae*). Some species are highly related in their morphology, for example, *L. leichtlinii* var. *maximowiczii* is similar to intermediate between *L. tigrinum* and *L. amabile*. *L. taliense*, *L. duchartrei*, and *L. lankongense* are closely related, whereby *L. duchartrei* bears its flowers in an umbel, the other two in racemes. Some species are stoloniferous (*L. bakerianum*, *L. lankongense*).

9.1.1.6 Section *Leucolirion*

Involved species:

6a. *L. leucantum*, *L. regale*, *L. sargentiae*, *L. sulphureum*, *L. Leucanthum*
6b. *L. fomosanum*, *L. longiflorum*, *L. neilgherrense*, *L. philippinense*, *L. wallichianum*, *L. wenshanense*

L. longiflorum Thunberg (Easter lily; Japan) is the common white lily of the florist trade. Many cultivars exist of which cultivars such as "Georgia" as cut flower and "Nellie White" as pot plant are among the most widely cultivated and commercially important lily. The plants of this section show a tubular flower shape with a white color and distinct fragrance. With *L. longiflorum* and *L. formosanum* Wallace (Formosa lily), interspecific hybrid "*L. formolongo*" are derived for annual seed lily, which is tall, has a vigorous growth habit and flowers at early stage. Other species, such as *L. philippinense* Baker (Philippine lily), are often tall and have a thin stem which is not an attractive characteristic for lily culture.

9.1.1.7 Section *Oxypetalum*

Involved species: *Lilium oxypetalum*, *L. amoenum*, *L. henrici*, *L. mackliniae*, *L. lophophorum*, *L. sempervivoideum*, for description see section *Sinomartagon*.

9.1.1.8 Crossability Throughout the Genus Lilium

Undoubtedly, the taxonomic classification based on phenotypic characteristics described above is a useful guide to delimit the species. But further refinement can be achieved through other methods such as interspecific hybridization as well as molecular phylogeny in order to determine species relationships. A crossing polygon (Fig. 9.2), which is constructed on the basis of species hybridizations carried out at Plant Research International, Wageningen University and Research Center, Netherlands can be used for this kind of refinements. Generally, the species within each section (included in each large circle) are easily crossable and the hybrids are fertile. On the contrary, hybridization between species of different sections (combinations indicated by arrows among large circles) is

very difficult to achieve because of pre- and post-fertilization barriers and high sterility of the hybrids. Nevertheless, through in vitro embryo, ovule or other rescue methods, successful intersectional species hybrids, shown in the crossing polygon, have been achieved. These interspecific hybrids have also been useful for assessing species relationships through the analysis of chromosome pairing during meiosis. Thus, the analyses of meiosis using genomic in situ hybridization (GISH) in the F_1 hybrids between Longiflorum × Asiatic, Asiatic × Oriental, *L. henryi* × *L. auratum*, among others, have provided valuable data regarding chromosome pairing relationships among different genomes (Lim et al. 2001a, b; Barba-Gonzalez et al. 2005a, b; Van Tuyl et al. 2005).

9.2 Conservation of *Lilium* Germplasm

As with other plant species, *Lilium* species are also facing the threat of genetic erosion, so more and more attention is being paid for conservation of *Lilium* germplasm as the genus represents one of the most important crops of cut flowers and pot plants worldwide which is also used as kind of vegetable and medicine in some places of the world. The conservation of lily germplasm is divided into two main strategies: in situ and ex situ.

9.2.1 Ex Situ Conservation

9.2.1.1 Cold Storage of Bulbs

Collections of bulb crops are usually preserved in the field or greenhouse by yearly planting, harvesting and storing of the bulbs, with high investments of labor, space, and risk of losses caused by diseases (Towill 1988; Withers 1991). Since many lily genotypes are unique and heterozygous, they should be preserved vegetatively as clones. By increasing the storage duration of the bulbs would make the maintenance of a field collection more efficient. Temperature is the most important factor in lily bulb storage. Normally, the lily bulbs are stored at 4°C. If a longer storage is required, the temperature must be decreased to −2°C and bulbs should be put in moist peat (Van der Salm and Van der Salm 1985; Beattie and White 1993). However, sometimes sprouts are damaged after bulb storage at −2°C (Boontjes 1983; Beattie and White 1993), and the regeneration ability of bulb scales will decrease after more than one year of storage and will be completely lost after 5 years (Bonnier et al. 1997). So increased freezing tolerance of lily would reduce the risk of freezing injury, and could make storage at a lower temperature than −2°C possible. Lower temperature (less than −2°C) would further minimize growth conditions and therefore bulbs could be stored for maximum time period. Freezing tolerance can be increased by: cold-acclimatization, abscisic acid treatment, partial dehydration, or by low atmospheric pressure (Halloy and Gonzalez 1993; Hincha 1994; Lang et al. 1994; Mantyla et al. 1995). Controlled atmospheric storage (CAS) and modified atmosphere packaging in closed bags (MA package) were also used to decrease the metabolism of clone material in bulb flowers (Prince et al. 1981, 1986; Bonnier et al. 1996). Ion leakage from lily bulbs measured by the electrical conductivity of external solution increased with damage caused by frost, heat or dehydration, and with viability loss during storage (Bonnier et al. 1992, 1994). Therefore, ion leakage could be used for measuring the viability and estimate maximum storage duration of lily bulbs.

9.2.1.2 Conservation in Gene Banks

The lily gene bank at Plant Research International has maintained several thousands of lily genotypes for more than 35 years. In gene banks, as a means of preserving genetic material, the storage of seed is the preferred method, but clone germplasm and recalcitrant seed species can also be kept in field plantings as field gene banks.

9.2.2 In Situ Conservation

9.2.2.1 Tissue Culture as In Vitro Gene Banks

An alternative method to preserve lily collections is conservation in vitro. In vitro stored collections need relatively small amounts of space, medium components can be used that minimize growth, plants can be

multiplied quickly, and there is often a possibility to eliminate viral diseases (Towill 1988; Withers 1991). Lily material could be stored more than 28 months at 25°C (Bonnier et al. 1997) before transfer to new medium is required. However, at each transfer event, there is a risk of contamination with microbial organisms (Withers 1991). Furthermore, the establishment of an in vitro collection is labor-intensive and genotypes may react differently under identical conditions (Towill 1988).

Slow growth increases the maximum storage duration. In vitro slow growth could be obtained by low temperature (−2°C), osmotic stress (Grout 1991; Withers 1991), or by a low concentration of nutrients (Engelmann 1991). However, the low temperature is commonly used to store lily bulbs (Beattie and White 1993; Bonnier and Van Tuyl 1997).

9.2.2.2 Cryopreservation in Liquid Nitrogen

Cryopreservation of lily meristems could be a suitable method for long-term preservation. Research by Bouman and De Klerk (1990) resulted in the survival of 8% of meristems of *L. speciosum*. By using the technique of vitrification, apical meristems from scale bulblets of *L. japonicum* had been successfully cryopreserved. The rate of shoot formation after cryopreservation was approximately 80% after 4 weeks. Later on, this vitrification method was also successfully applied to five other lily genotypes (Matsumoto et al. 1995). Although so many methods could be used for the preservation of lily germplasm, the existing challenge is to interface between the in situ and ex situ system. Until now, the two systems are more or less implemented independently by two different groups of people and institutions with a different basic conservation philosophy.

9.3 Role in Development of Cytogenetic Stocks and Their Utility

Because of the large size of their chromosomes (Bennett and Smith 1976), *Lilium* species have been used in cytological and cytogenetic studies for a long time. However, there has been very little interest in producing cytogenetic stocks such as tri- or monosomic series or alien addition or substitution lines as has been done in many other crop plants. There are a few main reasons for this. Lily is not a major leading crop when compared to wheat, maize, or tomato that are well known for such developments; secondly, the generation time in lily – from seed germination to seed production – exceeds two or more years. This obviously discourages the development of cytogenetic stocks in lily. Nevertheless, whenever *Lilium* species or cultivars are used in any significant investigation, the names of the species or cultivars are promptly mentioned so that they are available for verification when necessary. There are several examples of basic studies such as: chromosome identification, karyotype analysis (Stewart 1947), chiasma formation and crossing-over (Brown and Zohary 1955; Fogwill 1958), and time and duration of female meiosis (Bennett and Stern 1975), where the material used in the experiments is well documented. Later on, different banding pattern techniques such as C- and Q-banding pattern were used to study the chromosome structure of different *Lilium* spp. (Holm 1976; Von Kalm and Smyth 1984; Smyth et al. 1989). These banding patterns were also used to detect the nucleolar organizer regions (NORs) (Von Kalm and Smyth 1980, 1984; Smyth et al. 1989). Flow cytometry has been employed to estimate the genome size of different *Lilium* species (Van Tuyl and Boon 1997). Although systematic development of cytogenetic stocks is lacking in lily, there is evidence that alien additions and substitutions are possible in the case of *Lilium*.

In general, the karyotypes of individual plant species, but not their hybrids or progenies, are used for comparison of evolutionary trends, if any. But in the case of *Lilium* not only the individual species but also their hybrids and their progenies have been used for karyotype studies. Besides using conventional staining methods, DNA in situ hybridization has been used for distinguishing the chromosome constitution of the hybrids and the progenies. Obviously, these analyses have provided a great wealth of information on chromosome morphology as well as on homoeology and crossing-over relationships among the genomes of different species involved in the development of the most important lily cultivars. In this section, both conventional as well as molecular methods of karyotype analyses in the genus *Lilium* are described.

Among angiosperms, the species of the genus *Lilium* have the largest genomes with haploid DNA content (1C) ranging from 32.75 pg in *L. pyrenaicum* (Bennett 1972) to 47.90 pg in *L. canadense* (Zonneveld et al. 2005). The species are diploids with 24 chromosomes ($2n = 2x = 24$), in several species aneuploids were found having additional chromosomes or chromosome fragments (Stewart 1943). Tiger lily (*L. tigrinum*) is the only triploid species in the genus (Noda 1978; Kim et al. 2006). *Lilium* species represent interesting material for cytological study due to exceptionally large size of chromosomes, which ranges, in *L. longiflorum* from 18.1 μm to 34.4 μm (Lim et al. 2000, 2001a, b). There are many reports on chromosome morphology and karyotype analysis by using conventional cytological techniques (Stewart 1947; Darlington and Wylie 1995; Lighty 1960; Fedorov 1969; Marasek and Orlikowska 2003). However, chromosomes' morphology (length and centromere position) is highly conserved within and between species, therefore only a few chromosomes are recognizable on the basis of the above traits (Lim et al. 2001a, b; Marasek and Orlikowska 2003). Chromosomal markers in lily refer predominantly to the presence and the position of secondary constrictions (Uhring 1968; North and Wills 1969; Okazaki et al. 1994; Fernandez et al. 1996; Obata et al. 2000; Marasek and Orlikowska 2003). Chromosome morphology and the presence and position of secondary constriction have been also used to verify *Lilium* hybrids (Okazaki et al. 1994; Obata et al. 2000). In order to help in *Lilium* chromosome identification, banding techniques have been applied such as silver staining of nucleolar organizing regions (Ag-NORs) (Von Kalm and Smyth 1980; Smyth et al. 1991), staining of heterochromatin sections (C-bands) (Smyth et al. 1989; Smyth 1999), and fluorescent staining (Kongsuwan and Smyth 1977; Lim et al. 2001a, b; Marasek et al. 2005). Fluorescent in situ hybridization (FISH) is another technique producing chromosomal markers that show positions of specific genes thereby permitting chromosomal identification. The use of FISH with 5S rDNA and 25S rDNA probes has provided molecular cytogenetic markers for identification of somatic chromosomes in different *Lilium* species and has been used for verification of lily hybrids (Lim et al. 2001a, b; Marasek et al. 2004a, b). Genomic in situ hybridization (GISH) using labeled whole-genomic DNA enables unambiguous distinction between the different genomes in interspecific crosses, which makes it very useful for identifying plant hybrids and chromosome recombination. In the genus *Lilium*, GISH was successfully used for identification of parental chromosomes in hybrid (Lim et al. 2000a, b; Barba-Gonzalez et al. 2005a, b) and to trace recombination events in BC_1 and BC_2 progenies of LA and OA hybrids (Karlov et al. 1999; Lim et al. 2000a, b; Barba-Gonzalez et al. 2005a, b; Zhou et al. 2008). Based on the recombination sites identified through GISH cytological maps were constructed for four (L, O, and two times A in different backgrounds) lily genomes, which can be used as landmarks for assigning molecular markers or desirable genes to chromosomes of *Lilium* (Khan et al. 2009a). GISH has also been applied to reveal the restitution mechanisms (FDR – first division restitution, IMR – intermediate division restitution, SDR – second division restitution) that lead to different chromosome constitution in $2n$ gametes (Lim et al. 2001a, b, 2004; Barba-Gonzalez et al. 2005a, b; 2008).

Karyotype analysis of interspecific hybrids and their backcross progenies through GISH technique can provide highly useful information that cannot be obtained by conventional methods of analyses of chromosomes of species alone. In interspecific hybrids, the homoeologous chromosomes can be directly compared through GISH, as in the case of the F_1 hybrids of Longiflorum × Asiatic (LA), Oriental × Asiatic (OA), and *L. auratum* × *L. henryi* (AuH) (Lim et al. 2000a, b; Van Tuyl et al. 2002; Barba-Gonzalez et al. 2005a, b). In the backcross progenies, besides a direct comparison of homoeologous chromosomes of the parents, invaluable insight can be obtained on intergenomic crossing-over. For example, highly unequal distribution of crossovers among different pairs of homoeologous chromosomes in the BC progenies of LA and OA hybrids has been observed (Khan et al. 2009a). Analyses of karyotypes of triploid BC_1 progenies derived from the functioning of $2n$ gametes of LA and OA hybrids have been useful to determine the restitution mechanisms (viz., FDR and IMR), through which the sexual polyploid progenies originated. Furthermore, besides triploid BC progenies, in the case of LA hybrids, it has also been possible to produce diploid ($2n = 2x = 24$) BC_1 progenies through the use of haploid gametes from LA hybrids. Karyotype analysis of these diploid BC_1 progenies has yielded useful information on the extent of intergenomic crossing-over in the diploid LA hybrids (Khan et al. 2009b).

This investigation has conclusively established that, despite considerable genome differentiation between the karyotypes of different *Lilium* species of different taxonomic sections, the homoeologous chromosomes appear to be mostly homosequential as far as crossovers are concerned and they fully compensate for each other.

9.4 Role in Crop Improvement Through Traditional and Advanced Tools

Commercial cultivation of the three major groups of lilies, viz., Longiflorum, Asiatic and Oriental lilies, is practiced through vegetative propagation of bulbs. Also the parental wild species of *Lilium* from which these cultivars have originated reproduce through bulbs and thus are vegetatively propagated. There are certain constraints to breed such vegetatively propagated crops. These are: a high degree of heterozygosity, sterility of the hybrids and progenies, segregation for deleterious recessive genes and effort needed to produce large progeny populations that could facilitate selection. Some or all of these constraints may be relevant to lilies. Besides these limitations, if the final products, i.e., cultivars, are to be polyploids the breeding procedures have to be appropriately modified. Taking the above considerations into account, the possibilities of crop improvement in lily are highlighted in this section.

9.4.1 Overcoming Interspecific Crossing Barriers

Hybridization between the cultivars or species from different taxonomic sections in the genus *Lilium* is generally difficult and can succeed only through the use of special techniques. In the last decades, many different techniques have been developed to overcome pre-fertilization barriers. These include:

1. Cut-style method. This method has been utilized to produce interspecific hybrids in the genus *Lilium* (Myodo 1962; Van Tuyl et al. 1991), *Allium* (Doubouzet et al. 1994), and *Fritillaria* (Wietsma et al. 1994). This method comprises the deposition of pollen on the stylar surface after cutting the style with stigma (usually a millimeter above the ovary), and allowing pollen to circumvent stylar barriers, which normally inhibit pollen tube growth (Myodo 1962). A variation of this method is the stylar graft technique, in which pollen grains are deposited on a compatible stigma. After 1 day, the style of the pollen donor is cut 1–2 mm above the ovary and grafted on to the ovary of another plant. Style and stigma are joined in vivo using a piece of a straw filled with stigmatic exudate or are stuck together with only the exudate (Van Tuyl et al. 1991; Van Creij et al. 2000).
2. Pull-style method. This technique involves pulling out the receptive style, brushing its base with the desired pollen, and reinserting it into the ovary. It has been applied to overcome pre-fertilization barriers in the genus *Allium* (Doubouzet et al. 1994).
3. Intrastylar pollination. In this method, a compatible species' style is utilized as a pistil donor and it is pollinated on the stigma with pollen from another species. This has been utilized successfully to produce *Lilium*-interspecific hybrids (Asano and Myodo 1977a; Asano 1980).
4. Mentor pollen. This method consists of "inactivating" genetically by irradiation-compatible pollen (but it is still capable to germinate) and mix it in incongruent pollen. This method has been useful to overcome self-incompatibility but not in interspecific crosses in the genus *Lilium* (Van Tuyl et al. 1982). In the genus *Cucumis* when this method was utilized on interspecific crosses, embryo-like structures were developed but they were not able to germinate (Den Nijs and Oost 1980).
5. In vitro pollination. Several in vitro pollination methods have been developed in order to overcome pre-fertilization methods. These include:
   a. Stigmatic pollination. This method has been successful for the production of compatible embryos of lily and Nerine (Van Tuyl et al. 1992). It consists of normal pollination and the successive artificial cultivation of the entire pistil.
   b. Placental pollination. This method consists of performing a longitudinal cut in the ovary, exposing the ovules, and applying an abundant amount of pollen. These method has been utilized in the genus *Tulipa* (Van Creij 1997) and *Lilium* (Van Creij et al. 2000), with successful

penetration of the pollen tube into the ovaries, however, there was no embryo formation.

Once pre-fertilization barriers are overcome, hybrid embryo growth is restricted by post-fertilization barriers. Both embryo and endosperm have to develop an equilibrium for sharing nutrients in an undisturbed developmental process. In general, the first division of the zygote is delayed to favor the first division cycles of the endosperm cells. When the equilibrium in the development of the zygote and endosperm is disturbed, an abortion of the young embryo or disintegration of endosperm follows. This abortion can take place in various stages of development of the young seed. Depending on the stage of embryo abortion, various in vitro techniques can be applied to rescue the abortive embryo (Van Tuyl and De Jeu 1997).

In the last decades, the production of a wider number of interspecific hybrids was possible with the development of several in vitro methods that made possible to overcome the post-fertilization barriers, these methods include:

1. Ovary slice. This method has been applied mainly in the production of interspecific hybrids of the genus *Lilium* (Straathof et al. 1987; Kanoh et al. 1988; Van Tuyl et al. 1991; Arzate-Fernandez et al. 2006) and *Tulipa* (Van Creij et al. 2000). It can be applied when the maternal tissue does not have a negative effect on the development of the seeds (Van Tuyl and De Jeu 1997). It consists in slicing the ovary in placing it in vitro, where the seeds are allowed to grow until embryos can be dissected.
2. Ovary culture. This method has been utilized to produce interspecific hybrids in several genera, these include: *Allium* (Nomura et al. 2002), *Lilium* (Van Tuyl et al. 1982, 1991), *Nemesia* (Datson et al. 2006), and *Nerine* and *Tulipa* (Van Tuyl et al. 1990). This method consists of surface sterilization of ovaries and the excision of ovules and transfer to a substrate that allows them to either grow until the embryo can be excised or has germinated.
3. Embryo rescue. This method consists of surface sterilization of ovary and the excision of immature embryos out of the ovules. This method is one of the most effective in the production of interspecific hybrids, and it is utilized mainly when there is no endosperm in the seed and very small embryos are produced, which usually abort in early developmental stages (Myodo 1975; Asano and Myodo 1977b). This method has been applied in the production of interspecific and intergeneric hybrids of the genera *Allium* (Bino et al. 1989), *Alstroemeria* (Buitendijk et al. 1992, 1995), *Lilium* (Van Tuyl et al. 1991), *Pelargonium* (Denis-Peixoto et al. 2006), and *Primula* (Kato et al. 2001). One of the main problems in embryo culture in some cases is the size of the embryo itself; however, modifications to this method have been introduced that mitigate this. The ovaries are cut off into half and placed in liquid medium, in which the embryos are capable to germinate and the plantlets can be subsequently transferred to solid medium (Van Tuyl and De Jeu 1997). Using special pollination techniques, many interspecific hybrids have been produced in the genus *Lilium* and the examples are furnished in Table 9.1.

Whereas a number of interspecific hybrids have been produced through the use of special hybridization techniques, there is also a fairly long list of hybrids obtained between species within a section in the genus *Lilium* without special techniques as well (Table 9.2).

Until relatively recently, most of the cultivars in all the three major hybrid groups of lilies were diploid ($2n = 2x = 24$), and most importantly, they were *intrasectional* species hybrids such as Longiflorum (L), Asiatic (A), Oriental (O), and Trumpet (T), etc., in which closely related species were involved as parents. However, in recent years *intersectional* species hybrids such as LA, OT, and LO involving distantly related cultivars/species are most successfully cultivated (Lim et al. 2008). These are not only hybrids between the cultivars of different sections but also they are polyploids possessing distinctly differentiated genomes (i.e., allopolyploids). Obviously, these allopolyploid hybrids are most ideal for combining desirable horticultural traits available in the distantly related cultivars/species into new cultivars. A recent study of the LA group of the Dutch lily cultivars has shown that they are predominantly triploid ($2n = 3x = 36$) with one genome of L and two genomes of A (i.e., LAA) constitution (Zhou et al. 2008). The predominance of triploid cultivars also indicates that this particular ploidy level is the most ideal threshold for a successful cultivar. An important drawback of triploid cultivars is that they are not suitable for use in further breeding.

Table 9.1 Reports of successful intersectional crosses among different species of the genus *Lilium* after special pollination techniques and embryo rescue

Cross	References
L. alexandrae × *L. auratum*	McRae (1972)
L. alexandrae × *L. speciosum*	McRae (1972)
L. alexandrae × *L. rubellum*	McRae (1972)
L. alexandrae × *L. nobilissimum*	McRae (1978)
L. speciosum × *L. rubellum*	Matsumoto (1992)
(*L. auratum* × *L. japonicum*) × *L. rubellum*	McRae (1972)
L. nobilissimum × *L. henryi*	Asano (1982)
L. pyrenaicum × *L. pomponium*	North (1994)
L. henryi × *L. candidum*	Van Tuyl et al. (2002)
L. lankongense × *L. davidii*	Marshall (1983b)
L. lankongense × *L. cernuum*	Marshall (1983b)
L. lankongense × *L. leichtlinii*	Marshall (1983b)
L. lankongense × *L. duchartrei*	Marshall (1983b)
L. duchartrei × *L. davidii*	Marshall (1983b)
L. taliense × *L. davidii*	Marshall (1983b)
L. pumilum × *L. leichtlinii*	McRae and McRae (1985)
L. cernuum × *L. dauricum*	McRae and McRae (1979)
L. cernuum × *L. concolor*	McRae and McRae (1979)
L. tigrinum × *L. regale*	McRae (1991)
L. longiflorum × *L. cernuum*	Myodo and Asano (1977)
L. longiflorum × *L. henryi*	Myodo and Asano (1977)
L. formosanum × *L. speciosum*	Myodo and Asano (1977)
L. longiflorum × *L. dauricum*	Asano and Myodo (1980)
L. longiflorum × *L. pumilum*	Asano and Myodo (1980)
L. longiflorum × *L. brownii*	Van Tuyl (1980)
L. longiflorum × *L. martagon*	Van Creij et al. (1990b)
L. longiflorum × *L. rubellum*	Van Tuyl et al. (2000)
L. longiflorum × *L. Bulbiferum*	Van Tuyl et al. (2000)
L. longiflorum × *L. canadense*	Van Tuyl et al. (2000)
L. longiflorum × *L. concolor*	Van Tuyl et al. (2002)
L. longiflorum × *L. hansonii*	Van Tuyl et al. (2005)
L. longiflorum × *L. monadelphum*	Van Tuyl et al. (2005)
L. longiflorum × *L. kelloggii*	Van Tuyl et al. (2011)
L. longiflorum × *L. sempervivoideum*	Van Tuyl et al. (2011)
L. longiflorum × *L. pardalinum*	Van Tuyl et al. (2011)
L. longiflorum × *L. hansonii*	Van Tuyl et al. (2005)
L. longiflorum × *L. bakerianum*	Lim et al. (2008)
L. longiflorum × *L. hansonii*	Lim et al. (2008)
L. longiflorum × *L. lophophorum*	Wang et al. (2009)
L. regale × *L. leichtlinii*	Matsumoto (1992)

Therefore, starting from diploid intersectional cultivar/species hybrids (e.g., LA, OA), different breeding strategies can be envisaged including (1) breeding at the diploid level through the production of diploid BC progenies; (2) use of unreduced (2*n*) gametes to produce triploid cultivars (unilateral sexual polyploidization); (3) use of bilateral sexual polyploidization; and (4) use of somatically doubled allotetraploids.

9.4.2 Breeding at the Diploid Level

Hybrids between two diploid cultivars/species from two different taxonomic sections are, in almost all cases, completely sterile and they cannot be used as parents. Rarely however, such F_1 hybrids do produce normal haploid gametes and they can be used to generate diploid BC progenies as has been demonstrated

Table 9.2 Reports of successful intrasectional crosses without the help of special pollination techniques and embryo rescue among different species in the genus *Lilium*

Cross	Reference	Cross	Reference
L. speciosum × *L. auratum*	Yerex (1948)	*L. davidii* × *L. dauricum*	Marshall (1981)
L. speciosum × *L. japonicum*	Woodriff (1958)	*L. davidii* × *L. cernuum*	Marshall (1981)
L. speciosum × *L. rubellum*	Woodriff (1959)	*L. callosum* × *L. dauricum*	Marshall (1983a)
L. speciosum × *L. henryi*	Emsweller and Stuart (1948)	*L. davidii* × *L. wilsonii*	Marshall (1981)
L. speciosum × *L. nepalense*	Woodriff (1969)	*L. davidii* × *L. bulbiferum*	Marshall (1981)
L. auratum × *L. japonicum*	Pfeiffer (1952)	*L. davidii* × *L. leichtlinii*	Marshall (1981)
L. auratum × *L. nobilissimum*	Matsumoto (1992)	*L. davidii* × *L. amabile*	Marshall (1981)
L. sargentiae × *L. regale*	Yerex (1948)	*L. davidii* × *L. maculatum*	Slate (1968)
L. sargentiae × *L. henryi*	Yerex (1948)	*L. wilsonii* × *L. bulbiferum*	Marshall (1981)
L. henryi × *L. leucanthum*	Yerex (1948)	*L. wilsonii* × *L. dauricum*	Marshall (1981)
L. henryi × *L. regale*	Andel (1982)	*L. bulbiferum* × *L. dauricum*	Marshall (1981)
L. leucanthum × *L. sargentiae*	Yerex (1948)	*L. dauricum* × *L. maculatum*	Clas (1972)
L. henryi × *L. auratum*	Henningsen (1951)	*L. amabile* × *L. maculatum*	Fisher (1969)
L. hansonii × *L. martagon*	Lawrence (1950)	*L. candidum* × *L. chalcedonicum*	Lawrence (1949)
L. hansonii × *L. medeoloides*	Skinner (1949)	*L. tigrinum* × *L. amabile*	Knox-Finlay (1977)
L. martagon × *L. medeoloides*	Lawrence (1950)	*L. tigrinum* × *L. davidii*	Patterson (1955)
L. martagon × *L. tsingtauense*	Doak (1977)	*L. tigrinum* × *L. maculatum*	Slate (1968)
L. concolor × *L. callosum*	Slate (1968)		
L. concolor × *L. dauricum*	McRae (1978)	*L. longiflorum* × *L. formosanum*	Kline (1950)
L. concolor × *L. pumilum*	Preston (1948)	*L. parryi* × *L. kelloggii*	Walden (1962)
L. pumilum × *L. dauricum*	Marshall (1983a)	*L. parryi* × *L. pardalinum*	Kline (1948)
L. pumilum × *L. bulbiferum*	Marshall (1983a)	*L. bolanderi* × *L. kelloggii*	Beane (1957)
L. pumilum × *L. davidii*	Marshall (1983a)	*L. pardalinum* × *L. bolanderi*	Woodriff (1950)
L. pumilum × *L. amabile*	Marshall (1983a)	*L. canadense* × *L. parryi*	Showalter (1961)
L. pumilum × *L. cernuum*	Hager (1953)	*L. michiganense* × *L. canadense*	Wadekamper (1988)
L. callosum × *L. amabile*	Marshall (1983a)	*L. superbum* × *L. canadense*	Pfeiffer (1976)

in the case of LA hybrids (Khan et al. 2009b). This has opened up the prospect of breeding and selection at the diploid level after which the selected genotypes can be used to produce triploid cultivars. For this synthesis, either unilateral sexual polyploidization or diploid × (synthetic) tetraploid crossing can be used. This procedure is somewhat similar to breeding other vegetatively propagated autopolyploid crops like potato (Chase 1963; Mendiburu et al. 1974).

9.4.3 Use of Unilateral Sexual Polyploidization

Although F_1 hybrids between cultivars of different sections are mostly sterile, a small percentage of them can produce 2*n* gametes in reasonable frequencies. It is easy to detect such genotypes because the presence of larger pollen grains is a reliable indication. Detection of genotypes that produce 2*n* eggs is more difficult because it requires crossing with normal pollen and testing whether it leads to fruit and seed set. One of the important advantages of using unilateral sexual polyploidization is that the resulting progenies possess intergenomic recombination. As a consequence of the presence of recombinant segments in the BC_1 progenies, there is scope for the expression of recessive loci that might become nulliplex in the BC_1 triploids (Barba-Gonzalez et al. 2005a, b). This means that selection can be effective in the BC_1 generation itself as it might have been the case in Dutch cultivars derived from LA hybrids (Zhou et al. 2008). A cardinal feature of 2*n* gametes in distant hybrids such as LA or OA genotypes is that they originate predominantly through first division restitution (FDR) and because of this the heterozygosity of the parental hybrids is largely preserved in the 2*n* gametes and transferred to the progenies. This is in contrast to the use of 2*x* gametes derived from the somatically doubled tetraploids, which can lead to "inbreeding depression" giving rise to weakly performing polyploid progenies.

The important advantages of using 2*n* gametes are that they help to overcome the F_1 hybrid sterility, transfer hybrid vigor, facilitate intergenomic recombination, and directly give rise to triploid progenies that are preferred for cultivar selection.

9.4.4 Bilateral Sexual Polyploidization

As was pointed out earlier, only very few genotypes of F_1 *Lilium* hybrids produce either 2*n* pollen or 2*n* eggs in reasonable frequencies but none of the genotypes that have been examined so far produce both types of 2*n* gametes in appreciable frequencies. Therefore, it has never been possible to obtain a tetraploid through the functioning of 2*n* pollen and 2*n* egg from the same diploid hybrid parent. However, by using two separate LA hybrids as parents, one donating 2*n* egg and the other 2*n* pollen, it has been possible to produce tetraploid progenies (Khan et al. 2010). Such bilateral sexual (tetraploid) progenies have certain advantages for using them as parents in breeding. In the first place, they are expected to be reasonably fertile because of their allotetraploid constitution (LLAA). Secondly, they will not have the drawback of being "permanent hybrids" in which no recombination can occur. On the contrary, because they have originated through 2*n* gametes, these allotetraploids do possess recombinant segments in some pairs of chromosomes. This means, such genotypes have the potential for segregation of genetic traits that might be present on the distal parts of the crossover segments. Thus, there is scope for selection of sexual tetraploids, which may be repeatedly used as parents in order to produce triploid progenies.

9.4.5 Somatic Chromosome Doubling

One of the widely used methods for overcoming the F_1 sterility of distant hybrids was to double the chromosome numbers of such hybrids and produce allopolyploids that are mostly fertile. Chemicals such as colchicine and oryzalin, among others (van Tuyl et al. 1992; Barba-Gonzalez et al. 2006a), have been successfully used for this purpose. One of the drawbacks of allopolyploids produced through somatic doubling is that there will not be any intergenomic recombination between the parental genomes due to autosyndetic pairing of chromosomes (Lim et al. 2000a, b). Nevertheless, this method has been successfully used for producing polyploid cultivars of lily. Apart from the use of colchicine or oryzalin, the use of nitrous oxide (N_2O) for chromosome doubling in the germinal cells has proven to be effective in producing 2*n* gametes with certain amount of intergenomic recombination (Barba-Gonzalez et al. 2006a, b). This method can be a substitute in order to induce 2*n* gametes in those genotypes that normally never produce such gametes (or only in very low quantities). The potential of this method must be further evaluated for large-scale application.

So far, lily breeding has been carried out through traditional methods. These approaches are time-consuming, especially in this crop, because its generation time is about 2–3 years from seed germination to maturity of fruits and seeds. In such situation, it is attractive to apply molecular methods of tagging desirable traits and practice the so-called marker-assisted selection, which might reduce time. In this context, linkage maps have been constructed using amplified fragment length polymorphism (AFLP) and diversity array technology (DArT) markers (Van Heusden et al. 2002; Khan 2009; Shahin et al. 2010). Like in other crops, these molecular methods might be potentially useful in lily as well.

9.5 Genomics Resources Developed

Besides morphological or phenotypic resources, few molecular aspects have also been investigated in *Lilium*. Zhang et al. (2008) characterized one of the protease inhibitors, a trypsin inhibitor (17 kDa), in the bulb of *Lilium brownii*. The amino acid sequence of this protease showed similarity to a short fragment of a known trypsin inhibitor from *Populus tremula* and a putative trypsin inhibitor from *Arabidopsis thaliana*. Trypsin (protease) inhibitors are quite important compounds due to their role as defense proteins against pests. Another compound "free mannose" has been characterized in *L. longiflorum* bulbs (Miller 1989). Besides the starch, which is known to be the main storage carbohydrate in *Lilium*, glucomannan has been recorded (Tomoda et al. 1978) to comprise

about 15% of the carbohydrate in the bulb (Matsuo and Mizuno 1974). This carbohydrate is a water-soluble polysaccharide that is considered as a dietary fiber and used in food as an emulsifier and thickener.

Because the sexual reproduction system is highly important in plants, many studies were carried out in order to understand the complex process of transportation of sperm to egg cell. Kim et al. (2003) identified a chemotropic molecule "chemocyanins" in lily stigma, which is a small basic protein that shows sequence similarity to plantacyanins, cell wall proteins of unknown function (Nersissian et al. 1998) and that belongs to the ancient phytocyanin family of blue copper proteins (Ryden and Hunt 1993). Genes that encode histone proteins have been reported in male gametic cells within the pollen grain of *L. longiflorum*. Histones are highly conserved throughout the evolution and are encoded by multigene families (Xu et al. 1999). H3 and H2B have been identified as potential tissue-specific histones in the generative cell of lily (Ueda and Tanaka 1995). Later on Xu et al. (1999) isolated and characterized *gcH2A* and *gcH3* histone genes from a cDNA library of *Lilium* generative cells. These two genes are expressed specifically in the generative cell but not in microspores undergoing pollen mitosis I or in other dividing cells of *Lilium* somatic tissue (Xu et al. 1999). Other three core histones were specifically expressed in the generative cell of lily: gH2A, gH2B and gH3, which had been proposed to be specific core histones that contribute to chromatin condensation of male gametes or to chromatin remodeling, and resulted in the repression of gene expression in male gametes (Ueda et al. 2000, 2005). Expressed sequence tags (ESTs) of the generative cells were constructed from *L. longiflorum* with an aim to detect gametophyte-specific genes. About 886 ESTs were generated and clustered into 637 unique ESTs of which 70% showed significant similarity to *Arabidopsis* genes with known function. Among these, 129 ESTs showed significant similarity to male gametophyte-specific transcripts, and 55 ESTs appeared to have significant hits in both maize sperm cell ESTs and *Arabidopsis* male gametophyte-specific genes, suggesting that these genes are common across different plant species (Okada et al. 2006). The expression of 83% of the generative-cell genes was up-regulated in generative cells, which suggests their specialized function (Okada et al. 2007).

Lily (*L. longiflorum*) pollen tube contains two exo-β-glucanases, i.e., *LP-ExoI* and *LP-ExoII*, secreted into its cell walls. These two exhibited over 80% similarity to each other. *LP-ExoI* transcripts were abundant in pollen grains. However, *LP-ExoII* transcripts found in all lily tissues were tested. In addition, it has been suggested that 1,3:1,4-β-glucan was present in lily pollen tubes. LP-ExoI and LP-ExoII may be involved in the regulation of pollen tube elongation by hydrolyzing callose and 1,3:1,4-β-glucan within pollen tube walls (Takeda et al. 2004).

The mature pollen of *L. longiflorum* has stable oil bodies that contain a protein of 18 kDa, which is accumulated massively in the late stages of pollen maturation. Immunological and sequence analysis suggest that it is a putative oleosin that represents a distinct class in comparison with oleosins found in seed oil bodies and tapetum (Jiang et al. 2007).

Many physiological processes in plant cells are highly correlated with actin cytoskeleton, such as the elongation of pollen tubes tips. The dynamic of actin cytoskeleton Rop1Ps and its importance for pollen tube elongation characteristics in *L. davidii* were investigated and have been shown by Zhao and Ren (2006). Many compounds were detected in the pollen including ABP29, LlLIM1, and LLA23. *Lilium* ACTIN BINDING PROTEIN29 (ABP29, 29 kDa) is the smallest identified member of the villin/gelsolin/fragmin superfamily, and it is a splicing variant of *Lilium* villin that plays important roles in remodeling of the actin cytoskeleton (Xiang et al. 2007). LlLIM1, also an actin-binding protein (ABPs), was identified in *L. longiflorum* pollen. It plays an important role in regulating the actin microfilaments, which is essential for polar cell tip growth (Wang et al. 2008). LLA23 is an abscisic acid-, stress-, and ripening-induced protein, which was isolated from *L. longiflorum* pollen. It was shown that LLA23 protein plays an important role against drought. This protein mediates stress-responsive ABA signaling under elevated salt concentration or dehydration (Yang et al. 2005).

MADS box genes consist of three major groups: A, B and C, and a number of functional genes have been investigated in lily. LMADS1 is homologous to the *Arabidopsis AP3* gene and was cloned and characterized from *L. longiflorum*. The messenger RNA of this gene was detected in the petals and stamens only, suggesting that this gene is possibly post-transcriptionally regulated in lily (Tzeng and Yang 2001).

Two other MADS box genes (*LMADS2* and *EgMADS1*) characterized in *L. longiflorum* showed expression in the carpel, mainly in ovules and partly in style tissues, whereas they were absent from other flower organs or vegetative leaves (Tzeng et al. 2002). *LMADS3* and *LMADS4* are two AGL2-like MADS-box genes also characterized in *L. longiflorum* and their expression was detected in the inflorescence meristem and floral buds at different developmental stages and in all four whorls of the flower organ. However, *LMADS4* was also expressed in vegetative leaf and in the inflorescence stem (Tzeng et al. 2003). *LMADS5*, *LMADS6*, and *LMADS7* have been isolated and characterized from *L. longiflorum* (Chen et al. 2008). The expression of these three genes was similar, and their RNAs were detected in vegetative stem and inflorescence meristem. *LMADS5* showed high-sequence similarity to oil palm (*Elaeis guineensis*) *EgSQUA3*, and *LMADS7* is more close to an orchid gene *DOMADS2* (Chen et al. 2008).

Very little sequence information is available for *Lilium*. Some regions of *Lilium*'s chloroplast DNA (*trn*T–*trn*L, *trn*L–*trn*F, and *atp*B–*rbc*L) have been sequenced. These sequences were used to study the phylogenic relationships among different cultivars of the *Lilium* and *Archelirion* sections (Nishikawa et al. 2002). Besides that, Sentry and Smyth (1989) characterized a 9.35 kb transposable element with 2.4 kb long terminal repeats (LTRs) from *L. henryi*).

In near future, a considerable amount of DNA sequences will be available as they are currently regenerated for genetic mapping purposes.

9.6 Domestication History of *Lilium*

Lilium has a long history of cultivation. Madonna lily (*L. candidum*) and Tiger lily (*L. tigrinum*) are believed to be the oldest domesticated floral species. The artistic and botanical evidences suggested that the wild Madonna lily (*L. candidum*) had become a garden plant during the Late Minoan period. However, little is known where and when a garden Madonna lily became sterile and vegetatively propagated by man and consequently a domesticated plant (Negbi and Negbi 2000). *L. candidum* most likely originated from the Middle Orient and the first record on cultivation of this species dates back to 3000 BC. Probably during the Iron Age, the Madonna lily was introduced into Egypt by Greek colonists. In southern Europe, the Madonna lily was introduced by the Romans and until the middle of the fifteenth century this was the only cultivated species in European gardens, but by the end of the sixteenth century other European species were introduced, e.g., Orange lily (*L. bulbiferum* L.) (Pelkonen et al. 2007) or in the Netherlands the so-called rye lily (Bos 1993). During the seventeenth and eighteenth centuries, *L. canadense* and *L. speciosum* were introduced into European gardens from North America and Asia (Woodcock and Stearn 1950). China is considered as a second center of origin of domesticated lilies. *L. tigrinum* is believed to be cultivated in China since ancient times (Haw 1986).

9.6.1 *Lilium* as Traditional Medicinal Plant Species

In the Middle Orient and eastern Asia, various lily species have been widely used as important medicinal plants for more than 2,000 years. In "The Canon of Medicine" (980–1037 AD), the bulbs, leaves and oil of the flowers of *L. candidum* have been suggested to be useful for curing injures, burns, and inflammation (Avicena 980–1037 AD after Farsam et al. 2003). In China, the history of cultivation of lily for medicinal purposes can be traced back to the Han dynasty (202 BC–220 AD). One of the oldest Chinese medical books, *jin kui yao lue*, described that lilies were prescribed for such diseases as chronic coughing, for certain blood disorders and against sleeplessness (Zhang Zhongjing and Xiwen 1995). According to Chinese herbal records, the bulbs of *L. dauricum*, *L. pumilum*, *L. longiflorum*, and *L. brownie* var. *colchesteri* have been used as a medical material (Mimaki et al. 1992) where the later has been prescribed as an antitussive and sedative (Lin et al. 2003).

The fresh and dried bulbs of *L. candidum* have been suggested to be useful in gynecological disorders, ulcer problems, burns, injuries, and may be used as a diuretic (Gruenwald 2000). Furthermore, the bulbs of several other *Lilium* species exhibited a wide spectrum of biological activities (Duke 2002).

9.6.2 Lily as Human Food

Some lily species have a long history of being used as a food ingredient. The bulbs of *L. brownie*, *L. pumilum*, and *L. tigrinum* were already collected as vegetables around 900–960 AD in China (Zhao et al. 1996).

Nowadays, there are two main cultivation centers for vegetable lilies in China: Yixing of Jiangsu Province and Lanzhou of Gansu Province. Beside China, the bulbs of *L. tigrinum*, *L. pumilum*, *L. candidum*, and *L. brownii* are grown for food purposes on a large scale in Japan, Korea, and Vietnam (Simoons 1991; Chau and Wu 2006). Lily bulbs may be dried, eaten fresh, baked, made into soup, or processed to extract starch (Simoons 1991). Similarly, native Americans used the bulbs of many species of *Lilium* for food. The Cherokee made flour from *L. canadense* during famine whereas in Saskatchewan, the Cree dried the bulbs scales of *L. philadelphicum* as snack.

9.6.3 Chemical Composition

Numerous investigations have been carried out on chemical constituents of lily species (Mimaki and Sashida 1990; Mimaki et al. 1992; Nakamura et al. 1993; Farsam et al. 2003; Eisenreichová et al. 2004). *Lilium* have turned out to be a rich source of steroidal compounds, such as steroidal saponins (Eisenreichová et al. 2004), steroidal glycosides (Mimaki et al. 1992), and steroidal alkaloids (Mimaki and Sashida 1990). Steroidal saponins have been extracted mainly from bulbs, e.g., of *L. candidum* (Haladová et al. 1998), *L. brownii* (Mimaki and Sashida 1990) and *L. speciosum* × *L. nobilissimum* "Star Gazer" (Nakamura et al. 1993), as well as from flowers of *L. candidum* (Haladová et al. 1998) and from flowers and corm of *L. ledebourii* (Farsam et al. 2003). Furthermore, phenolic constituents have been isolated, e.g., from fresh bulbs of *L. brownii* (Mimaki and Sashida 1990) and *L. pumilum* (syn. *L. tenuifolium*, Mimaki et al. 1989). In flowers and leaves of numerous *Lilium* species, the presence of flavonoids such as kaempferol, quercetin and isorhamnetin was confirmed (Skrzypczakova 1967). The dried bulbs of *L. tigrinum* are rich in calories, iron, and ascorbic acid (Simoons 1991).

The bulbs of some *Lilium* species exhibited different biological activities, e.g., the extract from *L. brownie* var. *colchesteri* showed the inhibitory effect on monoamine oxidases (MAOs) (Lin et al. 2003). Ethanolic extracts of *L. candidum* L. were found to express antifungal and antiyeast activity (Eisenreichová et al. 2004). Some of the saponins present in the bulbs of this species can inhibit epidermal carcinogenesis promoter activity (Vachlkov et al. 2000).

9.7 Recommendations for Future Actions

Normally, all crop plants have the threat of pests and diseases. This includes the commercially grown ornamental plants as well. But in recent years, there is a new threat. This is the implementation of laws against the use of protective chemicals that will be harmful for the environment. When such laws are rigidly implemented, it can seriously affect the cultivation of ornamental crops. This means, in addition to all other horticultural traits, breeders will have to make serious efforts to introduce disease resistances into new cultivars of lily if it has to survive as a crop. Fortunately, the genus *Lilium* includes about 100 species that are distributed widely in the northern hemisphere extending up to some tropical areas as well. This indicates that there might be scope to discover more useful genetic variation than hitherto has been done. Keeping this in mind, the following lines of work may be contemplated for the future: (1) more in-depth screening of wild germplasm for (partial) disease resistance; (2) interspecific hybridization; (3) screening for haploid and 2*n* gametes in distant hybrids; (4) perfecting methods for polyploidization; and (5) development of molecular linkage maps and tagging of useful traits.

1. The diversity within and between wild species and the current cultivated assortment is very large and for many (disease) traits not fully explored leaving significant perspective for crop improvement under current and new culture conditions.
2. The importance of creating new breeding material through interspecific hybridization cannot be overemphasized. Fortunately, methods for producing hybrids between distantly related species have been

well developed and fairly successful in lily (Van Tuyl et al. 1991, 2000; Van Tuyl and De Jeu 1997). Besides the three important hybrids of groups of lily, viz., Longiflorum, Asiatic and Oriental, it might be desirable to extend to other groups as well and pay attention to the use of wild species of other sections that might possess disease resistances and other desirable traits.

3. Based on the experience gained from LA hybrids, it is apparent that distant hybrids can produce normal haploid gametes in spite of their genome differentiation. This certainly depends on the genotypes of the parents. Once such genotypes are identified, the benefits can be highly rewarding because it can open up the way for breeding at the diploid level and by using them for producing polyploids of desired level, i.e., triploids. Besides haploid gametes, it might be worthwhile to screen for genotypes that produce 2*n* gametes. This is because, as in numerous other ornamental plants (Van Tuyl et al. 2002), polyploid cultivars in lily will be the most successful ones.
4. Not all distant F_1 hybrids might be able to produce genotypes with 2*n* gametes. In such cases, it might be desirable to use traditional methods or preferably, through the use of nitrous oxide treatment, to restore fertility. But this later method may have to be refined further so as the results of the treatment will become more predictable.
5. It is imperative that molecular genetic maps might be potentially useful in breeding. At present, however, none of the molecular markers are assigned to chromosomes as yet and the number of linkage groups exceed the basic chromosome number (i.e., $x = 12$). In this context, the cytological maps of the three genomes of lily cultivars (Khan et al. 2009a) might be useful for assigning linkage groups to the respective chromosomes. Regardless of the availability of general high-quality linkage maps, however, it might be useful to develop markers to tag useful quantitative traits and increase the efficiency of selection in breeding. The use of sequences of the lily genome will become important in the near future for the construction of genetic maps, which are needed for molecular marker-assisted breeding (P Arens and JM van Tuyl, unpublished results).

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Chapter 10
Nicotiana

Ramsey S. Lewis

10.1 Introduction

Nicotiana is a relatively large genus that has been used widely in plant breeding and genetics research. One major use of *Nicotiana* species has been as a source of genetic diversity for improvement of the species of greatest economic importance, tobacco (*N. tabacum* L.), which is cultivated for its cured leaf used in the manufacture of cigars, cigarettes, pipe tobacco, and smokeless tobacco products consumed by more than one billion persons globally. Several classic research papers on gene introgression have been based on *Nicotiana* species. Some members of *Nicotiana* offer a number of research advantages including extensive phenotypic diversity, amenability to controlled hybridizations and ploidy manipulations, high fecundity, and excellent response to tissue culture. Consequently, several species have also played central roles in the generation of new knowledge related to inheritance, cytogenetics, and polyploid evolution. *Nicotiana* species have also been used as model systems for developing methodologies for plant transformation and in studies investigating gene function. The objectives of this chapter are (1) to present information related to the evolution, taxonomy, and cytogenetics of cultivated tobacco and its wild relatives, (2) to discuss the conservation and utilization of genetic diversity within *Nicotiana* for improvement of the cultivated form, and (3) to review the use of *Nicotiana* species as platforms for experimentation in basic plant genetics research.

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10.2 Basic Botany of *Nicotiana*

10.2.1 Genus Classification, Taxonomy, and Origin

The genus *Nicotiana* was established by Linnaeus in 1753 and it is the sixth largest member of the Solanaceae, or nightshade family, which includes many important crop plants including tomato, potato, pepper, and eggplant. Only the genera *Solanum*, *Lycianthes*, *Cestrum*, *Physalis*, and *Lycium* contain more species than *Nicotiana*. Plastid DNA restriction site mapping and sequence information have been used to study intergeneric relationships among members of this family (Olmstead and Sweere 1994; Olmstead et al. 1999). Tobacco is the primary *Nicotiana* species of commerce, although several additional species have also been cultivated on much smaller scales for smoking (*N. rustica* L., *N. repanda* Willd. ex Lehm., *N. attenuata* Torrey ex S. Watson, *N. quadrivalvis* Pursh), ornamental (*N. sylvestris* Spegazzini & Comes, *N. alata* Link and Otto, *N. langsdorffii* Weinmannm, *N. forgetiana* Hemsley, *N. sanderae* hort), or industrial purposes (*N. rustica* L., *N. glauca* Graham) (Lester and Hawkes 2001).

Initial presentations of phylogenetic relationships within *Nicotiana* were supported by geographical distributions, morphological similarities, chromosome pairing in various interspecific F_1 hybrids, and karyotypic observations. On the basis of this information, the genus was initially divided into three subgenera, 14 sections, and 60 species (Goodspeed 1945, 1954). DNA-based approaches were later applied to investigate intrageneric relationships. Methods included chloroplast restriction site mapping (Olmstead and

C. Kole (ed.), *Wild Crop Relatives: Genomic and Breeding Resources, Plantation and Ornamental Crops,*
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Palmer 1991), random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) marker genotyping (Bogani et al. 1997; Yu and Lin 1997; Ren and Timko 2001), plastid DNA sequencing (Aoki and Ito 2000; Clarkson et al. 2004), analysis of nuclear ribosomal DNA (Komarnitsky et al. 1998; Kitamura et al. 2001; Chase et al. 2003), and fluorescent and genomic in situ hybridization (FISH and GISH, respectively) (Lim et al. 2000; Kitamura et al. 2001; Chase et al. 2003). In general, results have been partially or largely congruent with those based on the traditional methods of Goodspeed (1954). Knapp et al. (2004) have provided the most recent taxonomic revision for the genus *Nicotiana*, after taking into consideration recent DNA sequence information, adding species unknown at the time of Goodspeed's monograph, and making a number of nomenclatural changes. This revision recognizes 76 naturally occurring (i.e., not synthetic) *Nicotiana* species and subdivides the genus into 13 sections (Table 10.1). Readers are referred to Knapp et al. (2004) for more details on the current outline of taxonomic relationships in this genus.

On the basis of continental drift considerations and natural distributions of species, Uchiyama et al. (1977) proposed the genus to be 75–100 million years old. Wikstrom et al. (2001), however, used DNA sequence information to date the split between *Nicotiana* and *Petunia* (a closely related genus) at 23–25 million years ago (Mya). *Nicotiana* is likely to have evolved in southern South America, east of the Andes, and was later dispersed to Africa, Australia, and southwestern North America (Clarkson et al. 2004). The progenitor of the current species of Australian origin likely migrated to this continent via Antarctica or by long-distance dispersal, perhaps via South Pacific island stepping stones (Goodspeed 1954; Olmstead and Palmer 1991). Of the current species, 50 are indigenous to North and South America and associated islands. Twenty-five members of section *Suaveolentes* are native to either Australia or the Australian archipelago. *Nicotiana africana* Merxmüller & Buttler is the only species found to be indigenous to the continent of Africa (discovered in Namibia) (Merxmüller and Buttler 1975).

Information on early history of *Nicotiana* use is scarce, but at least ten species of *Nicotiana* have been suggested to have been used by aboriginal persons in the Americas and Australia for ceremonial or medicinal purposes, and the natural ranges of most or all of these species were probably extended by cultivation before discovery of the New World (Setchell 1921; Garner et al. 1936; Gerstel and Sisson 1995). *Nicotiana tabacum*'s range was extended throughout much of South America and into Mexico and the Caribbean islands during the pre-Columbian era (Setchell 1921; Brand 1939; Gerstel and Sisson 1995). Although *N. tabacum* can be found in natural settings in South America, these plants are generally believed to be escapes from cultivation and the species is not thought to currently exist in a true wild state (Goodspeed 1954; Kawatoko 1998). Genetic drift in combination with natural and human-directed selection resulted in the differentiation of this species into distinct types. Although records on early types are few, four distinct forms were described in the West Indies as early as 1667 (Garner et al. 1936). North American colonists initially cultivated *N. rustica*, but this was soon replaced with *N. tabacum* because cured leaves of this species were much preferred by European importers. Tobacco was spread throughout the world after colonization (Garner et al. 1936; Tilley 1948; Spinden 1950; Brooks 1952; Tso 1990), and during the seventeenth through nineteenth centuries, different market classes emerged in response to consumer preferences for different tobacco uses. The current market classes of flue-cured, burley, Maryland, dark air-cured, dark fire-cured, cigar filler, cigar wrapper, and oriental tobaccos existed prior to the initiation of science-based tobacco breeding in the early part of the twentieth century.

10.2.2 Cytogenetics, Evolution, and Genome Organization

The basic chromosome number in *Nicotiana* is $x = 12$, and the most frequent chromosome number is $2n = 24$ (33 species) (Table 10.1). Approximately 46% of the species in *Nicotiana* are true allotetraploids or aneuploid derivatives thereof, with chromosome numbers ranging from $2n = 32$ to $2n = 48$. On the basis of morphological, cytological, and DNA sequence data, the likely parentage of all allotetraploid species has been determined (Goodspeed 1954; Aoki and Ito 2000; Chase et al. 2003; Clarkson et al. 2004),

Table 10.1 Sectional classifications, chromosome numbers, and geographical distributions of naturally occurring *Nicotiana* species and number of representative accessions held by the United States *Nicotiana* Germplasm Collection

Section/Species[a]	n[b]	Natural geographical distribution[b]	# of accessions (February, 2010)
Nicotiana Section *Paniculatae* Goodspeed			
Nicotiana benavidesii Goodspeed	12	Peru	1
Nicotiana cordifolia Philippi	12	Chile (Juan Fernandez Islands)	2
Nicotiana cutleri D'Arcy	12	S Bolivia	0
Nicotiana knightiana Goodspeed	12	Peru (S Coast)	1
Nicotiana paniculata L.	12	W Peru	5
Nicotiana raimondii J.F. Macbride	12	Peru, Bolivia	1
Nicotiana solanifolia Walpers	12	Chile (N Coast)	1
Nicotiana Section *Rusticae* Don			
Nicotiana rustica L.	24	Ecuador, Peru, NW Bolivia	87
Nicotiana Section *Tomentosae* Goodspeed			
Nicotiana kawakamii Y. Ohashi	12	Bolivia	1
Nicotiana otophora Grisebach	12	Bolivia, NW Argentina	4
Nicotiana setchellii Goodspeed	12	N Peru	1
Nicotiana tomentosa Ruiz and Pavon	12	S and C Peru, W Bolivia	3
Nicotiana tomentosiformis Goodspeed	12	Bolivia	1
Nicotiana Section *Nicotiana* Don			
Nicotiana tabacum L.	24	Cultivated worldwide	1,900
Nicotiana Section *Undulatae* Goodspeed			
Nicotiana arentsii Goodspeed	24	Peru, Bolivia	1
Nicotiana glutinosa L.	12	Peru, S Ecuador	9
Nicotiana thyrsiflora Bitter ex Goodspeed	12	N Peru	1
Nicotiana undulata Ruiz and Pavon	12	Peru, Bolivia, N Argentina	3
Nicotiana wigandioides Koch and Fintelman	12	Bolivia	1
Nicotiana Section *Trigonophyllae* Goodspeed			
Nicotiana obtusifolia M. Martens & Galeotti	12	SW United States, Mexico	2
Nicotiana palmeri A. Gray	12	SW United States, Mexico	0
Nicotiana Section *Sylvestres* Knapp			
Nicotiana sylvestris Spegazzini and Comes	12	Bolivia, NW Argentina	3
Nicotiana Section *Alatae* Goodspeed			
Nicotiana alata Link and Otto	9	Uruguay, Brazil, Paraguay, Argentina	3
Nicotiana azambujae L.B. Smith & Downs	?	S Brazil	0
Nicotiana bonariensis Lehmann	9	SE Brazil, Uruquay, Argentina	1
Nicotiana forgetiana Hemsley	9	SE Brazil	2
Nicotiana langsdorffii Weinmann	9	Brazil, Paraguay, Argentina	3
Nicotiana longiflora Cavanilles	10	Bolivia, Brazil, Paraguay, Uruguay, Argentina	4
Nicotiana mutabilis Stehmann & Samir	9	Brazil	0
Nicotiana plumbaginifolia Viviani	10	Peru, Bolivia, Argentina, Paraguay, Brazil	3
Nicotiana Section *Repandae* Goodspeed			
Nicotiana nudicaulis S. Watson	24	NE Mexico	1
Nicotiana repanda Willdenow ex Lehmann	24	S United States, N Mexico	2
Nicotiana stocktonii Brandegee	24	Mexico (Revillagigedo Islands)	3
Nicotiana nesophila Johnston	24	Mexico (Revillagigedo Islands)	0
Nicotiana Section *Noctiflorae* Goodspeed			
Nicotiana acaulis Spegazzini	12	Argentina	1
Nicotiana ameghinoi Spegazzini	12	Argentina	0
Nicotiana glauca Graham	12	Bolivia, N Argentina	5
Nicotiana noctiflora Hooker	12	Argentina, Chile	2

(*continued*)

Table 10.1 (continued)

Section/Species[a]	n[b]	Natural geographical distribution[b]	# of accessions (February, 2010)
Nicotiana paa Martinez Crovedo	12	N Argentina	0
Nicotiana petunioides (Grisebach) Millan	12	Argentina, Chile	1
Nicotiana Section *Petunioides* Don			
Nicotiana acuminata (Graham) Hooker	12	Chile, Andes Mountains of Argentina	4
Nicotiana attenuata Torrey ex S. Watson	12	W United States, NW Mexico	2
Nicotiana corymbosa Remy	12	Chile, Argentina	1
Nicotiana linearis Phillipi	12	Argentina, Chile	1
Nicotiana longibracteata Philippi	12	Andes Mts. of N Argentina and Chile	0
Nicotiana miersii Remy	12	Chile	1
Nicotiana pauciflora Remy	12	Chile	1
Nicotiana spegazzinii Millan	12	Argentina	2
Nicotiana Section *Polydicliae* Don			
Nicotiana clevelandii A. Gray	24	SW United States, NW Mexico	3
Nicotiana quadrivalvis Pursh	24	W United States	11
Nicotiana Section *Suaveolentes* Goodspeed			
Nicotiana africana Merxmuller and Buttler	23	Namibia	1
Nicotiana amplexicaulis Burbidge	18	E Australia	2
Nicotiana benthamiana Domin	19	NC and NW Australia	2
Nicotiana burbidgeae Symon	21	S Australia	0
Nicotiana cavicola Burbidge	23	W Australia	1
Nicotiana debneyi Domin	24	E Australia	10
Nicotiana excelsior J.M. Black	19	Australia	2
Nicotiana exigua H.-M. Wheeler	16	SE Australia	0
Nicotiana fragrans Hooker	24	South Pacific Islands	1
Nicotiana goodspeedii Wheeler	20	S Australia	1
Nicotiana gossei Domin	18	C Australia	1
Nicotiana hesperis Burbridge	21	Australia	0
Nicotiana heterantha Kenneally & Symon	24	W Australia	0
Nicotiana ingulba J.M. Black	20	Australia	0
Nicotiana maritima Wheeler	16	S Australia	1
Nicotiana megalosiphon VanHeurck & Mueller	20	E Australia	2
Nicotiana occidentalis Wheeler	21	Australia	5
Nicotiana rosulata (S. Moore) Domin	20	Australia	3
Nicotiana rotundifolia Lindley	22	SW Australia	2
Nicotiana simulans Burbidge	20	Australia	1
Nicotiana stenocarpa H.-M. Wheeler	20	Australia	0
Nicotiana suaveolens Lehmann	16	SE Australia	10
Nicotiana truncata Symon	?	W Australia	0
Nicotiana umbratica Burbidge	23	W Australia	1
Nicotiana velutina Wheeler	16	Australia	3
Nicotiana wuttkei Clarkson & Symon	16	NE Australia	0

[a]Sectional classification follows that presented by Knapp et al. (2004)
[b]Chromosome numbers and geographical distributions were taken from Japan Tobacco Inc (1994), Chase et al. (2003), and Knapp et al. (2004)

along with estimated dates for their speciation based on calibrated molecular clock analyses (Clarkson et al. 2005; Kovarik et al. 2008). Autoploidy seems to have been unimportant in recent evolution, and dysploid reduction has played a role in the evolution of several species in sections *Alatae* and *Suaveolentes* (Reed 1991).

The species of greatest economic importance, *N. tabacum*, is a classic amphidiploid ($2n = 48$) that probably arose on the eastern slopes of the Andes Mountains in Bolivia or northern Argentina by union of two unreduced gametes, or by chance hybridization between two $2n = 24$ progenitor species followed by chromosome doubling (Goodspeed 1954; Gerstel and

Sisson 1995). Although references to very recent origins for *N. tabacum* (6,000–10,000 years ago) can be found in various non-scientific writings, Okamuro and Goldberg (1985) used DNA re-association kinetics to estimate that speciation occurred much earlier, but within the last six million years. Kovarik et al. (2008) suggested that the initial hybridization event likely occurred less than 200,000 years ago based on molecular clock analyses.

Identity of species contributing to the evolution of *N. tabacum* is important because genome structure dictates the likelihood of successful gene transfers from wild relatives to this cultivated species. Cytological, molecular marker, and plastid sequence information strongly suggest that an ancestor of present-day *N. sylvestris* Spegazzini & Comes was the maternal parent of *N. tabacum* and contributed the chromosomes of the "S" genome as well as the cytoplasm to the original hybrid (Bland et al. 1985; Olmstead and Palmer 1991; Aoki and Ito 2000; Yukawa et al. 2006). All genetic evidence suggests a member of section *Tomentosae* as the donor of the paternal genome ("T" genome), but the precise ancestor has been debated (Goodspeed and Clausen 1928; Burns 1966; Sheen 1972; Gray et al. 1974; Narayan and Rees 1974; Gerstel and Sisson 1995). Goodspeed (1954) argued that *N. otophora* Grisebach was the most likely progenitor species as the present ranges of *N. otophora* and *N. sylvestris* overlap on the eastern slopes of the Andes Mountains in Bolivia and northern Argentina. Furthermore, synthetic *N. sylvestris* × *N. otophora* allotetraploids resemble *N. tabacum* morphologically and are at least semi-fertile. Gene segregation experiments of Gerstel (1960), however, supported *N. tomentosiformis* Goodspeed as the probable progenitor species. Murad et al. (2002) provided molecular cytogenetic evidence to indicate that the T-genome was derived from a particular lineage of *N. tomentosiformis*. Other molecular data suggest that the T genome may have been derived from an introgressive hybrid between *N. otophora* and *N. tomentosiformis* (Kenton et al. 1993; Riechers and Timko 1999; Lim et al. 2000; Kitamura et al. 2001).

It is not known how many initial interspecific hybridization events occurred in the formation of *N. tabacum*. Furthermore, it is not known if there may have been continual gene flow from the diploid species to the newly formed allopolyploid. Because the diploid progenitor genomes had diverged significantly at the time of hybridization (Okamuro and Goldberg 1985), a large amount of sequence diversity entered into the original *N. tabacum* genome. Lim et al. (2007) have described an array of genetic changes or "genome turnover" that likely occurred in *N. tabacum* during hundreds of thousands of years after polyploidization. These modifications likely included retrotransposon sequence deletion/amplification, changes in retroelement activity, intergenomic translocations, epigenetic adjustments, and evolution of new satellites. Some variations may exist among diverse tobacco strains with regard to genome structure, translocations, etc. (Mallah 1943; Cameron 1952; Kenton et al. 1993; Lim et al. 2004), and progenitor genomes have become interdependent as evidenced by the fact that most nullisomics do not survive.

10.3 *Nicotiana* Conservation

Knowledge, access to, and use of available genetic diversity in cultivated and wild *Nicotiana* species are essential for continued genetic modification and improvement of cultivated tobacco, as well as for many basic studies in plant biology. An extensive survey of areas of occupation for naturally growing *Nicotiana* species was recently conducted (Kawatoko 1998). Several ex situ germplasm collections around the world maintain thousands of accessions of *N. tabacum* and its relatives. In 2002, the Cooperation Center for Scientific Research Relative to Tobacco (CORESTA) generated a list of 6,479 *Nicotiana* germplasm accessions maintained among 12 international organizations (list available from the author, or from the Cooperation Center for Scientific Research Relative to Tobacco, 53, Quai d'Orsay, 75340 Paris, France, Cedex 07). In addition, a number of botanical gardens such as the Nijmegen Botanical Garden, Netherlands, have maintained a number of accessions of wild *Nicotiana* relatives (Barendse and Van der Weerden 1997).

Collection and maintenance of *Nicotiana* germplasm was largely initiated in the 1930s as early tobacco breeders sought to increase levels of disease resistance in commercial tobacco cultivars, and also because of the use of *Nicotiana* species in genetic and botanical studies by researchers at the University of California Botanical Garden (UCBG) in Berkeley, California. In 1934, United States Bureau of Plant

Industry plant pathologist E. E. Clayton organized an effort to collect diverse *N. tabacum* germplasm from regions representing this species' initial domestication and dispersal, and W. Andrew Archer and Raymond Stadelman were employed by the Bureau from 1935 until 1937 to collect seeds of native varieties in Central America, the Caribbean, and South America. By 1937, the size of the US collection was increased to 1,160 Tobacco Introductions, or TIs (Chaplin et al. 1982), and the accessions collected between 1935 and 1937 still constitute the major portion of the current US *Nicotiana* Germplasm collection. This initial set of TIs likely provides the best available representation of genetic diversity for *N. tabacum* that existed in the western hemisphere prior to wide-scale adoption of science-based plant breeding methodology. In addition to the efforts of the USDA, UCBG workers also conducted collection expeditions to much of South America from 1935 until 1958 (Goodspeed 1961). The collection of tobacco wild relatives (TWs) assembled by the United States Department of Agriculture in the 1950s and currently maintained by the US *Nicotiana* Germplasm Collection is largely a duplication of original materials collected from native sites by the UCBG.

The US *Nicotiana* Germplasm Collection is currently located in Oxford, North Carolina, and consists of 2,174 *Nicotiana* accessions that fall into one of five categories: 1,244 *N. tabacum* introductions of foreign origin (TI group), 656 tobacco cultivars or germplasm lines of historical importance (TC group), and 147 accessions of other *Nicotiana* species (TW group). This latter group of accessions has been the source of several economically important, simply inherited, disease-resistance genes that have been transferred to cultivated tobacco (Holmes 1938; Clayton 1947, 1969; Valleau et al. 1960; Apple 1962; Clayton et al. 1967; Lewis 2005), and it also contains several species, such as *N. benthamiana* Domin and *N. plumbaginifolia* Viviani, that have been frequently used in basic plant biology research. Eighty-six accessions of *N. rustica* assigned to the Tobacco *Rustica* (TR) group are also maintained in the Germplasm Collection. Finally, the Tobacco Hybrid (TH) group contains 50 miscellaneous interspecific hybrids and synthetic amphidiploids that were contributed by various tobacco breeders and geneticists. Genetic variability among accessions of certain species maintained by a number of germplasm collections/gardens around the world was studied by Moon et al. (2008) using microsatellite markers. Outside of *N. tabacum* and *N. rustica*, however, it can be assumed that genetic variability of almost all wild *Nicotiana* species is inadequately represented in ex situ germplasm collections (from 1 to 11 accessions per species).

Seedstocks in the US *Nicotiana* Germplasm Collection are maintained in 25 mL glass vials with screw caps in a controlled environment (refrigerators or refrigerated incubators) at 10°C, and 40–42% relative humidity. Approximately 15 g (about 200,000 seeds) is maintained for each accession of the TI and TC collections. Smaller and variable amounts are maintained for each member of the TR and TW collections. Subsamples of each accession in the collection are also kept on site in a freezer maintained at −20°C to serve as a local backup, and also in the long-term storage facility of the National Center for Genetic Resources Preservation (NCGRP) in Fort Collins, Colorado.

The United States *Nicotiana* Germplasm Collection is a component of the National Plant Germplasm Service (NPGS), a network of cooperating institutions, agencies, and research units of the federal, state, and private sectors coordinated by the USDA Agricultural Research Service (Shands et al. 1989). Each accession in the *Nicotiana* Germplasm Collection has been assigned a unique identifying Plant Introduction Number (PI) by the Plant Exchange Office, and a listing of all available NPGS accessions and associated data can be accessed from the Germplasm Resources Information Network (GRIN) database via the Internet at http://www.ars-grin.gov/npgs. The database contains data on the taxonomy of the genus and on the origin and characterization of most accessions. There is an extensive list of descriptors for the characterization of *N. tabacum*, and a tremendous amount of phenotypic variation exists among accessions for a number of these descriptive characteristics. The database containing this information can be searched from the GRIN website. Online queries can be made from the GRIN database to identify accessions that meet specified criteria for the various descriptive characteristics.

10.4 Cytogenetic Stocks and Their Utilities

10.4.1 Monosomics and Trisomics

Aneuploid stocks can be useful resources for genetic investigations in crop species and their relatives, and also for facilitating and characterizing alien gene introgressions. Although development and use of *Nicotiana* cytogenetic stocks are not as advanced as compared to those for *Triticum* species, a significant amount of work has been conducted. Because of their polyploid origin, species such as *N. rustica* and *N. tabacum* have a large degree of tolerance to some forms of aneuploidy. Monosomic individuals ($2n - 1$) are those in which a single chromosome is missing from the normal chromosome set. Lammerts (1932) was able to establish seven monosomics of *N. rustica* by backcrossing the F_1 hybrid between *N. rustica* and *N. paniculata* back to *N. rustica*. A complete series of 24 monosomics for *N. tabacum* was generated and characterized both cytologically and morphologically by Clausen and Cameron (1944) and Cameron (1959) in a "Red Russian" genetic background. These stocks were established as the result of spontaneous monosomy, from progeny derived from crosses between *N. tabacum* and *N. sylvestris*, and also through the utilization of asynaptic mutants. The letters "A" through "L" refer to the chromosomes of the T genome, while the letters M through Z correspond to the chromosomes of the S genome. The letters X and Y are not used. Other researchers have chosen to designate the chromosomes of the S genome as S1, S2, etc., and the chromosomes of the T genome as T1, T2, etc. (Kenton et al. 1993; Moscone et al. 1996). Monosomic stocks have been employed to assign a number of different *N. tabacum* and introgressed genes to specific chromosomes (Clausen and Cameron 1944; Gerstel 1945a; Smith 1979; Danehower et al. 1989).

Trisomic individuals are those possessing three copies of a specific chromosome, instead of the normal two. All primary trisomic types have been identified only in *N. sylvestris* (Goodspeed and Avery 1941). Eight of the nine possible trisomics have been identified in *N. langsdorffii* (Lee 1950).

10.4.2 Alien Addition and Substitution Lines

Alien chromosome addition and substitution lines have also been studied in *Nicotiana*, primarily because of their role as intermediates in foreign gene introgression efforts (Gerstel 1945a, b; Moav 1958; Bai et al. 1996; Lewis 2005). For example, Gerstel (1943, 1945a, b) studied tobacco substitution and addition lines carrying an intact chromosome from *N. glutinosa* L. in work to transfer the tobacco mosaic virus (TMV) resistance gene from this species to cultivated tobacco. Moav (1958) used a *N. tabacum* addition line monosomic for a *N. plumbaginifolia* chromosome carrying a dominant white seedling (*Ws*) marker to determine that gene introgression events to *N. tabacum* chromosomes were mostly due to crossing-over mediated by residual homology between two homeologous chromosomes rather than chance translocations. An *N. africana* chromosome added to the genome of *N. tabacum* has also been investigated for possible value as a "designer chromosome" into which multiple transgenes could be inserted and used as a transgene shuttle to rapidly transfer desirable linkage blocks from one tobacco line to another (Campbell et al. 1994; Lewis and Wernsman 2001). Alien addition lines have also been used to localize genes to specific chromosomes. For example, Suen et al. (1997) used a collection of *N. plumbaginifolia* monosomic alien addition lines possessing single *N. sylvestris* chromosomes to assign restriction fragment length polymorphism (RFLP) markers to specific chromosomes of the "S" genome. Aberrant alien addition lines were used to assign DNA markers to specific "S" chromosome arms (Chen et al. 2002).

10.4.3 Nullisomics

Nullisomic genotypes are of the $2n - 2$ condition and can be generated through self-pollination of monosomic individuals (Gerstel and Parry 1973) or chromosome doubling of nullihaploid plants generated via anther culture of monosomic plants (Mattingly and Collins 1974; Moore and Collins 1982). It is presumed

that most nullisomics are non-viable in diploid *Nicotiana* species and thus have been studied mostly in *N. tabacum* because of its allopolyploid nature and possible tolerance of the $2n - 2$ condition. It has been found, however, that most nullisomics are not viable in this species because of apparent development of genomic interdependence during the evolution of this species. Only nullisomics E and S have sufficient fertility to be maintained by self-pollination. Other nullisomics have included C, D, and H, but these must be propagated asexually (Reed 1991).

10.4.4 Haploids/Doubled Haploids

Haploid plants possess the gametic chromosome number and have been identified and characterized in a number of *Nicotiana* species (Goodspeed 1954). Most work with haploids in *Nicotiana* has focused on *N. tabacum*, however, which has been used as a model species for the development of haploid/doubled haploid breeding systems in plant breeding. Haploid plants can arise either from the egg cell via gynogenesis, from a male gamete nucleus via androgenesis, or from microspore nuclei during in vitro pollen or anther culture. They have been observed at low frequencies among progenies from interspecific crosses between *N. digluta* and *N. tabacum* (Clausen and Lammerts 1929), *N. tabacum* and *N. sylvestris* (Clausen and Mann 1924), and *N. tabacum* and *N. africana* (Burk et al. 1979). Kumashiro and Oinuma (1985) have also obtained *N. tabacum* haploids after fertilization of tobacco ovules with radiation-impaired pollen of *N. alata*.

The practical use of haploids in cultivar development requires efficient methodology for their generation and identification. An efficient hybrid lethality system was developed by Burk et al. (1979) to isolate haploid *N. tabacum* plants of maternal origin from seed. In this method, *N. tabacum* plants are pollinated with pollen from *N. africana*. This is a semi-lethal cross, and almost all true interspecific hybrids fail to survive past the cotyledonary stage. A significant fraction of surviving plants is haploid that, with experience, can be visually recognized at the seedling stage. The second factor required for practical use of haploids in plant breeding is the ability to double their chromosome number to produce doubled haploid individuals. Chromosome doubling can occur spontaneously in *Nicotiana* (Clausen and Goodspeed 1925; Kasperbauer and Collins 1972), but at extremely low frequencies. Treatment of young seedlings (Burk et al. 1972) or axillary buds (Tanaka and Nakata 1969) with colchicine, or in vitro culture of plant parts such as leaf midveins (Kasperbauer and Collins 1972) are methods frequently used to achieve chromosome doubling in *Nicotiana*.

10.5 Role in Classical and Molecular Genetic Studies

10.5.1 Interspecific Hybridization and Gene Transfer

The genus *Nicotiana* has been a favored material for studies on interspecific hybridization, inheritance, and gene transfer in higher plants partially because of the relatively large number of widely varying species in the genus, and also because of the presence of desired genes (primarily disease resistance genes) in the gene pools of certain species. Many species are among the easiest self-pollinating species to work with because of their sizeable flowers that can be manipulated without difficulty for directed crossing experiments. Additionally, large amounts of seed can often be obtained from single pollinations (more than 3,000 seeds per capsule, in some cases).

In 1761, the German botanist Josef Gottlief Kölreuter used *Nicotiana* species in the first experiments involving interspecific hybridization in plants when he produced a hybrid between *N. paniculata* and *N. rustica*. Kölreuter's conclusions from this and other hybridization experiments were that each species contributes equally to the characteristics of an offspring, and that interspecific hybrids are quantitatively intermediate between their two parents in phenotype. More than 300 interspecific hybrids have since been reported in the genus *Nicotiana* (East 1928; Kostoff 1943, Goodspeed 1954; Chaplin and Mann 1961; Smith 1968). Interspecific *Nicotiana* hybrids have been used to investigate a number of genetic abnormalities that occur in such materials including the spontaneous development of genetic tumors on stems, roots, leaves, or flower parts (Smith 1972), and the

generation of chromosomes of extraordinary length (termed megachromosomes) that were first observed in cells of *N. tabacum* × *N. otophora* hybrids and their progenies (Gerstel and Burns 1966). *Nicotiana* species and synthetic amphidiploids have also been used to study an array of genetic changes or "genetic turnover" that occurs in species post-allopolyploidization (Lim et al. 2007).

Nicotiana researchers were among the first to describe mechanisms of interspecific gene transfer from a wild relative to a crop species in a series of classic papers detailing the transfer of a TMV-resistance gene designated as *N* from *N. glutinosa* to *N. tabacum*. These papers outlined the isolation of a fertile, spontaneously chromosome-doubled interspecific hybrid between *N. tabacum* ($2n = 48$) and *N. glutinosa* ($2n = 24$) known as *N. digluta* ($2n = 72$) (Clausen and Goodspeed 1925). After backcrossing *N. digluta* to *N. tabacum* followed by several generations of self-pollination, a $2n = 48$ line designated as "Holmes Samsoun" was established that was genetically stable for TMV resistance (Holmes 1938). Genetic analyses suggested that introgression of *N* had initially occurred via substitution of an intact *N. glutinosa* chromosome carrying *N* for chromosome H of the *N. tabacum* genome (Mallah 1943; Gerstel 1943, 1945a). It was later demonstrated that gene introgression could be furthered via segmental substitution after extra backcrossing provided additional opportunity for recombination between the H chromosome of *N. tabacum* and the substituted *N. glutinosa* chromosome (Gerstel 1948; Gerstel and Burk 1960). Ultimately, the *N* gene was the first plant virus-resistance gene to be cloned (Whitham et al. 1994), which thus permitted the gene to be transferred via plant transformation (Lewis et al. 2007a).

10.5.2 Studies of Quantitative Variation

Because of the large degree of phenotypic variability among and within *Nicotiana* species, in addition to their ease of genetic manipulation, some have been used as model systems in classic experiments to better understand quantitative genetic systems. East (1916) used crosses between accessions of *N. longiflora* Cavanilles differing greatly with respect to flower dimensions in experiments to generate data to support the "multiple factor hypothesis." Measurements of floral parts in progeny were used to verify the main aspects of this model, which uses segregation of alleles at multiple, independent loci combined with superimposed environmental effects to explain continuous quantitative phenotypic variation in plants. Later, linkage between qualitative and quantitative characters was demonstrated in progeny of a cross between *N. langsdorffii* and *N. sanderae* (Smith 1937).

European quantitative geneticists selected *N. rustica* as a model species for several studies designed to validate the applicability of biometrical methods for investigating polygenic inheritance (Mather 1949; Mather and Vines 1952; Smith 1952). Analyses from this work allowed interpretations about the kinds of gene action (additive versus dominance) that contribute to total phenotypic variance. In general, additive effects were found to contribute more to the total variance than dominance effects.

10.5.3 Tissue Culture and Plant Transformation

Several *Nicotiana* species have played central roles in establishing tissue culture methodologies, which, in turn, helped pave the way for development of gene transfer methods via genetic engineering. Such techniques dramatically broadened the number of organisms that might be used as genetic resources for manipulation of *N. tabacum*. In one of the first successful demonstrations of in vitro tissue culture, White (1939) used the tissues originating from spontaneous tumors produced by the interspecific hybrid *N. glauca* × *N. langsdorffii* to show that undifferentiated plant tissues could be cultured indefinitely on agar-solidified nutrient medium. Skoog and Miller (1957) later reported that whole plants could be regenerated from tobacco tissues by manipulating relative concentrations of auxins and cytokinins in culture. Totipotency was soon after demonstrated through the regeneration of whole tobacco plants from single somatic cells or from protoplasts (Vasil and Hildebrandt 1965; Usui and Takebe 1969; Nagata and Takebe 1970; Takebe et al. 1971). In addition, the first report using in vitro anther or pollen culture to produce haploid plants of a cultivated crop species utilized *N. tabacum* (Bourgin

and Nitsch 1967; Nitsch and Nitsch 1969). Since this time, several members of *Nicotiana* have been the species of choice for extensive in vitro biological research. Cells and tissues from many *Nicotiana* species are totipotent and grow readily on simple tissue culture media (Li et al. 2003).

Because of *N. tabacum*'s amenability to tissue culture techniques, it was a preferred species for early plant transformation research, and was subsequently the first plant species to be the recipient of a foreign gene delivered via genetic engineering (Barton et al. 1983; De Block et al. 1984; Horsch et al. 1985; An et al. 1986). Initial transfers of foreign genes into the nuclear genome were mediated by *Agrobacterium tumefaciens*, but other methods involving the use of electroporation and biolistics have also since been used to transform this and other *Nicotiana* species (Birch 1997). *N. tabacum* may also provide the most effective system for plastid transformation (Svab and Maliga 1993; Bock 2001). These methods, in conjunction with recombinant DNA techniques that permit overexpression or silencing of target genes, now provide great opportunity for manipulating plant genetic systems and for studying gene function in plants. In addition, *N. benthamiana* has been utilized extensively as a host for virus-induced gene silencing (VIGS), a system in which viral vector-mediated expression of a foreign sequence is used to silence a target gene in order to gain insight on gene function (Baulcombe 1999).

10.6 Role of *Nicotiana* Species in Crop Improvement

10.6.1 Sources of Genes Affecting Qualitative Traits

The general objective of cultivar development is to increase yield, quality, and/or disease resistance without simultaneous degradation of other desired characteristics. Genetic variation is essential for continued improvement of any crop species, and the within-species gene pool is always the preferred source of genetic variability for improvement of cultivated tobacco. Often times, however, desired variability does not exist within the immediate germplasm pool and the option of utilizing wild relatives may be visited.

On paper, the genus *Nicotiana* appears to be an enormous source of genetic variability for improving cultivated tobacco for an array of characters, including disease resistance (Stavely 1979), and research has consequently been conducted to transfer a number of desired genes from *Nicotiana* relatives. The steps for conventional introgression of genes into *N. tabacum* include (1) identification of a species possessing the trait of interest, (2) hybridization with *N. tabacum*, (3) generation of fertile offspring, and (4) backcrossing to the cultivated form with hope of transferring the desired trait. The time and effort required to complete conventional interspecific gene transfers should not be underestimated, however, as many years are often required to reach a commercially viable end product. The probability of success is usually dependent on the degree of taxonomic distance between *N. tabacum* and the trait donor. As this distance increases, lethality or sterility of resulting materials may complicate introgression. In addition, frequency of recombination between tobacco chromosomes and those derived from the alien species is largely dependent upon the degree of genetic and cytological differentiation in the genomes of the parental species.

Some interspecific hybrids are relatively easy to generate. In general, these include hybrids between closely related species. Initial hybridizations for more distant crosses can often be challenging, however, because of failed fertilization, poor seed development, or seedling lethality. Possible routes for overcoming some of these problems have included use of a third *Nicotiana* species in a bridge-crossing scheme (Burk 1967), or use of monosomic *N. tabacum* parents (Burk 1972; Burk et al. 1979). Hybridization can also be extended sometimes to otherwise difficult to produce hybrids through in vitro culture of fertilized ovules (Reed and Collins 1978; Douglas et al. 1983), or use of parasexual techniques such as protoplast or microprotoplast fusion. Symmetric and asymmetric somatic hybrids have been produced between *N. tabacum* and a number of different *Nicotiana* species (Evans et al. 1980, 1981, 1983; Bates 1990; Sproule et al. 1991; Bui et al. 1992; Donaldson et al. 1995). Protoplast fusions have also been reported between *N. tabacum* and *Daucus carota* (Dudits et al. 1987; Kisaka and Kameya 1994), *N. plumbaginifolia* and *Petunia hybrida* (Hinnisdaels et al. 1991), and

N. plumbaginifolia and *Solanum tuberosum* (de Vries et al. 1987). Protoplasts carrying the donor genome have sometimes been irradiated with X- or gamma rays to achieve partial genome transfer (Bates 1990; Hinnisdaels et al. 1991; Kisaka and Kameya 1994). Microprotoplast-mediated chromosome transfer (MMCT) has been used to directly produce *N. tabacum* alien addition lines possessing single chromosomes from potato, *Solanum tuberosum* (Ramulu et al. 1996).

Crosses between *N. tabacum* and *N. sylvestris* or several species of section *Tomentosae* can result in somewhat fertile F_1 hybrids that can be backcrossed to *N. tabacum*. Most interspecific hybrids between *N. tabacum* and wild relatives are male and/or female sterile, however, and strategies involving chromosome doubling agents must thus be used to generate a fertile synthetic allopolyploid or sesquidiploid intermediate from which to initiate backcrossing (Burk and Chaplin 1979). Fertile allopolyploids can be produced by doubling the chromosome number of the F_1 hybrid between *N. tabacum* and the donor species. Fertile sesquidiploids can also be produced directly by doubling the chromosome number of *N. tabacum* to produce the allooctaploid (8*x*) form prior to hybridization with the donor species. These intermediates are then used as the starting points for a series of backcrosses to 4*x N. tabacum* accompanied with selection for the trait of interest. This leads to rapid elimination of chromosomes from the donor species. Alien chromosomes or chromosome segments can also be lost during mitotic divisions (Moav and Cameron 1960; Moav 1961; Smith 1968; Gerstel 1977). During backcrossing, the breeder hopes for gene transfer that may occur via three mechanisms (Clausen and Cameron 1957; Moav 1958): (1) formation of an alien chromosome addition line (24 *N. tabacum* pairs + 1 alien pair), (2) substitution of an entire alien chromosome for a *N. tabacum* chromosome, or (3) formation of segmental substitution lines where a donor chromosome segment replaces only a segment of a *N. tabacum* recipient chromosome. It should be noted that *N. tabacum* should be used as the maternal parent in initial interspecific hybridizations or in early backcross generations in order to reduce the possibility of producing end products exhibiting male sterility conferred by an alien cytoplasm.

Strong preferential bivalent pairing between *N. tabacum* chromosomes usually operates to exclude chromosome substitutions or genetic exchanges between alien and *N. tabacum* chromosomes. Homozygous alien chromosome addition lines are usually outside the limits of commercial acceptability, however, and the breeder's goal is thus to obtain the smallest possible segment in the position of least disturbance to the genetic constitution of *N. tabacum*. Alien chromosome substitutions and segmental substitutions may arise spontaneously from trivalent conjugations of two *N. tabacum* homologs and a single alien chromosome (Clausen and Cameron 1957) or because of imperfect meiosis (Mann et al. 1963). Although a given gene can be transferred to multiple *N. tabacum* chromosomes (Moav 1958; Stokes 1960; Lewis et al. 2005), residual chromosome homology likely causes there to be preferential transfer to one or two recipient chromosomes (Moav 1958). Techniques that might increase the probability of interchromosomal exchanges include tissue culture (Lewis 2005), the use of autotriploidy (Moav 1962; Schweppenhauser 1968, 1975), or radiation treatment (Niwa 1965). Techniques that involve chromosome breakage can often result in fusing of chromosome ends in new structural arrangements. Random translocations are most likely to be non-compensating, however, thus increasing the probability of generating end products that are not commercially viable.

A fair number of disease-resistance genes have been transferred to cultivated tobacco from wild *Nicotiana* relatives (Table 10.2), and some tobacco cultivars, particularly of the burley market class, have up to four introgressed alien chromosome regions carrying major disease-resistance genes. Some gene introgressions have had very significant effects on decreasing pesticide use and reducing economic loss from disease. Some have been difficult to deploy commercially, however, because of adverse linkage drag effects on yield and/or cured leaf quality. Examples include TMV resistance from *N. glutinosa* (Chaplin et al. 1961, 1966; Chaplin and Mann 1978) (Table 10.3), black shank resistance from *N. longiflora* and *N. plumbaginifolia* (Valleau et al. 1960; Johnson 1999) (Table 10.4), and black root rot resistance from *N. debneyi* Domin (Legg et al. 1981) (Table 10.5). Such unfavorable linkages can be very difficult to break even after many cycles of backcrossing (Stam and Zeven 1981; Young and Tanksley 1989). In many cases, disease-resistance tobacco

Table 10.2 Documented trait introgressions to *N. tabacum* from wild *Nicotiana* relatives

Trait	Donor species	Reference(s)
Tobacco mosaic virus (TMV) resistance	*N. glutinosa*	Holmes (1938), Ternovsky (1945), Gerstel (1945a), Kostoff (1948), Valleau (1952)
Wildfire (*Pseudomonas syringae* pv *tabaci* (race 0)) and angular leaf spot (*Pseudomonas syringae* pv *angulata*) resistance	*N. longiflora*	Clayton (1947)
Wildfire (*Pseudomonas syringae* pv *tabaci* (race 0 and 1)) and angular leaf spot (*Pseudomonas syringae* pv. *Angulata)*	*N. rustica*	Stavely and Skoog (1976), Woodend and Mudzengerere (1992)
Black shank (*Phytophthora parasitica* var *nicotianae*) (race 0) resistance	*N. longiflora*	Valleau et al. (1960)
Black shank (*Phytophthora parasitica* var *nicotianae*) (race 0) resistance	*N. plumbaginifolia*	Apple (1962), Chaplin (1962)
Black shank (*Phytophthora parasitica* var *nicotianae*) resistance (race 0)	*N. rustica*	Woodend and Mudzengerere (1992)
Black root rot (*Chalara elegans*; Syn. *Thielaviopsis basicola*) resistance	*N. debneyi*	Clayton (1969)
Blue mold (*Peronospora tabacina*) resistance	*N. velutina*	Clayton (1967), Clayton et al. (1967), Lea (1963)
Blue mold (*Peronospora tabacina*) resistance	*N. debneyi*	Wark (1963, 1970)
Blue mold (*Peronospora tabacina*) resistance	*N. goodspeedii*	Wark (1963, 1970)
Root knot nematode (*Meloidogyne javanica*) resistance	*N. longiflora*	Schweppenhauser (1968, 1975), Ternouth et al. (1986)
Root knot nematode (*Meloidogyne javanica*) resistance	*N. repanda*	Ternouth et al. (1986)
Root knot nematode (*Meloidogyne incognita*) (races 1 and 3) resistance	*N. tomentosa*	Clayton et al. (1958), Yi and Rufty (1998)
Powdery mildew (*Erysiphe cichoracearum*) resistance	*N. debneyi*	Smeeton and Ternouth (1992)
Powdery mildew (*Erysiphe cichoracearum*) resistance	*N. glutinosa*	Smeeton and Ternouth (1992)
Powdery mildew (*Erysiphe cichoracearum*) resistance	*N. tomentosiformis*	Smeeton and Ternouth (1992)
Tomato spotted wilt virus (TSWV) resistance	*N. alata*	Gajos (1987)
Potato virus Y (PVY) resistance	*N. africana*	Lewis (2005)
Potato virus Y (PVY) resistance	*N. tomentosiformis*	Legg and Smeeton (1999)
Tobacco cyst nematode (*Globodera tabacum*) resistance	*N. plumbaginifolia*	Apple (1962), Chaplin (1962), Johnson et al. (2009)
Increased leaf number and delayed flowering time	*N. tomentosa*	Clausen and Cameron (1944), Lewis et al. (2007b)

Table 10.3 Effects of homozygous *N. glutinosa N*-mediated TMV resistance on yield and quality in flue-cured tobacco. *N* was transferred to nine TMV-susceptible varieties using four backcrosses. Susceptible and resistant BC_4S_2 lines were evaluated in two environments (Chaplin et al. 1961)

Group	Yield (kg/ha)	Value ($/cwt)	Value ($/ha)
Mean of 18 TMV-susceptible BC_4S_2 selections (*nn*)	2,527	132.15	3316.15
Mean of 18 TMV-resistant BC_4S_2 selections (*NN*)	2,379	128.02	3041.86
% Difference (*NN* vs. *nn*)	−5.9%	−3.12%	−8.27%

cultivars have resulted from a trade-off between resistance and yield or quality. In practice, negative linkage drag effects can be lessened by deployment of alien disease-resistant genes in heterozygous condition in F_1 hybrids. Negative effects from alien genes flanking a transferred gene of interest are frequently additive in their effects. Full disease resistance can often be achieved in some hybrids with yield or leaf quality being intermediate or more similar to the susceptible parents (Wernsman and Rufty 1987). An example includes deployment of the *N* gene conferring resistance to TMV (Table 10.6).

Table 10.4 Effect of homozygous *N. plumbaginifolia Ph*-mediated black shank resistance on yield and quality in flue-cured tobacco. Doubled haploid lines were extracted from two crosses between a *PhPh* line and a *phph* line and were evaluated for yield and quality in a single environment (Johnson 1999)

Group	Yield (kg/ha)	Grade index
Mean of 38 black shank-resistant (*PhPh*) doubled haploid lines	2175	59.83
Mean of 31 black shank-susceptible (*phph*) doubled haploid lines	1976	57.93
% Difference (*PhPh* vs. *phph*)	−9.11%	−1.53%

Table 10.5 Effect of *N. debneyi*-derived black root rot resistance in burley tobacco. Resistance was transferred to eight black root rot-susceptible varieties using seven backcrosses. Susceptible and resistant BC_7S_2 lines were compared for yield and plant height in three environments (Legg et al. 1981)

Group	Yield (g/plant)	Plant height (cm)
Mean of eight black root rot-susceptible BC_7S_2 selections	142.7	122.4
Mean of eight black root rot-resistant BC_7S_2 selections	132.8	130.8
% Difference (resistant vs. susceptible)	−6.94%	+6.86%

Table 10.6 Effect of *N. glutinosa N*-mediated TMV resistance in homozygous and heterozygous condition in flue-cured tobacco. *N* was transferred to six TMV-susceptible varieties using six backcrosses. Susceptible (*nn*) and resistant (*NN*) BC_6S_2 lines were compared to *Nn* F_1 hybrids. Materials were evaluated in four environments (Chaplin et al. 1966)

Group	Yield (kg/ha)	Value ($/cwt)	Value ($/ha)
Mean of six TMV-susceptible BC_6S_2 selections (*nn*)	2,436	131.90	3,158.00
Mean of six TMV-resistant BC_6S_2 selections (*NN*)	2,213	127.71	2,844.18
Mean of six F_1 hybrids (TMV-resistant selection × TMV-susceptible variety)(*Nn*)	2,372	128.02	3,034.45
% Difference (*NN* vs. *nn*)	−9.20%	−3.18%	−9.94%
% Difference (*Nn* vs. *nn*)	−2.6%	−2.94%	−3.92%

10.6.2 Sources of Genes Affecting Quantitative Traits

Because of the difficult and time consuming nature of gene transfer from most wild *Nicotiana* relatives, they have been utilized almost exclusively as sources of simply inherited disease-resistance genes. It can be an extremely arduous task to transfer an oligogenic or polygenic system to cultivated tobacco from species other than those most closely related to *N. tabacum* because of the great difficulty in introgressing multiple chromosomal segments from more distant relatives. Nevertheless, diploid relatives have also been evaluated as sources of variation for improvement of quantitative traits in tobacco.

Mann and Weybrew (1958) were the first to evaluate hybrids between *N. sylvestris* and several *N. tabacum* cultivars for important quantitative characters and observed earlier flowering and faster growth rates in F_1 hybrids. It was concluded, however, that the advantage of faster growth rates was offset by reduced leaf number and increased sucker potential. Hybrids were generally lower yielding than their varietal counterparts and produced leaves with less-than-desirable cured leaf quality. Heterotic responses were examined in crosses between tobacco cultivars and five wild *Nicotiana* species (*N. otophora*, *N. tomentosiformis*, *N. sylvestris*, *N. glauca*, and *N. glutinosa*) and Kostoff's hybrid [4*x*(*N. sylvestris* × *N. tomentosiformis*)] by Matzinger and Wernsman (1967). Heterosis for yield, plant height, and number of leaves was greatest for crosses with the possible progenitor species *N. otophora* and *N. tomentosiformis*. Crosses between *N. tabacum* and Kostoff's hybrid exhibited little heterosis except for plant height. Interspecific hybrids possessed quality characteristics that would have made them unusable for direct commercial use, however.

Because of significant heterosis observed in F_1 hybrids between *N. tabacum* and several of its probable progenitor species, Wernsman and Matzinger (1966) proposed a breeding scheme to incorporate

desirable genetic variation from wild species into *N. tabacum* while maintaining the potential for retention of important quality attributes. The method involved initial hybridization between allooctaploid (8*x*) *N. tabacum* with *N. sylvestris*, *N. otophora*, or *N. tomentosiformis*. The authors then suggested that the resulting pentaploid hybrids be backcrossed as females to conventional varieties. Several steps could be taken during backcrossing to stabilize chromosome number and introduce desirable germplasm. If additive or intergenomic dominance effects were of primary importance, selected backcross progenies could be intercrossed to form a heterogeneous population from which inbred lines could be derived via self-pollination. If non-additive effects were of importance, recurrent selection for specific combining ability in derived heterogeneous populations could be conducted by simultaneous self-pollination of BC_1 plants and testcrossing them with the original variety.

This system was evaluated using material derived from crosses between 8*x* tobacco cultivar "SC58" and *N. sylvestris* (Wernsman et al. 1976). Two cycles of recurrent selection within the species-derived synthetic population for specific combining ability with SC58 produced positive results. First- and second-cycle populations yielded 7.7% and 12.9%, respectively, more than SC58. The authors also noted that inbred lines superior to SC58 could be extracted from the cycle 0 population using pedigree selection. Oupadissakoon and Wernsman (1977) also found that species-derived lines could yield better than the corresponding varieties from which they were derived. Yield increases, however, were accompanied with later maturity, increased plant height, and reduced leaf quality.

Research to evaluate incorporation of *N. rustica* germplasm into the *N. tabacum* genome was conducted by Legg and Mann (1961). In this study, hybridization of *N. rustica* ($2n = 48$) with allooctaploid (8*x*) *N. tabacum* was followed by three cycles of backcrossing to diploid *N. tabacum*. In field tests, many BC_3 and BC_3S_1 families exhibited reductions in yield, sugar content, plant height, and leaf number. The authors concluded that the chromosomes of the two species occasionally paired and exchanged germplasm, but that efforts to utilize variation from *N. rustica* for improvement of quantitative traits was not justified due to a very large number of negative effects. Chaplin (1987) later described work to transfer genes influencing alkaloid accumulation from *N. rustica* to *N. tabacum*.

Work to introduce favorable alien genes affecting yield from wild relatives has essentially ceased because of the degree of negative correlated effects on cured leaf quality (Oupadissakoon and Wernsman 1977). It may be possible, however, to utilize molecular markers to identify and retain favorable introgressed alleles while leaving the undesirable portion of the donor genome behind in breeding procedures. For example, Tanksley and Nelson (1996) proposed "advanced backcross QTL analysis," which can be used to simultaneously identify positive chromosomal region(s) from a donor parent and transfer them to a recipient elite line while selecting against the remainder of the undesirable donor parent genome.

10.6.3 Sources of Cytoplasmic Male Sterility

Alien gene introgression into *N. tabacum* has not been limited to nuclear genes. Cytoplasmic male sterility is frequently observed following transfer of the nuclear genome of *N. tabacum* via backcrossing into the cytoplasm of another *Nicotiana* species (Gerstel 1980; Nikova et al. 1997; Berbec 2001; Berbec and Laskowska 2005). This feature has been utilized to increase the efficiency of hybrid seed production of commercial tobacco cultivars. Male sterility provides a mechanism of plant variety protection and also facilitates the deployment of multiple disease-resistance genes in F_1 hybrids. Fertility restoration systems are not needed because the product of interest is vegetative (leaves) rather than reproductive. The impact of various alien cytoplasms on agronomic performance of cultivars has been variable. In general, some cytoplasms (*N. suaveolens*, *N. bigelovii*, *N. plumbaginifolia*, *N. megalosiphon*, and *N. undulata*) may contribute to depressions in growth rates and/or alterations of cured leaf chemistry (Mann et al. 1962; Aycock et al. 1963; Chaplin and Ford 1965; Hosfield and Wernsman 1974), but not necessarily yield or quality reductions.

10.6.4 Application of Newer Technologies to Use of Genetic Resources

10.6.4.1 DNA Markers

Molecular markers can be important tools for understanding phylogenetic relationships, investigating the genetic control of important traits, and for use in marker-assisted selection programs. A very large degree of DNA marker polymorphism among *Nicotiana* species has been reported (Ren and Timko 2001; Moon et al. 2008). Reported levels of nucleotide variation among diverse strains of *N. tabacum* as revealed by RFLP, RAPD, and AFLP markers have been comparatively low, however (Brandle and Bai 1999; del Piano et al. 2000; Ren and Timko 2001; Rossi et al. 2001). This may be attributed to a genetic bottleneck effect where only a fraction of the genetic variation that existed within the progenitor germplasm pools entered into *N. tabacum*. Limited work has been conducted to characterize DNA marker polymorphism among accessions of diploid *Nicotiana* relatives, however, primarily due to poor representation of these species in germplasm collections (Moon et al. 2008). Simple sequence repeat (SSR), or microsatellite, markers have been demonstrated to exhibit higher levels of polymorphism in *N. tabacum* than other tested marker systems, and Moon et al. (2008) have demonstrated transferability of SSR markers identified in *N. tabacum* (Bindler et al. 2007) for use in other *Nicotiana* species.

Early DNA marker–trait association research in *Nicotiana* was exclusively directed toward identification of DNA markers linked to major disease-resistance genes transferred to tobacco from wild *Nicotiana* relatives. This work was made possible because of the significant amount of marker polymorphism that was simultaneously introduced into *N. tabacum* within introgressed chromosome blocks. Simple bulked segregant analyses led to identification of RAPD, sequence characterized amplified region (SCAR), or AFLP markers linked to major disease-resistance genes introgressed into *N. tabacum* from *N. plumbaginifolia, N. longiflora, N. debneyi, N. glutinosa, N. africana, N. tomentosa,* and *N. alata* Link & Otto (Table 10.7). Suppressed recombination has been observed within some introgressed linkage blocks, particularly those transferred from species such as *N. debneyi*, *N. plumbaginifolia,* or *N. longiflora* (Bai et al. 1995; Johnson et al. 2002a), which are not closely related to either of the progenitors of *N. tabacum*. This is of value for marker-assisted breeding in segregating populations, but disadvantageous from the standpoint of precisely mapping resistance gene locations, and also for the potential of reducing the size of foreign introgressed chromosome segments. If recombination does occur at low frequency, DNA markers can be used to identify rare recombinatory events to perhaps reduce deleterious linkage drag effects (Young and Tanksley 1989). Markers have been used to examine variability among introgressed chromatin blocks transferred to *N. tabacum* from *N. glutinosa* (Lewis et al. 2005) and *N. africana* (Lewis 2005).

Research to identify DNA markers linked to genes influencing quantitative traits in *Nicotiana* has been much more limited (Nishi et al. 2003; Julio et al. 2006). In principle, mapping of quantitative trait loci (QTL) could be facilitated by introgression of foreign alleles with large effects on phenotype accompanied with introgression of substantial DNA polymorphism. Lewis et al. (2007b) investigated the potential of utilizing AFLP polymorphism introgressed from a species closely related to cultivated tobacco, *N. tomentosa*, to map a gene controlling the quantitative trait leaf number. Here, the authors observed sufficient recombination to map a QTL designated as *Many Leaves* to within a 3.1 cM region.

10.6.4.2 Plant Transformation

Because of *N. tabacum*'s high responsiveness to tissue culture techniques, it was a favored species for initial plant transformation research, and was the first plant species to be genetically engineered (see above). Development of plant transformation methods in combination with recombinant DNA technologies can allow circumvention of sexual incompatibilities and interchromosomal recombination problems that can be encountered during conventional interspecific gene introgression, and permits the transfer of genes to tobacco from virtually any other living organism.

Hundreds, if not thousands, of publications have documented transfer of foreign genes to *Nicotiana* species using plant transformation methods. Several papers have shown that cloned resistance genes from

Table 10.7 DNA markers associated with traits introgressed into *N. tabacum* from wild *Nicotiana* relatives

Trait	Marker type[a]	Source	Reference
Tobacco mosaic virus (TMV) resistance	Genic, AFLP	*N. glutinosa*	Whitham et al. (1994), Lewis et al. (2005)
Wildfire (*Pseudomonas syringae* pv *tabaci* (race 0)) and angular leaf spot (*Pseudomonas syringae* pv *angulata*) resistance	RAPD	*N. longiflora*	Yi et al. (1998a)
Black shank (*Phytophthora parasitica* var *nicotianae*) (race 0) resistance	RAPD	*N. plumbaginifolia*	Johnson et al. (2002a, b)
Black shank (*Phytophthora parasitica* var *nicotianae*) (race 0) resistance	RAPD	*N. longiflora*	Johnson et al. (2002a, b)
Tobacco cyst nematode (*Globodera tabacum*) resistance	RAPD	*N. plumbaginifolia*	Johnson et al. (2002a, 2009)
Blue mold (*Peronospora tabacina*) resistance	RAPD, SCAR	*N. debneyi, N. goodspeedii, N. velutina*	Milla et al. (2005)
Black root-rot (*Chalara elegans*; Syn. *Thielaviopsis basicola*) resistance	RAPD	*N. debneyi*	Bai et al. (1995)
Root knot nematode (*Meloidogyne incognita*) (races 1 and 3) resistance	RAPD	*N. tomentosa*	Yi and Rufty (1998), Yi et al. (1998b)
Tomato spotted wilt virus (TSWV) resistance	AFLP, SCAR	*N. alata*	Moon and Nicholson (2007)
Potato virus Y (PVY) resistance	RAPD	*N. africana*	Lewis (2005)
Increased leaf number and delayed flowering time	AFLP	*N. tomentosa*	Lewis et al. (2007b)

[a]*RAPD* random amplified polymorphic DNA; *AFLP* amplified fragment length polymorphism; *SCAR* sequence characterized amplified region

one Solanaceous species can function in the genetic background of another. For example, researchers have shown that the cloned resistance gene, *Pto*, from tomato confers resistance to *Pseudomonas syringae* pv *tabaci* in *N. tabacum* and *N. benthamiana* when the bacteria carries the corresponding avirulence gene *avrPto* (Rommens et al. 1995; Thilmony et al. 1995). Likewise, the tomato *Cf-4* and *Cf-9* genes also function in *Avr4* and *Avr9*-dependent fashions in *N. tabacum* and *N. benthamiana* to provide protection against the fungus *Cladosporium fulvum* (Thomas et al. 2000). In addition, the tomato genes *Sw5* and *Tm-2*^{2} have been shown to function in *N. tabacum* to provide resistance against tomato spotted wilt virus and tomato mosaic virus, respectively (Spassova et al. 2001; Lanfermeijer et al. 2004).

One major advantage of plant transformation is that the method can be used to bypass deleterious linkage drag effects because it allows for the introduction of specific genes without any accompanying foreign chromatin. For example, the TMV-resistance gene *N* was initially transferred to *N. tabacum* via chromosome substitution followed by recombination with its *N. tabacum* homolog (Holmes 1938; Gerstel 1948; Gerstel and Burk 1960; Lewis et al. 2005). TMV-resistant flue-cured cultivars possessing this resistance have not been widely grown, however, because of reduced yields and/or quality attributed to linkage drag effects caused by the presence of unfavorable genes of *N. glutinosa* origin linked to *N* that have been difficult to remove using conventional backcrossing (Chaplin et al. 1961, 1966; Chaplin and Mann 1978). The isolation of *N* (Whitham et al. 1994) has permitted comparison of tobacco lines containing the *N* transgene introgressed using conventional methods to those possessing the gene introduced via plant transformation. Results indicated that the plant transformation mode of gene transfer reduced the negative effects typically associated with the conventionally introgressed *N. glutinosa* chromosome segment (Lewis et al. 2007a).

10.7 Genomic Resources

Although *Nicotiana* species have played important roles in many areas of plant biology research, genomic resources for this genus have developed slowly relative to most other major crop species. Complete sequences are available for the chloroplast genomes of *N. sylvestris*, *N. tomentosiformis*, and *N. tabacum*

(Shinozaki et al. 1986; Yukawa et al. 2006), and the complete sequence of the *N. tabacum* mitochondrial genome has also been published (Sugiyama et al. 2005).

The vast majority of nuclear sequence information for *Nicotiana* in public databases is for *N. tabacum*, with the second largest amount being for *N. benthamiana*. Several projects are largely responsible for this data. Matsuoka et al. (2004) deposited the sequences of >9,000 expressed sequence tags (ESTs) for *N. tabacum* into GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Another major sequencing effort for *Nicotiana* was that of the "European Sequencing of Tobacco" (ESTobacco) project carried out as a collaborative arrangement between Advanced Technologies Ltd. (Cambridge) and the Institute du Tabac de Bergerac (Altadis) (http://www.estobacco.info). This project has deposited 46,546 EST sequences in GenBank.

The largest effort, however, has been that of the Tobacco Genome Initiative (TGI) funded by Philip Morris USA and carried out by researchers at North Carolina State University, USA (Gadani et al. 2003; Opperman et al. 2007). This project was concluded in 2008, and completed objectives included sequencing of >85,000 EST sequences for *N. tabacum* and >38,000 ESTs from *N. benthamiana* (Opperman et al. 2007) using mRNA collected from an array of different tissues from both species. A second major objective of the TGI was sequencing of methyl-filtered *N. tabacum* genomic clones in a strategy designed to selectively sequence gene-rich regions. Results suggested a nearly tenfold increase in gene discovery in methyl-filtered vs. non-filtered libraries (Gadani et al. 2003), and >1.4 million genomic sequences are now available in public databases (GenBank, and TGI website: http://www.pngg.org/tgi/).

Other databases containing *Nicotiana* sequence information include the Tobacco Gene Index (NtGI) (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=tobacco) that integrates research data from international EST sequencing and gene research projects and that it is maintained by the Dana-Farber Cancer Institute and Harvard School of Public Health. A *Nicotiana* transcription factor database has also been created by researchers at the University of Virginia (Rushton et al. 2008) and can be found at: http://compsysbio.achs.virginia.edu/tobfac/.

Genetic mapping in *Nicotiana* has also been limited compared to other major crops. The first genetic linkage map in this genus was based on RFLP and RAPD genotypes in an F_2 population derived from a cross between *N. longiflora* and *N. plumbaginifolia* (Lin et al. 2001). The most complete genetic map published to date for *Nicotiana* is that based on mapping 293 microsatellite markers into 24 linkage groups for *N. tabacum* (Bindler et al. 2007).

10.8 Conclusions

Nicotiana species have contributed significantly to the improvement of disease resistance in cultivated tobacco. In addition, male sterility conferred by alien cytoplasms has had a very significant impact on tobacco cultivar development. Their utility for improving other traits of consequence to tobacco production has yet to be demonstrated, however. Of probably greater importance is the role that species of this genus have played in the development of new knowledge and technology of great relevance to applied plant genetics, in general. It is likely that some *Nicotiana* species will continue to be used heavily into the future as model systems for investigating many aspects of general plant biology.

The future role of *Nicotiana* species for improving cultivated tobacco is not clear. Attempts to introduce desired traits from wild relatives into tobacco through conventional methods have been on the decline. Many traits can now be conferred to tobacco through gene cloning and plant transformation, and technologies related to trait manipulation are expected to grow rapidly in the next decade. Although knowledge regarding crossability, cytogenetics, and gene introgression in *Nicotiana* may be important into the future, research emphasis in these areas will likely be reduced.

New opportunities may exist for exploitation of *Nicotiana* genetic resources. For example, a considerable amount of recent research has been devoted to possible utilization of tobacco or *Nicotiana* species as "bioreactors" to produce plant-manufactured products such as enzymes, proteins, pharmaceuticals, antigens, etc. (Powledge 2001; Ma et al. 2003). Several patents have been issued or claimed for various hybrids

between *Nicotiana* species because of their high biomass, sterility (for transgenic containment), and/or their amenability to production of recombinant proteins via viral vector systems (Fitzmaurice 2002; Zaitlin and Mundell 2006).

Because of strong opinions about tobacco research, political obstacles exist for utilizing genetic resources for tobacco improvement. Public investment in this area has declined rapidly in recent years through retirements and lack of public support for such activities. Future efforts to modify *N. tabacum* for use in tobacco products will rely upon investment from the private sector. Past maintenance of *Nicotiana* genetic resources for use in applied and basic scientific research has mostly been financially supported through public funds. Conservation of *Nicotiana* germplasm is in a precarious position, however, because of a reluctance to use public funds in support of activities related to the politically charged species, *N. tabacum*.

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Chapter 11
Petunia

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11.1 Introduction

It is no wonder that Petunias continue to rank among the most popular flowering annuals. They are bright and lively, bloom from spring until frost, and scent the air with lovely fragrance. Above all, they are amazingly easy to grow, both in the garden and in containers. Petunia is considered to be the first cultivated bedding plant and has remained as a commercially important ornamental since the early days of horticulture and one of the favorite genera for developing new varieties. Hybrid seed production transformed the crop and continued domestication by flower breeders produced a wide range of flower colors and flower patterns.

Petunia got its name from French, which took the word "*petun*" meaning "tobacco" from a Tupi-Guarani language. Petunia is considered as a genetically interesting plant of applied value. It has remained a useful model system, particularly for studies of gene regulation and genome structure. Petunia is one of the few floriculture crops, which have well-established linkage maps, trisomics, transformation/regeneration protocols, and precise elucidation on taxonomic and cytogenetic relationships between taxa and advanced molecular genetics. In particular, elucidation of the genes involved in the flavonoid biosynthesis pathway has enabled precision in the creation of new flower colors or modification of pigment production and expression.

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Petunia offers numerous advantages as a model crop, including its simplicity to grow; abundant seed setting potential; easy self- and cross-pollination; short life cycle (4 months from seed to seed); relatively large flowers, which render biochemical sampling very simple; easiness in transformation and regeneration from leaf disk or protoplast. Besides, petunia harbors an endogenous, very active transposable element system, which is being used to great advantage in both forward and reverse genetics screens.

Use of petunia as a research model was very limited during the late 1950s of the last century. Up to the early 1980s, no more than five groups had developed classical and biochemical genetics, almost exclusively on flower color genes. In due course, the significance of petunia grew in leaps and bounds and at present around 20–25 academic groups around the world are using petunia as their main model system for a variety of research purposes, while a number of smaller and larger companies are developing further new varieties.

11.2 Basic Botany

11.2.1 Origin, Distribution, and Genetic Diversity

Petunia belongs to the family Solanaceae and is closely related to important crop species such as tomato, potato, eggplant, pepper, and tobacco. The geographic origin of petunia is South America, where various *Petunia* species have been found in Argentina, Brazil, Bolivia, Paraguay, and Uruguay. According to Bailey (1910), *Petunia axillaris* (*Petunia nyctaginiflora*) was first cultivated in 1823 and *Petunia*

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integrifolia (*Salpiglossis integrifolia*) first flowered in the Glasgow Botanical Garden (UK) in July 1831 ("from seeds sent the fall before from Buenos Ayres by Mr. Tweedie"). Fries (1911) recognized a subspecies of *P. integrifolia* subsp. *depauperata*, which occurred along the Atlantic coast of Brazil. The primary locations for species diversity of *Petunia* are mostly limited to the three Brazilian provinces of Parana, Santa Catarina, and Rio Grande do Sul, particularly along river banks and isolated areas (Sink 1984c).

Ando and Hashimoto (1993, 1994, 1995, 1996, 1998) described several new species of *Petunia* from uplands of southern Brazil based on the observation of native live plants. All the new species are unique in floral and other morphological traits and are readily distinguishable from previously known species including "*P. integrifolia* complex." Smith and Downs (1966) treated this taxon as a variety of *P. integrifolia*, while Wijsman (1982) regarded this as a variant growing in nutrient-poor soil. However, this plant produces extremely long prostrated stems that bear sparse small flowers and linear leaves, and such features in morphology are never found in any taxa of *Petunia* with the exception of *P. littoralis* L. B. Sm. & Downs.

To clarify morphological intergradation between the two subspecies of *Petunia axillaris,* viz., subsp. *axillaris* and subsp. *parodii*, Kokubun et al. (1997) studied the floral morphological characters on live plants grown from seeds collected from 102 localities in Uruguay. Five discriminant functions were calculated by stepwise discriminant analysis using typical individuals of the two subspecies (from 12 localities of subsp. *axillaris* and from 16 localities of subsp. *parodii*). Mean values of floral characters taken from individuals from 102 localities were substituted into these five functions to predict subspecies. Plants from some localities were predicted inconsistently or doubtfully by respective functions and were regarded as intermediate. The results helped to infer the distribution of the two subspecies and their intermediate forms with more detail and clarity. The two subspecies of *P. axillaris* occurred separately and the border between the two taxa roughly corresponded to the Rio Negro River. The localities of intermediate forms were mostly distributed in the area parallel to the Rio Negro and on the shore of the Rio de la Plata.

Ando et al. (2005) reported *Petunia interior* and *Petunia guarapuavensis* as new records for the Argentinean flora and their geographical distributions were updated. *P. interior* was best characterized by the unique morphology of anthers, viz., evidently channeled lobes of the dehisced anther. In *P. interior*, lateral stems developing from the base of the main stem are usually prostrate, unlike those of the other Argentinean species whose lateral stems are ascendant. The Brazilian specimens of *P. interior* possess densely glandular-pilose stems, but Argentinean specimens have almost glabrous ones.

In Brazilian territory, distribution range of *P. interior* is restricted to western Santa Catarina and northwestern Rio Grande do Sul. It grows exclusively on the elevated gently hilly terrain with few grasses, above 500 m, surrounded by the steep slopes eroded by the Uruguay river and its tributary streams. In the Argentinean territory, this species seems to be exclusive to a similar habitat in the high-altitude portion of Misiones, Sierra de Misiones.

Overall features in the morphology of *P. guarapuavensis* resemble those of *P. integrifolia*. In *P. guarapuavensis*, the interior of the corolla tube is whitish and completely covered with a marked, deep purple reticulation. In *P. integrifolia*, as well as in *P. inflata* and *P. interior*, the interior of the corolla tube is light to dark purple, and the rather obscure reticulation is restricted to the upper part of the tube.

The known habitat of *P. guarapuavensis* in Misiones is open roadsides within thick forest. Distribution range of *P. guarapuavensis* in Argentina is probably restricted to the northern lowlands of Misiones. In Brazil, it grows abundantly on the third high-plateau (Terceiro Planalto or Guarapuava High Plateau; after Maack 1968) of Parana and adjacent Santa Catarina. The elevation of the third high-plateau gradually decreases toward the west, and the frequency of *P. guarapuavensis* decreases proportionally.

Kokubun et al. (2006) reported that the Brazilian and Uruguayan subsp. *axillaris* differed in self-incompatible/self-compatible (SI/SC) status and floral morphology. *P. axillaris* occurs in temperate South America and consists of three allopatric subspecies: *axillaris*, *parodii*, and *subandina*. Previous studies have revealed that subsp. *axillaris* is SI, subsp. *parodii* is SC in Uruguay, and subsp. *subandina* is SC in Argentina. However, the SI/SC status over the entire distribution range is not completely understood. Kokubun et al. examined the overall SI/SC status of the respective subspecies in comparison with floral

morphology. The results confirmed that subsp. *parodii* and subsp. *subandina* were SC throughout the distribution range, and that subsp. *axillaris* was also SC in Brazil and in most of the Argentinean territory. The SI *P. axillaris* occurs in the natural population only between 34 and 36°S, along the eastern shore of South America. The Brazilian and Uruguayan subsp. *axillaris* differed in SI/SC status and floral morphology.

Recent reports (Aline et al. 2006) have indicated that the Serra do Sudeste region in the extreme south of Brazil is one of the centers of diversity of the genus *Petunia* and is characterized by the presence of species with different pollination syndromes. *Petunia exserta* has been found to occur only in four sandstone towers in a restricted area of this region (about 500 km) and has been characterized by its differentiated habitat and its floral characteristics adapted to ornithophily. Plants with intermediate floral morphology were found to occur in towers where *P. exserta* was sympatric with the sphingophilous *P. axillaris*, phylogenetically close to *P. exserta*, suggesting hybridization between them. Hummingbirds have been reported as responsible for the interspecific gene flow. Analysis of molecular variance revealed high interpopulation diversity among the towers. The low gene flow between populations is possibly related to the autochoric seed dispersion system.

11.2.2 Morphology and Anatomy

Petunias are tender perennials. They form self-branching mounds that are bushy to cascading, 1–2 ft high. Their leaves are ovate to ovate-lanceolate, 4–5 in. long. The trumpet-shaped flowers may be single or double, 2–5 in. across. They come in all shades and may be edged, striped, or starred with a contrasting color. Some are sweet smelling. Even though petunias are perennials, they are usually treated as annuals and raised from seed yearly.

Most of the varieties seen in gardens are hybrids (*Petunia* × *hybrida*). The origin of *P.* ×*hybrida* is thought to be by hybridization between *P. axillaris* (the large white or night-scented petunia) and *P. integrifolia* (the violet-flowered petunia). *P. axillaris* bears night-fragrant, buff-white blossoms with long, thin tubes and somewhat flattened openings. *P. integrifolia* has a somewhat weedy habit, spreading stems with upright tips, and small lavender to purple flowers. A wide range of flower colors, sizes, and plant architectures are available in both the hybrid and open-pollinated species.

Reis et al. (2002) studied the leaf anatomy of 16 species of *Calibrachoa* and eight species of *Petunia*. In *Calibrachoa* leaves, the vascular bundle sheath (endodermis) was formed by parenchymatous developed cells, different from those of the mesophyll. In *Petunia*, this sheath did not show a marked morphological differentiation. The *Calibrachoa* leaves could be separated according to the type of leaf margins, the distribution of the stomata on leaf surfaces, the organization of the mesophyll, and the morphology of the trichomes. Based on these results, an indented dichotomous identification key was elaborated for the species of the genus *Calibrachoa*. Ando et al. (2005) supported the separation of *Petunia* and *Calibrachoa* by chloroplast DNA analysis. Two groups in the *Calibrachoa* were also recognized with a high degree of confidence.

Petunias are generally insect-pollinated with the exception of *P. exserta*, which is a rare, red-flowered, hummingbird-pollinated species. The complexity and functional convergence of various traits within pollination syndromes (sets of floral traits that are adapted to particular groups of pollinators) are outstanding examples of biological adaptation. Petunia pollination syndromes are defined by four general aspects, viz., color, morphology, nectar, and fragrance. In the genus *Petunia*, complex pollination syndromes are found for nocturnal hawkmoths (*P. axillaris*) and diurnal bees (*P. integrifolia*), with characteristic differences in petal color, corolla shape, reproductive organ morphology, nectar quantity, nectar quality, and fragrance. Stuurman et al. (2004) dissected the *Petunia* syndromes into their most important phenotypic and genetic components. They appear to include several distinct differences, such as cell growth and cell division patterns in the basal third of the petals, elongation of the ventral stamens, nectar secretion and nectar sugar metabolism, and enzymatic differentiation in the phenylpropanoid pathway.

Ohya et al. (1996) identified and isolated three sucrose ester types from the leaf surface lipids of *Petunia hybrida*. They contained both unbranched and branched fatty acids (from C2 to C8). Further, one of these sucrose esters contained malonic acids.

The structures of the sucrose esters were as mentioned below:

(a) 2,3,4-*O*-tri-acyl-α-D-glucopyranosyl-4-*O*-malonyl-4-*O*- acyl-acetyl-β-D-fructofuranoside
(b) 2,3,4-*O*-tri-acyl-α-D-glucopyranosyl-4-*O*- acyl-acetyl- β-D-fructofuranoside
(c) 2,3,4-*O*-tri-acyl-α-D-glucopyranosyl-β-D-fructofuranoside

11.2.3 Taxonomy

Taxonomic hierarchy of petunia

Botanical name:	*Petunia* × *hybrida* Vilm.
Kingdom:	Plantae
Division:	Angiosperms
Order:	Solanales
Family:	Solanaceae
Genus:	*Petunia* Juss.

The genus *Petunia*, established by Jussieu in 1803, belongs to the family Solanaceae. Since the establishment of the genus, it has undergone constant restructuring and is still quite unsettled even today. The early literature on petunia is quite confusing, since the taxonomists of the era did not have ready access to foreign herbaria specimens or the scientific literature (Ando et al. 1992). The first *Petunia* species was collected by Commerson in Uruguay, but described by Lamarck (1793) as *Nicotiana axillaris*. Unaware of the description of *N. axillaris*, Jussieu in 1803 established the genus *Petunia* and described the taxon collected by Commerson in Argentina as *Petunia nyctaginiflora*. It was soon discovered that *N. axillaris* and *P. nyctaginiflora* were very closely related and *P. nyctaginiflora* was transferred to *Nicotiana* as *N. nyctaginiflora*. By 1825, it was well recognized that these two plants were the same taxon and were not *Nicotiana* species. The taxon was commonly known as *P. nyctaginiflora*. At this time, the modern nomenclature rules used today were not in place. It was in 1888 that Britton, Sterns, and Poggenburg properly changed the name of this taxon to *P. axillaris* (Lamarck).

Parodi discovered another taxon closely related to *P. axillaris* in Argentina. This taxon was distinguished from *P. axillaris* by its stamens of equal length and long narrow corolla tube and acute corolla apices, while *P. axillaris* has didynamous stamens and a short wide corolla tube. In 1930, Steere (1930) described this taxon as *Petunia parodii*. In 1979, Cabrera reduced the status of this taxon to a subspecies, *P. axillaris* subsp. *parodii* (Steere) Cabrera. Later, Ando (1996) described a second subspecies from Bolivia, *P. axillaris* subsp. *subandina* Ando. Its didynamous stamens and a long narrow corolla tube distinguished this subspecies.

This second *Petunia* taxon to be described was collected in Argentina by J. Tweedie. This taxon was described by W. Hooker (1831) as *Salpiglossa integrifolia*. It was clear that this taxon was not *Salpiglossis*; therefore in 1833, it was renamed *Nierembergia phoenicea* by Don (1833) and *P. violacea* by Lindley (1833). Once again because of nomenclature rules at that time, this species was commonly known as *P. violacea*. Later, Schiz and Thellung (1915) using modern rules changed the name to *P. integrifolia* (Hooker) Schinz et Thellung.

In the first petunia monograph (Fries 1911), division of the genus into two distinct subgenera, *Pseudonicotiana* and *Eupetunia*, was proposed. Species in the subgenera of *Pseudonicotiana* had long, narrow corolla tubes (hypocrateriform), while species in the subgenera *Eupetunia* had short, wide corolla tubes (infundibuliform) (Sink 1984c). Fries placed *P. axillaris* in *Pseudonicotiana* and *P. integrifolia* in *Eupetunia*. Fries also separated *P. integrifolia* into three distinct species (*P. violacea, P. inflata* Fries, and *P. occidentalis* Fries). *P. integrifolia* was not the accepted name in 1911. *Petunia violacea* had the largest flowers and pendent pedicels, while *P. occidentalis* had the smallest flowers and erect pedicels. The flower size and position of the pedicel of *P. inflata* were intermediate between *P. violacea* and *P. occidentalis*. In addition, Fries recognized a diminutive suspects of *P. violacea* (*P. violacea* subsp. *depauperata* Fries) with very small flowers and leaves.

Smith and Downs (1966) recognized *P. integrifolia* (*P. violacea*) and *P. inflata* as the same taxon (*P. integrifolia* var. *integrifolia*) and *P. violacea* subsp. *depauperata* and *P. occidentalis* as the same taxon (*P. integrifolia* var. *depauperata* (Fries) Smith et Downs).

Wijsman (1982) reported that both flower size and pedicel position in *P. integrifolia* could be characterized by geographical distribution. The more western ecotypes had smaller flowers and more erect pedicels. Therefore, he concluded that various taxons were

subspecies of a single broadly defined species. He recognized three different subspecies [*P. integrifolia* subsp. *integrifolia* (Fries) Wijsman, *P. integrifolia* subsp. *inflata* (Fries) Wijsman, and *P. integrifolia* subsp. *occidentalis* (Fries) Wijsman] and a single variety [*P. integrifolia* subsp. *integrifolia* var. *depauperata* (Fries) Wijsman]. Wijsman did not report on the relationship of *P. reitzii, P. saxicola, P. littoralis,* and *P. scheideana* to *P. integrifolia*.

In 1985, Wijsman and De Jong separated *Petunia* into two genera based upon chromosome number, flower morphology, and breeding behavior. The type species (*P. parviflora* Juss.) and all those species with a chromosome number $2n = 18$ remained in *Petunia*, while those species with a chromosome number of $2n = 14$ were transferred to *Stimoryne Rafin*. *Stimoryne* was selected because it was the oldest known name other than *Petunia* for a $2n = 14$ species. *P. integrifolia* was described by Rafinesque-Schmaltz (1836) as *Stimoryne purpurea*.

This resulted in the highly undesirable name change for the garden petunia. Therefore, Wijands et al. (1986) proposed that the second described species (*P. nyctaginiflora* Juss.) be conserved as the lectotype and it was agreed by the I.N.G. Committee (Brummit 1989). Wijsman (1990) then transferred the $2n = 18$ species to *Calibrachoa* Llave et Lex. *Calibrachoa* was selected because it was the oldest known name other than *Petunia* for a $2n = 18$ species; *Calibrachoa parviflora* was described by La Llave and Lexarza (1825) as *Calibrachoa procumbens*. Besides the obvious difference in chromosome number, *Calibrachoa* can also be distinguished from *Petunia* by their woody stems, conduplicate aestivation with two petals covering the other three, and seed coats with straight anticlinal walls. *Petunia* has non-woody stems, imbricate aestivation, and seed coats with wavy anticlinal walls.

Ando et al. (1995) completed an extensive morphological comparison using living material of all the described *Eupetunia* species. They concluded that all of the species, except *P. littoralis*, were clearly distinct. *P. littoralis* could not be distinguished from *P. integrifolia* subsp. *integrifolia* var. *depauperata*. In addition, all of the *P. integrifolia* subspecies in their native habitats were separately distributed and readily distinguished from one another.

During the 1990s, Ando and Hashimoto (1993, 1994, 1995, 1996) recognized four *P. integrifolia* taxa as distinct species (*P. bonjardinensis* Ando et Hashimoto, *P. altiplana* Ando et Hashimoto, *P. guarapuavensis* Ando et Hashimoto, *P. interior* Ando et Hashimoto, *P. bajeensis* Ando et Hashimoto, and *P. riograndensis* Ando et Hashimoto). In addition, they recognized a *P. scheideana* taxon as a distinct species (*P. mantiqueirensis* Ando et Hashimoto) (Ando and Hashimoto 1994).

The cultivated garden petunia is considered a complex hybrid between *P. axillaris* and *P. integrifolia*. The first report on the *P. axillaris* × *P. integrifolia* interspecific hybrid was by Atkins in 1834 and described by (Sweet 1935) as *Nierembergia atkinsiana*. Many forms of the hybrid using different parents and backcrosses quickly appeared in gardens (Loudon 1840). At this time, all the purple-flowered garden hybrids were called "*P. violacea*." By 1900, the true species had disappeared from cultivation (Ferguson and Ottley 1932). Even the herbarium specimen of *P. integrifolia* at the Royal Botanic Garden at Kew was determined to a *P. axillaris* × *P. integrifolia* hybrid (Anonymous 1918). In 1863, Vilmorin coined the term *P.* ×*hybrida* to represent the garden petunia.

Even today, there is still disagreement over whether many species of *Petunia*, like *P. inflata, P. occidentalis,* and *P. parodii*, are actually true species or are subspecies of either *P. integrifolia* or *P. axillaris* (Wijsman 1982; Griesbach et al. 2000; Mishiba et al. 2000).

Thus, *Petunia inflata* is treated taxonomically in various ways; it has been described as an independent species, treated as a synonym of *P. integrifolia*, and also regarded as a subspecies of *P. integrifolia*. To resolve the ambiguity involving the *P. integrifolia* complex (*P. integrifolia* plus *P. inflata*), Toshio Ando et al. (2005) analyzed 21 morphological characters of 113 natural populations of the *P. integrifolia* complex in Argentina, Brazil, Paraguay, and Uruguay and established the existence of a clear, statistically significant gap between the morphological measurements of the two groups, ensuring the accuracy of identification carried out in the field except for a probable hybrid swarm. The two groups were found to have geographically distinct distributions: the *integrifolia* group occurred in southern regions, whereas the *inflata* group occurred in northern regions. Based on the available evidence, it was concluded that the two groups are allopatric species, *P. integrifolia* and *P. inflata*, in agreement with the opinion of Fries (1911).

A taxonomic guide for the 14 currently recognized species of *Petunia*, some of which are restricted to very small geographic areas, has been provided by Stehmann et al. (2009). Such a guide is critical at a stage now where species diversity is in danger of diminishing significantly due to human intervention, particularly in the form of grassland destruction.

11.2.4 Cytology

The karyotype of *P. hybrida* was first studied by Marthaler (1936) who described seven pairs of chromosomes and concluded that they were easily distinguishable from one another. Later, Takehisa (1963) gave a karyotypic formula of *Petunia* chromosomes similar to that reported by Marthaler (1936). Thereafter, Maizonnier (1971) established a karyogram in which five chromosomes were rather easily distinguished, but chromosomes V and VI remained indistinguishable. Finally, chromosomes V and VI were discerned by Smith and Odu (1972) and Smith et al. (1973) through fluorescence and scanning reflectodensitometer analyses, which gave intensity curves for each of the seven chromosomes.

Pollen diameter (Ferguson and Collidge 1932), chloroplast number in guard cells (Mitchell et al. 1980), stomata length (Santos and Handro 1983), and microfluorimetry (Galbraith et al. 1981) have been used to simplify the determination of ploidy level. Under certain instances, these techniques may not be reliable. For example, chloroplast counts measure only the polyploidy level of the epidermis and will not be able to determine the ploidy of the gametes (Kamo and Griesbach 1989).

TAZ1 (tapetum development zinc finger protein 1; renamed from PEThy; ZPT3-2) cDNA was first isolated as an anther-specific cDNA from petunia. Kapoor et al. (2002) demonstrated an essential role for TAZ1 in the post-meiotic phase of tapetum development in petunia. TAZ1 showed a biphasic expression pattern. In the pre-meiotic phase, TAZ1 transcripts were found to accumulate in all cell types of the anther except the tapetum and gametophytic tissues, whereas the post-meiotic phase of anther development was characterized by expression exclusively in the tapetum. Silencing of TAZ1 by cosuppression resulted in aberrant development and precocious degeneration of the tapetum, followed by extensive microspore abortion that started soon after their release from pollen tetrads. A few pollen grains that survived showed reduced flavonol accumulation, defects in pollen wall formation, and poor germination rates.

11.2.5 Significance of the Species

11.2.5.1 Significance as a Research Species

The genus *Petunia* is associated with many and diverse biological and economic aspects. The use of petunia as a model plant species for biological investigations is due to the fact that plants can be readily grown from seed to flower in approximately 70–80 days, that the greenhouse or growth chamber cultural requirements are simple, and that abundant seeds are produced in about 4 weeks after pollination. Petunia may also be studied as a result of a culture problem or because it presents a unique biological system.

Since the genus was established in 1803 by Jusseau, the early studies were mainly concerned with taxonomic, cytological, and inheritance studies. Starting in the 1930s, papers were published on chromosomally induced variegation, mosaic color patterns, chromosomal behavior, self-incompatibility, and the biochemistry of flower color pigmentation. In the 1940s and 1950s, further genetic reports were issued on incompatibility, anatomical and reproductive studies, cytoplasmic inheritance, and the inheritance of flower color. Research reports in the 1960s were concerned with biochemical studies of the pigments responsible for flower color, in vitro fertilization, cytoplasmic sterility and restoration genes, karyotype analysis, inheritance of the grandiflora–multiflora character, air pollutant studies, refined biochemical analysis of incompatibility reactions, pollen grain composition, guidelines for seed germination, and early seedling growth.

From the early 1970s to the present time, studies were reported on the enzymatic and other molecular changes in styles of self-compatible and incompatible lines, ultrastructure of the female reproductive apparatus, and additional information on flower color pigmentation, genetics of air pollutant resistance, cytoplasmic sterility, and cytogenetics. Plant nutrient

levels were also established; the biochemical and metabolic response to pathogens and parasites, inheritance studies and tissue, anther, protoplast culture, and related genetic transformation studies were reported.

The various characters of the *Petunia* genus that facilitate studies in genetics and molecular biology have been reviewed by Bianchi and Dommergues (1979) and by Hanson (1980). For identical reasons, *Petunia* was chosen early to investigate the effects of mutagenic treatments (Cornu 1970, 1977). These characteristics include:

1. Low chromosome number ($x = 7$) and thus few linkage groups.
2. Ease of sexual reproduction. Crosses and selfs are easily performed. A single fruit contains hundreds of seeds. The large number of flowers per plant leads to a tremendous reproductive potential.
3. Short reproductive cycle of around 3 months, so that three to four generations can be obtained in one year.
4. Ease of asexual propagation which can be achieved by traditional horticultural techniques (e.g., cutting, grafting) or by in vitro techniques (apex, foliar, and floral explants). In this way, non-transmissible mutations can easily be maintained.

Considering the ease of using *Petunia* material and with the help of marker genes scattered on every chromosome, numerous research studies could be conducted including the study and the development of new mutagenesis techniques, the analysis of genetic effects of mutagens, and the use of induced mutations for different purposes.

Petunia has been used as a model system for many genetic studies, most notably in the analysis of genetic regulation of anthocyanin pigmentation (Napoli et al. 1990) and gene silencing (Metzlaff et al. 1997; Que et al. 1997). In addition to the availability of accumulated genetic data for well over 100 loci (Gerats et al. 1993), its ease of transformation, relatively short regeneration period (Napoli et al. 1990), and production of hundreds of progeny per cross have made petunia an attractive choice for transgenic research.

Garabagi and Strommer (2000) examined a variant of the gene encoding green fluorescent protein (GFP), *mgfp5* in petunia, for its ability to meet the critical attributes of a reporter gene viz., ease of scoring for activity and capacity for expression in all cell types. Under regulation of the cauliflower mosaic virus (CaMV) 35S promoter, GFP was detectable in all vegetative and most floral cell types. Promoters from petunia *adhl* and *adh2* allowed for production of GFP in those few cell types lacking GFP production from the CaMV 35S promoter, verifying its capacity for expression in all cell types. With the appropriate promoter, GFP fluorescence was thus readily detectable throughout the plant.

Some of the important qualities of the petunia model system as highlighted by Gerats and Vandenbussche (2005) are enlisted below.

- Easy transformation procedures for a defined set of varieties (including the species *P. axillaris*).
- Large and expanding set of functionally and molecularly well-characterized genes involved in, for example, flavonoid synthesis and diverse aspects of plant development.
- Availability of large sets of mutants, mainly caused by insertion of endogenous transposable elements.
- Sophisticated methods for forward and reverse screenings of such mutants.
- Intriguing genome behavior (genetic linkage maps are among the smallest on record, indicating huge blocks of recombination inhibition, which might be related to the hybrid origin of most cultivars).
- Amenity for cytogenetic analysis.
- Amenity for biochemical analysis because of its large leaves and flowers.

There are three useful greenhouse varieties namely the Mitchell variety, which is a doubled haploid from a complex hybrid between *P. axillaris* and the cultivar "Rose of Heaven" that exhibits superior fertility, growth, tissue culture, and transformation abilities; the line V26, a bluish purple line that has been used for antisense and cosuppression studies, flavonoid gene isolation, and ethyl methane sulfonate mutagenesis; and the line W138, which is practically untransformable but is renowned for its active endogenous dTph1 transposable element system and which has already produced many interesting mutants (Gerats et al. 1990; Koes et al. 1995; Vandenbussche et al. 2003). A recombinant inbred line collection of species crossed with W138 lines and a collection of 1,250 stabilized insertion lines is also available (Stuurman et al. 2004).

A major advantage of petunia is that the system combines so many excellent technical features with a broad range of research possibilities and topics.

Furthermore, petunia as a model excels in areas such as the use of endogenous transposable elements for forward and reverse genetics and the analysis of diverse topics such as branching patterns, volatile production, pollination syndromes, and mycorrhiza–plant interactions.

11.2.5.2 Significance as an Economic Plant Species

Petunia ranks first among bedding plants. It is generally grown as an annual from seed each year for outdoor decorative purposes in prepared ground beds and window boxes. Some cultivars can be grown in various types of containers, their pendulous growth-flowering habit adapting them as decorative plants often termed hanging baskets.

In the horticultural industry, petunias are found in a variety of forms for landscape, home garden, and container use as mentioned below:

Growth habit:	Upright, spreading, semi-trailing, trailing
Leaf color:	Light to dark green
Flower color:	Solid colors – Single colored flowers
	Bicolors – A colored flower with a white star, formed by white petal stripes joining at the center of the flower
	Picotees – The flower is edged or bordered with white. Some varieties feature fringed or ruffled petal edges instead of the usual smooth edged flower
Flower type:	Double and single
Flower size:	Grandiflora – plants typically having large flowers with wide sepals, thick filaments, and large stigmas
	Multiflora – plants typically having small flowers with narrow sepals, thin filaments, and small stigmas

11.2.5.3 *Petunia* spp. as a Weed

P. axillaris finds a place in the "Global Compendium of Weeds" (GCW). Given below are the citations of references to this species as a weed (Website for further information: mailto:webmaster@hear.org?).

- Alanen et al. (2004). Introduced Species in the Nordic Countries (Denmark) under Nordic Council of Ministers (NMR), subgroup Natur-og Friluftslivsgruppen (cultivation escape)
- Argentina AGRO Management Malezas Incluidas. Argentinian Software Development Company. (weed)
- Auld BA, Medd RW (1992) Weeds: an illustrated botanical guide to the weeds of Australia. Inkata, Melbourne. ISBN/ISSN:0909605378 (weed)
- Germplasm Resources Information Network, GRIN (naturalized)
- Holm LG, Pancho JV, Herberger JP, Plucknett DL (1979) A geographical atlas of world weeds. Wiley, NewYork, USA (weed)
- Introduced (Naturalized) Species to the United States [USDA, NRCS 1999]. The PLANTS database. National Plant Data Center, Baton Rouge, LA 70874-4490, USA (naturalized)
- John Hosking, NSW Department of Agriculture, Weed Database 30 April 2003 (agricultural weed, naturalized)
- Marzocca A (1994) Guia Descriptiva De Malezas Del Cono Sur. Instituto Nacional De Tecnologia Agropecuaria (weed)
- Richardson FJ, Richardson RG, Shepherd RCH (2006) Weeds of the South-East. An identification guide for Australia. Meredith, Victoria. ISBN 0958743932, 438 pages (weed)
- Wildlife Atlas CAPS plant taxa – Census of Plants in New South Wales National Parks? New South Wales National Parks and Wildlife Service (weed)

11.3 Role in Elucidation of Origin and Evolution of Allied Crop Plants

11.3.1 Related Crop Plants

The Solanaceae members have held great interest for many researchers, breeders, and consumers for a long time. The Solanaceae family is composed of more than 3,000 species, including the tuber-bearing potato (*Solanum tuberosum*), a number of fruit-bearing vegetables: tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and peppers (*Capsicum annuum*), ornamental plants (petunias – *P. hybrida*, *Nicotiana*), plants with edible leaves (*Solanum aethiopicum, Solanum macrocarpon*), and medicinal plants (*Datura*, *Capsicum*) (Knapp et al. 2000). The Solanaceae is the third most important plant taxon economically, the most

valuable in terms of vegetable crops, and the most variable of crop species in terms of agricultural utility. In addition to their role as important food sources, many solanaceous species have a role as scientific model plants, such as tomato and pepper, for the study of fruit development (Gray et al. 1992; Fray and Grierson 1993; Hamilton et al. 1995; Brummell and Harpster 2001; Alexander and Grierson 2002; Adams-Phillips et al. 2004; Giovannoni 2004; Tanksley 2004), potato for tuber development (Prat et al. 1990; Fernie and Willmitzer 2001), petunia for the analysis of anthocyanin pigments, and tomato and tobacco (*Nicotiana tabacum*) for plant defense (Bogdanove and Martin 2000; Gebhardt and Valkonen 2001; Li et al. 2001; Pedley and Martin 2003).

11.3.2 Molecular Characterization

A method was developed by Griesbach et al. (2000) to characterize the genetic heterogeneity of the chalcone synthase gene intron within the *P. integrifolia* (Hook.) Schinz & Thell. species complex. The DNA from wild species collected from known locations was used to amplify the chalcone synthase gene intron through the polymerase chain reaction (PCR). The resulting PCR product was then characterized by *Rsa*1 restriction, revealing a degree of heterogeneity that could be used to characterize the species genetically. Of the four different species that were characterized, two could be placed in the same genetic grouping. The study led to the inference that variation in the intron of the *ChsA* gene may be species-specific.

Kulcheski et al. (2006) compared representatives from 11 *Petunia* species in South and Southeast Brazil with two *Calibrachoa* La Llave & Lex., one *Bouchetia* Dunal, and two *Nierembergia* Ruiz & Pav. taxa in relation to DNA molecular variability. A total of 4,532 bp related to one nuclear, five plastidial and one mitochondrial systems was investigated. *Petunia* and *Calibrachoa*, although separated among themselves, clearly differentiate from the two other genera. Despite the fact that the *Petunia* species do not show marked molecular differences, they can be separated in two complexes, in good agreement with altitude data. *Petunia* + *Calibrachoa* should have diverged from other clades at about 25 million years before present.

Zhang et al. (2008) reported on molecular discrimination among taxa of *P. axillaris* complex and *P. integrifolia* complex based on *PolA1* sequence analysis. The authors sequenced the 19th intron and the 20th exon of the *PolA1* gene, a single gene encoding the largest subunit of RNA polymerase I, and showed clear differences between *P. axillaris* complex and *P. integrifolia* complex, and also among all the taxa within each complex.

11.4 Role in Development of Cytogenetic Stocks and Their Utility

11.4.1 Ploidy

The basic chromosome number of *P. hybrida* is $x = 7$, $2n = 14$. As early as in 1937, Levan published a summary of chromosome numbers found in *Petunia* progenies by different workers; all chromosome numbers from 14 to 35 were reported, with the maxima at the diploid, triploid, and tetraploid levels. Octoploidy and, later, haploidy completed the list of viable ploidy levels in *Petunia*. Thus, ploidy in *Petunia* may be classified into three groups: haploidy, polyploidy, and aneuploidy.

11.4.1.1 Haploidy

A very small frequency of haploidy (0.04%) was reported to occur when triploid females were crossed with diploids (Straub 1973). Maizonnier (1973) reported 20 haploids, of both androgenetic and parthenogenetic origins that appeared following different types of mutagenic treatments and also in their untreated controls. Singh and Cornu (1976), using seedling markers, obtained 16 androgenetic haploids with maternal cytoplasm, which determined pollen sterility, with a frequency of $1–2 \times 10^{-4}$. Some haploids also appeared in the material of the Genetics Institute of Amsterdam (Smith et al. 1973; Wiering and de Vlaming 1973). On the basis of these results, one may suppose that haploidy in *Petunia* is spontaneous and occurs with a frequency of about 10^{-4}.

From his analysis of meiosis in petunia haploids, Maizonnier (1974, 1976a, b) observed that generally the seven chromosomes yield univalents, but sometimes associations forming bivalents and even chains of three to six chromosomes may occur. If in certain cases one may suspect that heterologous associations involving several chromosomes are due to segmental homologies, some bivalents seem to present undisputable chiasmata. In AI, most of the pollen mother cells (PMCs) show an unequal distribution yielding unbalanced end products. However, in a proportion of meiocytes, the univalents split instead of distributing and give rise to dyads with two viable pollen grains. In some cases, after splitting, the resulting 14 chromosomes do not migrate to the poles; they produce a restitution nucleus and finally a diploid spore. The same phenomena – at least production of egg cells with seven chromosomes – must exist during megasporogenesis as evidenced by the diploid progeny from selfed haploids (Maizonnier 1976b). Thus, haploid *Petunia* is not fully sterile and can yield as high as 30% stainable pollen grains.

Sen (1969) observed non-specific pairing between non-homologous chromosomes during pachytene and bivalents and trivalents at diakinesis and metaphase I. He observed the splitting of univalents in 8% of the PMCs.

Among the 20 haploid plants cultivated in Dijon, Maizonnier (1973) observed different behavior with respect to doubling ability. Some haploids doubled spontaneously, yielding easy-to-isolate diploid sectors. Periclinal cytochimeras were also identified; these were from plants with small stomata and small flowers but contained the usual amount of pollen for a diploid plant. Diploid progeny was obtained by selfing such haploid–diploid chimeras. In other cases, doubling was artificially obtained by applying colchicine–DMSO mixtures to haploid buds.

Polyploidization has been obtained also by means of in vitro cultures of haploid pedicels (Pelletier and Delise 1969), which regenerated diploid and even tetraploid buds. However, some haploidy remained despite attempted doubling treatments. Thus, not all haploid plants appear to have the same doubling ability.

Haploid petunias are not sterile and can produce up to 30% viable pollen (Maizonnier 1974). Nearly all of the progeny is diploid, the result of unreduced gametes. Diploid plants can also arise through tissue culture. In culture, haploid plants are unstable and frequently give rise to diploid shoots. These "doubled-haploid" diploids are extremely useful for genetic studies for they are homozygous for all genes. A "doubled-haploid" petunia, "Mitchell," was widely distributed during the 1980s as a model plant for genetic studies (Ausubel et al. 1980). "Mitchell" arose from the anther culture of a plant from the cross *P. axillaris* × (*P. axillaris* × *P. hybrida* "Rose du Ciel"). The use of "Mitchell" as a model plant was superseded by *Arabidopsis*.

Hanson (1984) observed in his studies on pollen and anther culture that depending upon the genotype, up to 10% of cultured anthers can produce plantlets and that most of the regenerated plantlets are not haploid, but triploid. In different studies (Engvild 1973; Raquin 1973; Wagner and Hess 1974; Sangwan and Norreel 1975), the frequency of haploid plant ranged from 0 to 30%. Due to these difficulties, haploids are best obtained through triploid × diploid crosses.

11.4.1.2 Polyploidy

Tetraploidy

The first known tetraploid *Petunia* appeared in California in 1888 and was the starting point of the giant forms cultivated at the beginning of the century (Bailey 1910). Other spontaneous tetraploid plants were described by De Vilmorin and Simonet (1927), Kostoff (1930), Dermen (1931), Steere (1932), and Matsuda (1934). The colchicine induction of tetraploids in petunia has been reported by many authors (Blakeslee and Avery 1937; Dansereau 1938; Gyorffy 1938; Levan 1938; Nebel and Ruttle 1938; Simonet and Simonet 1938; Nishiyama 1938). Maizonnier (1976a) described a genetic system yielding natural tetraploids.

Most naturally occurring polyploids are the result of unreduced or 2*n* gametes (Matsuda 1927). In petunia, a few chromosomes frequently remain at the plate during metaphase of the first meiotic division. If there are more than one or two lagging chromosomes, the nuclear membrane forms around them and then expands to include the chromosomes at the poles. The resulting gamete is diploid. The frequency of unreduced gametes depends upon the genotype and the

environment. It is not uncommon to find 3% of the pollen to be diploid (Matsuda 1927).

As in most other species, tetraploidy in *Petunia* may lead to an increase in plant and flower size (Levan 1938); however, in certain cases, chromosome counts were absolutely necessary to distinguish them from diploids in a mixed population of the multiflora type (D Maizonnier unpublished data). Furthermore, some genes for flower size and shape, for instance *Un*, yield an increase in flower size and, with such a genetic background, polyploidy effects are difficult to distinguish from genetic ones.

Induced and spontaneous tetraploids have the same behavior at meiosis. They both form typical meiotic configurations and result in classical meiotic disturbances, aneuploidy, and poor fertility (Matsuda 1934; Levan 1938). Due to seed production problems and also to slow growth habits, tetraploids remain rather scarce among cultivars, and it seems that attempts to improve them by Muszynski (1975) have been unsuccessful.

Triploidy

Spontaneous triploids found among diploid progenies may be attributed to the functioning of unreduced gametes, which is a frequent phenomenon in the species (D Maizonnier unpublished data). Triploids have been obtained by crossing tetraploids with diploids and vice versa (Dermen 1931; Steere 1932; Matsuda 1935; Levan 1937). Among these results, it is worth mentioning the occurrence of a great number of diploid progeny with the combined parental characters when the seed parent was diploid (Steere 1932).

Meiosis of triploids has been reported as surprisingly regular with only trivalent configurations (Steere 1932) or rather abnormal with a mixture of trivalents, bivalents, and univalents (Dermen 1931).

Dermen (1931), Matsuda (1935), and Levan (1937) analyzed the progeny of selfs and crosses among triploids. Their results constitute the basis of most of the chromosome number series reported by Levan (1937) and others.

11.4.1.3 Aneuploidy

Presence of odd number of chromosomes in the complement rather than a multiple of the basic number is referred to as aneuploidy. The significance of aneuploids in general and trisomics in particular has been greatly appreciated for its value in fundamental genetics.

Monosomics

According to Rick (1971), *P. hybrida* is "an example of extreme tolerance of aneuploidy." Rick (1943) found three monosomics among 33 mutants with gross chromosomal deviations and following X-ray treatment. In one monosome, he attributed the pairing of the odd chromosome with another pair to the presence of a duplication.

Among the numerous mutants cytologically observed at Dijon since 1970, D Maizonnier (unpublished data) observed only one undisputable monosomic; the plant was a sectorial chimera. The fifth chromosome was involved and the monosomic was revealed since this chromosome carried dominant markers. The monosomic part of the chimera was propagated by cuttings, but selfed and crossed progeny did not yield monosomics. Maizonnier (1976b and unpublished data) analyzed numerous cases of partial monosomy, that is, deletions of varying size and involving several different chromosomes. Semi-sterility of pollen was generally observed but no deleted gamete was transmitted.

Trisomics

The usefulness of trisomics in cytogenetics and breeding researches of diploid species is well known and has been adequately discussed (Khush 1973). Trisomic series are being produced in an increasing number of crops to localize genes on to specific chromosomes and to determine phenotypic effects of individual chromosomes. Trisomics are of practical use in genetic studies because they produce progeny with modified segregation ratios for marker genes present on the trisomic chromosome, facilitating the localization of genes, establishment of linkage groups, and to identify chromosomes involved in translocations.

Although Khush (1973) mentioned the existence of Levan's complete trisomic set, these were never fully described. Heseman (1964) tried to distinguish trisomics by such characters as number of pollen grains

with four pores and number of chloroplasts in stomata guard cells. But the results are not convincing and not confirmed in Maizonnier's material (D Maizonnier unpublished data). Some trisomics were used to localize six mutations, but at that time the only recognizable chromosome was the second, with its satellites, on which Heseman (1964) supposed the self-incompatibility *S* locus to be located.

The first analysis of all seven primary trisomics was performed by Maizonnier (1976b), who established the correlation between each supplementary chromosome and its floral morphology and some other characters in homogeneous material of the *Multiflora* type. These trisomics were used to localize genes on all chromosomes except chromosome I (Maizonnier and Moessner 1979).

The trisomics reported by Levan (1937) originated from crosses involving trisomics with disomics and diploids with triploids. The trisomics of Heseman (1964) and Smith et al. (1975) originated among progenies of triploids. Maizonnier (1976a, b) described no less than seven possible sources, including crosses between diploid individuals. Once trisomics were known well enough to be easily recognized, they were found among most of the progenies grown for mutagenesis or genetic experiments. Their appearance is likely due to non-disjunction, and in certain cases their frequency could be enhanced by induced deletions on the same chromosome (Cornu and Maizonnier 1979).

Maizonnier (1976b) demonstrated that the transmission rate of the supplementary chromosome through the female gamete varied from one trisomic to another (3–30%) for a given trisomic and from one experiment to another (about 4–37% for trisomic IV). No transmission was observed at all when the seven trisomics were used as pollen parents (D Maizonnier unpublished data). *Petunia* is one of those species in which a trisomic for a given chromosome may yield in its progeny trisomics for other chromosomes (Maizonnier 1976b).

Levan (1937) and Smith et al. (1975) reported the occurrence of plants with 16 chromosomes when triploid plants were pollinated with pollen of a diploid individual. One could think that these plants were tetrasomics ($2n + 2$), but experiments reported by Maizonnier (1976b and unpublished data) suggested that they are most often double trisomics ($2n + 1 + 1$). Maizonnier provided results of segregations for double trisomies II–VII and I–IV in his 1976b report.

China Pullaiah and Padmaja (1992) reviewed the information on aneuploids of petunia with particular emphasis on the origin, identification, utility, and characterization of primary trisomics based on cytomorphological as well as biochemical parameters.

According to them, primary trisomics reported in *P. hybrida* show significant morphological differences from those of the corresponding trisomics of *P. axillaris*. Identification of the extra chromosome involved in trisomy was mainly based on studies at pachytene stage since the pairing pattern at later stages did not reveal consistency. Biochemical studies mainly contributed to the localization of genes governing the enzymes under study on to the particular chromosomes. Multiple trisomics confirmed additive gene action of some of the genes governing plant stature.

White and Rees (1985) described the somatic hybrid between *P. parodii* ($2n = 14$) and *P. parviflora* ($2n = 18$) as an aneuploid with 31 chromosomes. The missing chromosome was a product of interchange between a large, satellited *P. parodii* chromosome and a medium sized chromosome from *P. parviflora*. Chromosome pairing at meiosis in hybrid PMCs is almost exclusively between strictly homologous chromosomes, that is, the hybrid behaves as an allotetraploid. The mean bivalent chiasma frequency in the hybrid is achieved in an unusual way, by increase in the bivalent chiasma frequency of *P. parodii* chromosomes relative to that in the parent PMCs, and decrease in the bivalent chiasma frequency of *P. parviflora* chromosomes relative to that in the parent. The distribution of chiasmata in the hybrid also differs from that in the parent. In the *P. parodii* chromosomes, there is a very pronounced increase in the frequency of interstitial chiasmata. In respect of both chiasma frequency and distribution, the results show that the *P. parodii* and *P. parviflora* chromosomes respond differently to control exercised by the hybrid genotype, in other words control is chromosome-specific.

Chromosome Fragments

According to Levan (1937), chromosome fragments in *Petunia* were discovered in 1933 in crosses involving diploid and tetraploid plants. Matsuda (1935) found fragments in the progeny of a triploid plant. Malinowski (1935) described B-chromosomes, which were probably only fragments. Finally, Maizonnier

(1976b) found 43 telotrisomics in several progenies of trisomic plants and later (D Maizonnier unpublished data) found other fragments in which the centromere was not inserted at the ends of the arms. The true telosomes are probably due to the phenomenon of misdivision described by Darlington (1939), but the others, with a subterminal or submedian insertion, are explained by a meiotic misfunctioning with breakages of chromosomes, probably due to chromosomal rearrangements. The fragments and especially the telosomes will be very useful for marking the 14-chromosome arms of the *Petunia* karyogram. Such fragments already have been utilized by Brewbaker and Natarajan (1960) to demonstrate their effects on the breakdown of self-incompatibility in *P. inflata* as a result of competition interaction.

Aneusomaty

In addition to aneuploidy found among individual plants within populations, aneuploidy also exists among somatic cells of an individual (aneusomaty) in *Petunia*. Takehisa (1961) reported chromosome numbers ranging from 14 to 28 in leaves. Furthermore, a peculiar case of aneusomaty was also observed by Maizonnier (unpublished data) in a plant with $2n = 13 +$ telo I, originating from a γ-ray treatment. The aneusomaty has been attributed to a selective endoreduplication.

11.5 Role in Classical and Molecular Genetic Studies

11.5.1 Use in Classical Genetic Studies

Petunia has a considerable body of genetic information and molecular tools. Its classical linkage maps comprise more than 100 markers (Gerats et al. 1993), and it was among the first plants to be transformed by *Agrobacterium* (Horsch et al. 1985). A non-autonomous transposable element (d*Tph*1) has been used extensively for reverse genetics and cloning of tagged mutations (Koes et al. 1995; Souer et al. 1996; Stuurman et al. 2002; Vandenbussche et al. 2003) and ranks among the most aggressive mobile elements known in plants. Many flower color genes have been isolated, including the regulatory locus that underlies the white color of *P. axillaris* (Quattrocchio et al. 1999).

The various characters of the *Petunia* genus that facilitate studies in genetics and molecular biology have been reviewed by Bianchi and Dommergues (1979) and by Hanson (1980). For identical reasons, *Petunia* was chosen to investigate the effects of mutagenic treatments (Cornu 1970, 1977). These characteristics include:

1. Low chromosome number ($x = 7$) and thus few linkage groups.
2. Ease of sexual reproduction. Crosses and selfs are easily performed. A single fruit contains hundreds of seeds. The large number of flowers per plant leads to a tremendous reproductive potential. The reproductive cycle is short, around 3 months, so that three to four generations can be obtained in one year.
3. Asexual propagation can be achieved by traditional horticultural techniques (e.g., cutting, grafting) or by in vitro techniques (apex, foliar, and floral explants). In this way, non-transmissible mutations can easily be maintained.

Considering the ease of using *Petunia* material and with the help of marker genes scattered on every chromosome, numerous research studies could be conducted including the study and the development of new mutagenesis techniques, the analysis of genetic effects of mutagens, and the use of induced mutations for different purposes.

11.5.2 Use in Cytological Studies

Petunia has been found ideal for studies of the mechanisms that result in the diversification of plant architecture because its body architecture is different from that of other common plant models (e.g., maize, *Antirrhinum*, and *Arabidopsis*) and because it lends itself well to molecular genetic studies (Angenent et al. 2005). Research using petunia has had a substantial share in the enormous progress that has been made in the understanding of the molecular and genetic control of meristem growth, maintenance, and differentiation into organs in plants. Integration of

information obtained from petunia has given clues about the common and diverged pathways underlying the formation and functioning of plant meristems.

For continuous growth and production of differentiated organs, plants rely on a well-balanced program of maintaining meristematic activity and cell identity determination. Many studies of this subject using multiple model species have increased the knowledge about the control of plant meristems. Although many of the molecular and genetic control mechanisms are conserved between distinct species, there are several reasons why plant scientists should continue this type of research with different model plants. First, the position and timing of differentiation in meristems from distinct species might differ, as observed by Angenent et al. (2005) for the racemose and cyme inflorescence structures for *Arabidopsis* and *Petunia*, respectively. Second, there are many examples of redundancy in genes controlling meristem development. Some are unique in *Petunia* but highly duplicated in *Arabidopsis* and vice versa, which favor paralleled approaches in both model plants. The transposon system in *Petunia* with corresponding populations comprising innumerable insertions will be a powerful tool for these studies. Further, the relatively large meristems make *Petunia* an ideal system for physical, (bio)chemical and hormonal experiments, which would reveal more information on meristem functioning.

11.5.3 Inheritance and Biochemistry of Pigments

11.5.3.1 Genetics of Flower Pigmentation in Petunia

Wiering and De Vlaming (1984) reviewed the reports related to the pigments in petunia. Since the introduction of *P. axillaris* and *P. integrifolia* in Europe between 1823 and 1835, crossings have been made between the two species (Bailey 1896; Ferguson and Ottley 1932). The first results of these experiments, with regard to flower color, were pictured in 1837 by both Harrison and Hooker. It is impossible to explain those results in terms of genes, although the pictures allow us to attribute some presently known alleles to them. Also, the experiments of Naudin (1865) and Hoffmann (1869), done before the rediscovery of Mendelian laws, have only historical value.

The first attempt to analyze the inheritance of flower color was made by West-gate (1911). Further experiments were executed by Lotsy (1912), Malinowski (1914), Malinowski and Sachs (1916), Rasmuson (1918), Ferguson and Ottley (1932), Ferguson (1934), Stormer and von Witsch (1938), Dale (1942), and Mather and Edwardes (1943). These authors explained the inheritance of flower and pollen color without examining the pigments involved. The translation of their results to presently used alleles remains very difficult.

A new period in flower color genetics in *Petunia* began with the introduction of paper chromatography in the analysis and identification of plant pigments. The first results using this method came from Kazuo (1952) and Werckmeister (1954), who published some paper chromatographical analysis of flower pigments. Paris and Haney (1958), Paris and Goldsmith (1959), and Paris et al. (1959) described 13 genes responsible for corolla pigmentation. Muller (1958) mentioned a gene for pollen color. Mosig (1960), Meyer (1964), and Van Wyk (1964) together reported 12 genes controlling the synthesis of flavonoid compounds. Bianchi (1960) reported two genes regulating the nature of the anthocyanidins in the corolla. Meynet et al. (1971) found six genes for which the influence on the phenotypic floral color and also chemical activity was described. Some of these six genes had already been mentioned by Chenault et al. (1968), but not all of them were indicated by the same symbols. The work of Meynet et al. (1971) was a continuation of work done by Dommergues and Cornu (1974) and Dommergues et al. (1974); they described 14 genes involved in the formation of flower color in *Petunia*. Meynet et al. (1971), Dommergues et al. (1974), and Vallade and Cornu (1979) established the existence of two new genes for flower color.

Hess (1969, 1970) also mentioned some genes involved in anthocyanin synthesis in petunia. Some work on the flower color genetics of *Petunia* has been done by El Gamassy et al. (1972) and Hussein and Misiha (1978). Wiering (1974) has described 23 genes of which 19 regulate the nature and the distribution of flavonoid compounds and four affect flower color without changing the flavonoids. Some of these genes are discussed in detail by Wiering and de Vlaming (1973, 1977). The collaboration between the

Genetical Institute of the University of Amsterdam and the Station d' Amelioration des Plantes, Centre de Recherches de Dijon has produced a list of genes found in *Petunia* (Wiering et al. 1979a). In this list, 32 genes are mentioned, which affect flower pigments, flower color, and color of the pollen.

11.5.3.2 Biochemistry of Flower Pigmentation in Petunia

The first authors who analyzed flower pigments in petunia were Willstatter and Burdick (1917). They found the diglucoside petunin and its aglycon petunidin. Beale et al. (1941) investigated the anthocyanins in *P. integrifolia*. Kazuo (1952) and Werckmeister (1954) published paper chromatographic results of their investigations on flower pigments in some *Petunia* cultivars. Birkofer and Kaiser (1962, 1963) and Birkofer et al. (1963, 1965) analyzed flavonoid compounds in petunia. They were able to determine the chemical structure of different glycosides of flavonoids.

The color of the corolla in *Petunia* is chiefly caused by the presence of anthocyanin compounds dissolved in the vacuole of epidermal cells. Under the influence of flavonols, also present in the vacuole, the color may become modified (copigmentation). Carotenoids play only a minor role in determining ultimate floral color. Anthocyanins consist of an aglycon, an anthocyanidin linked with one or more sugar residues and occasionally with one or more acid groups. The most important anthocyanidins are pelargonidin, peonidin, delphinidin, petunidin, and malvidin. All these anthocyanidin pigments occur in petunia, but pelargonidin is found only rarely and if so, it is present only in a very small quantity (Cornu et al. 1974; Wiering and De Vlaming 1984). Some important differences in color between the various anthocyanidins found in petunia appear to be caused by variations in the degree of glycosylation and by acylation.

Of the flavonols, myricetine is of fairly rare occurrence in *P. hybrida*; it is found only in some white flowering mutants. Flavonols are colorless to pale yellow and by themselves do not contribute much to the floral color. However, they form complexes with the anthocyanins present, which results in a marked bluing of the anthocyanidin color (copigmentation). For actual floral color, the pH in the vacuoles of the epidermis cells also seems to be important: the higher the pH of homogenates of the flower limb, the bluer the color. Concomitant with this bluing, a fading of color takes place. In pollen of some inbred lines, a yellow pigment can be found. This pigment has been identified by de Vlaming and Kho (1976) as 2′,4′,6′,4-tetrahydroxychalcone.

The groups of floral color genes distinguished in petunia are listed in Table 11.1.

Anthocyanins have the C15 skeleton, which is characteristic of flavonoids in general. The biosynthetic pathway of flavonoids has been thoroughly studied (Hahlbrock and Griesebach 1975, 1979).

A schematic outline of the biosynthetic pathway of flavonoids is given in Fig. 11.1.

The main precursor for all flavonoids is phenylalanine, which by the action of the enzyme phenylalanine ammonia-lyase is converted to cinnamic acid. This compound is further hydroxylated to *p*-coumaric acid. By condensation of *p*-coumaroyl-CoA with malonyl-CoA, the C15 skeleton of a chalcone is synthesized. Isomerization leads to a flavonone, naringenin, which is hydroxylated to dihydrokaempferol, a dihydroflavonol. The colorless dihydroflavonols are the direct precursors of anthocyanins. Further hydroxylation of dihydrokaempferol to dihydroquercetin or

Table 11.1 Groups of flower color genes distinguished in petunia

S. No.	Gene	Symbol
1	Anthocyanin genes	*An*
2	Hydroxylation genes	
	– Genes controlling substitution at position 3′	*Ht*
	– Genes controlling substitution at positions 3′ and 5′	*Hf*
3	Glycosylation genes	
	– Genes regulating the addition of a rhamnosyl group at position 3	*Rt*
	– Genes responsible for the introduction of a glucosyl group at position 5	*Gf*
4	Methylation genes	
	– Genes for substitution at position 3′	*Mt*
	– Genes for substitution at positions 3′ and 5′	*Mf*
5	Acylation genes	*Ac*
6	Flavonol genes	*Fl*
7	Genes responsible for a venation pattern on the corolla limb	*Ve* and *Fn*
8	Genes regulating the intensity of flower color	*In* and *Fa*
9	Genes regulating the pH of the vacuoles of the epidermis	*Ph*
10	Genes for pollen color	*Po*

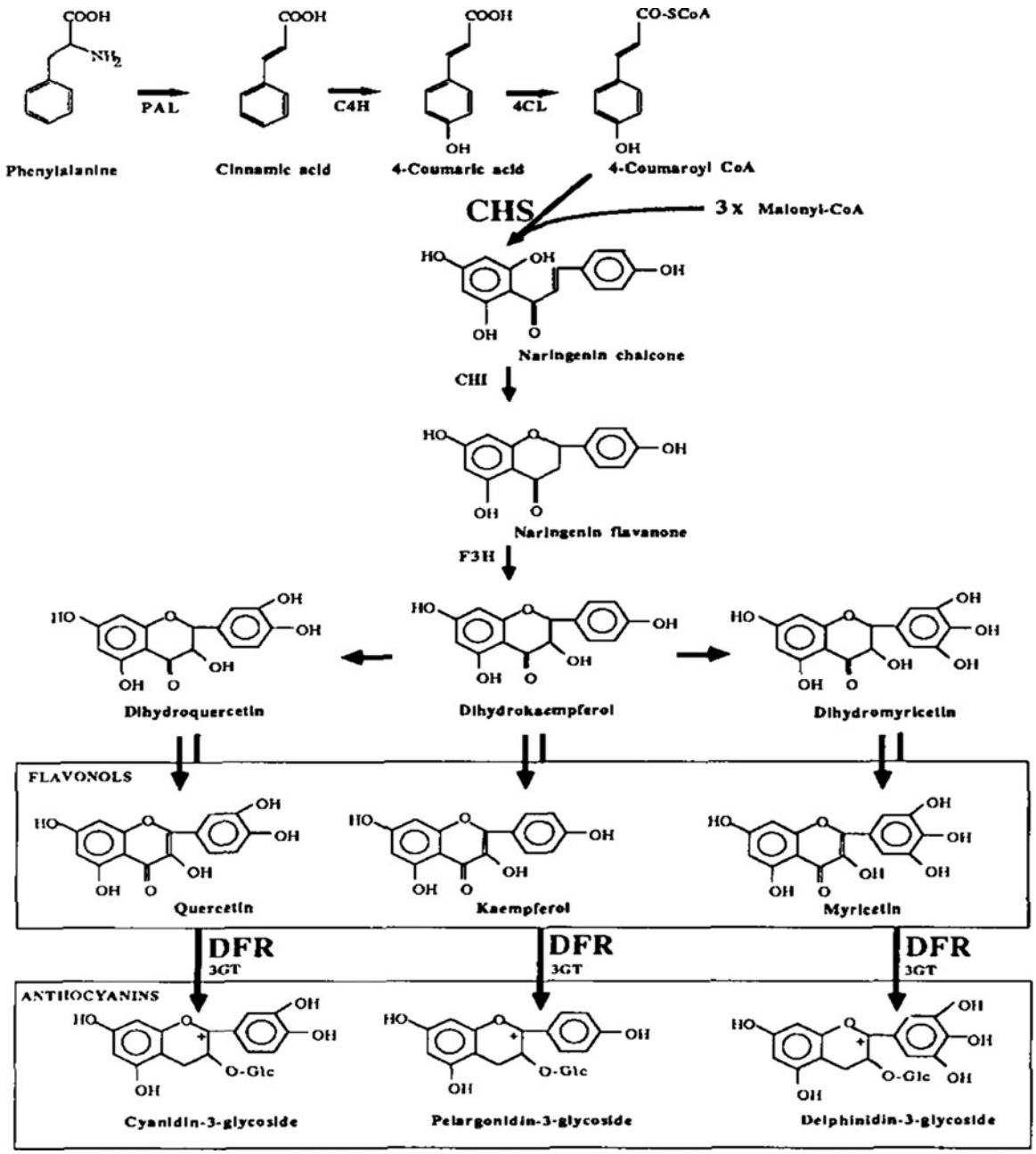

Fig. 11.1 Schematic representation of the flavonoid biosynthesis pathway (Adapted from van der Krol et al. 1990). Enzymes are abbreviated as follows: *PAL* Phenylalanine ammonia-lyase; *4CL* 4-Coumarate:CoA ligase; *CHI* Chalcone flavanone isomerase; *DFR* Dihydroflavonol-4-reductase; *C4H* Cinnamate 4-hydroxylase; *CHS* Chalcone synthase; *F3H* Flavanone 3-hydroxylase; *3GT* 3-*O*-flavonoid glucosyltransferase. Of the compounds shown, only the tetrahydroxychalcone (also named naringenin-chalcone) and the anthocyanins are pigmented. Tetrahydroxychalcone is yellow; the color of the anthocyanins depends on substitution of the flavonoid ring structure and ranges from red (cyanidin derivatives) to orange (pelargonidin derivatives) and purple (delphinidin derivatives). Pelargonidin derivatives are not synthesized in petunia because the petunia DFR enzyme does not recognize dihydrokaempferol as a substrate

dihydromyricetin provides substrates for conversion to the anthocyanidins cyanidin and delphinidin.

11.5.3.3 Molecular Events in the Anthocyanin Biosynthetic Pathway of Petunia

The expression of the structural genes encoding the enzymes of the anthocyanin pathway appears to be regulated differently in distinct species (reviewed in Mol et al. 1998; Weisshaar and Jenkins 1998). *P. integrifolia* and *P. axillaris* bear flowers with different shapes and colors that appear to be visited by different insects. *P. integrifolia* and *P. axillaris* subspecies occur naturally in South America in partially overlapping areas. Yet, both species remain genetically separated, apparently because their flowers are visited by different insects (Wijsman 1982, 1983). *P. integrifolia* flowers have a purple corolla with a short wide tube and are pollinated by bees, whereas *P. axillaris* flowers have a white corolla with a long narrow tube, which is typical of moth-pollinated flowers (Wijsman 1982). Manual cross-pollination, however, readily produces fertile progeny. In fact, the garden petunia (*P. hybrida*) is thought to be derived from such interspecific crosses (Wijsman 1983; Sink 1984c; Koes et al. 1987).

The *anthocyanin2* (*an2*) locus, a regulator of the anthocyanin biosynthetic pathway, is the main determinant of color differences. Quattrocchio et al. (1999) reported an analysis of molecular events at the *an2* locus that occur during *Petunia* spp. evolution. They isolated *an2* by transposon tagging and found that it encodes a MYB domain protein, indicating that it is a transcription factor. Analysis of *P. axillaris* subspecies with white flowers showed that they contain $an2^{-}$ alleles with two alternative frameshifts at one site, apparently caused by the insertion and subsequent excision of a transposon. A third $an2^{-}$ allele has a nonsense mutation elsewhere, indicating that it arose independently. The distribution of polymorphisms in $an2^{-}$ alleles suggests that the loss of *an2* function and the consequent changes in floral color were not the primary cause for genetic separation of *P. integrifolia* and *P. axillaris*. Rather, they were events that occurred late in the speciation process, possibly to reinforce genetic isolation and complete speciation.

11.5.4 Genetic Linkage and Mapping

The first available data pertaining to linkage relationships between genes of *Petunia* concerned the main anthocyanin genes that control flower color, for example, *An2, An4, Fl, Hfl, Po,* and *Rt*. It has readily been demonstrated that *An2, An4, Fl, and Hfl* segregate independently, while close linkage has been found to exist between *An2* and *Rt* and between *Po* and *Hf2* (Meynet et al. 1971; Dommergues and Cornu 1974; H Wiering and P de Vlaming personal communication). In connection with their studies on glycosylation and methylation genes, Wiering and de Vlaming (1973, 1977) observed independent inheritance for the following gene pairs: *Hf1*/*Gf, Hf1*/*Hf2, Hf1*/*Rt,* and *Mf1*/*Mf2* and linkage between *Gf*/*Hf2, Ht1*/*Mf1, and Ht2*/*mf2*. Besides research on the "*grandiflora*" character, dependent on the dominant gene *Un,* the independent inheritance of *Un* with regard to *Do* and *Gp* (Sink 1973) and to *Rt* (Smith et al. 1975) has been shown. These first results have led to easily recognizable phenotypes, namely *Hf1, Fl, Hf2, Rt,* and *An4*. Later on, *Htl* and *Bl* were chosen as representatives of the two remaining groups.

Various methods have been used by cytogeneticists to assign genes on chromosomes. For example, segregation analysis in progenies originating from trisomics identifies which chromosome carries a particular gene. Such results were first achieved by Smith et al. (1975) who assigned the locus *Un* to chromosome V, distinguished from chromosome VI by fluorescence and scanning reflectodensitometry. Thereafter, Maizonnier (1976b) who described the whole set of primary trisomics of petunia, subsequently determined the chromosomal localization of the seven linkage groups (Maizonnier and Moessner 1979). The trisomic method was also used by Van den Berg and Wijsman (1981b) to localize genes coding for several enzymes (Wijsman 1983). More accurate assignment of chromosomes could be carried out by using induced chromosomal rearrangements (Maizonnier 1976a, b).

Dana and Ascher (1986) studied a previously identified *S*-linked stylar-inactivation pseudo-self compatibility (PSC) factor (Flaschenriem and Ascher 1979) for its location relative to *S*. Plants exhibiting complete stylar-inactivation PSC were those with higher multigenic PSC background level than plants with only *S*-linked partial stylar-inactivation PSC. A pollen-

mediated pseudo-self-compatibility (PMPSC) adjustment factor was offered as a device to focus on stylar-inactivation PSC by removing some male origin, multigenic PSC. The stylar inactivation factor was not tightly linked to *S* but affected expression of only the allele to which it was linked. A three part interacting association of genetic material governing self-incompatibility (SI) was proposed. The parts of *S* are the SI identity gene, *S*-specific PSC genes and, finally, PSC genes, which are not *S*-specific in action. The complete association is termed the SI-complex.

Several attempts have been made to understand parts of the phylogeny of *Petunia* sensu Jussieu using molecular data, such as restriction fragment length polymorphism (RFLP) analysis of ribosomal DNA (Kabbaj et al. 1995), DNA amplification fingerprinting (Cerny et al. 1996), and polymorphism in the chalcone synthase intron sequence (Griesbach et al. 2000).

Strommer et al. (2000, 2002) used three segregating populations of petunia for RFLP mapping of genes and merged 27 loci with 11 previously mapped morphological and biochemical markers. In total five maps were constructed from two mapping populations, with placement of more than 800 markers. A phylogenetic analysis of *Petunia* using chloroplast DNA RFLP done by Ando et al. (2005) supported the separation of *Petunia sensu* Wijsman and *Calibrachoa*. Two groups in the *Calibrachoa* were also recognized with a high degree of confidence.

Hoballah et al. (2007) in their efforts toward identification and characterization of the genetic changes that caused the evolution of divergent pollination syndromes in closely related plant species, focused on *ANTHOCYANIN2* (*AN2*), a well-defined *myb*-type transcription factor that is a major determinant of flower color variation between *P. integrifolia* and *P. axillaris*. Analysis of sequence variation in *AN2* in wild *P. axillaris* accessions showed that loss-of-function alleles arose at least five times independently. DNA sequence analysis was complemented by functional assays for pollinator preference using genetic introgressions and transgenics. These results show that *AN2* is a major determinant of pollinator attraction. Therefore, changes in a single gene cause a major shift in pollination biology and support the notion that the adaptation of a flowering plant to a new pollinator type may involve a limited number of genes of large effect.

11.5.5 Male Sterility

Male sterility in petunia is expressed as the lack of pollen grains in the anthers of otherwise normal flowers. The lack of pollen is a result of breakdown in microsporogenesis or gametogenesis.

Male sterility types in *Petunia* can be classified as being under nuclear or under cytoplasmic-nuclear control:

Type 1: The first type of male sterility includes different male sterile phenotypes occurring spontaneously by selfing of plants from outcrossing populations. These phenotypes may be partially or fully male steriles. As an example, Izhar (1973) described the occurrence of cell budding and other abnormal divisions in young microspores of several highly inbred lines of *Petunia*. No viable pollen grains were produced as a result of these divisions.

Genetically, these sterilities may be caused by accumulation of recessive deleterious alleles as a result of homozygosity. An indication of that effect comes from the fact that fertility is restored immediately when these male steriles are crossed with normal lines. In some advanced, inbred lines, even female sterility can be observed. This type of sterility is not useful in practice for hybrid seed production because of its instability and the female sterility and association with general weakness of the plant.

Type 2: The second type of male sterility consists of genic male steriles (GMS) in which certain defined alleles were recognized as controlling male sterility. The first case of monogenic male sterility was described by Frankel (1962, 1971). A male-sterile plant appeared in a F_2 population of plants derived from selfing of a fertile scion grafted on a cytoplasmic male sterile (CMS) stock.

Type 3: The third type of male sterility is cytoplasmic–nuclear, commonly referred to as cytoplasmic male sterility (CMS). Sterility is conditioned by interaction between plasmon and the proper non-restorer alleles in the nuclear genome.

Izhar and Frankel (1976) attempted to determine whether cms in *Petunia* is conditioned by a single (S) plasmon or there are more than one as in maize (Duvick 1965), or *Nicotiana* (Gerstel 1980). Comparative study of several cms sources obtained from different *Petunia* geneticists, using restorer genes strongly indicated that, unlike maize or *Nicotiana*,

there is only a single (S) plasmon in *Petunia* (Izhar and Frankel 1976).

One of the reasons *Petunia* is becoming a model plant for somatic hybridization studies is the fact that CMS was shown to be a very convenient extranuclear marker (*cms*) after it had been successfully used in asexual transmission via grafting (Frankel 1956; Edwardson and Corbett 1961; Bianchi 1963) and by protoplast fusion (Izhar and Power 1979; Izhar and Tabib 1980).

A mitochondrial gene that encodes CMS in petunia has been cloned by Bentolila et al. (2002). The gene encodes an abnormal protein that disrupts mitochondrial activities. A nuclear gene (the restorer fertility or *Rf* gene) is known to interact with this mutant mitochondrial gene, reducing its expression and thereby restoring normal fertility to male sterile genotypes. The group has identified the *Petunia Rf* gene by cloning candidate genes from map position and demonstrating that such a gene is able to confer fertility to CMS *Petunia* lines. Subsequently, genes highly similar to the *Petunia Rf* have been identified as fertility restorers in *Brassica* and rice (Hanson and Bentolila 2004).

11.5.6 Self-Incompatibility

In several plants, reproductive mechanisms have evolved to prevent self-pollination. One such mechanism is self incompatibility (SI). SI can be due to either the gametophyte or sporophyte genotype. In sporophytic SI, the pollen's response is determined by its parental genotype, while in gametophytic SI the pollen's response is determined by its own genotype. Only the *Eupetunia* expresses gametophytic SI (Dowd et al. 2000; Robbins et al. 2000). The *Pseudonicotiana* species are self-compatible producing seed upon self-pollination. SI is controlled by a multiallelic *S*-locus. Fertilization is prevented when the *S*-alleles are expressed in the pistil. Incompatible pollen usually germinates, but does not grow past the upper third of the style.

In *Petunia*, 19 different *S* alleles have been described (Mather 1943; Linskens and Straub 1978; Broothaerts et al. 1989; Ai et al. 1991; Robbins et al. 2000; Wang et al. 2001). Phylogenetic analysis suggested that the different alleles arose through intragenic recombination (Wang et al. 2001). The *S*-alleles within the pistil encode *S*-RNases, which have two highly variable regions (HV_a and HV_b) and five conserved regions (C1 through C5). The exact nature of the pollen *S*-alleles has not yet been elucidated; however, they are physically linked to the pistil *S*-alleles (McCubbin et al. 2000). A potential pollen *S*-allele encoded protein has been identified (Sims and Ordanic 2001). This protein (PhSBP1) is only expressed in pollen and binds to *S*-RNases with a high degree of specificity. PhSBP1 contains a RING-HC domain, which functions as an E3 ubiquitin ligase. This suggests that phSBP1 might function by degrading *S*-RNases.

P. axillaris has been known to display a type of gametophytic self-incompatibility, which is controlled by a single multiallelic locus, called the *S*-locus. Survey by Tsukamoto et al. (1999) in Uruguay revealed that of more than 100 natural populations of *P. axillaris* in the region, the majority of the populations of subspecies *axillaris* were comprised of virtually all self-incompatible individuals. The rest were "mixed populations," which contained mostly self-incompatible and some self-compatible individuals. Analysis of the *S*-genotypes of the few self-compatible plants and the possible causes for the breakdown of their self-incompatibility revealed that the breakdown was caused by suppression of the production of S-RNase from the *S*-allele they all carry. The authors have termed this phenomenon "stylar-part suppression of an *S*-allele" or SPS. Further, the authors have shown that the failure to produce S-RNase in the self-compatible individuals was not caused by deletion of the S-RNase gene or by mutations in the promoter of the S-RNase gene, but rather by a modifier locus that suppressed the expression of the S13-RNase gene (Tsukamoto et al. 2003).

RNA differential display was used by McCubbin et al. (2000) to identify pollen cDNAs of *P. inflata*, a self-incompatible species, which exhibited restriction fragment length polymorphism (RFLP) for at least one of the three *S*-haplotypes (*S1*, *S2*, and *S3*) examined. The genes corresponding to ten groups of pollen cDNAs were found to be genetically tightly linked to the *S-RNase* gene.

11.5.7 Mutagenesis and Mutability

The various characters of the *Petunia* genus that facilitate studies in genetics and molecular biology have

been reviewed by Bianchi and Dommergues (1979) and Hanson (1980). For identical reasons, *Petunia* was chosen early to investigate the effects of mutagenic treatments (Cornu 1970, 1977). These characteristics include:

1. Low chromosome number ($x = 7$) and thus few linkage groups.
2. Ease of sexual reproduction. Crosses and selfings are easily performed. A single fruit contains hundreds of seeds. The large number of flowers per plant leads to a tremendous reproductive potential. The reproductive cycle is short, around 3 months, so that three to four generations can be obtained in one year.
3. Asexual propagation can be achieved by traditional horticultural techniques (e.g., cutting, grafting) or by in vitro techniques (apex, foliar and floral explants). In this way, non-transmissible mutations can easily be maintained.

Considering the ease of using *Petunia* material and with the help of marker genes scattered on every chromosome, numerous research studies could be conducted including the study and the development of new mutagenesis techniques, the analysis of genetic effects of mutagens, and the use of induced mutations for different purposes.

The first pollen treatments with X-rays showed that *Petunia* pollen is extremely radio-resistant (Gilissen 1978). Rick (1943) and Muller (1958) assessed the effects of radiation on chromosomes of petunia. With the aid of lines carrying marker genes involved in the anthocyanin syntheses, Dommergues and Cornu (1974) could measure directly the frequencies of induced mutations after pollen irradiations, which correspond directly to loss or change of physiological functions as detected on the flower.

The use of mutagenesis in *Petunia* is very positive as more than 40 loci were discovered in this way. At present, numerous mutations are used as genetic markers of chromosomes. Among them, chlorophyll mutations have an advantage as seedling markers and they are scattered on all the chromosomes.

Induced mutations at the level of anthocyanin loci may yield new flower colors for plant breeding. New alleles induced at loci *Rt* and *An4* give rise to new anthocyanin equilibria, which are transmissible to F_1 hybrids. By means of mutagenesis, lines with an increased amount of pelargonidin, a pigment which is scarce in *Petunia*, were obtained (Cornu et al. 1974). Kashikar and Khalatkar (1981) reported on the potential of physical (gamma rays) and chemical (EMS) mutagens to generate mutants of *P. hybrida* with altered flower color.

11.5.8 Genetic Instabilities

Genetic instabilities in *Petunia* are rather frequent. The most-documented cases concern floral pigmentation, which was first studied by Malinowski (1935). Two types of genetic instabilities exist:

1. Chromosomal changes whose somatic fate leads to an unequal distribution of genetic markers through breakage-fusion-bridge cycles (Maizonnier and Cornu 1971, 1979; Maizonnier 1976b)
2. A genic instability which generally gives rise to reversions to a normal activity of the gene

Petunia is particularly suitable for studies on instability phenomena, because there is extensive information on the genetics of anthocyanin biosynthesis and linkage groups. Furthermore, plants can easily be regenerated from variant sectors cultured in vitro. Most investigations on instability concern anthocyanin loci, that is, *Anl* (Bianchi et al. 1978), *An2,* and *Rt* (Cornu 1977), but an unstable dwarfing gene is also known (Bianchi et al. 1974), and among the chlorophyll variegations observed in *Petunia*, many cases of nuclear unstable genes probably exist.

Genetic instability in *Petunia* generally arises through the mutation of a dominant allele to a so-called unstable allele. The mutation may be spontaneous or induced. Instability for an anthocyanin gene is phenotypically characterized by frequent sectors, which are more deeply colored than the background. These sectors are due to "reversions" of the mutated allele toward a new, generally stable state.

11.6 Role in Crop Improvement Through Traditional and Advanced Tools

11.6.1 Breeding Efforts in Petunia

Breeding of petunias began about 30 years after the plant's discovery. One of the early hybridizers was

a Californian, Theodosia Burr Shepherd, who produced petunias with huge flowers. They were given the name Giants of California. This type of petunia is not often seen today, but seeds are still obtainable from a few specialty seed companies. "Can Can" and "Dwarf Ruffled Giants of California" are two varieties of this large-flowered type. The extensive range of modern hybrids is derived from just a few *Petunia* species native to South America.

The goal of petunia plant breeding is to develop new, unique, and superior petunia plants. The breeder initially selects and crosses two or more parental lines, followed by repeated selfing and selection, producing many new genetic combinations. The breeder can theoretically generate billions of different genetic combinations via crossing, selfing, and mutations. The breeder has no direct control at the cellular level. Therefore, two breeders will never develop the same line, or even very similar lines, having the same petunia traits.

The development of commercial petunia hybrids typically requires the development of homozygous inbred lines, the crossing of these lines, and the evaluation of the crosses. Pedigree breeding and recurrent selection breeding methods are used to develop inbred lines from breeding populations. Breeding programs combine desirable traits from two or more inbred lines or various broad-based sources into breeding pools from which inbred lines are developed by selfing and selection of desired phenotypes. The new inbreds are crossed with other inbred lines and the hybrids from these crosses are evaluated to determine which have commercial potential.

Commercially available petunia varieties are primarily F_1 hybrids. In F_1 hybrid varieties, pollen from an inbred "male" line is used to pollinate an inbred, but genetically different "female" line. The resulting F_1 hybrids are both phenotypically highly uniform and vigorous. In addition to this hybrid vigor, hybrids also offer opportunities for the rapid and controlled deployment of dominant genes. A homozygous dominant gene in one parent of a hybrid will result in all F_1 hybrids expressing the dominant gene phenotype. Within the seed trade industry, F_1 hybrids command the prominent role because of their superior vigor, uniformity, and performance.

11.6.2 Intergeneric, Interspecific, and Somatic Hybridization

Within the two subgenera (*Pseudonicotiana* and *Eupetunia*), geographic isolation is the primary reproductive isolating mechanism. Within a subgenus, the species are allopatric producing fertile progeny between any interspecific combination (RJ Griesbach unpublished data). The species in the different subgenera are syntopic. The range of the *Pseudonicotiana* and *Eupetunia* species overlaps, but hybrids are not commonly found. Lack of hybridization is due to the difference in pollinators (Ando et al. 2001). The *Pseudonicotiana* species are pollinated by nocturnally active hawkmoths (*Manduca contracta* and *M. diffusa*), while the *Eupetunia* species are pollinated by a diurnally active bee (*Hexantheda* sp.).

All *Pseudonicotiana* × *Eupetunia* hybrids are fertile, but vary in pollen viability (Watanabe et al. 1996). Even within a subgenus, pollen viability of interspecific hybrids can vary from 100% to less than 50% that of a sib-mated species control (Tsukamoto et al. 1998). Interestingly, the fertility of hybrids between species in the subgenera is not significantly less than that of hybrids among species within a subgenus.

P. axillaris × *P. integrifolia* is the most studied *Pseudonicotiana* × *Eupetunia* hybrid. The cultivated petunia (*P.* ×*hybrida*) was derived from this hybrid. Significantly higher success rates occur when *P. axillaris* is used as the female parent. Morphologically, the hybrid is intermediate between the parents except in flower color. In most instances, the hybrid produces purple flowers with the same intensity as the *P. integrifolia* parent. In advanced generations, aberrant segregation ratios occur. It was concluded that these aberrant ratios were caused by differential pollen growth (Mather and Edwardes 1943). Aberrant segregation can also be caused by recombination genes (Robert et al. 1991). A major gene (*Rm1*) and several modifier genes have been found, which enhance recombination frequencies of specific chromosome fragments.

Reibel et al. (2006) have invented a new allele designated "*gc1-1*" in the genus *Petunia* that is phenotypically expressed in altered flower color and/or flower color pattern. This mutant allele has been

determined to be a single dominant or partially dominant gene. The invention further provides plants, seeds, and other plant parts such as pollen and ovules containing the mutant allele. The invention also provides methods for evolving plants with altered flower color by employing the invented mutant allele. Further, the invention provides a method for producing an intergeneric *Calibrachoa–Petunia* hybrid comprising crossing first a *Petunia* parent with a second *Calibrachoa* parent and harvesting the resultant hybrid *Calibrachoa–Petunia* plant, wherein one or both parents contain the mutant allele. A representative sample of said seed containing said *gc1-1* allele has been deposited with the American Type Culture Collection (ATCC) at 10801 University Boulevard, Manassas, Va 20110–2209 on Dec 19, 2006 with the Deposit Accession Number PTA-8092 (http://www.faqs.org/patents/app/20080256658). The petunia mutant of the present invention was an unexpected result that arose from a spontaneous mutation in a "Supercascade White" commercial F_1 hybrid petunia population in May 2003.

Petunia is considered one of the model plants for tissue culture (Hanson 1980). Techniques for plant regeneration from almost any tissue have been reported (Izhar and Zeleher 1984). Unlike in most plants, petunia protoplasts are relatively easily regenerated into whole plants (Cocking 1981). Several wide hybrids between sexually incompatible species have been created, but only three have resulted in whole plants (Sink 1984a, b). The widest hybrids created were *P.* ×*hybrida* + *Salpiglossis sinuata* and *P. axillaris* subsp. *parodii* + *S. sinuata* (Lee et al. 1994). Putative hybrids were identified by chromosome and isozyme analysis.

The other wide hybrid between sexually incompatible species was *P. axillaris* subsp. *parodii* ($2n = 14$) × *C. parviflora* ($2n = 18$) (Power et al. 1980). The two most widely studied somatic hybrids (*P. axillaris* subsp. *parodii* × *P. integrifolia* subsp. *inflata* and *P. axillaris* subsp. *parodii* × *P.* ×*hybrida*) are between sexually compatible species (Powers et al. 1976, 1979). Both hybrids can be sexually produced and are fully fertile. The shape of the flowers and leaves of the somatic hybrids are slightly different from that of tetraploid forms of the sexually produced hybrids.

A large number of different *P. axillaris* subsp. *parodii* × *P.* ×*hybrida* somatic hybrids were analyzed by Izhar and Zelcher (1980). In the most common type, the hybrids contained both nuclear genomes. In the second type, only a single genome was present. In the hybrids with a single parental nuclear genome, the cytoplasmic genome contained chloroplast DNA (cpDNA) and mitochondrial (mtDNA) genomes from both parents. The term "cybrid" was coined for these hybrids. Cybrids have been used to determine the inheritance of mtDNA. The *P. axillaris* subsp. *parodii* × *P. integrifolia* subsp. *inflata* somatic hybrids were not as stable as their sexually produced counterparts (Schnabelrauch et al. 1985). Many of the plants produced branches with slightly abnormal leaves and flowers. A few plants were unstable aneuploids producing varying chromosome numbers over time. These plants expressed various degrees of abnormal floral and leaf morphology, as well as floral anthocyanin and leaf chlorophyll variegation. It was proposed that the instability was due to cytoplasmic/nuclear incompatibility analogous to hybrid dysgenesis. In plants, with abnormal leaf and flower development, an occasional branch is produced with normal flowers and leaves. cpDNA and mtDNA rearrangements and segregation was found to occur over time (Clark et al. 1986). All of the hybrids contained only the *P. axillarisi* subsp. *parodii* cpDNA genome. On the other hand, most of the hybrids contained only the *P. integrifolia* subsp. *inflata* mtDNA genome. Additional evidence for hybrid dysgenesis is found in the fact that the sexual hybrid can only be created when *P. axillaris* subsp. *parodii* is used as the female parent. The sexual hybrid is completely stable.

Glimelius and Bonnett (2004) fused protoplasts of a chloroplast-defective cultivar of *N. tabacum* with γ-irradiated protoplasts of *P. hybrida*. Over 100 photoautotrophic plants were regenerated; of these 94 were tested for *Petunia* chloroplast traits and all but one had *Petunia* chloroplasts based on their sensitivity to the fungal toxin, tentoxin. cpDNA was analyzed for 3 of the sensitive plants and was shown to be identical to *Petunia* cpDNA. Most of the plants (about 70%) appeared to be normal *N. tabacum* plants, based on morphology and chromosome number. They were fully fertile with normal pollen viability, seed set, and seed viability. The remaining 30% of the plants showed varying degrees of vegetative and reproductive abnormalities.

11.6.3 Genetic Transformation

11.6.3.1 Flower Color

One of the first practical examples of plant genetic engineering involved the development of a novel flower color in *Petunia* through the engineering of dihydroflavonol reductase (DFR). The *Petunia* DFR has an extremely low substrate specificity for dihydrokaempferol; therefore, pelargonidin is rarely found (Huitts et al. 1994). Mutants with a defective *Htl* gene would be expected to produce unpigmented flowers. Mutants with pelargonidin were generated through transformation (Griesbach 1993; Tanaka et al. 1995; Johnson et al. 1999).

A *Zea mays* DFR gene (*A1*) was introduced into RL01, a *Petunia* mutant with one-tenth the normal amount of anthocyanin and a negligible amount of pelargonidin (Meyer et al. 1987). The *Zea* enzyme recognizes both dihydroquercetin and dihydrokaempferol as a substrate, but has a much stronger affinity for dihydroquercetin. Pelargonidin is only produced in the absence of dihydroquercetin. When *A1* was expressed in RL01, there was a tenfold increase in the total amount of anthocyanin, as well as an increase in the relative amount of pelargonidin (Griesbach 1993). Introduction of a *Rosa* DFR gene into Skr4 × Sw63 resulted in a tenfold increase in the total amount of anthocyanin, as well as an increase in the relative amount of pelargonidin (Tanaka et al. 1995).

Chimeric DFR genes were constructed and introduced into *P.* ×*hybrida* "W 80," so as to identify the DNA sequence leading to substrate specificity (Johnson et al. 1999). It was determined that the substrate binding region was between amino acids 132 and 158 with amino acid 134 critical in substrate specificity. A switch from asparagines to leucine at position 134 caused a change in substrate preference from dihydroquercetin to dihydrokaempferol.

To evaluate the effect of increased expression of genes involved in flower pigmentation, van der Krol et al. (1990) transferred additional dihydroflavonol-4-reductase (DFR) or chalcone synthase (CHS) genes to *Petunia*. In most transformants, the increased expression had no measurable effect on floral pigmentation. Surprisingly, however, in up to 25% of the transformants, a reduced floral pigmentation, accompanied by a dramatic reduction of DFR or CHS gene expression, respectively, was observed. This phenomenon was obtained with both chimeric gene constructs and intact CHS genomic clones. The reduction in gene expression was independent of the promoter driving transcription of the transgene and involved both the endogenous gene and the homologous transgene. The gene-specific collapse in expression was obtained even after introduction of only a single gene copy. The similarity between the sense transformants and regulatory CHS mutants suggests that this mechanism of gene silencing may operate in naturally occurring regulatory circuits.

11.6.3.2 Alteration of Plant Form

Christopher Winefield et al. (1999) transformed Mitchell *Petunia* [*P. axillaris* × (*P. axillaris* × *P. hybrida*)] plants with the *rolC* gene from *Agrobacterium rhizogenes* under the control of the CaMV 35S promoter. Five transgenic lines expressing differing levels of *rolC* transcript were characterized in detail. The severity of phenotypic alterations were found to increase with increasing *rolC* transcript abundance with respect to reductions in plant height, leaf, and flower size, a break in apical dominance leading to increased branching and decreased male and female fertility. Time to flowering was also reduced in the *rolC* transgenic plants. The authors inferred that the *rolC* gene appears to be a useful tool for the development of cultivars of ornamental crops with alterations in several commercially important traits.

11.6.3.3 Extended Flower Longevity

Petunia × *hybrida* was transformed with *boers*, a mutated allele of *BOERS*, an ethylene receptor sensor gene of *Brassica oleracea* (Shaw et al. 2004). The allele *boers* was obtained by removing an *Eco*RI cutting site with a silent mutation at Gly-521 and introducing a point mutation at Ile-62, replacing isoleucine with phenylalanine. Transformation was *A. tumefaciens* mediated. Flowers of transgenic plants retained turgidity and pigmentation considerably longer than those of untransformed controls, whether left undisturbed on plants or excised and placed in water. Furthermore, flowers were unaffected by exposure to exogenous ethylene. Excised shoots of transgenic

plants released considerably more ethylene than those of untransformed plants. Transformed plants also produced apparently larger flowers. Unexpectedly higher mortality was observed, suggesting that the ethylene insensitive petunia plants were also lower in disease resistance.

11.6.3.4 Alteration of Response to Ethylene

Transgenic petunias generated by incorporating the plant expression vector of antisense *ETR1* cDNA from *Rosa* "Texas," pBinETR1D3 showed reduced levels of ethylene sensitivity, opening the possibility to obtain ethylene insensitive cut rose cultivars through molecular breeding (Zheng et al. 2005).

Using transgenic ethylene insensitive (44568) and Mitchell diploid petunias, Beverly et al. (2005) demonstrated that multiple components of emission of volatile organic compounds (VOCs) are regulated by ethylene. Expression of benzoic acid/salicylic acid carboxyl methyltransferase (*PhBSMT1* and *2*) mRNA is temporally and spatially down-regulated in floral organs in a manner consistent with current models for post-pollination ethylene synthesis in petunia corolla. Emission of methylbenzoate and other VOCs after pollination and exogenous ethylene treatment parallels a reduction in *PhBSMT1* and *2* mRNA levels. Under cyclic light conditions (day/night), *PhBSMT* mRNA levels are rhythmic and precede emission of methylbenzoate by approximately 6 h. When shifted into constant dark or light conditions, *PhBSMT* mRNA levels and subsequent methylbenzoate emission correspondingly decrease or increase to minimum or maximum levels observed during normal conditions, thus suggesting that light may have a more critical influence on cyclic emission of methylbenzoate than a circadian clock. Transgenic PhBSMT RNAi flowers with reduced *PhBSMT* mRNA levels showed a 75–99% decrease in methylbenzoate emission, with minimal changes in other petunia VOCs. These results implicate *PhBSMT1* and *2* as genes responsible for synthesis of methylbenzoate in petunia.

11.6.3.5 Prevention of Leaf Senescence

Using a promoter from a senescence-associated gene (*SAG 12*) fused to the isopentenyl transferase (*IPT*) gene, which catalyzes the rate-limiting step of cytokinin production, Dervinis et al. (1998) produced transgenic petunia plants with delayed leaf senescence. Bai et al. (2009) attempted prevention of leaf senescence of petunia. The cytokinin biosynthetic gene isopentenyl transferase (*ipt*) was placed under the control of CaMV 35S promoter and introduced into petunia. PCR analysis showed an expected 0.5 Kb fragment of *ipt* gene in transgenic petunia. RT-PCR analysis indicated the expression of *ipt* gene in the transgenic lines. Leaves from transgenic plants remained green and healthy in normal culture condition, while the non-transformed plants turned to yellow. Transgenic plants showed a reduction in height and smaller leaf sizes. In transgenic lines, the internodes were shorter, and the roots grew slower than the non-transformed plants.

11.7 Conservation Initiatives and Genomic Resources

11.7.1 The SOL Genomics Network (SGN; http://sgn.cornell.edu/)

The SOL Genomics Network (SGN; http://sgn.cornell.edu/) is a genomics information resource for the Solanaceae family and related families, with the aim of building a comparative bioinformatics platform for answering questions regarding adaptation, evolution, development, defense, biochemistry, and other facets pertaining to plants belonging to the Solanaceae family and related families. The efforts of SGN are focused primarily on the following four areas:

1. Cataloging and maintaining genetic maps and markers of the Solanaceae species.
2. Disseminating sequence information for the different species of Solanaceae, mostly in the form of expressed sequence tags, for which SGN generates and publishes unigene builds.
3. Cataloging and publishing phenotypic information.
4. Assembling, analyzing and publishing data from the recently commenced sequencing of the tomato (*S. lycopersicum*) genome.

The Solanaceae genomes have undergone relatively a few genome rearrangements and duplications and therefore have very similar gene content and

order. This exceptionally high level of conservation of genome organization at the macro- and micro-levels makes this family a model to explore the basis of phenotypic diversity and adaptation to natural and agricultural environments. Recognizing these unique features of the Solanaceae, the International Solanaceae Project (SOL) was launched, and the project addresses two key questions:

1. How can a common set of genes/proteins give rise to the wide range of morphologically and ecologically distinct organisms that occupy our planet?
2. How can a deeper understanding of the genetic basis of plant diversity be harnessed to better meet the needs of society in an environmentally friendly and sustainable manner?

11.7.2 The Petunia Platform (http://www.petuniaplatform.net/)

A database has been created, which holds over 5,000 sequence tags, many of which are in reading frames. The development of the Petunia Platform (http://www.petuniaplatform.net/) has brought together most groups working on Petunia.

11.7.3 The Solanaceae Germplasm Bank at the Botanical Garden of Nijmegen, Netherlands

The Botanical Garden of Nijmegen has built a germplasm collection of non-tuber bearing Solanaceae consisting of about 1,800 accessions, which represent 50 genera and approximately 550 species and subspecies of the Solanaceae (Gerard and van der Weerden. 1999). A major objective of the Nijmegen Botanical Garden is to create a Solanaceae Information Network database, which collates the accessions with the dispersed taxonomic as well as other relevant information on Solanaceae in the literature.

The Solanaceae germplasm collection of the Botanical Garden of Njjmegen was initiated more than 30 years ago and is the most extensive germplasm collection of non-tuberbearing Solanaceae in the world (Barendse and Van der Weerden 1996, 1997).

11.7.3.1 Ex Situ Conservation

Ex situ conservation of the genetic resources of Solanaceae is achieved through maintenance of a large living plant collection in connection with a seed bank. The living plant collection is especially useful to maintain perennial, recalcitrant, and vegetatively propagated species which often produce little or no seed. For some species they are the only available option to maintain germplasm for conservation. An important reason for maintaining a living collection is the production of seed for genebanking, the collection of herbarium material, and the ability to study the plants for proper identification and documentation. Seedbanking is the most cost-effective way in providing genetic resources for the long-term ex situ conservation and is mostly employed by botanic gardens and gene banks (Heywood 1989; Touchell and Dixon 1997). An important advantage of ex situ conservation is that the genetic resources of Solanaceae from the dispersed habitats are brought together on one or a few location(s), which make them more accessible to comparative taxonomic and other studies as well as to plant breeders.

In order to build a valuable germplasm collection of Solanaceae, seeds, and/or plant material, preferably from known wild origin, are acquired through collection and exchange, raised to seed for genebanking, taxonomically identified, and documented by description, photography and by collecting herbarium material. Over the years, the quality of the germplasm bank has been enhanced by taxonomic (re)identification and by replacing many of the accessions with material obtained from known wild origin. In order to enhance the gene pool (Swanson 1996) and to reduce genetic erosion, whenever possible, several accessions from different wild locations were acquired and maintained.

The need to improve accessibility and hence utilization of the genetic resources is widely recognized and has led to the concept of "core" collections (Hodgkin 1991). The Botanical Garden has built a specialized collection of Solanaceae, which approaches this concept of a core collection and which is internationally recognized. A core collection assumes a minimum of repetitiveness, which contributes to the development of an integrated conservation approach, ensures the conservation of a significant part of the diversity present in nature and is an indispensable complement to in situ conservation.

The core collection is also an important basis for research (e.g., molecular taxonomy as well as other studies) by the immediate availability of the same plant material to researchers worldwide. Moreover, plants in cultivation lend themselves to studies, which cannot be achieved in the dispersed and remote wild populations.

Quite a few of the accessions were derived from seeds collected in the wild many years ago (e.g., by R. N. Lester from Birmingham University, UK) and would be very hard to acquire again and include some rare and threatened species.

11.7.3.2 Computer-Based Reference System

Besides the database containing the Solanaceae accessions, an extensive computer-based reference system consisting of a bibliography of literature on Solanaceae has also been built.

11.7.4 *Working Groups on* Petunia

In the early 1980s, there were only a few laboratories that focused on *Petunia* as a model system, mainly the Genetics Institute at the University of Amsterdam (Netherlands) and a group at the INRA (Dijon, France). These two groups started to exchange material and data in 1978, which led to a merging of information on genetic maps and of mutant collections (including trisomics for each of the seven chromosome pairs). However, despite the passage of time, the genetic map of petunia has remained enigmatically small, probably because of the hybrid nature of the genome of most cultivars. One of the main outcomes of the collaboration between the two groups was the genetic and biochemical description of structural and regulatory genes involved in flavonoid synthesis. In the early 1980s, the classical description of endogenous transposable elements also took shape.

Around the same time, a new group was set up at the Free University of Amsterdam that ventured into the field of plant molecular biology with *Petunia* as the model system. Over the years, the group has contributed to the cloning and characterization of many flavonoid genes, antisense and cosuppression, and the isolation and use of endogenous transposable element systems. Although not a widely recognized model system of choice, *Petunia* has been useful, for example, for developing transformation methods, to map transgenes genetically as stable inherited units, to map transgenes by in situ hybridization, or to change flower color using transgenes.

From the late 1980s onwards, *Petunia* research proliferated further with progressively more work on various aspects of plant and flower development. Currently, there are 15–20 groups worldwide that employ *Petunia* as their main model plant system. A brief overview of active research areas in petunia is presented in Table 11.2.

Table 11.2 Overview of active research areas in petunia with some key references

Research areas	References
Taxonomy	Wijsman (1982)
	Ando (1996)
	Watanabe et al. (1999)
Flavonoid synthesis	Wiering and de Vlaming (1984)
	Schram et al. (1984)
	Koes et al. (1995)
Meristem activity	Angenent et al. (2005)
	Stuurman et al. (2002)
Floral development	Angenent et al. (1995)
	Vandenbussche et al. (2004)
	Nakagawa et al. (2004)
Genetic maps	Strommer et al. (2002)
	Robbins et al. (1995)
Transposons	Gerats et al. (1990)
	Stuurman et al. (2004)
	van Houwelingen et al. (1999)
Forward genetics	Zubko et al. (2002)
Epigenetics	Kooter et al. (1999)
	Meyer (2001)
Volatiles	Verdonk et al. (2003)
	Negre et al. (2003)
	Boatright et al. (2004)
Pollination syndromes	Stuurman et al. (2004)
Self incompatibility	Clark et al. (1990)
	Robbins et al. (2000)
Male sterility	Bentolila et al. (2002)
Transformation of plastids	Zubko et al. (2004)
Senescence	Clevenger et al. (2004)
	Clark et al. (2004)
Information on *Petunia* × *atkinsiana* D. Don ex Louden [*axillaris* × *integrifolia]*	USDA Natural Resources Conservation Service
An overview of the major research themes in petunia	Gerats and Vandenbussche (2005)
An exhaustive and valuable overview of research topics and publications on petunia up to 1980	Sink (1984c)

An overview of groups working with *Petunia* can be found at http://www.petuniaplatform.net where keyword descriptions of the research of each group are presented, together with links to their respective websites.

11.8 Future Thrust

Species diversity of *Petunia* has been reported to be in danger of diminishing significantly due to human intervention, particularly in the form of grassland destruction (Stehmann et al. 2009). Some of the 14 currently recognized species are restricted to very small geographic areas of South America, the native of petunia. Intensive conservation measures are the urgent need of the hour.

11.9 Conclusion

Petunia species has played a central role in transformation research since the earliest reports of plant transformation. Subsequently, it played an important role in elucidating many of the characteristics of plant transformation, including unique sites of insertion, variable expression levels, and modified T-DNA structures among independently derived transformants. It was central in the demonstration of transient expression immediately following cocultivation and transgene-induced silencing of gene expression, two phenomena currently of great importance in studies of gene function. Ease of transformation, coupled with other favorable biological characteristics, ensures that *Petunia* will remain a valuable model system for studies of gene function in plants.

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Chapter 12
Rosa

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12.1 Basic Botany of the Species

12.1.1 *Basic* Rosa *Taxonomy*

The genus *Rosa* has attracted considerable attention from taxonomists and numerous species have been described. Presently, about 100–250 species are usually recognized. Many of these species are now thought to have arisen by hybridization, often accompanied by polyploidization. In addition, extensive anthropogenic impact has led to the development of many new semi-wild and/or cultivated rose varieties. Consequently, taxonomy is not straightforward! Although much criticized in, e.g., numerous recent DNA-based analyses (see below), the classification system of Rehder (1940) or variations thereof (e.g., Wissemann 2003) still constitute the standard taxonomic treatment of this genus. Rehder (1940) recognized four different subgenera: *Hesperhodos* (two species), *Hulthemia* (one species), *Platyrhodon* (one species), and *Rosa*. Subgenus *Rosa* is furthermore divided into ten sections: *Pimpinellifoliae*, *Carolinae*, *Cinnamomeae*, *Synstylae*, *Caninae*, *Gallicanae*, *Indicae*, *Banksiae*, *Laevigatae,* and *Bracteatae*, but the five latter sections have only one to three species each. According to the nomenclatural code, it is advised to change sect. *Cinnamomeae* into sect. *Rosa* (McNeill et al. 2006) due to the generic type designation of *R. cinnamomea*. However, before this designation in 2006, the generic type was *R. centifolia* phylogenetically located in sect. *Gallicanae*, thus this name has also been much used for sect. *Gallicanae* (e.g., Wissemann 2003). To avoid confusion based on nomenclatural reasons, it is therefore avoided altogether in the following treatise to use sect. *Rosa*.

The wild ancestors of our domesticated ornamental roses are found mainly in the sections *Synstylae* (*R. moschata*, *R. wichurana,* and *R. multiflora*), *Gallicanae* (*R. gallica*), *Indicae* (*R. chinensis* and *R. gigantea*), and *Pimpinellifoliae* (*R. foetida*) (Wylie 1954). A smaller but still noticeable contribution has been made by, e.g., *R. spinosissima* in sect. *Pimpinellifoliae* and by *R. cinnamomea* and *R. rugosa* in sect. *Cinnamomeae*. *R. damascena* (sect. *Gallicaneae*) is also worth mentioning due to its considerable influence both as an ornamental and for the commercial production of rose oil. This rather small set of species has thus been instrumental in producing the enormous cornucopia of shape, color, and fragrance that we now enjoy in gardens and parks, and as pot plants and cut flowers. In addition, wild or semi-wild genotypes in, e.g., sect. *Caninae* (dogroses) are used as rootstocks and landscape plants. A possibly expanding area is the production of rose hips for culinary and medicinal purposes based mainly on species in sections *Caninae* and *Cinnamomeae*, and the chestnut rose *R. roxburghii* in subgenus *Platyrhodon*.

12.1.2 *Morphometry*

Traditionally, rose species have been defined according to quantitative and qualitative morphological characters like shape, size, and color of petals, sepals, and hips; inflorescence architecture; length of pedicel; presence or absence of glandular hairs; shape and size of leaves, leaflets, and leaflet indenture; and

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number, shape, and color of prickles. Quantitative (morphometric) characters show a continuous variation and are likely to be polygenically controlled whereas qualitative (descriptive) characters produce major discrete categories and are more likely to be monogenic in nature. Descriptive characters are often easier to evaluate in naturally growing populations and/or on herbarium sheets but may overemphasize the underlying genetic differentiation. Moreover, strong linkage among a few such genes may lead to parallel combinations of characters as evidenced in dogrose taxonomy (Nilsson 1999). Recent taxonomic studies have made use of morphological characters evaluated by hand or by automated image analysis, sometimes also involving electron microscopy (e.g., pollen and seed morphology). Most of these studies have, however, targeted either a restricted number of closely related taxa and/or a restricted geographic area. Several of these studies have concerned the allopolyploid and taxonomically very controversial sect. *Caninae*. In one study on morphometric variation in Nordic dogroses, only 65% of all evaluated plants could thus be referred to a single species according to a canonical variates analysis (Nybom et al. 1996). Another study on Nordic dogroses using Fourier coefficients of leaflet shape also detected some differentiation among six taxa but without clear-cut boundaries (Olsson et al. 2000). Similarly, much overlapping was found among seven investigated dogrose taxa in Belgium, investigated with a set of 17 morphometric and descriptive characters (De Cock et al. 2007, 2008). In all of these studies, considerable differentiation was, however, found between taxa belonging to different subsections (subsections *Caninae*, *Rubigineae,* and *Vestitae*) suggesting that these entities are rather well defined.

Allopolyploid speciation has also taken place in sect. *Cinnamomeae*. When Joly and Bruneau (2007) studied five diploid and three tetraploid taxa in eastern North America, they could not discern distinct groups when all samples were studied together using a set of 25 morphometric characters. Analysis of each ploidy level separately did, however, provide evidence of differentiation between some of the described taxa.

A general finding in the above-mentioned morphology-based studies is that single characters generally cannot discriminate completely among species. A multivariate approach is needed, and preferably both ordination and cluster analyses. Still, considerable overlapping is usually encountered, and many samples cannot be unambiguously classified into discrete species.

12.1.3 DNA-Based Taxonomy

Numerous DNA-based analyses have been applied to investigations of species differentiation and relationships in *Rosa*. The division into four different subgenera does not have much support in the DNA-based data. The first major study was published by Jan et al. (1999) who used random amplified polymorphic DNA (RAPD) markers and demonstrated that *Platyrhodon* and *Hesperhodos* should be placed within subgenus *Rosa*. In another study, Koopman et al. (2008) applied amplified fragment length polymorphism (AFLP) markers and showed that *Hulthemia* and *Platyrhodon* should be included in subgenus *Rosa*. The lack of a sound genetic basis for a subgeneric division has been demonstrated also in studies based on the sequencing of regions in the chloroplast-DNA (Matsumoto et al. 1998; Wisseman and Ritz 2005; Bruneau et al. 2007) as well as in the nuclear-DNA (Wisseman and Ritz 2005).

Division of species among the different sections in subgenus *Rosa* has also been problematic. The largest section in the genus is sect. *Cinnamomeae* (approximately 80 species), which should be merged with sect. *Carolinae* (approximately five species) according to analyses based on RAPD (Jan et al. 1999), AFLP (Joly and Bruneau 2007; Koopman et al. 2008), simple sequence repeat (SSR or microsatellites markers, Scariot et al. 2006), and sequencing data (Wisseman and Ritz 2005; Bruneau et al. 2007). In addition, sect. *Pimpinellifoliae* (approximately 15 species) is clearly polyphyletic (Matsumoto et al. 1998; Wisseman and Ritz 2005; Koopman et al. 2008), and some of its species apparently belong together with sections *Carolinae* and *Cinnamomeae* (Scariot et al. 2006; Bruneau et al. 2007).

Two major clades have been identified in the genus, with *Carolinae*, *Cinnamomeae,* and parts of *Pimpinellifoliae* in one clade and most of the other sections in the other clade (Jan et al. 1999; Bruneau et al. 2007). In this second major clade, sect. *Synstylae* (approximately 25 species) shows considerable similarities with sect. *Gallicanae* (Koopman et al. 2008) and

with sect. *Indicae* (Wu et al. 2000; Wisseman and Ritz 2005). Removal of the only European species in section *Synstylae*, *R. arvensis*, would result in an apparently monophyletic section (Matsumoto et al. 2000; Wu et al. 2000; Koopman et al. 2008). However, the largest member in this second clade is the sect. *Caninae* (approximately 50 species, also known as dogroses). Early in the last century, several hundreds of taxa were described but more critical evaluations, both in the field and in herbaria, have later prompted a reduction to approximately 50 dogrose species (Wissemann 1999, 2002, 2003). DNA analyses suggest that this section constitutes a well-circumscribed monophyletic group (Matsumoto et al. 2000; Wisseman and Ritz 2005; Scariot et al. 2006; Koopman et al. 2008). Although sharing some internal transcribed spacer (ITS) sequence types with species in other sections, thereby confirming their hybridogenous origin, the *Caninae* species also have one unique ITS sequence type, which is further evidence of their monophyly (Ritz et al. 2005; Kovarik et al. 2008). Of the five described subsections in sect. *Caninae*, only subsection *Rubigineae* is well defined according to AFLP data (De Cock et al. 2008; Koopman et al. 2008).

Interestingly, almost all of the horticulturally important species belong to this second clade, thus suggesting that the wealth of cultivated roses has a surprisingly narrow genetic basis (Matsumoto et al. 1998).

Only a few in-depth studies of species delimitations have been carried using DNA markers. In sect. *Caninae*, ordination analyses based on RAPD and AFLP, respectively, produced fewer but larger groups of samples compared to results obtained with morphological characterization, and several commonly recognized taxa overlapped completely in their DNA profiles (Olsson et al. 2000; De Cock et al. 2008). A set of five diploid and three tetraploid and probably hybridogenous species in sect. *Cinnamomeae* were studied using AFLP and morphometry (Joly and Bruneau 2007) as well as sequencing of the nuclear *GAPDH* gene (Joly et al. 2006). Similar patterns for species delimitations were found, but evidence for the exact origination of the allopolyploid species was rather inconclusive.

The relationships among cultivated and wild material have been investigated in several studies. AFLP analysis thus showed that most cultivars grouped either into a European cluster related to *R. damascena* and *R. gallica*, or into an Oriental cluster related to *R. moschata*, *R. wichurana,* and *R. multiflora* (Koopman et al. 2008). The first cluster contained mainly European cultivars belonging to the Alba, Centifolia, Damask, Gallica, Moss, and Portland cultivar groups. These groups are derived from the old European garden roses in sect. *Gallicanae*, but often with some contribution also from the Hybrid China roses. This European cluster also showed affinity with sect. *Caninae*, which may contain the seed parent of the Alba roses. The Oriental cluster instead contained cultivars that belong mainly to the Bourbon, Moschata, Multiflora, Noisette, Polyantha, and Tea groups together with *R. moschata*. Another cluster with *R. wichurana* and *R. multiflora* was sister to this group. The Hybrid perpetuals, which are derived from crosses between Hybrid China roses and *R. gallica*/*R. damascena* hybrids, appeared to be closer to the latter.

Similarly, SSR analysis of both wild species and cultivars produced a large and mainly European cluster, which also contained the Hybrid China roses and one Noisette cultivar (Scariot et al. 2006). This cluster showed affinities to both the dogroses in sect. *Caninae* and to the Alba roses. Species and cultivars involving the remaining sections, *Indicae*, *Carolinae* and *Cinnamomeae*, and *Synstylae*, were further apart in the dendrogram.

12.1.4 Phylogeny

Both morphological and DNA-marker differentiation among rose species involve mainly novel character combinations caused by gene flow instead of novel character states caused by the amassing of mutations over a long time period. Similarly, the extremely low levels of DNA sequence divergence found across the genus (Matsumoto et al. 1998; Wisseman and Ritz 2005; Bruneau et al. 2007) suggest that this is a young genus where much speciation has taken place after the last glaciation. Poor phylogenetic resolution and commonly occurring contradictions between chloroplast and nuclear gene phylogenies also suggest that hybridization has been a strong driving force in the evolution of roses.

In contrast, the fossil record points to an old origin of *Rosa*. Fossil record including hips date back into the

Miocene and Oligocene, so approximately 30 million years ago (Kvacek and Walther 2004). It is therefore conceivable that the origin of *Rosa* is quite ancient, but the radiation and diversification process is recent. Clearly, further research into the age of *Rosa* is required.

So far, the wealth of genomic data has not been successfully used to produce a comprehensive phylogeny of *Rosa* species. As mentioned earlier, one of the reasons is that divergence is recent, thus making it difficult to use coding gene sequences for the production of well-supported trees. Nonetheless, at least one partial phylogenetic tree has been published based on *OOMT* 1 and 2 (Scalliet et al. 2008), reflecting the history of a gene duplication in Chinese and European roses. Because they evolve faster than coding sequences, non-coding sequences (intronic or promoter regions of genes) should be more useful for phylogenetic inference in *Rosa*. Characterizing haplotypes could also be useful to understand past hybridization and/or polyploidization events; for example, based on alcohol dehydrogenase (*ADH*) haplotypes, it was possible to conclude that the tetraploid *R. gallica* may have originated from an interspecific hybridization between one *Cinnamomeae* and one *Synstylae* species (O. Raymond unpublished data). Future research should focus on the evolution of the regulatory regions of some key genes to morphological and/or architectural innovation such as the transcription factors controlling floral organ identity (e.g., Kitahara and Matsumoto 2000).

12.2 Conservation Initiatives

Cultivated roses have a very ancient history and the first selections were reported already in the early sixteenth century. Later on, artificial crossing led to what is today perceived as the "modern rose cultivars." However, the genetic basis on which these modern rose cultivars are established is poorly understood. It is thought that only between 8 and 20 species out of about 200 have contributed to the origin of our present cultivars (de Vries and Dubois 1996; Reynders-Aloisi and Bollereau 1996; Gudin 2001).

Martin et al. (2001) tried to clarify the domestication history by DNA analysis with RAPD markers of 100 cultivars of old roses. They showed that selection resulted in the retention of only a small number of alleles during the process of rose domestication. These alleles probably originate from *R. chinesis* for characters concerning color and recurrence, and from European groups for those concerning hardiness and flower complexity. Hence, genetic diversity in wild species may be used to increase the diversity for specific traits in cultivated roses. This has already been done to some extent but there are many more traits that could prove very valuable for cut and garden rose improvement. It is one of the reasons why genetic diversity in these wild species should be conserved.

For in situ conservation, we would need to know how large the variation is in wild *Rosa* populations. For *Rosa canina*, Jürgens et al. (2007) found a high level of genetic variation within populations whereas also population differentiation between regions was very high, as can be expected given the breeding system of this species. Hence, populations should be conserved across a large region. De Cock (2008) and De Cock et al. (2008) describe the genetic diversity within and between populations of various *Rosa* species in Flanders and in some western European countries, as determined using AFLP. They showed that the European *R. spinosissima*, *R. gallica*, *R. majalis*, *R. pendulina*, *R. arvensis,* and *R. sempervirens* populations showed strong geographical genetic differentiation. However, in many cases there was no consistent differentiation based on taxon or on geographical pattern. For instance, the three taxa *R. canina*, *R. corymbifera*, and *R. balsamica* showed a higher interspecific similarity when sampled at the same location compared to their congeners sampled at other localities in Flanders. Apparently, for these taxa the locality is a more accurate predictor of genetic relatedness than the taxonomical determination. This is perhaps not so surprising considering the taxonomic problems in the genus, but it means that in situ conservation efforts should try to cover as many populations as possible.

Given that the taxonomy is not always clear, it is not straightforward to predict how much genetic variation resides within the wild species. The good cross-species transferability of SSR markers across the genus *Rosa* (another indication that it is a young genus) will enable the study of genetic diversity across the whole genus (Scariot et al. 2006) and a reasonable balanced assessment of the levels of diversity in the different species groups.

Ex situ conservation takes place by collecting plants and maintaining them in botanical gardens and, typical for roses, in rose gardens. There are many rose gardens in the world. However, they contain relatively large collections of cultivated roses. The wild material has been described taxonomically, but there is no overview of how accurately this has been done. As rose gardens exchange material, just as botanical gardens and genebanks, they collectively may conserve only a tiny amount of the variation present in the wild.

12.3 Role in Elucidation of Origin and Evolution of Rose

12.3.1 The Origin of Damask Roses

Damask roses are well known for their strong fragrance (Widrlechner 1981). From a historical and geographical perspective, the Damask roses are considered to originate from Persia (today Iran). By the fourteenth century, the Damask roses were already available in West Europe (Beales et al. 1998). Some Damasks have been maintained in West European rose collections as garden roses ("York and Lancaster," "Quatre Saisons," "Quatre Saisons Blanc Mousseux," "Kazanlik," and others). During the nineteenth century, the Damask roses are thought to have played a substantial role in the improvement of the modern European hybrid roses. The most significant Damask rose from a commercial point of view is the 30-petalled *R. damascena* "Trigintipetala" which is cultivated for rose oil production in Bulgaria, Turkey, Iran, India, China, and northern Africa.

In 1941, Hurst classified the Damask roses into two groups according to their flowering time: Summer Damasks that bloom only in early summer and Autumn Damasks that have a second blooming in the autumn. This classification was based on morphological and general botanical observations, which can often be misleading. In-depth investigations of the actual existing genetic diversity in this group of roses based on DNA genotyping have only recently been conducted. Iwata et al. (2000) analyzed two Summer Damask varieties ("Kazanlik" and "York and Lancaster") and two Autumn Damasks ("Quatre Saisons" and "Quatre Saisons Blanc Mousseux") and found no difference in their DNA profile using 24 RAPD primers. Agaoglu et al. (2000) found no difference among accessions of *R. damascena* plants in Turkey using RAPD markers. Baydar et al. (2004) demonstrated that 15 *R. damascena* plants brought from 15 different plantations in Isparta province, which is the main rose growing region in Turkey, possess identical genotypes based on AFLP markers and nine microsatellite loci. Rusanov et al. (2005a) characterized a total of 40 Damask rose accessions of which 25 originated from Bulgaria (the collection of the Institute of Roses and Aromatic Plants, Kazanlak) using microsatellite markers derived from *Rosa wichurana* and *Rosa hybrida*. The results showed that all analyzed "Trigintipetala" accessions and the old garden Damask rose varieties "York and Lancaster" and "Quatre Saisons" (in confirmation of Iwata et al. 2000) possess identical genotypes. In Iran more than one genotype was found, but the genotype in the main production area was identical to "Trigintipetala" (Babaei et al. 2007). In conclusion, it appears that the industrial production of rose oil in Bulgaria, Turkey, and to a great extent in Iran is based on a single genotype (and mutants thereof). An interesting observation in this study was the reported high somatic stability of the analyzed microsatellite loci as the allele sizes of the 33 assayed SSR loci had remained unchanged in accessions, which have been vegetatively propagated for centuries in different geographical regions.

The studies of Babaei et al. (2007) and Kiani et al. (2008) identified non-"Trigintipetala" genotypes, mostly in the mountainous northwestern part of Iran, with microsatellite alleles that are not present in the Bulgarian and Turkish genotype. They are therefore not the result of self-pollination. This may suggest that the center of diversity may be in Iran, but detailed sampling of wild populations has not been carried out in the whole distribution area of the species.

So far the only in-depth DNA based study on the parental origin of the Damask roses was published by Iwata et al. (2000). They compared the sequences of the ITS of the ribosomal DNA and the *psb*A–*trn*H spacer sequence of the chloroplast genome of four Damask varieties possessing an identical genotype ("Kazanlik," "York and Lancaster," "Quatre Saisons," and "Quatre Saisons Blanc Mousseux") with those from the species that had been suggested by Hurst (1941) as parents of the Damask roses: *R. gallica*,

R. phoenicia, and *R. moschata*. The results rejected *R. phoenicia* as a potential parent in the initial crossing. The authors further included *R. fedschenkoana* in their analysis and proposed that the actual crossing that led to the formation of the genotype found in all four Damask varieties is (*R. moschata*♀ × *R. gallica*♂) × *R. fedschenkoana*♂. On the other hand when Rusanov et al. (2005b) analyzed the genetic similarity among various oil-bearing roses, they found that *R. damascena* differs in all alleles at several microsatellite loci from the profiles of the analyzed accessions of *R. moschata* and *R. gallica*. As microsatellites are polymorphic within species this does not immediately preclude these species as parents. It will be necessary to assay a number of genotypes that are closely related to *R. damascena* and its putative ancestors with molecular markers allowing easy allele scoring.

12.4 Ploidy Levels and How to Manipulate Them

12.4.1 Ploidy Levels

Almost all presently grown rose cultivars are tetraploid and usually interfertile. Most of them are derived from several generations of spontaneous or man-made crosses and no doubt contain several different wild species in their ancestries. By contrast, most of the wild rose species are diploid and have a regular meiosis with seven bivalents. Polyploid species are found mainly in sections *Cinnamomeae* and *Carolinae*, which have only made minor contributions to the cultivar gene pool. Some polyploids are, however, found also in other sections like *R. chinensis* (sect. *Indicae*), which has been reported as 2*x*, 3*x,* and 4*x*, and the tetraploid *R. gallica* (sect. *Gallicanae*). Interfertility among wild species is generally high as long as the crosses are undertaken between species at the same ploidy level. Still, the prevalence of tetraploidy in cultivars suggests that hybridization has been more successful at this higher ploidy level although other desirable traits like increased plant vigor may also have played a part.

One section deviates from the remainder; all species in sect. *Caninae* are characterized by the peculiar *canina* meiosis (Lim et al. 2005). Regardless of ploidy level (usually 5*x*, but some 4*x* and 6*x* taxa also occur), only seven bivalents are formed in the first meiotic division. The remaining chromosomes form univalents and are not included in viable pollen grains, which therefore contain only the seven divided bivalent chromosomes. All univalents are transmitted to one of the daughter cells in the female meiosis, and are finally included in the viable egg cells, which therefore contain 21, 28, or 35 chromosomes depending on ploidy levels. SSR-based analyses of different species and offspring from controlled crosses suggest that bivalent formation involves one biparentally inherited, highly homozygous diploid genome, whereas the remaining 2, 3, or 4 haploid and often highly differentiated genomes are transmitted only from the seed parent (Nybom et al. 2004, 2006). Interfertility is very high among dogroses, and they can also be used in crosses with species on other levels, behaving as a polyploid species when used as seed parent, and as a diploid species when used as pollen parent.

Hybridization between diploid and tetraploid roses results in triploid hybrids that are generally sterile or have very low fertility. Two different strategies can be envisaged to overcome this ploidy barrier: haploidization of tetraploid cultivars and polyploidization of wild, mostly diploid genotypes.

12.4.2 Haploidization

Up to now, the only successful haploidization method has resulted from in situ induction of parthenogenesis using irradiated pollen and subsequent in vitro culture of immature seed.

12.4.2.1 In Situ Parthenogenesis in Roses

Dihaploid derivatives from tetraploid rose cultivars are produced by in situ parthenogenesis induced after pollination by irradiated pollen and subsequently in vitro embryo rescue (Meynet et al. 1994). For this, anthers are collected from flower buds of the male parents 1–2 days prior to flower opening. The anthers are dried for 2 days in an incubator at 30°C. The pollen is sifted and stored in a desiccator at 4°C for the duration of the hybridization period, e.g., from April to June in European countries. All the flower buds on

the maternal plants are emasculated 2 days prior to anthesis by removing the calyx, petals, and anthers with a forceps. Dry pollen samples are exposed to gamma irradiation from a $Cobalt^{60}$ source for a total exposure of 600 Gy. Pollinations with irradiated pollen are made on the day of irradiation or the following day. After pollination, the flowers are protected with paper bags.

Eight weeks after pollination the hips formed are collected and achenes are extracted from each hip. The enlarged achenes are plunged into water. The probability that achenes contain an embryo is approximately 15 times higher in achenes of density >1 than in floating ones. The achenes that sink are disinfected in 30 g/l $CaCl_2$ for 20 min, and subsequently rinsed three times for 5 min in sterile distilled water. Embryos are aseptically removed from the endocarp. Embryos are placed on a solid culture medium in darkness at 4°C for 4 weeks, and then transferred to a 16 h photoperiod provided by daylight fluorescent illumination [90 μmol/(m^2 s)] and at 22°C for 2 weeks to obtain rooted plantlets. At the end of incubation, the embryos germinate and the rooted plantlets are planted into a classical horticultural medium and grown under greenhouse conditions. The ploidy level of the plants obtained is then determined by flow cytometry (FCM) or vegetative meristem chromosome counts.

12.4.2.2 Characteristics of Dihaploid Roses

Characteristics such as the guard cell length, chloroplast number, stem length, leaf and flower size are reduced in dihaploids as compared with their tetraploid donors. Male fertility of the dihaploids is usually very low; 76% of them were characterized by pollen viability lower or equal to 5% (El Mokadem 2001). However, three dihaploids showed pollen viability greater or equal to that of their tetraploid donors. Female fertility of the dihaploids was variable.

Many progenies have now been obtained from a hybridization program between dihaploids of rose cultivars, used as female parents, and diploid wild species (e.g., *R. gigantea*, *R. roxburghii*, *R. rugosa*, and *R. wichurana*), used as male parents (El Mokadem 2001). Although some of these dihaploids were fertile, their gametogenesis often revealed abnormalities and resulted in the frequent production of 2*n* gametes, i.e., gametes with the somatic chromosome number (El Mokadem et al. 2002a, b). Unreduced gametes or 2*n* gametes are mainly formed in two ways (1) an incomplete first meiotic division (first division restitution, FDR), or (2) an incomplete second meiotic division (second division restitution, SDR). Unreduced gametes via FDR are comprised mainly of the non-sister chromatids of each homologous pair of chromosomes, whereas in SDR the sister chromatids are included in the same gametes. As a result, 2*n* gametes formed by FDR transmit more of the parental heterozygosity into progenies than those formed by SDR.

The formation of 2*n* gametes can be detected by analyzing ploidy level (e.g., with FCM) of progenies resulting from crosses between dihaploids and diploid species, as well as by analyzing the size of the pollen grains produced by a dihaploid rose. The nature of the mechanisms underlying male and female 2*n* gametes produced by the dihaploids was determined by a cytological study of male meiosis and by estimating the heterozygosity level transmitted by female and male 2*n* gametes into the triploid progeny resulting from crosses made with diploid species, using AFLP markers (Crespel et al. 2002b). Among meiotic abnormalities leading to 2*n* pollen production, triads (containing a 2*n* microspore at one pole and two *n* microspores at the other) resulting from abnormal spindle geometry were frequently observed (El Mokadem et al. 2002a). There were various types of meiotic nuclear restitution leading to 2*n* pollen production: second division restitution with crossing-over (SDR-CO), first division restitution without crossing-over (FDR-NCO), and first division restitution with crossing-over (FDR-CO) transmitting ±40%, 100% and ±80% of the parental heterozygosity, respectively. The proportion of different 2*n* gamete types produced was mainly genotype dependent with some seasonal effects (Crespel et al. 2002b, 2006; Crespel et al. 2003). Since the ability to produce 2*n* gametes was transmitted to the offspring, a return to tetraploid level can be envisaged by meiotic polyploidization via 2*n* gametes.

12.4.3 Polyploidization

Polyploidization can be obtained via three different methods: mitotic, meiotic, and somatic polyploidization.

12.4.3.1 Mitotic Polyploidization

For mitotic polyploidization, a chemical substance is applied that transiently blocks mitotis, resulting in DNA replication without chromosome separation during anaphase. Amphidiploids, i.e., allotetraploids, are more useful than autotetraploids in such procedure, notably to restore the fertility of diploid interspecific hybrids, but also to avoid the typical infertility of primary autotetraploids. There are two practical ways to produce amphidiploids from diploid rose species (1) to double the diploid species and cross the resulting autotetraploids; and (2) to cross the diploid species and double the resulting diploid interspecific hybrid.

Since the middle of the twentieth century many authors have tried to improve the chromosome doubling technique, with varying success depending on genotypes, type and concentration of chemicals used, duration of application, and type of exposed explant. Both in vivo and in vitro assays have been made in roses. The most frequently used chemical is colchicine, an alkaloid obtained from *Colchicum autumnale*. Dinitroaniline compounds, which are herbicides, have also been tested: oryzalin, amiprophosmethyl (APM), and trifluralin. Chromosome doubling has been successfully performed using three applications of 0.5% colchicine solution at alternating days (Semeniuk and Arisumi 1968), or application of 0.06% colchicine solution each day during 4 days on the top lateral bud in active growth (4–8 leaf stage) of seedlings (Basye 1990; Byrne et al. 1996), on different genotypes (e.g., *R. laevigata* and *R. laevigata* hybrids, *R. roxburghii* and *R. roxburghii* hybrids, *R. bracteata* or *R. wichurana*). However, the results remain erratic. Trifluralin assays were also performed in vivo, by dropping 0.086% trifluralin solution on the shoot apex of *R. rugosa* hybrid germinating seedlings (Zlesak et al. 2005). These assays resulted in some polyploids, but with a quite low success rate (around 5% doubled plants) and a high level of chimerism.

In vitro assays have also been performed using colchicine. However, in this case an in vitro multiplication phase of plantlets before treatment, and an in vitro regeneration phase of treated explants afterwards, is necessary. Optimal application was achieved by growing plantlets on medium containing 1.25 mM colchicine (Ma et al. 1997), or by soaking nodal sections in 1.25 mM or 2.5 mM colchicine solution before transfer to solid culture medium (Roberts et al. 1990; Ma et al. 1997). Soaking assays on *R. wichurana* in vitro plantlets gave up to 30% of doubled cells, but the number of doubled plants was not reported (Roberts et al. 1990). Amphidiploids from the hybrids *R. wichurana* × *R. roxburghii* and *R. wichurana* × *R. setigera* were obtained at the end of the 1980s in these ways, but overall success rate was lower than 5% (Basye 1990; Byrne et al. 1996). A similar study was performed in the middle of the 1990s on five interspecific diploid hybrids involving the diploid species *R. wichurana*, *R. roxburghii*, *R. banksiae*, *R. rugosa rubra*, and *R. setigera* (Ma et al. 1997). Some amphidiploids were detected, but success rates remained below 5%.

Faced with the low efficiency of colchicine and its carcinogenic nature, other anti-microtubule chemicals have been explored as an alternative for polyploidization since the early 1990s. Oryzalin, APM, and trifluralin have actually been shown to have greater affinity for binding to plant microtubule, and so are used at micromolar concentrations, a thousand times lower than colchicine (Zlesak et al. 2005). Compared to colchicine, the efficiency of such herbicides in vitro for polyploidization of plants has been variable but promising. The first attempts with oryzalin were performed on in vitro plantlets from diploid *R. hybrida* "Thérèse Bugnet" (Kermani et al. 2003). Treatment of the shoot apex in liquid medium at 5 μM during 24 h, followed by 13 days on semi-solid medium at the same concentration, resulted in 40% doubled plants. The best results were obtained by exposing nodal section to oryzalin on solid medium at a concentration of 5 μM during 24 h, with a success rate of 66.6% doubled plants and a survival rate of 20%.

The use of oryzalin, APM, or trifluralin in vitro treatments on triploid *R. hybrida* "Iceberg" nodal sections showed similar efficiency (Khosravi et al. 2008). Application of the chemicals was done on a two-phase (liquid and semi-solid) shoot proliferation medium, at 6 μM concentrations, during 24 h. The same treatment on diploid *R. persica* and tetraploid *R. hybrida* "Akito" explants resulted in 6.3% and 0% chromosome doubling, respectively, which suggests that chromosome doubling is genotype dependent (Khosravi et al. 2008).

The above-mentioned approaches are limited to rose genotypes that can easily be grown in vitro. Concentrations of spindle inhibitor and exposure time are critical factors to success, but whatever chemical is used, chromosome doubling is genotype dependent,

and standard conditions that work for all rose species cannot be specified.

12.4.3.2 Characteristics and Use of Amphidiploids

Induced tetraploidy is visually detected by "gigas" characteristics: slower growth, thicker leaflets with a greater width-to-length ratio, greater overlapping of the leaflets, and larger flower size (Byrne et al. 1996; Ma et al. 1997; Kermani et al. 2003; Allum et al. 2007). Other indicators are a larger guard cells and larger pollen grains. These two parameters are typically used for ploidy level estimation of rose genotypes, due to the easiness of use in comparison to chromosome counts, but FCM analyses are currently performed for ploidy level assessment.

Since antimitotic agents work on a single cell level, the original plant is likely to be a chimera, a plant with sectors of doubled and non-doubled tissue. Thus, care needs to be taken to examine the plant thoroughly and isolate the doubled sector.

Fertility of amphidiploids depends on fertility of the initial diploid hybrid. The general rule is that the less fertile the interspecific diploid hybrid, the more fertile (and so usable in breeding program) the amphidiploid derived from it (Byrne and Crane 2003). The most widely used amphidiploid in modern rose breeding is *R. kordesii*, a spontaneous tetraploid seedling of the sterile diploid hybrid (*R. rugosa* × *R. wichurana*) "Max Graf," which gave rise to the Kordesii hybrid roses in European and Canadian breeding programs. Some of Basye's (1990) amphidiploids have also been hybridized with commercial rose germplasm in order to transfer general blackspot resistance to the breeding population.

12.4.3.3 Meiotic Polyploidization

Sexual polyploidization is the process by which a polyploid zygote is formed by natural fertilization, involving $2n$ gametes. Two cases are possible (1) unilateral polyploidization occurring in interploidy crosses ($2x \times 4x$), in which case one $2n$ gamete (from the diploid parent) fertilizes a reduced gamete (from the tetraploid parent); (2) bilateral polyploidization occurring by fusion of two $2n$ gametes coming from two diploid parents (in a $2x \times 2x$ cross). Production of $2n$ gametes in rose was first shown on the dihaploids obtained via in situ parthenogenesis using irradiated pollen (see above), but some tetraploid cultivars have been found to produce $2n$ gametes as well (Crespel and Gudin 2003).

In roses, $2n$ gametes produced by dihaploid rose cultivars and their diploid hybrid are used to obtain new tetraploid genotypes through unilateral and bilateral polyploidization. Crosses between dihaploids of rose and rose cultivars have been realized. The percentage of tetraploid progenies obtained varied from 14.2 to 100% according to the dihaploid parent used. A strong gametic selection is observed, in favor of $2n$ gametes, especially when a tetraploid partner is involved (Crespel and Meynet 2003).

12.4.3.4 Somatic Polyploidization

Finally, it is also possible to perform polyploidization by fusing somatic cells as protoplasts. This approach is described later, together with other biotechnological approaches.

12.5 Role in Crop Improvement Through Traditional and Advanced Tools

12.5.1 Role in Crop Improvement

Rose is the most ancient ornamental species. There is evidence that roses were already cultivated 5,000 years ago in China, western Asia, and northern Africa. The first acts of rose domestication corresponded to the cultivation and multiplication of wild rose species. Spontaneous hybridization between these collected species formed the start of new genetic variation (Gudin 2000). Some of these spontaneous hybridizations were only possible because of human activity as the original species involved were collected by plant hunters in distant parts of the world and brought together in gardens.

The voyages of discovery from Europe to Asia brought together the species that have formed the genetic basis of the modern cultivated rose assortment. Controlled rose breeding really started in the nineteenth

century and is nowadays practiced by 25–30 international commercial companies and by many amateur breeders. The activities of controlled and formerly spontaneous hybridizations have led to the modern rose cultivars that correspond to a complex artificial species, often referred to as *R. hybrida* (Gudin 2003).

Some of the important characteristics that were introduced from the progenitors of the modern cultivars in the rose cultivar gene pool were recurrent flowering from *R. chinensis* (around 1800), cold resistance from *R. wichurana*, and yellow flower color from *R. foetida* (around 1900). Recurrent flowering was also introduced from *R. rugosa*, *R. fedschenkoana,* and *R. bracteata*. For specific usages other species have been involved during the twentieth century. For instance, ramblers are mostly hybrids from *R. arvensis*, *R. wichurana*, *R. multiflora*, *R. pendulina,* and *R. sempervirens*. Species used in ground cover roses are *R. wichurana*, *R. davidii elongate,* and *R. bella* (Wylie 1954). For shrub roses, *R. rugosa* has attracted attention from breeders for over 100 years, with recent renewed interest. Also *R. moschata*, *R. multiflora*, *R. spinosissima*, *R. rubiginosa*, *R. moyesii,* and *R. multibracteata* have been used (Wylie 1954).

An extensive overview of interspecific hybrids from natural and artificial crosses that are mentioned in the literature has recently been provided by Spethmann and Feuerhahn (2003). Species belonging to the same section are easier to cross with each other than species from different sections. Sometimes unilateral incongruity is observed. In spite of the specialized cytology of the pentaploid species belonging to the section *Caninae*, these species might be successfully crossed both with species of the same section and with species belonging to other sections (Spethmann and Feuerhahn 2003). Also in nature several interspecific hybridizations between these species have been observed.

Interspecific crosses between species and cultivars are hampered by several factors, including taxonomic distance and ploidy differences. Another bottleneck is that hybrid seeds obtained from crosses between modern cultivars and wild species often show seed dormancy. Therefore germination starts only in the second year. The obtained F_1 progenies also frequently show some dominant characteristics of the wild parent: no flowers are formed during the year of germination, and the growth habit is in general more wild (Van Huylenbroeck et al. 2007). Thus, incorporating wild species in a breeding program implies that several backcross generations are made to obtain a "modern rose" with an additional "wild" characteristic. As a consequence breeding programs with wild species are usually enormously time-consuming and expensive. Especially for cut roses the gap between the desired high quality level of a commercial cultivar and the morphological features of the wild relative is enormous. These morphological differences make it difficult to use wild species in the breeding program. As a result the genetic background in these commercial breeding programs becomes narrow. For garden roses, the genetic and morphological distance between modern cultivars and the wild species is still narrower, which makes the use of wild species more feasible.

12.5.2 Desirable Agricultural Traits

Depending on the trait, different wild species might be valuable to broaden the genetic base of cultivated roses. Examples of interesting traits in species are the yellow flower pigmentation of *R. hugonis*, *R. ecae,* and *R. primula*; thornlessness in populations of *R. blanda*, or, quite the contrary, the ornamental prickles of *R. sericea* ssp. *omeiensis* var. *pteracantha*. Winter hardiness exists in *R. laxa* from central Siberia, *R. suffulta* possesses drought resistance, while *R. arvensis* is known for its shade tolerance (Spethmann and Feuerhahn 2003). Several species as *R. moschata*, *R. rubiginosa*, *R. canina*, *R. gallica,* or *R. damascena* could improve hip quality for specific medicinal or food uses (see below) or add aesthetical value to garden roses and to cut rose stems for flower arrangements. Evergreen rose species from the *Bracteatae* section might be of interest for improvement of shelf-life, i.e., sustained flowering of pot rose plants under room conditions (De Vries 2003).

The highest priority in rose breeding research is the development of disease resistant roses. The two major diseases in roses are black spot (*Diplocarpon rosae*) and powdery mildew (*Podosphaera pannosa*). Different resistance mechanisms for both black spot (Blechert and Debener 2005) and powdery mildew have been described in roses (Dewitte et al. 2007). Interesting fungal resistance is found in wild rose species. Since the discovery of resistance genes in several crops a lot of effort in breeding research has

been directed towards this monogenic or so-called qualitative or vertical resistance. However, an easy and systematic combination of traits as in diploids cannot be expected in tetraploid rose cultivars.

Resistance to powdery mildew varies among rose species and cultivars, and is often pathotype specific (Linde and Debener 2003; Leus et al. 2006). Results of natural and artificial infections with this pathogen show that only few cultivars are highly resistant. In wild species, there is no distinction between sections concerning resistance. *R. agrestis*, *R. glutinosa,* and *R. omeiensis* var. *pteracantha* are mentioned as highly resistant (Linde and Shishkoff 2003). Most modern cultivars are susceptible to black spot, but several species show resistance: *R. banksiae*, *R. carolina*, *R. laevigata*, *R. multiflora*, *R. rugosa*, *R. roxburghii,* and *R. wichurana* (Drewes-Alvarez 2003). A concern while using wild species for ameliorating disease resistance is that these traits are often genotype specific. As a consequence, resistance for a specific pathogen should be tested on individual plant genotypes. Results of interspecific crosses between wild species and resistances for black spot (*D. rosae*) and powdery mildew (*P. pannosa*) of obtained progeny are presented by Spethmann and Feuerhahn (2003).

Resistances to populations of the root-knot nematodes *Meloidogyne arenaria*, *M. incognita,* and *M. javanica* can be found in *R. multiflora* and *R. indica*. For *M. hapla* resistances found in rootstocks of the mentioned rose species were more variable. Experiments on *R. multiflora* indicate that resistance to *M. hapla* is polygenic (Wang et al. 2004b). *R. manetii* appears to be highly resistant, whereas the often-used *R. canina* rootstocks have, in general, a good nematode resistance. For root-lesion nematodes (*Pratylenchus penetrans*) resistances were identified in *R. multiflora* "K1," one accession of *R. virginiana*, *R. laevigata anemoides* and some accessions of *R. glauca*. Resistances in these species were higher compared to the resistance found in the popular rootstock *R. corymbifera* "Laxa" (Peng et al. 2003).

12.5.3 Improving Damask Rose

Currently the only Damask rose, which has a significant commercial and industrial importance and is widely grown in Bulgaria, Turkey, and Iran for production of rose oil, rose water, rose absolute, and rose concrete is *R. damascena* "Trigintipetala." The quality and composition of the distilled rose oil is controlled by implementation of an international standard (ISO 9842:2004), which corresponds to the oil from "Trigintipetala." Breeding of *R. damascena* has been mostly limited to clonal selection of best performing plants in the rose fields, e.g., the cultivars "Eleina" and "Janina," which show elevated tolerance to freezing and rust (*Phragmidium mucronatum*), were created by chemical and radiation mutagenesis, respectively (Raev 1984). Although crosses with other rose species such as *R. gallica* have been implemented by some rose breeders (Staikov and Kalaijiev 1980) the obtained hybrid roses never made their way to industrial cultivation for the perfumery and cosmetics industry because of the altered composition of the distilled rose oil from their petals.

According to Iwata et al. (2000) the old varieties of *R. damascena* (genotypically identical to "Trigintipetala") have a triparental origin. "Trigintipetala" generally produces no or just a few seeds with low germinating potential when forced to self- or cross-pollinate. However, Rusanov et al. (2005c) found that 24 seeds collected from "Trigintipetala" plantations in Bulgaria are the result of self-pollination or cross-pollination with neighboring plants (which possess the same genotype). On the basis of SSR allele inheritance they concluded that *R. damascena* "Trigintipetala" is most probably a segmental allotetrapoid species and that the type of inheritance (tetrasomic or disomic) depends on the chromosomal location. This suggests that it would be relatively easy to create a self-pollinated segregating population through collecting seeds from oil rose plantations, but that creating a genetic map and identifying quantitative trait loci (QTL) in "Trigintipetala" may not be straightforward.

During the last decade a number of studies have been published involving the elucidation of genes that are responsible for the rose scent formation (Guterman et al. 2002; Lavid et al. 2002; Scalliet et al. 2002, 2006, 2008; Shalit et al. 2003). Databases including sequences of thousands of petal-expressed genes have been established providing a solid ground for development of single nucleotide polymorphism (SNP) markers (Channelière et al. 2002; Guterman et al. 2002; Jung et al. 2004, 2008; http://www.bioinfo.wsu.edu/gdr/). Recently, at the AgroBioInstitute a strategy for development of SNP markers related to key genes involved in the rose scent formation was implemented.

The results (K. Rusanov unpublished data) demonstrated that data obtained through sequencing of PCR products derived from genomic DNA could easily be converted into gene-specific SNP markers. The developed markers could be used in a breeding program in order to follow the segregation of alleles of key genes that are responsible for the composition of rose oil. When applied to a population of self-pollinated "Trigintipetala," the developed markers might be used to identify a minimum set of allele configurations that can be used to screen for genotypes with genes for improved rose oil quality.

The most desirable traits for "Trigintipetala" breeding include flower yield, rose oil content, and resistance to diseases. Drought tolerance has also been recognized as a desirable trait due to the insufficient and irregular rainfall in rose plantation regions over the last decade (Gunes 2005). As was recently reported (Pirseyedi et al. 2005; Babaei et al. 2007; Tabaei-Aghdaei et al. 2007; Kiani et al. 2008) Iran represents a center of genetic diversity of the Damask roses. One of the main tasks would be to evaluate the reported non-"Trigintipetala" genotypes in Iran as well as other naturally occurring genotypes of oil-bearing roses such as "Stambolska" in Bulgaria (Rusanov et al. 2005b) for both their rose oil production potential and as a natural source of desirable traits.

12.5.4 Expanding the Gene Pool of Cultivated Rose by Biotechnology

In case of combinations between species that do not form hybrids readily, tissue culture-based techniques, e.g., embryo rescue, can be used to overcome post-fertilization barriers, such as premature embryo abortion. Embryo rescue has been applied in rose (El Mokadem et al. 2000), as described earlier. In this section, the focus is on two other methods based on modern biotechnology, namely somatic hybridization (or cell fusion) and genetic modification (through transformation).

12.5.4.1 Somatic Hybridization

The full procedure of obtaining hybrid plants through cell fusion is composed of several, equally important steps. For each step good-working protocols need to be established. Protocols for sterilization, protoplast isolation, cell fusion, cell division, callus growth, regeneration, rooting, and transfer to soil followed by hybrid identification are all required before thinking of using somatic hybridization. In rose, nearly all these requirements are met.

Increasing vigor, introducing fungal resistance, and improving fertility were the aims of Mottley et al. (1996) and Squirrell et al. (2005) in their attempts to produce hybrids from intergeneric fusions of three rose cultivars with a cherry rootstock *Prunus avium* × *pseudocerasus* "Colt" or a blackberry *Rubus laciniatus* "Thornless Oregon." They also performed self-fusion with a wild rose hybrid, *Rosa persica* × *xanthina* to increase the ploidy level to tetraploid. Hybrid callus lines were obtained and from them plants were regenerated. Some of those plants showed aberrant phenotypes, however, and further cytogenetic and molecular screening could not confirm the true hybrid nature of the plants. The aberrant phenotypes can be explained by somaclonal variation induced by the in vitro procedure.

The introduction of resistance to blackspot, *D. rosae*, was the target of Schum and Hofmann (2001) and Schum et al. (2002). Wild rose species that were studied for suitability to use in somatic hybridization were *R. canina*, *R. caudata*, *R. corymbifera*, *R. indica*, *R. majalis*, *R. multiflora*, *R. nutkana*, *R. roxburghii*, *R. rubiginosa*, *R. spinosissima*, *R. sweginzowii macrocarpa*, and *R. wichurana*. Recipients were two *R. hybrida* cultivars and the hybrid *R. persica* × *xanthina* in the ultimate fusions that were done with diploid accessions of *R. multiflora*, *R. roxburghii,* and *R. wichurana*. FCM and AFLP analyses on callus lines obtained after fusions indicated that hybrids between the cvs. "Heckenzauber" and "Pariser Charme" (+) *R. wichurana* had been formed. Unfortunately, the final step of shoot regeneration turned out to be difficult. Plants were obtained only from the combination "Pariser Charme" (+) *R. wichurana* (Schum et al. 2002), but FCM showed loss of chromosomes during culture and upon regeneration. No further details were given about continued testing on what remained of the chromosomes of the wild parent, on applications or on disease assays.

To widen the gene pool of *R. damascena,* Pati et al. (2008) fused this species with *R. bourboniana*. They reported on obtaining hybrid calli, as confirmed by molecular analysis, but not on hybrid plants.

The conclusions for somatic hybridization in *Rosa* are:

- Protoplast isolation, culture, and regeneration are possible.
- Parent knock-out treatments work (IOA, R6G, and irradiation).
- Cell fusion is possible, as are divisions and hybrid callus formation.
- Plants were obtained but there is no proof for hybrid nature.
- Phenotypical aberrations were probably caused by somaclonal variation; no proof for transfer of traits.

12.5.4.2 Genetic Modification

In genetic modification, genes from other organisms can be used to expand the pool of available genes in breeding and improvement of crops. In the production of genetically modified (GM) plants, two major processes are involved, i.e., gene transfer and regeneration. The plant cell, usually present within a multicellular explant (plant part), has to be amenable to accepting foreign DNA either transferred by *Agrobacterium tumefaciens* or by biolistics, and subsequently integrating it into its own nuclear genome. The preferred result is integration of one copy of transferred foreign DNA in a site allowing desired expression patterns and stability without negative interference on the expression of resident genes. This has to be ascertained in later stages. After successful integration of foreign DNA, this particular, altered cell needs to be capable of entering a stage of sustained cell divisions and development into a plant.

In rose, several groups have tested many explant types and dedifferentiation and regeneration media combined with *Agrobacterium* inoculation or particle bombardment, however without much success. Ming et al. (2007) used nodal and leaf explants for direct regeneration in their gene transfer experiments. They reported on transient GUS expression as a reporter system aimed at optimization of conditions for gene transfer, but they did not obtain plants that could be molecularly analyzed. Earlier, Derks et al. (1995) used stem slices from the rose rootstock "Moneyway." Here, plants were obtained and tested. Recently, rose petals were used to test a transient expression system for gene function analysis. The expression of the reporter gene *gus* was checked after agroinfiltration (Yasmin and Debener 2010). This transient expression system avoids the necessity to obtain fully regenerated new plants.

All other literature reports on the use of regeneration through somatic embryogenesis. Embryogenic callus material, either obtained after induction on roots, leaf explants, petioles, or filaments, is used primarily in transformation of rose. Somatic embryogenesis is a long and tedious technique entailing an induction phase, often of several months, a maintenance phase for growth and multiplication, a maturation phase and a germination phase followed by the outgrowth of rooted plants. However, because of the lack of efficient, more direct alternatives, somatic embryogenesis is the main regeneration method in rose.

Protocol development for gene transfer and GM plant production using reporter genes such as *gus* (glucuronidase), *gfp* (green fluorescent protein), or *luc* (luciferase) has been reported by Firoozabady et al. (1994), Derks et al. (1995), van der Salm et al. (1996), Marchant et al. (1998a), Li et al. (2002), Condliffe et al. (2003), Kim et al. (2004), Ming et al. (2007), and Vergne et al. (2010). The reports vary in the cultivars that were used, in the tissue source of the embryogenic callus, the reporter gene, or the gene delivery system. In all cases except for Ming et al. (2007), regeneration of transgenic rose plants was achieved and plants could be assayed for gene presence and expression.

The goals that were aimed for in rose improvement by genetic modification were quite diverse. Impairing bacterial growth in stems in vases was achieved by the introduction of the gene coding for cecropine (derived from the giant silkmoth *Hyalophora cecropia*; Derks et al. 1995). Improvement in yield in stem production was obtained by the introduction of *rol* genes from *Agrobacterium rhizogenes* in the rootstock "Moneyway" (van der Salm et al. 1997, 1998), however overall flower quality was poorer (De Jong and Visser 2000). Changing plant architecture was the aim of introducing the *ipt*-gene from *A. tumefaciens* involved in cytokinin biosynthesis (Condliffe et al. 2003). Transgenic plants carrying the gene have been produced; however, no obvious change in phenotype could be observed (F. Krens personal observations). Disease resistance, particularly resistance against

blackspot or powdery mildew was the goal of introducing chitinase or glucanase genes (derived from rice or from barley) or of genes coding for the T4 lysozyme (from T4 phage) or for a Type I ribosome inhibiting protein (RIP from barley) by Marchant et al. (1998b) and Dohm et al. (2001, 2002). Marchant et al. (1998b) found reductions of 14–43% in lesion sizes indicative of a reduction in the severity of blackspot symptom development, and Dohm et al. (2002) demonstrated a reduction in susceptibility to blackspot of up to 60% after introduction of the barley RIP. The antimicrobial protein of *Allium cepa* (Ace-AMP1) was successfully used by Li et al. (2003) to introduce resistance against powdery mildew (*Spaerotheca pannosa*) in rose. A significant reduction in disease symptoms could be demonstrated in multiple transgenic plant lines both in a detached leaf assay as well as in the greenhouse.

Another very important trait in ornamental crops is flower color. Souq et al. (1996) and Katsumoto et al. (2007) introduced genes involved in anthocyanin production or constructs aimed to silence endogenous anthocyanin production-related genes, and succeeded in altering the color of transgenic rose flowers. The origin of the genes used was respectively rose itself (the full length of the rose chalcone synthase [*CHS*] gene in antisense orientation) or rose (siRNA *DFR*) and iris/viola (*DFR*/*F3′5′H*) and plants expressing the genes did contain altered anthocyanin patterns and color.

None of the GM roses mentioned earlier has reached the market, but it is clear that genetic modification as a technology opens up possibilities to introduce new traits in tetraploid rose cultivars. The origin of the genes that were used varied from *Agrobacterium* to silkmoth and to plant species such as rice and onion. However, broad spectrum resistance genes from non-related species might be far less efficient to the specific diseases attacking rose than specific resistance genes aimed at the specific rose pathogens from wild *Rosa* species, especially when multiple genes can be pyramided within elite cultivars. Acceptance of such GM roses, carrying rose species derived "cisgenes" (Schouten et al. 2006), by growers, retailers, and European consumers may also be higher. With the full genomes of Rosaceae family members, *Prunus*, *Fragaria,* and *Malus*, sequenced and perhaps in the near future the genome of a diploid rose itself, the identification and availability of wild rose-derived new genes or better alleles coding for interesting traits will increase, thus contributing significantly to the improvement of cultivated *R. hybrida*.

12.6 Role in Classical and Molecular Genetic Studies

12.6.1 Use in Classical Genetic Studies

The basic chromosome number (x) of *Rosa* is 7, with the DNA content varying from 0.78 pg/2C in diploids ($2n = 2x = 14$) to 3.99 pg/2C in octaploids ($2n = 8x = 56$; Yokoya et al. 2000; Roberts et al. 2009). This genome is small in comparison to many other crops but still large compared to the *Arabidopsis* genome (0.085–0.215 pg). Despite the low chromosome number and small genome size, relatively little is known about the genetic basis of important traits (Gudin 2000; Hibrand Saint Oyant et al. 2008). The rose breeders, nevertheless, have managed to combine many favorable specific plant characters and were able to produce highly heterozygous, vegetatively produced cultivars with the desired combinations (Rajapakse et al. 2001). However, the changing global climate, the growing conditions in state-of-the-art greenhouses, together with demands for control of diseases in order to produce with a lower environmental impact all necessitate more insight into genetics of traits and into the interactions between genes and environment.

12.6.2 Molecular Marker Maps and Physical Maps

The main applications of molecular markers are genotype or cultivar identification, phylogenetic studies, construction of chromosome maps, and mapping of morphological and physiological characters (Debener et al. 2003). The commercially used hybrid rose and garden rose germplasm are tetraploid, but the inheritance of traits and the generation of a molecular map are much easier studied at the diploid level. For that reason mapping started using crosses between diploid parents, and still more marker–trait associations are

being analyzed at the diploid than at the tetraploid level.

The first diploid linkage map in rose was constructed by Debener and Mattiesch (1999) using over 300 RAPD and AFLP makers. A population of 60 F_1 hybrids from a cross between the diploid rose genotypes 93/1-117 and 93/1-119 was used as mapping population. This F_1 population showed segregation for double versus simple flowers and pink versus white flower color, and perpetual flowering. The parental genotypes were half-sibs from an open-pollination of the diploid genotype 81/42-15. The genotype 81/42-15 was derived from a breeding program in which diploid hybrids between *R. multiflora* and garden roses were produced (Reimann-Philipp 1981).

Debener and Mattiesch (1999) were able to map two morphological traits: double versus single flower (*Blfo*) and pink versus white flower color (*Blfa*). The blackspot resistance gene *Rdr1* was mapped in the same population by von Malek et al. (2000). This map was further saturated by Debener et al. (2001a, b) with additional AFLPs, SSR, restriction fragment length polymorphisms (RFLPs), and sequence characterized amplified regions (SCARs). Linde et al. (2004) mapped the dominant resistance gene for powdery mildew (*Rpp1*), and identified several AFLP makers closely linked to *Rpp1*. Yan et al. (2005) added AFLP, SSR, protein kinase (PK), resistance gene analog (RGA), RFLP, and SCAR markers to this map, as well as four morphological makers: pink flower color (*Bfla*), double flower (*Bflo*), resistance to black spot (*Rdr1*), and resistance to powdery mildew (*Mildew*). Thus, they constructed a map with a total of 520 molecular markers and a total length of 490 cM for P117 and 487 cM for P118, with an average chromosome length of ca. 70 cM. They concluded that both maps may cover more than 90% of the rose genome. Moreover, both parental maps were integrated as a map with seven linkage groups having a total length of 545 cM and an average chromosome length of ca. 78 cM.

A second genetic linkage map based on a diploid population was constructed using AFLP markers by Crespel et al. (2002a, b). A population of 91 F_1 hybrids from a cross between H190, a dihaploid rose, and the diploid species *R. wichurana*, was used as mapping population. H190 resulted from the haploidization of the 4*x R. hybrida* "Zambra." H190 is a recurrent blooming, double-flowered, and thornless rose, whereas *R. wichurana* is single blooming, single flowered, and thorny rose. In H190, 68 AFLP markers were mapped in eight linkage groups with an average length of 29.8 cM and a total length of 238.4 cM for the map. In *R. wichurana*, 108 AFLP markers were mapped on six linkage groups with an average length of 47.8 cM and a total length of 287.3 cM for the map. The average marker interval was 3.4 cM for both parental maps, which were not integrated since they did not include biparental multiallelic markers. They identified genes underlying a major QTL (*t4*) and minor QTL (*t4b*) for number of thorns on the stem, and located the QTL for recurrent blooming (*r4*) and double corolla (*d6*) that was previously known as *Blfo*.

A third diploid population was analyzed with RAPD and SSR markers by Dugo et al. (2005). A population of 96 F_1 plants from an interspecific cross between diploid roses, "Blush Noisette" (D10), one of the first seedlings from the original "Champneys' Pink Cluster," and *R. wichurana* (E15), was used as mapping population. The maternal parent (D10) is a pink double-flowered (more than five petals), thorny, and spreading rose with recurrent blooming, and susceptibility to powdery mildew. The paternal parent (E15) has white flowers with five petals, and is a ground covering thornless bush rose with single blooming and resistance to powdery mildew. A total of 133 markers (mainly RAPDs) were mapped on seven linkage groups for both parental maps. Four linkage groups could be integrated since they contained common biparental markers. The map length was 388.3 cM for D10 and 260 cM for E15. The average marker density was one every 5.7 cM for both parental maps. The map of the maternal parent D10 contains almost twice as many polymorphic markers as the one of E15, probably due to the presence of a China rose and *R. moschata* genetic background. Moreover a low number of biparental markers were included, but more microsatellite markers will be tested. They were able to putatively map QTL loci controlling flower size, days to flowering, leaf size, and resistance to powdery mildew.

The first two tetraploid linkage maps were produced by Rajapakse et al. (2001) using the F_2 population from a cross between "Basye's Blueberry" (82-1134), a moderately susceptible tetraploid plant, and 86-7, a black spot resistant amphidiploid, and AFLP and SSR markers. The maternal parent of the cross, 82-1134, is a spreading bush with pink flowers

consisting of ten or more petals. This tetraploid is free of prickles on both stems and petioles. Amphidiploid 86-7 contains genomes of two highly black-spot-resistant species, *R. wichurana* "Basye's Thornless" and *R. rugosa* var. *rubra* (Byrne et al. 1996). The 86-7 bush is a sprawling ground cover with white flowers of five petals. Its stems and petioles have prickles, traits inherited from the *rugosa* parent. From the F_1 hybrid 90-69 an F_2 mapping population of 52 individuals was obtained by open-pollination, showing a high level of field resistance to black spot, pink flowers, and prickles on stems and petioles. Simplex uni-parental markers were used to construct both parental maps separately. Resistance to black spot, growth habit, absence of prickles on the stem petiole (*Petiole Pr*) and the locus for malate dehydrogenase (*Mdh-2*) were mapped.

The maps were later expanded and integrated by Zhang et al. (2006) with the help of additional SSR markers. The final maps for 82-1134 consist of 256 markers assigned to 20 linkage groups with a total length of 920 cM, and for 86-7, 286 markers were mapped along 14 linkage groups with a total length of 770 cM. Based on shared SSR markers, consensus order maps for four out of seven rose chromosomes were generated. However, map distances could not be calculated yet since the homeologous linkage groups originating from different species may not be of the same size.

Yan et al. (2005, 2006) identified QTLs for powdery mildew resistance in a segregating tetraploid population with 181 genotypes derived from a cross between two tetraploid cultivars, P867 and P540. The map requires more markers in order to be integrated into other tetraploid and diploid maps. Koning-Boucoiran et al. (2009) are currently saturating Yan's genetic linkage map with nucleotide binding sites (NBS) profiling and SSR markers.

Spiller et al. (2011) recently published the first integrated consensus map for diploid rose. It was based on the information of four diploid maps, which were linked via 59 bridge markers. The integrated map comprised 597 markers distributed over 530 cM on seven linkage groups, which is close to the value from Yan et al. (2005), and about 17% larger than the values from the recalculated single maps. The map established a standard nomenclature for the rose genetic map. It also included the location of ten monogenic traits, including recurrent blooming, double flowers, pink flower color, prickles, self-incompatibility, and black spot resistance.

The integrated consensus map will be useful in synteny studies with related Rosaceae genomes. This is particularly interesting, as the genomes of apple, peach, and strawberry have now been sequenced. As for establishing the genome sequence of rose as well: using next generation sequencing technology it is not a large task to generate sufficient DNA sequences to cover several fold the complete rose genome. However, a major technical challenge in assembling the rose genome will be the heterozygosity.

12.6.3 Characterization of Disease Resistance Genes

Markers tightly linked to QTLs will allow an early selection of progeny having the desired trait. Because it is well known from breeders that first generation hybrids between highly developed rose varieties and wild roses rarely comprise genotypes suitable for variety development, Debener et al. (2003) showed the advantages of marker-assisted background selection in rose breeding to reduce the genetic background of wild rose species in introgression programs, when they introgressed the gene *Rdr1* conferring resistance to black spot into the genetic background of cultivated tetraploid roses. *Rdr1* originated from a diploid *R. multiflora* hybrid whose chromosomal set was doubled via colchicine treatment. It was subsequently crossed to tetraploid hybrid tea varieties according to a modified backcross strategy. Each new generation was screened for disease resistance, and the molecular marker fraction originating from the original donor was determined in order to select interesting progeny for further breeding. This strategy proved to select interesting genotypes, which would not have been identified by scoring of morphological characters alone or in conventional breeding programs.

Yan et al. (2005) identified QTLs for powdery mildew resistance in a segregating tetraploid population with 181 genotypes derived from a cross between two tetraploid cultivars, P867 and P540, showing partial resistance to powdery mildew. Multiple marker loci were found to be associated with powdery mildew resistance, which suggests that quantitative resistance

to powdery mildew is controlled by multiple genes in rose.

Linde et al. (2006) mapped QTL for resistance to powdery mildew in six different environments over 3 years. They included AFLP, RGA, SSR, SCAR markers, the *Rdr1* locus (Hattendorf et al. 2004; Hattendorf and Debener 2007), and four morphological markers (*prickles t4* and *t4b*, *double flowers d6*, and *white stripes*). The map was made on a diploid population that resulted from a cross of the diploid line 95/13-39 (resistant against powdery mildew isolate 9) and the susceptible diploid male parent Sp3 (82/78-1). Both genotypes are open-pollinated seedlings from a breeding program aimed at the introgression of genes from tetraploid garden roses into *R. multiflora* (Reimann-Philipp 1981). This enabled them to detect resistance QTLs that were stable over different environments and pathogen races as well as race- and environment-specific QTL. Highly significant QTLs (LOD scores of 18 and 12.5) were mapped near a RGA marker on linkage group 4. However, they could not be detected again on year three while a new QTL (LOD of 5.4) was detected on linkage group 7 where no significant gene action could be located in the previous 2 years. The authors speculated that in year three the infection pressure was lower hampering the detection of the QTL on group linkage 4. There could also have been a change of pathogenic strain allowing the detection of a new QTL on linkage group 7.

In parallel to genetic mapping, a bacterial artificial chromosome (BAC) library was constructed to serve as a tool for the physical mapping and positional cloning of rose genes (Kaufmann et al. 2003). By high resolution mapping of the *Rdr1* locus previously identified and mapped (von Malek et al. 2000) and the use of the BAC library, both genetic and physical maps for the *Rdr 1* genomic region have been aligned.

12.6.4 Characterization of Genes for Other Traits of Interest

Debener (1999) analyzed the inheritance of important morphological and physiological characters in diploid roses. He concluded that the presence of pink flower color, double flowers, and prickles are inherited as dominant genes whereas recurrent flowering is inherited as a recessive gene. He also mentions earlier studies that showed that recurrent flowering phenotype, dwarf character, and the moss character are inherited monogenically. Debener et al. (2001a, b) confirmed this in tetraploid populations. In addition, resistance to blackspot was also found to be inherited as a monogenic dominant character. They also concluded that when pink flower color was visually scored versus white, a dominant inheritance could be inferred, but when the total anthocyanin content in the pink flowers was measured using a photometric assay, two classes of genotypes were identified confirming a codominant inheritance in the pink flower.

Baudino (2003) identified a molecular marker linked to fragrance intensity in the progeny of a cross between tea hybrids. Scalliet et al. (2002) identified phenolic methyl ether 3,5-dimethoxytoluene as a major compound responsible for tea scent of many of the modern roses varieties, which have lost most of their scent, whereas the phenolic methyl ether pathway is restricted to Chinese roses.

Hibrand Saint Oyant et al. (2008) showed that petal number was controlled by a major gene and QTL as previously proposed by Debener (1999). Recently, Dubois et al. (2010) used a candidate gene approach to demonstrate that the double flower phenotype is associated with a deregulation of expression of the rose ortholog of AGAMOUS (RhAG). Furthermore, this deregulation of RhAG expression appears to have occurred in double flower roses during rose domestication, suggesting that man has tinkered with the regulation of a unique regulatory gene to obtain double flowers (Dubois et al. 2010). A strong QTL controlling blooming date was also located, but on a different linkage group from the two (*Df1* and *Df2*) detected by Dugo et al. (2005). Thus, the process of blooming may be different from one population to another.

12.7 Genomics Resources Developed

Traditional genetic mapping has been successful so far to identify QTLs and even loci responsible for a given phenotype. However, it is not always easy to find adequate segregating F_1 populations for given traits. Furthermore, the establishment of a genetic experiment is a time- and money-consuming process, as very often relatively large F_1 and backcross

populations are required, and phenotyping can be tedious (the plants need to be adult, and the phenotype studied for three consecutive years). Global "omics" approaches combined with traditional genetics could prove to be a powerful approach.

From an economic point, a small number of rose traits are very important. These include plant architecture, flower development, function and senescence, scent biosynthesis, reproduction and resistance to biotic and abiotic stresses. There is no or very little information available on the molecular mechanisms that control these traits. This dearth of information is a handicap, limiting the scope of rational selection for improvement of ornamental plants. Furthermore, several aspects (i.e., scent production, recurrent blooming, color) cannot be addressed using model species such as *A. thaliana*, or at least only in a limited manner. Rose represents an ideal ornamental model species to address some of these aspects.

Here we focus on the recent advances in molecular tools and their potential use to improve our understanding of the molecular bases of rose traits as well as to help in addressing and identifying molecular markers associated with different traits.

12.7.1 "Omics" Resources

12.7.1.1 Expressed Sequence Tags Libraries

Before 2002, only about 180 rose gene sequences were available in the public databases. During the past few years, several research groups have initiated projects for expressed sequence tag (EST) sequencing in order to obtain a good representation of genes expressed in roses. Channelière et al. (2002) and Guterman et al. (2002) provided the first global overview of genes in the rose genome through EST sequencing. These EST sequences represent mainly genes expressed in petals because for centuries, the economic importance of the rose relied on flower architecture and on natural fragrances, mainly determined by the petals. These studies provided the first annotated rose EST database comprising 2,977 unique sequences. Recently, Foucher et al. (2008) sequenced 5,000 new ESTs corresponding to about 2,336 genes expressed in vegetative and floral meristems of *Rosa* sp. Presently, there are about 9,289 ESTs, representing about 4,834 uni-sequences, available in the different databases (URGI: http://urgi.versailles.inra.fr/; GDR: http://www.bioinfo.wsu.edu/gdr/).

Rose ESTs have enabled the identification of many genes with potential roles in flower development and senescence, recurrent flowering, scent and pigment biosynthesis (Channelière et al. 2002; Guterman et al. 2002; Scalliet et al. 2002; Foucher et al. 2008). Furthermore, these studies provide a starting point for understanding some of the molecular, genetic, and biochemical processes associated with traits that greatly influence rose quality, especially flower quality. Furthermore, the above studies demonstrated the advantages of global EST sequencing approaches to identify genes of interest in non-model plants, such as the rose.

The available EST sequences (about 5,000 genes) represent only about a fifth of the expected number of expressed genes in rose. We are far away from having information on all *Rosa*-expressed genes. Recently, a French consortium performed a large-scale targeted rose EST sequencing program using novel technologies such as 454 pyrosequencing technology. cDNAs from a range of rose organs (root, leaves, flower, etc.) at different developmental and physiological stages (biotic and abiotic stresses) were used in this sequencing program (M. Bendahmane and collaborators unpublished data). The newly obtained EST database is expected to provide sequence information on the majority of genes expressed in rose. It also represents a resource to identify genes whose expression correlate with certain physiological characters as well as developmental and morphological characters. Furthermore, such resource can also serve as a base for the rose genome sequencing.

12.7.1.2 Proteomics

Proteomics has been also used to increase knowledge on rose petal development (Dafny-Yelin et al. 2005). In this study, authors generated stage-specific petal protein maps. They studied nearly 1,000 protein spots in closed buds, mature flower, and flower at anthesis and found that 30% of these proteins were development stage specific. Interestingly, they obtained 15 proteins with unknown function and seven novel proteins.

12.7.2 Identification and Functional Characterization of Genes Associated with Important Traits in Rose

12.7.2.1 Usefulness of Rosa Resources

ESTs were used as source to identify novel genes whose expression is associated with several rose traits. They have enabled the identification of a few rose scent-associated genes such as *O*-methyltransferases and rose alcohol acetyltransferase encoding genes (David et al. 2002; Scalliet et al. 2002, 2006, 2008; Shalit et al. 2003; Guterman et al. 2006). Recently, Foucher et al. (2008) used an EST approach to identify genes associated with recurrent blooming in rose. EST sequences were also used to generate the first rose DNA microarray comprising 2,100 uni-sequences (Guterman et al. 2002). The use of this microarray led to the discovery of several novel flower scent-related candidate genes (i.e., germacrene D synthase and *O*-methyltransferases encoding genes; Guterman et al. 2002). A new rose microarray chip comprising about 4,800 genes was recently developed and used to identify genes whose expression is associated with several flower characters such as double flower (M. Bendahmane's group unpublished data) and scent (S. Baudino's group unpublished data).

Application of suppression subtractive hybridization (SSH) to cloning differentially expressed cDNA enabled the isolation of eight novel scent-associated genes (RhMYB, OOMT, a geranylgeranylated protein-encoding gene, and five ESTs with no sequence homology; Jirong et al. 2008). Ahmadi et al. (2008) used a cDNA differential display approach to isolate an ethylene-induced cDNA homologous to a laccase whose expression is associated with abscission of petioles and flowers.

12.7.2.2 Identification of Rose Genes Using a Candidate Gene Approach

In *Rosa* a candidate gene approach was used to identify homologs of genes known to be involved in pathways, which are crucial for understanding the molecular mechanisms of several traits. These studies have targeted a few major rose traits, such as flower development and longevity. Homologs of genes associated with flower organ initiation and development have been identified (i.e., *MASAKO C1*, *MASAKO D1*, and *Rh-PIP2*; Kitahara and Matsumoto 2000; Ma et al. 2006). Post-harvest quality, including a long vase-life of cut flower roses, has always been a major selection criterion for rose breeders. For that reason, several genes putatively involved in senescence of rose petals have been isolated, for example ACC synthase and ethylene receptors (Wang et al. 2004a; Ma et al. 2006; Pan et al. 2005; Xue et al. 2008), protein kinases (Müller et al. 2002), lipoxygenase (Fukuchi-Mizutani et al. 2000), and delta-9-desaturase (Fukuchi-Mizutani et al. 1995).

12.7.3 Perspectives

One of the major tasks to be accomplished within the coming few years will be the rose genome sequencing. A complete rose genome sequence is now conceivable, even realistic and necessary in order to improve genomics research in the field of woody ornamentals. Rose represents an ideal model species for genome sequencing because of (1) its economic importance in the ornamental plant sector accounting for approximately 30% of the market, (2) the small size of its genome (approximately 560 Mbp or four times the size of the *A. thaliana* genome), (3) its amenability to transformation, and (4) its well-documented genetic history.

Sequencing technologies have made enormous progress in the recent years. As each genome sequencing technique has its specific drawbacks, in terms of sequence length and fragment assembly as well as sequence quality, a combination of different sequencing technologies will allow sufficient genome coverage and sequence quality, at a reasonable cost. It should be noted that heterozygosity is an important issue in the perspective of rose genome sequencing. Previously, de novo genome sequencing of a hybrid grapevine cultivar, Pinot Noir (Velasco et al. 2007) and an inbred Pinot Noir cultivar (Jaillon et al. 2007) was undertaken at about the same time. Better and faster results were obtained from sequencing the inbred line rather than the hybrid. This indicates that a wild diploid species, rather than a tetraploid cultivar, with low heterozygosity will be more suitable for

a rose genome sequencing project. Another option would be to create a dihaploid from a diploid hybrid cultivar. Both solutions are labor-intensive but necessary steps to provide good starting material for a successful genome sequencing project in rose.

12.8 Scope for Domestication and Commercialization

The chemistry of rose flowers involves highly diversified secondary metabolites that participate in their interactions with the environment and contribute to reproductive success through pollinator attraction. This diversity of molecules has also been harnessed during the domestication process, ultimately resulting in scented and colorful varieties displaying valuable ornamental flowers. For this reason, the chemistry of floral pigments and volatile compounds in roses has been extensively characterized.

12.8.1 Polyphenols

Anthocyanins are the main pigments of red *Rosa* species. In this family of molecules sharing the same biosynthesis pathway, flavonols act as copigments in order to protect anthocyanins against hydrophilic attacks. In wild *Rosa* species, these polyphenolic pigments and copigments are produced from four molecular skeletons: cyanidin and paeonidin for the anthocyanins and quercetin and kaempferol for the flavonols. Chromatography techniques (TLC and HPLC) have been used to determine the polyphenolic profiles of wild and cultivated species. The quantitative data produced by HPLC have been analyzed with multivariate methods, such as PCO, resulting in the definition of chemotypes that could be correlated to the taxonomy of *Rosa* species (Mikanagi et al. 1995).

The *Cinnamomeae* species display almost the whole range of existing chemotypes, in good agreement with the notion that diversification of *Rosa* has been especially pronounced in this large section. Three major chemotypes can be distinguished: paeonidin 3,5-diglucoside associated with quercetin and kaempferol 3-sophorosides as in *R. rugosa* and *R. canina*; cyanidin 3,5-diglucoside associated with quercetin and kaempferol 3-glucoside as in *R. chinensis* and in *R. gallica*; and quercetin and kaempferol 4-glucosides as in *Pimpinellifoliae*.

12.8.2 Carotenoids

A complete and highly diversified carotenoid metabolism provides the characteristic yellow color in petals of *Pimpinellifolia*e roses such as *R. foetida* (Eugster 1985). This ability to accumulate yellow pigments was introduced in hybrid cultivars around 1900 when a cross between a Hybrid Tea and *R. foetida* cv. "Persian Yellow" produced the cultivar "Soleil d'Or." Carotenoids are not only important for petal pigmentation, they are also related to the synthesis of minor volatile compounds, such as beta-damascenone, which is an oxidative cleavage product of beta-carotene, and – although it is produced in extremely low quantities – provides the typical fragrance found in *R. damascena* flowers and in the essential oil derived from them (Auldridge et al. 2006).

12.8.3 Volatiles

Volatile molecules are important cues for pollinators and therefore contribute to reproductive success in entomophilic plant species. In roses, these molecules are produced by the petal epidermis and/or by pollen (Dobson et al. 1999; Bergougnoux et al. 2007). The major scent components of roses include 2-phenyl ethanol, monoterpenes, and phenolic methyl ethers such as 3,5-dimethoxytoluene (DMT) or 1,3,5-trimethoxybenzene (TMB). However, characteristic odors can be due to minor compounds: in *R. damascena*, beta-damascenone accounts for most of the scent but only comprises 0.1% of the weight of volatiles. The peri-Mediterranean progenitors of modern hybrid rose varieties (*R. gallica*, *R. damascena*) mainly emit 2-phenylethanol and monoterpenes, whereas the Chinese progenitors (*R. chinensis*, *R. gigantea*) mainly produce DMT and/or TMB (Flament et al. 1993; Joichi et al. 2005). The ability to synthesize DMT or TMB is restricted to Chinese roses, since these, due to a recent gene duplication in the clade of *R. chinensis*, possess

a unique combination of two orcinol *O*-methyl transferases (OOMT) able to achieve this enzymatic sequential biosynthesis (Scalliet et al. 2008).

12.8.4 Rose Oil and Other Components from Damask Roses

Although some Damask roses have been maintained for centuries in West European garden rose collections, commercial cultivation of Damask roses is undertaken mainly for production of rose oil and rose water, obtained by steam distillation of rose petals, and "rose absolute" and "rose concrete" produced by solvent extraction. Since rose oil mixes well with other odors and prolongs the duration of the perfume aroma, it is used as a basic component in a number of products in the perfumery and cosmetics industry. The main rose oil producing countries are Bulgaria, Turkey, and Iran while India, China, and the countries from northern Africa produce mainly rose water and a minor fraction of the rose oil used by the perfumery industry. The world production in 2006 consisted of approximately 3,000 kg rose oil (of which 1,900 kg was produced in Bulgaria), 5,000 kg absolute and dozens of tons of rose water (http://www.biolandes.com/biolandes-rose-2007-lettre-65-gb.pdf). The price of rose oil has gradually increased during the last years, and reached €5,000 per kg in 2007.

Rose oil consists of a small number of major compounds including citronellol, geraniol, nerol, phenethyl alcohol, linalool, farnesol, eugenol and eugenol methyl ether, and more than 275 minor constituents. A major part of the rose oil odor is derived from two minor constituents, beta-damascenone and beta-ionone (Kovats 1987; Ohloff 1994). The composition of the distilled rose oil can vary significantly from year to year depending on the climatic conditions and the geographic regions where cultivation takes place (Nikolov et al. 1977, 1978; Topalov 1978). Therefore, rose oil is produced in only a few areas with favorable climatic conditions. In Bulgaria, the rose plantations are situated in the so-called Rose valley between the Balkan Mountains and the Sredna Gora Mountains where winters are mild and summers only moderately hot. In Turkey and Iran, the main rose growing regions are the provinces of Isparta and Isfahan, respectively.

In addition to the application of rose oil and rose water in the perfumery and cosmetics industry, these products are also included as minor components in a number of foodstuffs like gelatines, sweets, dairy desserts, etc. Dry petals from Damask roses are included in various preparations of traditional herbal medicine and homeopathic products.

Rose oil and other flower extracts have potential as a source of therapeutic compounds and dietary supplements. Rose water obtained from petals has been known for its soothing effect and was also found to be beneficial in ophthalmopathy (Kiritikar and Basu 1987; Biswas et al. 2001).

Aridogan et al. (2002) analyzed the antibacterial activity of *R. damascena* essential oil as a composite mixture against *Staphylococcus aureus*, and the antimicrobial activity of some components of the essential oil such as citronellol, geraniol, and nerol against *S. aureus* and *Escherichia coli*. They found that the tested compounds have more potent antimicrobial activity individually than in the mixture form of the rose essential oil. Basim and Basim (2003) determined the antibacterial effects of rose essential oil against three strains of *Xanthomonas axonopodis* ssp. *vesicatoria* and found that the essential oil may be a potential control agent in the management of the disease caused by *X. a. vesicatoria* in tomato and pepper plants.

Ozkan et al. (2004) determined the antibacterial activities of fresh and spent *R. damascena* flower extracts. The authors determined the antibacterial activity of the extracts against 15 species of bacteria, and found both extracts to be effective against all tested bacteria except *E. coli* O157:H7, the fresh flower extract being more effective than the spent flower extract.

Achuthan et al. (2003) reported that fresh juice of *R. damascena* flowers exhibits promising in vitro antioxidant potential and may protect against carbon tetrachloride (CCl4)-induced hepatotoxicity, possibly through its free radical scavenging activity. Boskabady et al. (2006) observed a potent relaxant effect of ethanolic extract and essential oils of *R. damascena* on guinea pig tracheal chains. Mahmood (1996) even observed that water and methanol extracts of *R. damascena* petals exhibited moderate anti-HIV activity.

12.8.5 Useful Dogroses

In contrast to their flashy cousin, *R. damascena*, species of *Rosa* section *Caninae*, the dogroses, have single short-lived flowers, not so much fragrance and are seldomly used for ornamental purposes. They are long-lived woody plants growing in woodland margins and disturbed habitats such as roadsides and open pastures. The plants are upright or climbing with more or less prickly and bristled shoots. During autumn these plants show their real value: an abundance of rosehips containing various bioactive contents. Unfortunately, scientists performing studies on the chemical contents of rosehips are seldom aware of the taxonomic confusion within this section. Expressions like, e.g., "dogrose" or "*canina* rose" or even "*R. canina*" are often used for any dogrose species, or at least any species within subsection *Caninae*. It is also almost impossible to compare reported concentrations between different studies, since the concentration of various compounds in the plant may depend on horticultural procedures and on environmental factors (Kovacs et al. 2004; Ercisli 2007). In addition, the extraction efficiency differs considerably between different laboratory protocols and may depend on the technical equipment used.

12.8.5.1 Rose Hip Components

Dogrose rosehips have high levels of vitamin B and C, carotenoids, polyphenols, and the minerals K and P. Polyphenols, vitamin C (ascorbic acid), and carotenoids are powerful antioxidants. In a study by Halvorsen et al. (2002) in which different fruits and berries were screened for total antioxidants, the dogroses were in the top with 39.46 mmol/100 g FW (fresh weight). The second best, crowberry (*Empetrum hermaphroditum*), had only 9.17 mmol/100 g FW.

Vitamin C level is estimated to be 300–4,000 mg/100 g DW (dry weight) (Ercisli 2007) depending upon species, genotype, and environmental factors. Vitamin C is an essential ingredient to our bodily functions, but has also been reported as a protection against cardiovascular diseases and atherosclerosis. However, vitamin C may not be the only component responsible for prevention and eventually apoptosis of, e.g., cancer cells; a synergistic action of several other compounds or metabolites in berries and fruits has also been suggested (Eichholzer et al. 2001; Vecchia et al. 2001; Olsson et al. 2004; Halliwell 2006).

Vitamin B_9 or folate is an essential vitamin required for DNA and RNA formation, and for cellular replication. It is recommended that pregnant women increase their daily dose of folate to prevent neural tube defects in their babies. Rosehips and strawberries are rich sources of this vitamin, with levels of 100–180 μg/100 g FW and 70–90 μg/100 g FW, respectively.

Polyphenols are the most abundant antioxidants in the human diet. The main dietary sources are fruits and berries and plant-derived beverages such as juices, tea, and red wine. The polyphenols commonly occur as complex mixtures in plants, such as the flavonoid anthocyanin (provides red and purple color in fruits) and tannins. The antioxidative effect of rosehips is caused mainly (up to 90%) by polyphenols (Gao et al. 2000). The tannin in rosehips is ellagic acid, also present in strawberries, which is said to have antimutagenic and anticarcinogenic effects (Mertens-Talcott et al. 2005).

Rosehips also contain large amounts of carotenoids of which the carotenes are the most interesting. Here, we find beta-carotene (precursor of vitamin A) and lycopene. Lycopene is usually associated with tomatoes and tomato products, but Hornero-Méndez and Mínguez-Mosquera (2000) found up to 390 mg/kg DW in rosehips from Chile, which is more than in most fresh tomatoes. They suggested that rosehips could be used as an alternative to tomatoes in the food industry.

The seeds of rosehips have often been regarded as a waste product, but they do contain the unsaturated oils alpha-linolenic and linoleic acid (omega-3 and omega-6), which can be used for medicinal applications (Szentmihályi et al. 2002). In South America this oil has been used for centuries as a cure for different skin diseases. Now, it is being exported all over the world as a treatment for eczema and as cosmetic oil. However, there are so far no scientific reports on the action or results of this treatment.

Rosehips are very seldomly eaten raw; they must be processed one way or the other. Many of the bioactive compounds are sensitive to light and heat, and many are also water soluble, and can therefore easily be

destroyed by the processing, or leech out into the surrounding water used for canning or cooking. It is vital that the processing is performed as fast as possible to retain the valuable compounds.

12.8.5.2 Dogrose Hip Uses

The dogrose plant has been used since the Middle Ages as a medicinal plant. All parts of the plant were then used – leaves, flowers, hips, and roots – in the form of concoctions and infusions as a cure for all sorts of ailments. The main use was for various stomach troubles and as a remedy for all sorts of infections. Rosehips from section *Caninae* have a very special aroma and taste, and they have therefore been used also for various foodstuffs. Today they are used as jam, jelly, marmalade, and teas, and they are also ingredients in, e.g., ice cream and yogurt. In Sweden, rosehip soup has been a well-known dessert and snack for hundreds of years. The soup is made from dried rosehips and it is served either warm or cold, often with whipped cream and sweet biscuits. The current interest in dogroses is, however, not directed to their culinary properties. Rosehips are instead being commercialized for their potential in functional food, i.e., food products that contain biologically active components with a potential of enhancing human health or reducing risk of disease, and nutraceuticals with a medicinal effect on human health. A nutraceutical is normally ingested as a capsule or powder in a prescribed dose.

In Turkish medicinal folklore, rosehips have been used as a remedy for all kinds of stomach problems, and several of the present day studies also focus on the gastrointestinal tract. Rats that were fed extracts from *R. canina* showed a 100% protection from ulcerogenesis caused by ingestion of 96% ethanol (Gürbüz et al. 2003). In a recent study, extracts from *R. canina* combined with a *Lactobacillus* bacterial strain decreased the ROS (reactive oxygen species) activity in injured rat colons (Håkansson et al. 2006). The authors suggested that this combination could be useful as a treatment in colonic and vascular surgery and also in organ transplantations. The polyphenols in rosehips from *R. canina* have shown to be effective ROS scavengers in human blood (Daels-Rakotoarison et al. 2002). In yet another study, extracts of *R. canina* (fruit flesh and seeds) showed an inhibitory effect on body weight gain in mice (Ninomiya et al. 2007). The authors have now applied for a patent with this extract as the active ingredient of a method for improving fat metabolism in biological tissues (http://www.freepatentsonline.com/y2008/0003312.html).

The best-studied effect of rosehips is the influence on osteoarthritis. Patients with diagnosed osteoarthritis have been subjected to double-blind, randomized, and placebo-controlled clinical tests as they orally ingested a powder made from ground rosehips containing seeds and dried fruitflesh, called Litozin® (or LitoMove®). The patients improved overall mobility as well as the capacity of knee and hip joints. Several of these patients were also able to reduce their pain-killing medication (Warholm et al. 2003; Winther et al. 2005). The effect was apparently mediated by the galactolipid "GOPO" ((2S)-1,2-di-*O*-[9Z,12Z,15Z]-octadeca-9,12,15-trienoyl-3-*O*-beta-D-galactopyranosyl glycerol). A more recent study has shown that the observed effect on osteoarthritis by rosehips might also be due to fatty acids in the seeds: alpha-linolenic and linoleic acids (Jäger et al. 2008). There are also indications that rosehip extracts together with minor amounts of blueberry, blackberry, and grapevine extracts may diminish the risk of cardiovascular diseases by lowering the levels of inflammatory substances associated with early heart infarctions (Kornman et al. 2007).

12.9 Some Dark Sides and Their Addressing

Roses have a long history as horticultural plants and have as such been introduced into several countries outside their native range. Unfortunately, some of these *Rosa* species escaped gardens or parks and invaded natural habitats and threatened the native flora. Four *Rosa* spp. are so far known to be invasive globally (*R. canina*, *R. multiflora*, *R. rugosa,* and *R. rubiginosa*). Management is cost-intensive, since once established as alien plants *Rosa* spp. are difficult to eradicate due to their ability to resprout after cutting, browsing, or even fire.

12.9.1 Invasive Species of the Genus Rosa

While the *Rosa* genus has its origins north of the equator, these four species have already invaded habitats in the southern Hemisphere (Weber 2003). With their ability to grow root suckers and well armed spiny shoots and branches they form impenetrable thickets in their invasive range (Epstein and Hill 1999; Amrine 2002; Yates et al. 2004; Bruun 2005).

R. multiflora and *R. rugosa* are both native to temperate Asia, yet nowadays they are invasive in Europe and they have both been introduced to several countries in the southern Hemisphere as well as in North America. *R. canina* is native to Europe, temperate Asia, and northern Africa. *R. rubiginosa* is native to Europe and temperate Asia as well and both species have likewise been introduced to several countries in the southern Hemisphere as well as in North America (Fig. 12.1). Another wild rose from Asia, *R. bracteata*, is noted as being invasive in North America, but this is yet to be recognized on a global scale (ISSG 2008).

12.9.2 Management Strategies: The Example R. multiflora

Formerly planted as a windbreaker or as a natural fence, *R. multiflora* became the most detrimental weed in the 1960s in the eastern United States (Amrine 2002). From then on it spread rapidly throughout the USA and is expected to become even more abundant in the future (Robertson et al. 1994; Hunter and Mattice 2002; Merriam 2003). *R. multiflora* stands cause severe degradation of land used for grazing or recreation through the formation of dense multi-crowned thickets (Epstein and Hill 1999). It is also locally common in four provinces of Canada, where it was cultivated as a garden plant and nowadays grows in old fields, forest clearings, and along shores and roadsides (Darbyshire 2003). Furthermore, *R. multiflora* has been reported for northern Europe, New Zealand, southern Africa (Bean 1951; Weber 2003), and central Argentina (H. Zimmermann unpublished data).

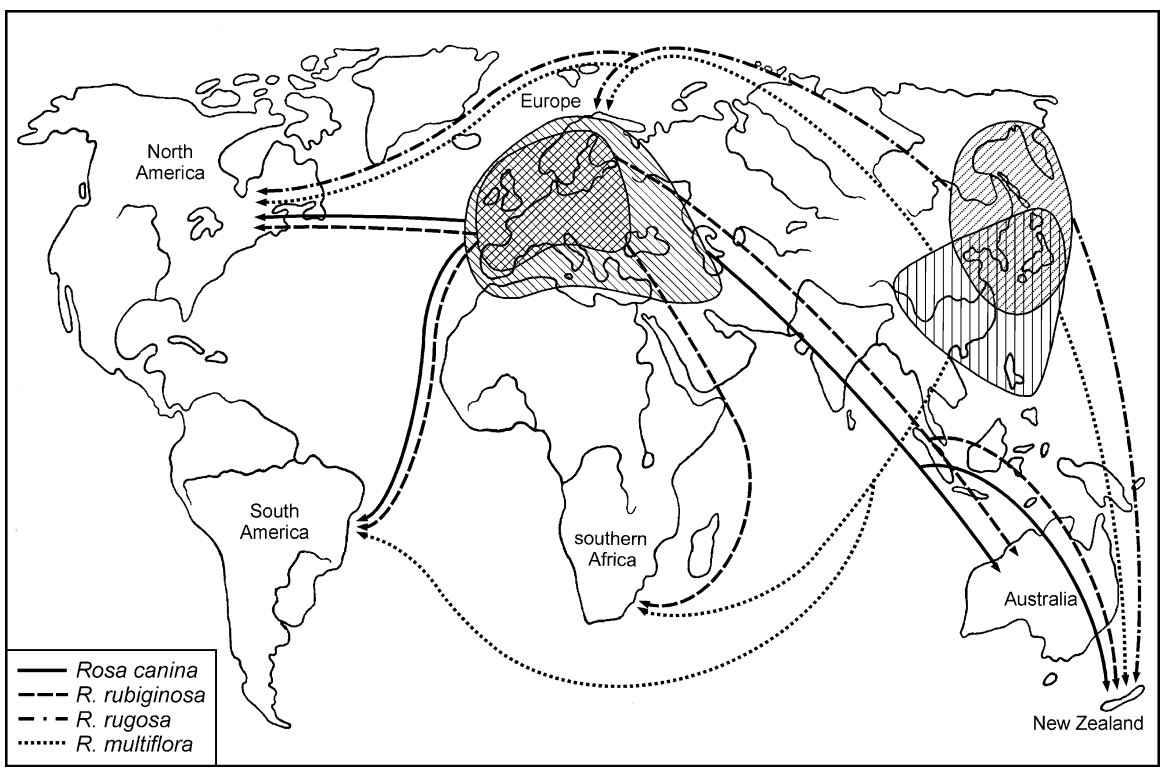

Fig. 12.1 Four rose species are currently invasive worldwide, *Rosa canina*, *R. rubiginosa*, *R. multiflora,* and *R. rugosa*. The native ranges are indicated by *shaded areas*, and represent only a rough outline, rather than an exact distribution range

In North America, numerous studies have investigated possible methods to eradicate *R. multiflora*. Loux et al. (2005) provide an overview of several control methods. As an alternative to biocontrol agents, herbicides, and mechanical control, they also propose a well-balanced grazing management by goats. They do, however, emphasize that the control of *R. multiflora* is a long-term task and that any success achieved in 1 year will be largely negated by reinfestation within the following 2 or 3 years. Amrine (2002) estimated that a herbicide-driven eradication in West Virginia alone would cost about $80 million. Since herbicide applications are cost- and labor-intensive, and herbicides pollute the environment, research in the USA has mainly focused on biocontrol agents for *R. multiflora*. The rose rosette disease (RRD) and the seed chalcid, *Megastigmus aculeatus*, seem to be the most promising candidates. So far the causal agent of RRD is not known but it can be transmitted with a 100% success by grafting (Amrine et al. 1988). Epstein and Hill (1999) propose the RRD as a biocontrol agent since it is native to North America, is lethal to *R. multiflora*, seems to be restricted to the genus *Rosa*, and implementation of this management method is feasible by landowners. No symptoms on ornamental roses in the surroundings were found in the 5 years following application (Epstein and Hill 1999), however, since a comprehensive screening of all available rose cultivars has not been conducted the risk of RRD is of concern to the rose industry (Jesse et al. 2006). Moreover, Hartzler (2003) objects that RRD alone will not provide a permanent solution, since plants in shaded habitats are less susceptible to infection and thus may act as new source populations. The eradication of *R. multiflora* plants alone cannot be deemed successful, since persistent *R. multiflora* seeds might still be in the soil ready to replace the recently removed adults. The ample production of rose hips as food for wildlife was one reason for planting this species in the USA (Epstein and Hill 1999). The second promising biocontrol agent, the seed chalcid (*M. aculeatus*), could resolve this problem. *M. aculeatus,* to which *R. multiflora* seems to be especially susceptible (Amrine 2002), lays its eggs in the receptacle and the developing larvae consume and kill the rose seeds.

12.9.3 R. rugosa: *A Threat to Nature Conservation*

R. rugosa has invaded habitats in northwestern Europe, northeastern North America, and New Zealand (Darbyshire 2003; Weber 2003; Bruun 2006). Its expansion is well demonstrated on the British Isles (Bruun 2005), where it was introduced in 1845 (Bean 1951). The species' preference for sandy coastline habitats (Ohba et al. 1973) made it a preferred candidate in the nineteenth-century shoreline protection schemes (Schlätzer 1974; Dubra and Olšauskas 2002). Shortly thereafter its invasive potential rendered it uncontrollable along the coast of the Baltic-, North-, and Atlantic Seas (Bruun 2006). In particular, the long distance dispersal of fruits and seeds by sea currents may have contributed to its success (Bruun 2005; Isermann 2008b). Moreover, it can be found inland in forest fringes and along railroad tracks (Brandes et al. 2003).

The presence of *R. rugosa* on coastal dunes represents a severe conservation problem as species richness, and more so the number of rare species, declines; thereby reducing species diversity of the dunes both on the local and regional scales (Isermann 2008b). Comparable dune shrub-communities, e.g., colonized by *Hippophae rhamnoides* or *Empetrum nigrum*, contain higher numbers of species (Isermann 2008a). Thus, this Asian rose is possibly a stronger competitor compared to *H. rhamnoides* and *E. nigrum*, since it shades the surrounding vegetation more effectively and has a stronger developed root system (Isermann 2008a, b). Furthermore, *R. rugosa*-dominated stands contain a higher number of neophytes (Isermann 2008a). Not only is *R. rugosa* a threat to species richness through competition, but it also hybridizes with native roses, thus triggering their genetic assimilation (Mercure and Bruneau 2008). Vanderhoeven et al. (2005) discovered that *R. rugosa* also alters soil properties, namely by increasing concentrations of exchangeable essential nutrients beneath its canopy.

Native Japanese *R. rugosa* communities are more species rich and are probably analogous to European *H. rhamnoides* communities, possibly due to the broader environmental niche in its new range (Isermann 2008a). While adult plants effectively

shade the surrounding vegetation, *R. rugosa* seedlings seem to prefer small-scale disturbed sites (Kollmann et al. 2007), and are probably dependent on mycorrhiza (Gemma and Koske 1997).

R. rugosa is difficult to control either mechanically or with herbicides (Bruun 2006); however, releasing potential biocontrol agents may constitute a threat to cultivated roses. Burning does not seem to be a permanent management option either, as *R. rugosa* plants constantly resprout after fire (Tsuda et al. 1999).

12.9.4 *Unsuccessful Invasion or Missing data?* R. canina

Despite the fact that *R. canina* has a wider distribution in Eurasia and is more abundant than, e.g., *R. rubiginosa* (Meusel and Jäger 1965; Timmermann and Müller 1994), it is not as equally successful an invader. Notwithstanding, it has been introduced to North America, Australia, New Zealand, and South America (Parsons 2001; Amrine 2002; Weber 2003; Damascos and Bran 2006); however, we did not find evidence of any capacity to build up such dominant stands as the other three species. Studies on invasive *R. canina* are few and a comparison with the other invasive European rose species would be desirable.

12.9.5 *Causes for the Invasion Success:* R. rubiginosa

R. rubiginosa is currently the most successful invader of these four rose species, being invasive in North America, Australia, New Zealand, southern Africa, Chile, and Argentina (Kissel et al. 1987; Hatton 1989; Damascos and Gallopin 1992; Amrine 2002; Weber 2003). Invasive species most certainly pose a threat to the local biodiversity, yet they also provide an opportunity for the study of evolutionary and biogeographic processes on a global scale. A comparison between native European *R. rubiginosa* populations in Germany and Spain with invasive Argentinean populations is currently underway which will enhance the understanding of invasion processes (H. Zimmermann et al. unpublished data). It is uncertain when *R. rubiginosa* was introduced to Argentina, however, it is assumed to have been at least 100 years ago (Damascos et al. 2004). Spanish immigrants are often held responsible for bringing this species to the New World, however, preliminary genetic analysis points to a Central European origin (Zimmermann et al. 2010).

R. rubiginosa shrubs in Argentina outgrow their European ancestors in both number and size, and populations cover much larger areas than in Europe (Zimmermann et al. 2008, 2010). The invasion success of *R. rubiginosa* could be due to special characteristics of this species (invasiveness) or attributes of the new environment (invasibility). Much like the other invasive roses, *R. rubiginosa* spreads clonally, resprouts after cutting or fire, is able to produce apomictic seeds, and its spiny leaves and branches form an efficient defense against herbivores. In contrast to Germany and Spain, propagule pressure is high in Argentina, simply because bigger shrubs produce more rose hips. Moreover, seeds are not only dispersed by birds and small mammals, but also by horses and cattle (Damascos et al. 2005). Reciprocal transplantation experiments in Europe and Argentina will clarify whether Argentinean roses are extremely successful ecotypes due to rapid genetic changes (Blossey and Nötzbold 1995).

It seems unlikely that the release from natural herbivores or parasites could be an attribute of the new environment as *R. rubiginosa* is equally well defended against herbivory in the invasive and native ranges. In addition, there seems to be no differences in the spectra of further parasites and fungi (Havrylenko 1995). Preliminary analysis of abiotic soil parameters and climatic variance did not point to more favorable conditions in Argentina (H. Zimmermann unpublished data). Since European *R. rubiginosa* populations are always intermixed with other *Rosa* species, the absence of competing roses in the same habitat could be an advantage for *R. rubiginosa* in Argentina.

Our geographic approach highlights that land-use history is an important factor determining population sizes on a continental scale (Zimmermann et al. 2008). Within the old cultural landscape of Europe, suitable *R. rubiginosa* habitat is limited to a few protected areas, where it is cut in order to promote plant biodiversity. This implies that Argentinean populations might simply be relatively large, because European populations are managed. All invaded habitats in Argentina had experienced anthropogenic disturbance

as well, however, disturbance was a singular event which enabled the establishment of the species.

In southern Argentina, *R. rubiginosa* is already falsely accepted as a typical Patagonian plant. Its image can be found on postcards, and fruits of naturalized *R. rubiginosa* are harvested to fabricate teas, marmalade, and rose oil (Damascos et al. 2004). Therefore, it is especially important to raise awareness that this species is invasive and to offer alternative indigenous replacements.

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Chapter 13
Theobroma

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13.1 Introduction

Theobroma cacao L. ($2n = 2x = 20$), commonly known as cacao (raw plant material), cocoa (processed material) or chocolate, is an important tropical rainforest tree in the family Sterculiaceae (alternatively Malvaceae *senso lato*); which is native to tropical South America (Cuatrecasas 1964; Alverson et al. 1999; Bayer et al. 1999; Wood and Lass 2001). This species comprises a large number of highly morphologically variable populations, which can all be crossed with each other (Cheesman 1944; Bartley 2005).

The ancient cultigens of cacao were only present in Mesoamerica, where cultural elaboration and use of cacao can be traced back several thousand years (Gómez-Pompa et al. 1990; Sauer 1993; Young 1994; Coe and Coe 1996; Henderson et al. 2007). The use of the sweet pulp that surrounds the seeds for fermented drink is a likely reason for domestication. Recent archeological discoveries in Honduras showed that the Olmec people in the Ulua Valley fermented the sweet pulp of cacao to make an alcoholic drink at least 3,000 years ago, well before the grinding of the bitter seeds to produce a chocolate drink (Henderson et al. 2007). Ultimately, the diverse nature of cacao's uses as well as its use as currency led to it being widely grown in Mesoamerica before the Spanish arrived (Bergmann 1969; Young 1994). Nevertheless, only a very small fraction of the diversity was dispersed from the Amazon to Mesoamerica and thus the domesticated cacao has a narrow genetic background (Dias 2001; Bartley 2005).

In the Amazonian rainforest, the evidence of cacao cultivation by different indigenous groups has been minimal (Sánchez et al. 1989; Dias 2001; Bartley 2005). It was suggested that Amazonian tribes might not have had the need to formally cultivate a tree that occurred in some abundance (Dias 2001; Bartley 2005). The "wild relatives of cacao," therefore, include two different categories of germplasm. The first one is the large spectrum of wild populations that spontaneously grow in the Amazonian rainforest, ranging from the French Guiana to Bolivia. There is no reproductive barrier between the cultivated and wild cacao trees. Even for the ancient cultigens of cacao – the Criollo cacao from Mesoamerica, most of their morphological characteristics are not distinctive from their wild counterparts. There is little difference between cultigens and wild cacao in terms of their agronomic traits, as shown in some breeding experiments (Iwaro et al. 2006). The adoption of cacao as a crop by man utilized native trees found in nature with minimal deliberate changes to phenotypic features. Compared with many other domesticated field crops, cacao has the advantage that wild germplasm can be directly used in breeding or commercial production, either as progenitors or as clones (Dias and Resende 2001; Eskes and Efron 2006).

The second category of wild cacao refers to the 22 related *Theobroma* species (Cuatrecasas 1964), but none of these have made an actual contribution to the cacao improvement yet due to the interspecific crossing barrier. Therefore, conservation efforts have focused on the wild *T. cacao* populations, whereas the resources allocated to the related *Theobroma* species have been scarce, except for *T. grandiflorum* (cupuassu), which is considered an important fruit crop in various

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Amazonian countries. The majority of the research work on the related species has focused mainly on cupuassu, which includes: a breeding program, germplasm collection (Alves et al. 2007), germplasm characterization and preservation, new product development, interspecific hybridization (Silva et al. 2001) and phylogenetic studies (Silva et al. 2004; Silva and Figueira 2005).

Today, *T. cacao* is cultivated extensively as the unique source of cocoa butter and powder for the confectionery industry. Approximately 3.7–3.9 million tons of cocoa beans are produced annually (International Cocoa Organization 2006), of which 95% are produced by 5–6 million smallholder farmers (FAOSTAT 2005). Worldwide, cocoa (the product obtained from dried fermented cacao seeds) is a $5–$6 billion export commodity (FAOSTAT 2005). The chocolate industry has grown to an approximately $53.2 billion market in 2005 (Research and Markets Guinness Centre 2009). Moreover, cacao is mainly produced in important biodiversity regions; where the cultivation of cacao can create environmental benefits such as enhanced carbon sequestration, as well as biodiversity and watershed conservation.

As with many other tropical perennial species, cacao seeds are recalcitrant, i.e. they will not survive the drying process and/or storage at low temperature. Therefore, conservation of cacao germplasm requires maintaining living trees ex situ in field genebanks in tropical regions. There are at least 45 major cacao collections around the world. Each accession is usually represented by multiple trees through clonal propagation. There are two global collections that are in the international public domain, one of which is the International Cocoa Genebank, Trinidad (ICG, T) managed by the Cocoa Research Unit (CRU) of the University of West Indies and the other is International CATIE Collection (IC3), by the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE) in Costa Rica (Turnbull et al. 2004). Currently, there are a more than 3,500 accessions held in these two international collections, which are made available to plant breeders via the international quarantine center located at the University of Reading in the UK. These genebanks are supported by both public and industrial funding. In addition, there are a large number of wild cacao accessions maintained in several major national genebanks, including the ones from "Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC)" and "Empresa Brasileira de pesquisa Agropecuária" (Embrapa) collections, both from the Brazilian Ministry of Agriculture, in northern Brazil (Silva et al. 2004) and the INIAP collection in Ecuador (ICGD 2007).

The cacao germplasm collections are often vulnerable due to the potential damages caused by biotic and/or abiotic stresses. Cryopreservation (storing germplasm usually in the form of tissue, mainly somatic embryos in liquid nitrogen at −196°C) could be a promising complementary approach (Fang et al. 2004), but further research is still needed to ensure the genetic integrity through the tissue culture process and while being stored at low temperature. Research on in vitro conservation has also been carried out in the International Cocoa Quarantine Center, Reading (ICQC, R), which serves as the source for the dissemination of disease-free (postquarantine) accessions for the global cacao community. Information on cacao germplasm held in the various ex situ collections is currently managed in the International Cacao Germplasm Database (ICGD) hosted at the University of Reading in UK (Wadsworth et al. 2003; Turnbull et al. 2004) and CocoaGenDB at the French Agricultural Research Center for International Development (CIRAD; Ruiz et al. 2004). This information network combines comprehensive passport data and characteristics of individual accessions. In addition, a research symposium is regularly organized by the International Group for Genetic Improvement of Cocoa (INGENIC, http://ingenic.cas.psu.edu), a network that includes cacao breeders, geneticists and germplasm curators from 35 countries. The activities of international cacao germplasm conservation is coordinated by CacaoNet (www.cacaonet.org), a network hosted by the Bioversity International, which contributes to the sustainability of the global cocoa economy by optimizing the conservation and use of cacao genetic resources.

13.2 Wild Cacao Populations in the Amazon Basin

The stretch of the Amazon River between its formation by the confluence of the Marañón River and the frontier with Brazil is known as "Alto Amazonas," from which the term "Upper Amazon" has been used to describe most of the known wild cacao populations

from this area. In this region, a series of major river systems in Peru, Ecuador, Colombia and Brazil flow into the Marañón River and the Amazon River. Among others, these include the rivers Santiago, Morona, Pastaza, Nucuray, Nanay, Urituyacu, Chambira, Tigre, Nanay, Napo, Huallaga, Ucayali, Javary, Putumayo, Japura, Purus and Negro. Wild cacao populations are found in these river basins in both spontaneous (without human interference) and subspontaneous (wild cacao trees exploited by man) prior to European occupation (Dias 2001; Bartley 2005). Wild cacao germplasm samples from the expeditions in the Amazon were predominantly collected along the banks of navigable rivers (Pound 1938; Lachenaud and Sallée 1993; Lachenaud et al. 1997; Almeida and Dias 2001). It was asserted that each natural cacao population was founded by a limited amount of reproductive materials and has a narrow genetic base (Pound 1945; Bartley 2005).

So far, the spatial genetic structure of the natural cacao populations remains poorly understood. Analysis based on morphological and molecular variation showed that the diversity of natural cacao populations is stratified by the major river valleys in the Amazon (Pound 1938; Almeida and Dias 2001; Bartley 2005). Within each river basin, wild cacao is usually grouped in patches and separated by large spatial distances between patches. It is hypothesized that gene flow of cacao is limited and mating is likely confined within patches (Chapman and Soria 1983) due to the short-distance seed dispersal by rodents and monkeys and short-distance pollen dispersal by midge species (*Forcipomyia* spp). Nevertheless, gene flow in the wild or within germplasm collections have not been investigated to determine the range of midge activity and the number of pollen donors, except for the ongoing research on mating system and gene flow in an ancient subspontaneous populations located in Cameta, near Belem, Brazil.

Significant departure from Hardy–Weinberg Equilibrium (HWE) was detected in the French Guiana wild populations (Lachenaud and Zhang 2008) and in the semi-wild population in the Ucayali river valley in Peru (Zhang et al. 2006a, b). In addition to the likely short-distance gene dispersal, cacao has a gametosporophytic self-incompatibility system (Cope 1962), which works in a quantitative manner. The lack of self-incompatibility in some genotypes is partially responsible for the high fixation index in several natural populations. Indeed, fully homozygous genotypes were frequently found in the populations from the Nanay and Pastaza River in Peru (D. Zhang et al. unpublished data). Some wild cacao trees are found in the form of single plants, but the majority of them formed a "clump" (plants with several trunks at different development stages and overlapped generations). The apparent generation overlap within a patch is likely another factor contributing to mating between relatives thus increases the level of inbreeding. The multiple trunks can also come from self-propagation by chupon production, which increased the chance of inbreeding by self-mating as opposed to inbreeding by mating between family members.

Despite the commonly perceived short-distance gene flow and limitation in effective population size in wild cacao, isolation by distance was only detected over a long geographical range (e.g., a few hundred km), but not in a local basin or short distance (Zhang et al. 2006a, b). Whether the lack of isolation by distance is due to the rapid range expansion or unknown mechanism of long-distance gene dispersal (e.g., pollen dispersal by long-distance pollinator) remains to be understood. Sereno et al. (2006) reported that in the natural or semi-natural populations sampled in four regions of Brazil (Acre, Rondonia, lower Amazon and upper Amazon), most of the genetic diversity was allocated within populations rather than between populations, indicating a typically high gene flow. Therefore, some of the apparently isolated populations may actually belong to the same metapopulation in terms of gene dispersal, which impacted their genetic differentiation.

The collection of cacao germplasm that has most influenced cacao breeding came undoubtedly from Trinidad. During the Spanish Colonial rule, Trinidad cacao planters grew mostly Criollo cacao. After the destruction, in 1727, of the majority of the cacao crop by an unknown "blast," Forastero material was introduced most likely from Venezuela and resultant natural hybridization led to the Trinitario germplasm, which is noted for its fine flavor (Toxopeus 1985). Hybrid vigor resulted in vigorous planting material, which was then introduced to Venezuela; a few fruits from Trinidad were sent to Ecuador about 1890 from which the "Venezolanos" were derived. Much later, at the then Imperial College of Tropical Agriculture in

Table 13.1 Diversity indices of primary germplasm groups from major cacao collecting expeditions

Major collecting expeditions	River basins	No. of mother trees	Gene diversity	Observed heterozygosity	Allele richness
Pound collection (1938–1943)	Morona, Nanay, Ucayali, Maranon	32–48	0.610	0.430	11.4
Anglo-Colombia collection (1952–1953)	Apaporis, Caqueta Caguan Cauca, Infrida Negro, Putumayo Vaupes	191	0.718	0.554	8.9
IBPGR-Bolivian collection (1974)	Rio Beli	21–43	0.540	0.460	5.1
Brazilian collection (1965–1967; 1981–1984)	Overall average	144	0.658	0.285	4.2
	Jari, Amapá	10	0.350	0.341	2.8
	Maicuru, Pará	10	0.499	0.352	2.9
	Jamari, Rondonia	15	0.479	0.140	2.3
	Ji-paraná, Rondonia	9	0.312	0.170	2.3
	Acre, Acre	22	0.321	0.285	2.8
	Iaco, Acre	10	0.454	0298	3.0
	Tarauacá, Acre	15	0.459	0.325	3.4
	Purus, Acre/Amazonas	14	0.580	0.291	3.1
	Japurá, Amazonas	19	0.506	0.261	3.2
	Solimões, Amazonas	14	0.581	0.334	3.6
	Baixo Japurá, Amazonas		0.525 (*H_{nb})	0.327	3.2
French Guiana collection	Oyapok, Camopi Euleupousing, Tanpok, Yaloupi	189	0.370	0.160	4.9
Chalmers collection (1968–1973)	Curaray, Coca, Napo, Putumayo	184	0.643	0.467	8.6
LCT EEN collection (1979–1987)	Curaray, Coca, Napo, Putumayo	255	0.670	0.440	9.5
ICA and IBPGR Colombia collection	Colombia	151	0.630	0.414	8.2
Peruvian collection (1987–1989)	Ucayali	51	0.740	0.490	9.8
INCAGRO/USDA collection (2008)	Pastaza, Nucuray Urituyacu	126	0.522	0.360	9.7

Trinidad, an extensive survey of the Trinitario population was conducted resulting in the selection of approximately 100 ICS clones selected principally for yield characteristics. However, the impact of witches' broom disease (WBD) led to the planning of expeditions to collect disease-resistant germplasm from the upper Amazon region (Pound 1938). Since then several collecting expeditions have been undertaken in the Amazon basin. Bartley (2005) and Lockwood and End (1993) provide a historical account of the collecting missions with the latter listing the primary collections of wild cacao (Table 13.1). These collecting activities obtained a substantial number of genetic groups of wild cacao which are now maintained in the various national and international collections. The major wild populations/genetic groups include but are not limited to the collections discussed hereafter.

13.2.1 The "Pound Collection"

This was the first cacao germplasm collecting expedition into the upper Amazon (Pound 1938, 1945; Bartley 2005) and the collecting sites included part of the tributaries of Rio Ucayali, Rio Morona and Rio Marañón. This led to the establishment of the germplasm collection in Iquitos, Peru, known as the "Pound Collection," named after the collector FJ Pound. Pound's expeditions were aimed at searching for genotypes resistant to WBD, caused by the fungus *Moniliophthora perniciosa* (Stahel) (Aime and Phillips-Mora 2005), and these germplasm accessions have therefore served as the foundation for breeding programs around the world for resistance to WBD. This collection comprised the IMC, MO, NA, PA, POUND and SCA accessions, which are all considered

upper Amazon Forasteros. Eighty half-sib families were collected from west Ecuador, 25 half-sib families from Peru and Colombia and 32 clones from Peru. The 80 half-sib families yielded 1,185 Ecuadorian Refractario that are in the ICG, T and were the result of the first expedition (Lockwood and End 1993). The 25 half-sib families yielded 250 fruits (Lockwood and End 1993) and the seed lot sent to Barbados for quarantine yielded about 2,500 seedlings (Toxopeus and Kennedy 1984) from which bud wood was later taken and vegetatively propagated onto rootstock in Trinidad as 486 accessions. The 1942 expedition in Peru yielded 32 clones and the majority of these accessions collected by FJ Pound are maintained ex situ in the ICG, T (Lockwood and End 1993; Motilal and Butler 2003).

13.2.2 The Anglo-Colombian Cacao Collecting Expedition

This expedition was organized by the University of West Indies and the Colombian government to explore parts of the Colombia to collect wild and cultivated *T. cacao*, as well as other species in the genus *Theobroma* and the allied genus *Herrania* (Baker et al. 1953). Several collecting trips were implemented from August 1952 to October 1953. The first explored areas were parts of the rivers Caquetá, Apaporis, Vaupes, Negro, Infrida and their tributaries in the provinces of Amazonas and Vaupes (1°30′S–3°N; 67°W–71°W). The second areas were parts of the rivers Putumayo, Caquetá and Caguan in the provinces of Caquetá and Putumayo (0°20′S–2°N; 74°W–77°W). The third areas were parts of the trans-Andean provinces of Valle del Cauca and El Chocó (3°N–6°N; 76°W–78°W). In addition, other scattered areas in the provinces of Antioquia, Norte de Santander, Magdalena Santander and Huila were also explored (Baker et al. 1953; Bartley 2005). While the main interests of the expedition centered on *T. cacao*, the opportunity was taken to study the diversity distribution of other relative species as well as the major pathogens of *T. cacao*. A total of 191 accessions were collected, of which 63 were living material sent to Trinidad. In addition, seven related species in *Theobroma* and ten species in the genus *Herrania* were collected. The *Theobroma* materials were identified by José Cuatrecasas and the *Herrania* materials were identified by Richard E Schulte (Baker et al. 1953).

13.2.3 The "Chalmers Collection"

The Chalmers Collection was the result of the INIAP-UWI (Instituto Nacional Autónomo de Investigaciones Agropecuarias – University of West Indies) expedition. This expedition collected *T. cacao* in the Oriente region of Ecuador between 1968 and 1973. Wild cacao trees were collected as budwood and pods from the primary forest. Data accumulated from Chalmers' Collection were summarized in a series of seven unpublished Reports by the CRU, University of the West Indies (Chalmers 1973, 1974). The expeditions covered a large part of the Oriente region of Ecuador, including the airstrip of Putumayo, Lago Agrio, Rio Aguarico, Rio Bobonaza, Rio Coca, Rio Curaray, Rio Napo, Rio Nushino, Rio Payamino upriver from Rio Napo, Rio Pushino, Rio San Miguel, Rio Tiputini, Rio Villano, Santa Cecilia and Shushufunde. The first five trips yielded approximately 200 clones as budwood and numerous pods. The sixth and final collection trip was made in March–April 1973 in which both fruit and budwood from tree lacking WBD symptoms were collected and half of the seeds were sent to Trinidad (Chalmers 1974). The expeditions collected some Amazonian Forastero material with a high percentage of white beans (a presumed Criollo characteristic) but unfortunately a large proportion of this collection was lost in nurseries (Lockwood and End 1993).

13.2.4 The London Cocoa Trade – Estacion Experimental Napo Collection

The London Cocoa Trade – Estacion Experimental Napo (LCT EEN) collection started in 1979 and was supported by the London Cocoa Terminal Market and the Cocoa Association of London. The plan was to make a systematic collection of wild cacao in the Amazon region of Ecuador. The expeditions were undertaken by John Allen and his colleagues in two phases

between 1979 and 1987, with the full support by the Instituto Nacional Autónomo de Investigaciones Agropecuarias (INIAP) of Ecuador.

During the first phase of the project, a total of 23 collecting trips were made, which resulted in 281 accessions (represented as either seedlings, clones or both) being established at the Estacion Experimental Napo (EEN) near San Carlos. The project adopted a collecting strategy to capture genetic diversity with a well-defined geographic zone, which covered a large part of the Amazon region of Ecuador. In the second phase of this project, the LCT EEN germplasm from the field collection established at San Carlos were sent to various cacao quarantine stations including Kew (England), USDA-Miami (USA), Montpellier (France) and the BCQS (Barbados). However, the overall survival rate of the germplasm was low, especially for the transferred budwood used for vegetative propagation. This was partially caused by the excessive delays between the time of collecting budwood and delivering it to the quarantine stations, due to the remote location of San Carlos. Based on the standard morphological descriptors, Allen did a cluster analysis using 281 samples from Ecuador and 36 samples from Colombia. Results of the analysis showed that all the Ecuador material was grouped into one cluster, whereas the Colombian material formed two more distinct clusters. All three clusters appeared to differ from Pound's Peruvian materials (Allen and Lass 1983). Some of the remaining material from this collection is also maintained at Babaoyo Station near Guayaquil, Ecuador (Allen and Lass 1983).

13.2.5 The Ucayali Population, Peru

The collection was made along the Ucayali River in 1987–1989 as part of the cacao rehabilitation project funded by the Peruvian government and the Food and Agriculture Organization (FAO) of the United Nations (Coral 1988; Evans et al. 1998). The major objective of this expedition was to collect material with resistance to WBD. The Ucayali clones were semi-spontaneous cacao trees collected from the Ucayali River valley as well as the Urubamba River and its tributaries (Coral 1988; Evans et al. 1998), including Contamana, San Carlos, Ucayali, Cushabatay and Lake Chiatipishca. These clones were collected as budwood and were maintained in the National Agricultural University at Tingo Maria. Part of the collection was also established in Sahuayacu near Quillabamba, Peru, and some was also sent to Brazil to the Instituto Agronomico de Campinas, São Pauo. However, a fraction of the collections was lost due to the social turmoil in Peru in the following years. In 1998, a recollection was carried out in Sahuayacu to restore this collection. Today, a total of 51 Ucayali clones are maintained in Tingo Maria, Peru (Evans et al. 1998; Zhang et al. 2006a, b) and Bahia (Figueira 1997). In recent years, this germplasm has increasingly attracted attentions as a potential source of disease resistance.

13.2.6 The French Guiana Collections

Three collecting expeditions were carried out by CIRAD in 1987, 1990 and 1995 in the southeastern French Guiana, where 215 mother-trees were collected (Lachenaud and Sallée 1993; Lachenaud et al. 1997). During the first expedition, in the upper Camopi and upper Tanpok rivers, a total of 243 pods were collected from 147 wild mother-trees (and two semi-wild trees), whilst two mother-trees were collected in budwood form. A pod from each mother-tree was preserved in French Guiana; the seedlings obtained were planted in a collection, and then studied individually for 10 years. Other pods, representing only 92 mother-trees, were sent to Montpellier (France) and, after quarantine, budwood was taken from the seedlings and sent to seven countries (Cameroon, Costa Rica, Ivory Coast, UK, Philippines, Togo and Trinidad and Tobago) between 1988 and 1990. In other words, each wild mother-tree collected in 1987 should be currently represented by a family of trees, in the form of seedlings (in French Guiana) and/or clones (in French Guiana and elsewhere).

During the second expedition (Kérindioutou and Upper Oyapok rivers in 1990; Lachenaud and Sallée 1993), 27 mother-trees were collected, in three populations (Borne 7, Kérindioutou and Pina). The seedlings and budded plants obtained were planted in two plots in Sinnamary, French Guiana.

The third expedition (Eulepousing, Oyapok and Yaloupi rivers in 1995; Lachenaud et al. 1997) was an international expedition (with CRU) and yielded pods from 34 mother-trees (46 pods were collected,

21 sent to Barbados). As in the two previous collecting trips, the germplasm was planted in a plot in Sinnamary and studied for 10 years.

From 1989 onward, the GU prefix (for "Guyane," i.e., French Guiana) was the code used at the CIRAD Paracou-Combi station (Sinnamary, French Guiana) for all of the cacao germplasm in the collection and covered quite diverse origins. Nevertheless, all the GU clones that have been sent out from French Guiana were collected in 1987 from the Camopi and Tanpok river basins (Lachenaud and Sallée 1993). This GU germplasm only accounts for part of the wild cacao trees of French Guiana: other accessions come from the basins of the Kérindioutou (clones with prefixes KER and B7), Oyapok (OYA, PINA), Euleupousing (ELP) and Yaloupi (YAL) rivers (Lachenaud and Sallée 1993; Lachenaud et al. 1997, 2005, 2007).

Descriptions of the activities undertaken on wild cacao trees from French Guian, including collection expeditions, germplasm characterization, evaluation and distribution, are presented in Lachenaud et al. (2005). A synopsis of the research activities undertaken in French Guiana and elsewhere, and the main results, can be found in Lachenaud et al. (2007).

13.2.7 The ICT-USDA Expedition

This expedition is the first of a series of collecting expeditions to survey the full cacao genetic diversity in the Peruvian Amazon. Several collecting expeditions will be carried out in areas lacking representation in the ex situ cacao germplasm collections. The upper Amazon region, as the putative center of diversity for cacao will be the primary target of the collecting expeditions. The first geographical focus will be the major tributaries of Rio Marañón, including Rio Santiago, Rio Pastaza, Rio Nucuray, Rio Urituyacu, Rio Tigre and Rio Putumayo. Within each subbasin, the identification of collecting sites will be assisted by GPS mapping tools. Habitat descriptions will be examined and the target area will be chosen based on the potential of complementary diversity.

The first expedition took place in July–August 2008 by a team of researchers from the Instituto de Cultivos Tropicales (ICT), in Tarapoto, Peru, and researchers from Sustainable Perennial Crops Laboratory and the Systematic Mycology and Microbiology Laboratory at Beltsville, Maryland. The expedition was supported by a Peruvian development project, INCARGRO and by the USDA. The objectives of this expedition were to collect (1) wild trees, (2) fungi that cause diseases on cacao and (3) beneficial fungi that might be natural enemies of these fungal pathogens. A total of 198 samples from seven rivers were collected and 120 living trees were propagated in the ICT facilities in Tarapoto, Peru. The goal is to carefully evaluate all of these wild cacao plants to see if any possess traits that could be used to improve resistance to diseases or to give the chocolate industry new quality or flavor traits. The beneficial and pathogenic fungi will be used to identify potential new biocontrol agents and to better understand the diseases that plague this crop.

13.2.8 The Brazil Populations

The Brazilian Amazon holds a large number of wild cacao populations (Almeida et al. 1987; Almeida and Dias 2001). Collecting expeditions in the region were initiated with the collaboration of international experts from 1965 to 1968, resulting in the populations BE, MA, RB, OB, CA, CSUL and CJ (Soria 1970).

After that, a systematic collection of the genetic diversity of wild and semi-wild cacao from the whole Brazilian Amazon region was conducted by the Brazilian government from 1976 to 1991 (Almeida et al. 1987, 1995) with the objectives to preserve and characterize the diversity of cacao in ex situ germplasm repositories in the region. The collection established at the "Estação de Recursos Genéticos do Cacau José Haroldo" (ERJOH), located in Marituba, Pará state, currently holds more than 1,800 accessions, of which 940 were derived from clonal propagation, while 877 were derived from open-pollinated seedlings, from 36 river basins of the 186 Brazilian Amazon basins (Almeida et al. 1995). Other field genebanks were established at Ouro Preto D´Oeste, Rondonia and Medicilandia, Para, which hold duplicate accessions from Marituba. Many spontaneous or subspontaneous trees have been collected from all over the Brazilian Amazon basin, including Rio Solimoes, Rio Japura, Rio Ica, Rio Javari, Rio Tarauaca and Rio Embira. The accessions collected after 1976 were named as CAB (Cacau da Amazonia Brasileira). All CAB material were collected under a provisional

local name and given a CAB number at a later date. Numbers given above 5,000 indicate seedling progeny from a collected pod; numbers below 5,000 are budwood collections. Materials in the CAB series were collected on various expeditions by numerous collectors (Almeida et al. 1995; Bartley 2005).

There were several other collecting expeditions in the Americas during the last few decades. However, these populations or genetic groups were not listed in this chapter either because it is not sure if they were truly wild populations or little molecular characterization has been done on these groups. For example the ICA and IBGRI Collecting Expedition in 1983 and 1986 were significant collecting efforts carried out in Colombia, but a significant fraction of the trees found in the river valleys appeared as subspontaneous or cultivated (Bartley 2005) thus they were not included in this chapter. A complete summary of all the historic collecting activities, including both cultivated and wild germplasm, was reviewed by Lockwood and End (1993).

13.3 Molecular Characterization of Wild Cacao Germplasm

Significant progress has been made in the past decade, owing to the availability of molecular markers, especially the development of microsatellite markers (Lanaud et al. 1999). The availability of high or mid-throughput genotyping facilities, in combination with data processing computing capacity, now enables systematic assessment of genetic identity and diversity in the national and international cacao gene banks, which has brought significant economic benefits and a broader insight into the genetic composition of the germplasm. Currently, the molecular characterization of cacao germplasm collections includes the following research issues.

13.3.1 *Identification of Duplicated and Mislabeled Accessions*

Duplicates or synonymous groups are common in cacao germplasm. The simple sequence repeat (SSR) technique allows verification of duplicates by matching multilocus DNA profiles between potential duplicates (Zhang et al. 2006a). The effectiveness of using SSR markers for identification of duplicated cacao accessions has been substantiated (Zhang et al. 2006a, 2009a; Motilal et al. 2008). Accessions with different names that are fully matched at a given number of SSR loci are designated duplicates or synonymously mislabeled accessions. Statistical rigor to determine whether two individuals may share the same multilocus genotype by chance (Waits et al. 2001) is also assessed for all match declarations. Another important tool that significantly enhances the effectiveness of germplasm identification is the newly available statistical method for assignment test (e.g., Bayesian approach), which does not require prior assumptions of population boundaries (Pritchard et al. 2000). The result of the assignment test is a posterior probability value that can be interpreted directly as the probability of origin of each individual from each population sampled (assuming the true population of origin has been sampled). Since the majority of the cacao germplasm was collected from different geographical regions and belongs to different populations, the assignment test in combination with SSR markers is a very powerful tool in detecting mislabeled accessions for cacao (Zhang et al. 2006b, 2009b; Motamayor et al. 2008).

13.3.2 *Verification of Genealogical Relationship*

The wide application of SSR (or microsatellite) markers in population genetics has spurred the development of new statistical methods for inferring genealogical relationships from molecular data. Parentage and kinship analysis is now routinely applied to verify the recorded pedigree in cacao germplasm (Schnell et al. 2005; Takrama et al. 2005; Zhang et al. 2009b). In addition to parentage inference, reconstruction of kinship can also be performed using maximum likelihood-based methods that assign individuals in a sample to full- and half-sib families (Blouin 2003; Wang 2004). These methods are particularly useful for populations known to have family structures but lacking parentage and kinship data (i.e., most of the cacao germplasm collected from upper Amazon in the 1930s). Using 15 SSR markers, Zhang et al. (2009b)

genotyped 612 accessions in the Pound collection and reconstructed the hidden full- and half-sib families in the wild cacao collected in the 1930s. Likelihood simulation also identified eight probable parents that are responsible for 117 pairs of mother–offspring relationships in this collection. The clarified genealogical relationship will significantly enhance the efficiency in both conservation and utilization of these upper Amazon wild cacao germplasm.

13.3.3 Investigating the Diversity Distribution in the South America Cacao Gene Pool

The spatial pattern of biodiversity and the historical and climatic effects on the distribution of genetic diversity in wild cacao have been studied in the last few years. Sereno et al. (2006) compared samples from natural populations from the Brazilian Amazon (Acre, Rondonia, lower Amazon and upper Amazon). The Brazilian upper Amazon population was found to have the largest genetic diversity and therefore was confirmed to be part of the center of diversity for the species. Lachenaud and Zhang (2008) fingerprinted 189 wild trees in 18 natural populations collected from French Guiana. They reported a major structure of genetic diversity in the metapopulation of French Guiana wild cacao trees ($Fst = 0.20$). The results suggest that the French Guiana populations, as a border region of the whole cacao gene pool in the Amazon, has experienced significant genetic drift and climate events that affected the Guiana forests in the Quaternary period. The relationship among the different populations existing in the various ex situ collections was assessed by Motamayor et al. (2008). A total of 1,241 accessions were genotyped with 106 SSR markers. Based on the Bayesian clustering analysis, these authors proposed that the collected cacao germplasm can be grouped into ten genetic clusters.

13.4 Related Wild Species of *T. cacao*

Theobroma is of exclusive Neotropical origin, with natural dispersion in tropical lowland rainforests extending from the Amazon basin through southern Mexico (18°N–15°S) (Cuatrecasas 1964). The genus *Theobroma* contains 22 species, classified into six sections (Table 13.2), which may have a great importance as gene reservoir for cacao improvement and an immense potential as new crops. The phylogenetic relationships of *Theobroma* species had been defined by the classical method of comparative morphology (Cuatrecasas 1964), based mainly on pod and floral structure, and relevant vegetative characters (mode of germination; growth type; presence of hairs in young leaves and branching pattern). Section *Glossopetalum* was considered the most ancestral of the genus (Table 13.2), while sections *Rhytidocarpus*, *Oreanthes* and *Theobroma* were considered more derived, with section *Theobroma* exhibiting the most apomorphic characters (Cuatrecasas 1964). Section *Telmatocarpus* displays some plesiomorphic characters, similar to *Glossopetalum*, while exhibiting other more apomorphic characters, similar to section *Theobroma* (e.g., glabrous leaves; partially woody pericarp) (Cuatrecasas 1964).

The genus *Herrania* is closely related to *Theobroma*, being distinguished by a few morphological traits, such as the unbranched trunk; compound palmate leaves and the long petal-lamina, exceeding the length of the petal-hood (Schultes 1958; Cuatrecasas 1964). *Herrania* contains 17 species divided into two sections based on floral morphology (*Herrania* and *Subcymbicalyx*), and previously to the revision of the genus, it had been considered a section of *Theobroma* (Schultes 1958).

The phylogenetic relationships of *Theobroma* and *Herrania* species have been investigated using sequences of the vicilin seed storage protein gene (Whitlock and Baum 1999); of the trypsin inhibitor gene (Silva and Figueira 2005); and from a few members of the *WRKY* gene family of transcription factors (Borrone et al. 2008). Phylogenies of the genes were congruent with the phylogeny of the *Theobroma* and *Herrania* species based on morphology. *Herrania* and *Theobroma* were considered monophyletic and sister genera, but only the monophyly of *Herrania* was strongly supported in two of the studies (Whitlock and Baum 1999; Silva and Figueira 2005), corroborating the previously described absence of obvious synapomorphies for *Theobroma*. The monophyly of all *Theobroma* sections was supported, except for section *Glossopetalum*, which was paraphyletic to section *Andropetalum* (Whitlock and Baum 1999; Silva and

Table 13.2 *Theobroma* species and respective section, according to the classification proposed by Cuatrecasas (1964)

Section and species	Common name
Section *Andropetalum*	
T. mammosum Cuatr. & León	
Section *Glossopetalum*	
T. angustifolium Moçiño & Sessé	"cacao de mico"
T. canumanense Pires et Fróes	
T. chocoense Cuatr.	
T. cirmolinae Cuatr.	
T. grandiflorum (Willd. ex Spreng.) Schum.	"cupuassu"
T. hylaeum Cuatr.	
T. nemorale Cuatr.	
T. obovatum Klotzsch ex Bernoulli	"cabeça de urubu"
T. simiarum Donn. Smith.	
T. sinuosum Pavón ex Hubber	
T. stipulatum Cuatr.	
T. subincanum Mart.	"cupuí"
Section *Oreanthes*	
T. bernouillii Pittier	
T. glaucum Karst.	
T. speciosum Willd.	"cacaui"
T. sylvestre Mart.	"cacau azul"
T. velutinum Benoist	
Section *Rhytidocarpus*	
Theobroma bicolor Humb. & Bonpl.	"mocambo," "patashte"
Section *Telmatocarpus*	
T. gileri Cuatr.	
T. microcarpum Mart	"cacaurana"
Section *Theobroma*	
Theobroma cacao	"cacao"

The other species are usually called "cacao del monte"

Figueira 2005; Borrone et al. 2008). In all studies, the classification of *Theobroma* and *Herrania* into sections was corroborated, with species divided in both genera into two clades. The *Herrania* clade was subdivided into the relatively plesiomorphic section *Subcymbicalyx* and the section *Herrania*, with apomorphic characteristics. The *Theobroma* clade was subdivided into a group containing sections *Andropetalum* and *Glossopetalum*, which exhibit many plesiomorphic characteristics, and a second group of the morphologically derived sections *Oreanthes*, *Rhytidocarpus*, *Telmatocarpus* and *Theobroma* (Whitlock and Baum 1999; Silva and Figueira 2005; Borrone et al. 2008).

All *Theobroma* species produce fruits of commercial value, mainly because of the sweet seed-surrounding pulp, but only *T. cacao*, *T. grandiflorum*, *T. bicolor* and *T. angustifolium* are cultivated (Silva et al. 2004; Silva and Figueira 2005). *T. cacao* is the only species planted on a large scale worldwide, but the importance of *T. grandiflorum* has increased in recent years in Brazil, and is grown on more than 30,000 ha. *Theobroma* and *Herrania* species are diploid with $2n = 20$ chromosomes (Muñoz Ortega 1948). Similarly to *T. cacao*, seeds of all the other *Theobroma* species are recalcitrant, restricting conventional ex situ preservation of genetic resources, requiring the establishment of germplasm collection in situ or ex situ. There are only a few collections with a limited number of species in the world, which represent a restricted sample of the natural *Theobroma* populations. Most of the research activities on the related species have been carried out in Brazil, which maintains two major ex situ collections of *Theobroma* species in Belém and Marituba, in the state of Pará, Brazil (Silva et al. 2004; Silva and Figueira 2005). In addition to the natural species, the two collections also maintained natural and artificial interspecific hybrids. A description of the

existing collections and detailed information about the interspecific hybrids was summarized by Silva et al. (2004).

Some of the *Theobroma* species contain valuable characteristics that could be introgressed into *T. cacao*. One trait of major interest would be the natural abscission of mature fruits that occurs in *T. grandiflorum, T.grandiflorum, T. bicolor* and to some extent *T. obovatum*, it would greatly facilitate cacao harvesting, but there are few reported attempts to obtain such trait transference. Changes in plant architecture, either by reducing lateral branching or plant canopy size, could be derived from *T. microcarpum*. Pest and disease resistance would be another major goal, but there are no reports of well-characterized resistance observed for any of the wild species. More attention has been given to characterization of seed quality attributes. All species of *Theobroma*, with exception of *T. cacao*, present white cotyledons. Seeds of *T. cacao* contain around 53% fat in average and from 15 to 20% protein by dry weight (defatted seeds), which is composed mainly of an albumin fraction (52% of the total proteins), and a globulin fraction that represents 43% (Voigt and Biehl 1993). Cacao seed cotyledons also contain high levels of polyphenols (14–20% dry weight), which have also been implicated in the formation of specific flavor notes (Afoakwa et al. 2008). Seeds from other *Theobroma* species have been less characterized, but all contain high levels of fat, ranging from around 38% in *T. bicolor* to 64% in *T. obovatum* (Carpenter et al. 1994), and significant amounts of proteins, ranging from 15 to 28% based on the dry weight of defatted seeds (Silva et al. 2001). Seeds of *Theobroma* species contain less polyphenol than cacao seeds, with values ranging from 0.3% (*T. speciosum*) to 8% (*T. bicolor*), while some species, particularly seeds from *T. speciosum* contain large amounts of polysaccharides in the cotyledons (Martini et al. 2008).

The seed fat composition of *Theobroma* and *Herrania* has been analyzed for the profile of fatty acids and for levels of sterols, tocopherols and tocotrienols (Carpenter et al. 1994). Cocoa butter fatty acids and triacylglycerols profiles significantly differ from the seed fats from other *Theobroma* species (Carpenter et al. 1994; Gilabert-Escrivá et al. 2002). In general, the fatty acid composition of *T. sylvestre* and *T. speciosum* (section *Oreanthes*) are the most similar to cocoa butter, but both had significantly higher palmitate content and significantly lower stearate. *Theobroma bicolor* (*Rhytidocarpus*) seed fat is also similar to cocoa butter, but contained significantly higher levels of stearate and significantly lower palmitate. The fatty acid profiles of seed fat from species of the *Glossopetalum* (*T. subincanum, T. grandiflorum* and *T. obovatum*) are highly similar among themselves. Stearate levels are not significantly different from *T. cacao,* but all had significantly lower levels of palmitate and significantly higher levels of arachidate. These fats are softer than cocoa butter and less stable, due to the high level of unsaturated acids (Gilabert-Escrivá et al. 2002). The fatty acid composition of *T. microcarpum* and *Herrania mariae* are the most divergent, both presenting the highest levels of linoleic acid. Furthermore, *T. microcarpum* contains significantly higher levels of linolenic and behenic acids, while the *Herrania* species have significantly elevated arachidate. A partial assessment of purine alkaloids in 11 species of *Theobroma* and nine of *Herrania* indicated that only *T. cacao* contains detectable levels of caffeine and theobromine in seeds (Hammerstone et al. 1994). However, tetramethyl urate (theacrine) was found in all *Theobroma* and *Herrania* species, with the exception of *T. obovatum,* which has no alkaloid at all. Some of these seed quality traits could become useful in *T. cacao*.

Although, natural hybrids between the species of *Theobroma* are rare, there are reports of occurrence, mainly between species of the *Glossopetalum* section (Silva et al. 2004). Some of these natural putative hybrids (five *T. grandiflorum* × *T. subincanum* trees) still exist in a germplasm collection in Marituba, Pará state (Silva et al. 2004). Experimental hybridization between species of the genus *Theobroma* was first attempted in 1937 in Trinidad, but only a few preliminary results were reported (Posnette 1945). A series of hybridization experiments between Brazilian species of *Theobroma* were performed between 1945 and 1951, by George O'Neill Addison and Rosendo Tavares in Belém, Pará, Brazil, and to date is the most comprehensive work in the area, which includes a detailed illustration of those hybrids (Addison and Tavares 1952). The crosses involved all of the Brazilian *Theobroma* species and the related genus *Herrania* (*H. marie*).

The hybridization between *Theobroma* species from distinct sections, first thought to be extremely difficult, has been proved possible. There are reports

of mature trees from interspecific hybrids between species of sections *Glossopetalum* and *Andropetalum* (hybrids between *T. angustifolium* and *T. mammosum*; and between *T. simiarum* and *T. mammosum* obtained at CATIE, Costa Rica) (Cuatrecasas 1964) and between *T. grandiflorum* (*Glossopetalum*) and *T. cacao* (section *Theobroma*) (Martinson 1966). Successful interspecific crosses involving *T. cacao* have been obtained according to reports (Silva et al. 2004), as hybrid pods between *T. cacao* × *T. mammosum*, *T. cacao* × *T. simiarum* and *T. cacao* × T. *speciosum*; as hybrid seedlings from *T. cacao* × *T. microcarpum* and *T. cacao* × *T. angustifolium*; and adult hybrid plants from *T. cacao* × *T. grandiflorum* (Martinson 1966).

These, interspecific hybrids involving *T. cacao* and other *Theobroma* species have reached various degrees of fruit and plant development (Silva et al. 2004). In general, *T. cacao* has been used as the mother plant, and successful putative hybrids seedling have been obtained at least from crosses involving *T. microcarpum* (*Telmatocarpus*), *T. angustifolium*, and *T. grandiflorum* (*Glossopetalum*); but plants have shown arrested development after reaching around 10–15 cm. Reports of hybrid adult plants are restricted to the work with *T. cacao* × *T. grandiflorum* by Martinson (1966), who used grafting onto either parental species to overcome the arrested growth. Curiously, there are no successful reports of hybridization between *T. cacao* and *T. bicolor* (a species from the closer *Rhytidocarpus* section), while crosses with *T. speciosum* (section Oreanthes) produced ill-formed seeds (Silva et al. 2004). Only two compatible groups have been described from grafting: one confined to members of the section *Glossopetalum* and the other composed of members of the sections *Rhytidocarpus* and *Oreanthes* (*T. bicolor*, *T. speciosum* and *T. sylvestre*).

13.5 Use of Wild Cacao in Breeding

Cacao breeding started as early as the beginning of the twentieth century with phenotypic selection of local populations with narrow genetic background in most of the cacao producing countries (Trinidad, Indonesia, Ghana, Nigeria, Costa Rica and Brazil) for improved yields, while maintaining commercial quality (Toxopeus and Kennedy 1984; Bartley 2005). The outbreak of witches' broom in Trinidad in the late 1920s led to the search for genetic resistance in the upper Amazonian region. During 1930s–1940s, wild germplasm was collected from the upper Amazon basin of Ecuador and Peru (Pound 1938, 1945; Wood and Lass 2001), which provided a much broader genetic variation for breeding (Lockwood and Gyamfi 1979; Iwaro et al. 2003; Bartley 2005). The first case of heterosis in cacao was described in the 1950s when introduced Trinitarios in West Africa were crossed and compared with selfed progenies from local selections. Later, crosses among upper Amazonian accessions were introduced into Ghana, and heterosis was observed in the progenies from these crosses. Interpopulation heterosis was demonstrated for crosses between upper Amazonian Forastero and Trinitario, Criollo and an unrelated Forastero, which formed the basis for all modern cacao breeding. Selected upper Amazonian parents are usually crossed with local selections and/or a Trinitario selection (Table 13.3), in order to retain traditional quality and improve yield and resistance to current pests and diseases. A thorough review of the breeding methods and achievements has been summarized by Posnette (1986), Dias and Resende (2001) and Eskes and Efron (2006). Disease resistance in wild cacao has been the primary interests for cacao breeders, although other traits including yield efficiency, growth vigor, and tolerance to abotic stresses were also objectives for the utilization of wild germplasm. On average, a 30% annual yield reduction is typically estimated and individual farm losses can approach 100% because of the three main cacao diseases (frosty pod – *Moniliophthora roreri*, witches' broom – *M. perniciosa* and black pod rot – *Phytophthora* spp.). Other important diseases and pests include the cocoa pod borer (*Conopomorpha cramerella* Snellen) in Asia (Day 1986; Santoso et al. 2004) and cocoa swollen shoot virus and mirid bugs (*Sahlbergella singularis* Haglund and *Distantiella theobroma* Distant) in West Africa (Hanna and Nicol 1954; Muller and Sackey 2005).

Witches' broom disease, which destroyed the cocoa industry in Suriname, Trinidad, Ecuador and Brazil, have remained the primary focus for gene introgression from wild cacao. Among the various upper Amazon wild cacao germplasm, two Peruvian accessions, "SCA 6" and "SCA 12," have been extensively used in breeding programs due to their resistance to WBD

Table 13.3 List of major wild cacao germplasm from upper Amazon distributed since the 1950s, their utilization status and genetic notes

Accession	Number of times used as parent[a]	Trees sampled leading to accession	No. of global collections with accession[a]
IMC 47	M(0), F(1)	IMC group from 2 trees	19
IMC 60	M(3), F(3)		11
IMC 67	M(71), F(72)		38
NA 31	M(1), F(1)	NA group from 31 trees	7
NA 32	M(24), F(17)		14
NA 33	M(3), F(9)		22
NA 34	M(2), F(2)		14
POUND 7	M(16), F(7)	POUND group from 32 trees	28
POUND 12	M(3), F(3)		14
POUND 18	M(11), F(7)		8
PA 7	M(9), F(11)	PA group from 7 trees	16
PA 35	M(6), F(3)		15
PA 46	0		9
SCA 6	M(113), F(77)	SCA group from 1 tree	39
SCA 12	M(37), F(48)		29

[a]Data obtained from Wadsworth et al. (2003) and ICGD (2007), *M* acted as maternal parent, *F* acted as paternal parent; other information from Lockwood and End (1993), Toxopeus and Kennedy (1984), Bartley (1984) and Wood and Lass (2001)

(Pound 1943). According to the records in the International Cacao Germplasm Database, "SCA 6" has been used 113 times as a male parent and 77 times as the female parent in various hybridization crosses. At least 300 improved varieties or advanced breeding lines have been selected through these crosses. Resistance in the SCA clones appeared to be durable based on observation in Trinidad over 50 years (Bartley 2005). However, a strong location effect was also observed in SCA clones and their derived progenies. Quantitative trait loci (QTL) mapping of the WBD resistance suggested that the resistance was caused by a single major gene, which is homozygous in SCA-6. A major QTL was identified on chromosome 9 near the SSR locus mTcCIR35, using the mapping population derived from "SCA 6" and other susceptible clones. The detected QTLs explained 35–51% of the phenotypic variance for resistance (Queiroz et al. 2003; Brown et al. 2005; Faleiro et al. 2006). In addition to the SCA clones, high resistance to WBD exists in other Forastero germplasm groups. Recent study in Brazil demonstrated that the wild germplasm originally collected from distinct river basins, including the Jamari river; Acre; Javari; Solimões and from the Purus river basin, have several new sources of resistance to WBD and these resistant accessions are "CAB 0208" and "CAB 0214" (Figueira et al. 2006). During the recent collection expeditions in the Peruvian Amazon, it was recorded that the general incidence of WBD as 14.7% on cushions, 13.7% on flushes and 1% on the fruits, indicating a large phenotypic variation of WBD resistance in these wild cacao populations Enrique Arevalo, personal communication. Further exploration of resistance from different germplasm groups is essential to expand the genetic background of WBD resistances and obtain a more durable and stable resistance to WBD, because its known that there is geographical variation in populations of *M. perniciosa*, which may explain the qualitative host–pathogen interaction reported in *M. perniciosa* isolates from different producing regions.

The wild cacao germplasm is also an important source of resistance to black pod rot diseases, caused by various *Phytophthora* species and strains. The disease caused by *Phytophthora palmivora* occurs globally, whereas *Phytophthora megakarya* is restricted to West Africa, and *P. capsici* and *P. citrophthora* occur in the Americas. Host resistance is a key component for an effective and economically sustainable control method (Rocha 1974; Soria 1974; Iwaro et al. 2003, 2006), but development of resistant varieties has been slow, partially due to the narrow genetic base in most cacao breeding programs (Iwaro et al. 2006). Iwaro et al. (2006) evaluated resistance to *Phytophthora* pod rot (black pod disease) in 816 cacao accessions from different genetic groups, including both wild and cultivated cacao. Thirteen percent of the tested accessions were found to be resistant. The Forastero group, consisting of many wild accessions, was found to contain

more resistant (18.0%) genotypes than the Trinitario (4.8%). Among the Forastero populations, the largest percentage of resistant (24.2%) and moderately resistant (28.8%) genotypes were found in the Parinari group. Thevenin et al. (2005) conducted field observations from 1998 to 2001 at the International Cocoa Genebank, Trinidad, on 57 cacao clones for resistance to black pod and WBDs. Eight clones (IMC 6, MAN 15/60, PA 67, PA 195, PA 218, PA 296, PA 303 and POUND 32/A) showed less than 10% infection for black pod and no symptoms of WBD were observed on the branches. All of them, except MAN 15/60, are upper Amazon wild cacao from Pound's collection. This group suggested that screening of clones should first assessed the level of resistance to WBD (on shoots), followed by the screening of the most promising individuals for black pod resistance with a detached pod assay.

Recently, Paulin et al. (2008) reported new sources of resistance to *P. megakarya* in wild cacao populations from French Guiana. Among 59 clones, they found seven "highly resistant" clones (12%) and 29 "resistant" (49%), with some of the clones displaying an even greater resistance than the resistant clone IMC 47.

Breeding varieties with resistance to *Phytophthora* pod rot has become a high priority in Côte d'Ivoire since 1990. The approach of overall population improvement (recurrent and reciprocal selection) has been taken up with the use of upper Amazon and lower Amazon + Trinitario populations. Among the 48 parental clones in use, nine had good levels of resistance (Lachenaud and Oliver 2001). The estimated heritability of resistance to black pod in Côte d'Ivoire was 0.34 and 0.67 for narrow sense and 0.60 and 0.67 for broad sense, for the diallel and the factorial design, respectively. The source of resistance is either wild accessions from upper Amazon (PA 150, P7 and SCA 6) or progenies with wild Forastero background (Tahi et al. 2000, 2006). QTL mapping of host resistance to *Phytophthora* species and isolates has been carried out in 12 mapping populations in Côte d'Ivoire, Costa Rica, Cameroon, Trinidad and France. Phenotypic data were obtained by field observation (pod rot losses), artificial inoculation of attached pods or by inoculation of leaf disks. QTLs have been identified in numerous accessions with upper Amazon wild population background, including resistance against *P. palmivora* in "UPA 402" (Lanaud et al. 2004); "UPA 134" (Flament et al. 2001), "IMC 78" (Clément et al. 2003); "Pound 12" (Crouzillat et al. 2000) and "Scavina 6" (Risterucci et al. 2003). The identified QTLs explained between 7.5% and 48.2% of the phenotypic variance. However, accuracy of phenotyping remains the most important factor affecting the reliability of QTL mapping, especially for the horizontal type of resistance exhibited for *Phytophthora* spp.

Frosty-pod rot is another extremely destructive disease in cacao in the Americas. The causal fungus *Moniliophthora roreri* is endemic in the Americas but has recently expanded its geographic range (Evans et al. 2003). Resistant genotypes have been reported in various genetic groups (Suárez-Capello 1996; Argüello 1997; Phillips-Mora and Wilkinson 2007). Host resistance to frosty pod was screened against *M. roreri* at CATIE by artificially inoculating nearly 600 clones from the international germplasm collection. Only six clones (UF-273, PA-169, UF-712, EET-75, ICS-43 and ICS-95) showed good levels of resistance (Phillips-Mora et al. 2007). From their progenies, a few high-yielding resistant genotypes have been selected and are being tested in a multilocation trial in central America against different variants of the pathogen (Phillips-Mora et al. 2007). Combining ability for resistance was investigated in some of the resistant clones using a partial full-sib design. The resistance, as reflected by the percentage of healthy pods and percentage of pods with frosty pod, showed predominantly additive gene action (Cervantes-Martinez et al. 2006). From a mapping population based on "Pound 7" × "UF 273," three major QTLs for resistance were identified using artificial inoculations with *M. roreri* (Brown et al. 2006). Meanwhile, diversity analysis and phylogeography study of *M. roreri have* shown that the pathogen has the highest levels of variability in Colombia, suggesting that *M. roreri* probably originated near middle Magdalena, Colombia, and appears to have dispersed elsewhere forming new genetic groups (Phillips-Mora et al. 2007). These results suggest that intensive screening of wild cacao germplasm from Colombia may lead to the identification of more resistance germplasm.

In West Africa and Southeast Asia, the adoption rate of varieties or hybrid families with wild Forastero parentage is much higher than that in the Americas. The prevalence of upper Amazon Forastero in West Africa and Southeast Asia is well explained by their narrow genetic background due to the early dispersal

of a small amount of diversity from the new world. The very small number of varieties from the Americas led to low on-farm diversity before the upper Amazon Forastero cacao arrived in the 1940s. West Africa was largely dependent on the narrow scope of West Africa Amelonado introduced from Brazil (Van Hall 1932), whereas Southeast Asia depends primarily on Criollo and Trinitario cacao from Venezuela (Van Hall 1932; Bartley 2005). The various traits of resistance/tolerance to biotic and abiotic stresses offered by the upper Amazon Forastero translates into better adaptability in the major cacao producing regions in Asia and Africa. Using 12 microsatellite loci, Aikpokpodion et al. (2005) made a survey of on-farm genetic diversity in Nigeria. Their work showed that upper Amazon wild cacao, especially the "Parinari" populations, was the main source of upper Amazon material utilized in the development of improved cultivars adopted in Nigeria. The contribution from clones IMC 47, IMC 60, IMC 76, NA 31, NA 32, NA 33, NA 34 and SCA 12 were also significant, in terms of parentage in the farmer selections. Their survey demonstrates the successful adoption of wild cacao in West Africa, where previously Amelonado had been dominant in production (Posnette 1986; Bartley 2005). The accessions PA 35 and NA 32 generated the best crosses in Nigeria that allowed for seedling establishment without shade in the dry season (Toxopeus 1985). Other West African countries imported material from Ghana, which was derived from wild germplasm of the upper Amazon (Toxopeus 1985). A similar survey in Cameroon found that among the elite farmer selections, 54% can be assigned as lower Amazon Forastero, 33% were upper Amazon Forastero, 7% was Trinitario and Criollo types, and 6% could not be decisively assigned (Efombagn et al. 2006).

Southeast Asia shared a similar experience of germplasm introduction as West Africa. In Indonesia, cacao production was started with a small number of Criollo varieties introduced from Mexico and Venezuela, whereas the Malaysia Commercial cacao production was facilitated by the introduction of Amelonado material from Ghana. The presence of vascular streak die back in Southeast Asia led to the establishment of polyclonal seed gardens that utilized upper Amazon accessions, including IMC 60, NA 31, NA 32, and PA 7. In Indonesia, breeding of bulk-cocoa varieties using parental clones of upper Amazon Forastero started in the 1950s, due to the demand for resistance to vascular streak die back disease (VSD). In the early 1970s, several upper Amazonian clones from the genetic groups of Scavina, Parinari and Nanay were introduced and a number of these clones were used in breeding programs in the mid-1970s. In addition, hybrids of upper Amazon Forastero were introduced from Malaysia in the mid of 1970s and were widely grown in North Sumatra (Mawardi et al. 1994). Based on these introduced materials, a series of varieties with tolerance to *Phytophthora* pod rot were developed (Napitupulu 1992). Since 1997, the Indonesian Ministry of Agriculture officially released RCC 70, RCC 71, RCC 72 and RCC 73 from the RISPA Research Station in North Sumatra, which strongly supported the rapid expansion of cacao planting in Indonesia in recent years (A. Susilo personal communication). In PNG, upper Amazon clones were introduced in the 1970s and screened by exposure to the naturally occurring diseases in PNG and those survivors were subsequently used as male parents together with Trinitario female parents to produce hybrids having a useful degree of resistance. These are now widely grown throughout PNG. Less susceptible genotypes still become infected, but symptoms are less severe and the pathogen grows more slowly and only rarely sporulates, while invasion into larger branches and the main trunk appears to be restricted in these genotypes. Resistance is durable and is inherited quantitatively (A. Susilo personal communication). In PNG, breeding with the surviving genotypes has been very successful, and has relegated VSD to a relatively minor disease in most years. Cacao in Sulawesi also appears to have a degree of resistance to VSD, probably because of natural selection operating in the genetically diverse cacao germplasm there. The disease occurs very commonly in the outer branches but rarely kills the trees.

Arguably, the most successful cacao breeding programs were conducted in Trinidad from 1930 to 1980 and were initiated by WE Freeman who made 864 selections during 1953–1976 from hybrid seedlings derived from crosses designed to restrict the genetic base to four parents ICS 1, IMC 67, SCA 6 and POUND 18 (Posnette 1986; Shripat 1995; Gonsalves 1996). The selection criterion was early and high-yielding, disease-resistant with large bean weight (Freeman 1969). Successive generations of intercrossings and selection of superior cacao generated the Trinidad Selected Amazon (TSA) and the Trinidad

Selected Hybrids (TSH). At least 58 TSH and TSA lines have been released to various countries including Brazil, Jamaica, Colombia, Costa Rica, Dominican Republic, Honduras, Indonesia, Malaysia, Puerto Rico and University of Reading, UK (ICGD 2007).

13.6 Future Prospects

Inadequate representation of genetic diversity in the germplasm collections remains a major constraint for cacao germplasm conservation. The wild germplasm in the existing collections were primarily acquired during a few collecting expeditions that only covered a small fraction of the upper Amazon. Deforestation, resulted from hydroelectric projects, logging, mining, and the expansion of the agricultural production has put the wild cacao populations under imminent risk of genetic erosion and extinction. Since 1970, over 600,000 km^2 (16%) of Amazon rainforest have been destroyed. New collecting expeditions into the center of origin of cacao are imperative to ensure that a representative sample of the cacao gene pool be collected, conserved and used for the present and future.

On the other hand, the use of available wild cacao germplasm for cacao breeding has been fragmented so far. It is imperative that the cacao breeders and germplasm curators take full advantage of the available tools of genomics and bioinformatics, combined with phenotypic evaluation, to fully utilize the potential of the wild cacao germplasm. The genomics and information revolution has enabled the generation of very large amounts of information about this species. These new tools now provide the means to exploit genetic diversity in the wild cacao populations (i.e., identify new alleles) with a much higher precision than ever before.

The completion of the large expressed sequence tag (EST) database (Argout et al. 2008) represented a critically important step toward the development of new molecular tools for assessing genetic diversity in wild cacao populations. The project sequenced and annotated 200,000 ESTs from 45 cDNA libraries constructed from mRNA isolated from different cacao tissues, as well as transcripts induced by a variety of biotic and abiotic stimuli. This new molecular cacao resource allowed the identification of hundreds of new microsatellite markers and thousands of single nucleotide polymorphisms (SNPs). It will facilitate the identification of unigene sets suitable for all functional genomic studies in cacao. Another large effort in cacao genomics resources development is that the entire genome of "Matina 1-6" (Kuhn et al. 2010; Scheffler et al. 2009) and "Criollo" (Argout et al. 2010), which was sequenced using a combination of various sequencing strategies. The annotated genome sequence will be deposited in a publicly available database, which can serve as a source for unlimited SNPs and other types of molecular tools for germplasm characterization.

Meanwhile, large numbers of genes with specific functions have been identified in other model species, including *Arabidopsis*, tomato, rice and *Medicago*. A large proportion of these genes shared conserved sequences across many regions of the genome (or within coding regions) among different species. This synteny among different species means a large number of candidate genes are readily available for assaying genetic diversity and to discover new alleles of important genes in cacao. With the high-throughput genotyping facilities, the functional characterization of genetic diversity in large cacao collection may soon become practical. Association analysis using phenotypic data obtained under specified spatial and temporal conditions will detect patterns of functional diversity changes related to environment, pest, pathogen and ancestral origin of the germplasm, which will reveal previously undetected useful variations (e.g., new alleles for disease resistance) in wild cacao populations.

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