DEVELOPMENT AND APPLICATION OF RAPD ANALYSIS FOR INTRA- AND INTERSPECIFIC CHARACTERIZATION WITHIN THE GENUS *AMELANCHIER*

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SUMMARY OF DISSERTATION

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Development and Application of RAPD analysis for intra- and interspecific characterization within the genus *Amelanchier*.

The development and application of RAPD analysis for intra- and interspecific characterization within the genus *Amelanchier* was the main objective of this study. Distinguishing among saskatoon cultivars (*A. alnifolia* Nutt.) and among *Amelanchier* species is difficult based on morphology. The ability to clearly identify cultivars is important in terms of maintenance, distribution and identification of genetic variability. An understanding of the taxonomy of the *Amelanchier* is important because it offers a system of nomenclature which allows for comparative references and is useful for the enhancement and development of germplasm.

A protocol for the extraction of DNA suitable for amplification by the polymerase chain reaction (PCR) from *Amelanchier* leaves was established. This protocol consistently yielded suitable DNA, regardless of plant growing conditions or leaf age.

A protocol for the reliable PCR-amplification of saskatoon DNA was established by optimizing the reaction components. The reproducibility of amplification products was affected by variation in any of the reaction components examined. Variation in the magnesium concentration by as little as 0.5 mM had a dramatic effect on the intensity and numbers of amplification products.

RAPD analysis was used to distinguish among 16 cultivars of saskatoon. Eight 9-base primers generated reproducible polymorphic markers which uniquely characterized twelve cultivars and two pairs of 2 cultivars. Polymorphism was not detected among five sources of the cultivar Thiessen, whereas variability was

found among seedlings from self-pollinated Thiessen. The cultivars Regent and Parkhill could be distinguished from one source, but were indistinguishable from another, suggesting the latter source had mislabelled these cultivars.

RAPD analysis was also used to assess the genetic relationships among 56 taxa representing 16 *Amelanchier* species. Amplification products were analyzed using cluster analysis, principal coordinates analysis, and Wagner parsimony. Based on these analyses, the bulk of the taxa were divided into these three groups: (1) *A. arborea, A. laevis, A. canadensis, A. intermedia*, and *A. x grandiflora*; (2) *A. alnifolia, A. florida, A. cusickii, A. oxyodon*, and *A. gaspensis*, and (3) *A. spicata, A. sanguinea*, and *A. stolonifera*. These results suggest the third group may have arisen through hybridization between the first two groups. Generally, the genetic relationships presented here can be supported by the literature.

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ABSTRACT

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RAPD analysis was also used to assess the genetic relationships among 56 individuals representing 16 species of *Amelanchier*. Amplification products were analyzed using cluster analysis, principal coordinates analysis, and Wagner parsimony. Based on these analyses, taxa were divided into these three groups: (1) *A. arborea, A. laevis, A. canadensis, A. intermedia*, and *A. x grandiflora*; (2) *A. alnifolia, A. florida, A. cusickii, A. oxyodon*, and *A. gaspensis*, and (3) *A. spicata, A. sanguinea*, and *A. stolonifera*. These results suggest the third group may have arisen through hybridization between the first two groups. Generally, the genetic relationships presented here can be supported by the literature.

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DEDICATION

This thesis is dedicated to my grandfather, R.N. Gooding, who took me picking saskatoons and shared his love of horticulture with me.

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CHAPTER 1

INTRODUCTION

The saskatoon (*Amelanchier alnifolia* Nutt.), which is native to central North America, is a relatively new horticultural crop. At present, there is good financial return for the crop, and demand for fruit exceeds production (St. Pierre, 1992). It is anticipated that the developing industry will expand to about 4000 ha (Pruski *et al.*, 1991). At least 19 different saskatoon cultivars have been described by various authors (Darrow, 1975; McConkey, 1979; St. Pierre, 1991b; Stushnoff, 1990; Wallace and Graham, 1976). Although cultivar differences are known to exist (Davidson and Mazza, 1991; Davidson and St. Pierre, 1994), distinguishing among cultivars, even mature cultivars, is very difficult based on morphological characters (St. Pierre, 1990b). The ability to distinguish among these cultivars using a molecular marker is important in the continuing development and domestication of the saskatoon as a crop plant. Saskatoon cultivars are maintained asexually, thus there is an immediate need for accurate identification of individual cultivars to monitor clones for genetic variation and eliminate duplication of cultivars in field trials.

A similar problem is encountered when trying to distinguish among

the species within the genus *Amelanchier*. Early students of North American flora thought the genus consisted of one or only a few highly variable species (Jones, 1946). This number had grown to at least 25 species by 1950 (Fernald, 1950; Jones, 1946) with an additional 17 putative natural hybrids reported (Fernald, 1950). Most of the taxonomic confusion was attributed to the variation of the foliage that occurs within species, and which is manifested throughout the developmental stages and in various habitats. Morphological peculiarities of the flowers and fruits were considered to be the most useful taxonomic characters (Jones, 1946). However, later authors suggested that floral characters tended to vary independently rather than in combination (Hitchcock and Cronquist, 1993). In addition, the complete integration of many previously named species (Cruise, 1964; Hitchcock and Cronquist, 1993) has lead to the recognition of fewer North American species (Cruise, 1964; Gleason and Cronquist, 1961; Hitchcock and Cronquist, 1993; Landry, 1975). The most current treatment of vascular flora of North America recognizes 14 species and three interspecific hybrids for the genus *Amelanchier* (Kartesz, 1994).

In other species, problems of intra- and interspecific characterization have been resolved using molecular markers such as isozymes, restriction fragment length polymorphisms (RFLP), and variable number of tandem

repeats (VNTR). Although isozymes have been the most widely used approach in molecular systematics (Moritz and Hillis, 1990), all of these markers have proven useful in a variety of genetic studies including: (1) screening for germplasm variation; (2) identification of hybrids; (3) as markers for commercially important traits; (4) population structure such as allele frequency and distribution; (5) paternity studies; and (6) origin of polyploid plants (Dowling *et al.*, 1990; Murphy *et al.*, 1990; Nybom and Schaal 1990a, 1990b; Weeden, 1989).

There are certain limitations to isozyme, RFLP and VNTR analyses. Isozyme analysis often fails to uniquely characterize individual cultivars (Bailey, 1983; Weeden, 1984), can be influenced by environmental factors (Bailey, 1983), and tends to underestimate genetic variability (Murphy *et al.*, 1990). Developing RFLP or VNTR probes can be costly, radioactive materials are usually necessary (Salm, 1991), and Southern blot hybridizations are very laborious (Williams *et al.*, 1990). Recently many of these limitations were overcome when randomly amplified polymorphic DNA (RAPD) analysis was used to generate genomic fingerprints and distinguish between cultivars of rice (Welsh and McClelland, 1990), corn and soybean (Williams *et al.*, 1990).

Since the introduction of RAPD analysis in 1990, this technique has been used to detect genetic variation essential to a number of comparative genetic research endeavours. These include cultivar or germplasm

identification in cocoa (Wilde *et al.*, 1992), cauliflower, broccoli (Hu and Quiros, 1991), and apples (Koller *et al.*, 1993); gene mapping in conifers (Carlson *et al.*, 1991); parentage determination in iris (Arnold *et al.*, 1991); population genetics in tomato (Williams and St. Clair, 1993); and molecular systematics in papaya (Stiles *et al.*, 1993), wheat (Joshi and Nguyen, 1993) and *Brassica* (Demeke *et al.*, 1992).

RAPD analysis involves the use of the polymerase chain reaction (PCR) which is a method of amplifying or copying segments of DNA *in vitro*. The basic steps of PCR are the denaturation of the template DNA, the annealing of oligonucleotide primers to the separated strands of template DNA, and the synthesis of new strands of DNA starting from the primer ends by a DNA polymerase. Newly synthesized strands of DNA and the original DNA template can serve as templates in subsequent cycles of denaturation, annealing and synthesis, which results in an exponential increase of the amplified segments (Linz, 1991). Only one primer is used in RAPD analysis, its sequence is arbitrarily chosen and sequence knowledge of the genomic DNA is not required. This results in a number of anonymous (ie. not previously determined) but reproducibly amplified fragments (Bowditch *et al.*, 1993).

Optimization of the PCR is essential; there is no one optimum protocol for all species (Munthali *et al.*, 1992). The reaction buffer, for example, contains magnesium, which effects both DNA-DNA and DNA-

protein interactions (Blanchard *et al.*, 1993), which will vary according to the species studied and choice of enzyme and primers. Therefore, it is important to optimize the magnesium concentration (Blanchard *et al.*, 1993; Innis and Gelfand, 1990; Sambrook *et al.*, 1989). Other components of the reaction include the DNA template, DNA polymerase, primers, buffers, and enzyme stabilizers such as gelatin or bovine serum albumin, all of which may need to be optimized (Innis and Gelfand, 1990). Additionally, the interacting components of the PCR include not only the reaction itself, but extraction of the DNA (Linz, 1991).

Current literature indicates that extraction of DNA is not always simple or routine, and that published protocols are not necessarily reproducible for all species (Rogers, 1994; Stein, 1993; Sytsma, 1994). RAPD analysis requires DNA of suitable purity for the enzymatic polymerase chain reaction (PCR), and it is often difficult to separate DNA from naturally occurring plant cell contaminants. In particular, polysaccharides (Do and Adams, 1991) and phenolic compounds (Newbury and Possingham, 1977) can form a complex with, and become irreversibly bound to, nucleic acids during extraction (Varadarajan and Prakash, 1991). Some of these contaminants can inhibit the activity of DNA modifying enzymes (Draper and Scott, 1988).

The main objective of this study was to develop and apply RAPD technology to solve the problems of intra- and interspecific characterization

with the genus *Amelanchier* discussed earlier. To achieve this objective it was necessary to develop a reliable extraction method and to optimize the PCR-amplification of *Amelanchier* DNA. Once these development stages were complete, RAPD technology was used to generate reproducible genetic markers to distinguish among saskatoon cultivars and survey genetic relationships among species within the genus *Amelanchier*.

CHAPTER 2

LITERATURE REVIEW

2.0 DESCRIPTION OF THE GENUS *AMELANCHIER*

The genus *Amelanchier* belongs to the subfamily Pomoideae of the Rosaceae and includes about 24 species distributed across North America, Europe, northern Africa, and eastern Asia. Over the years, *Amelanchier* has fallen under a number of different generic names including *Amelanchus, Amelancus, Aronia, Crataegus, Malus, Mespilus,* and *Pyrus* (Blanchard, 1907; Wiegand, 1912). This genus is distinct among the Pomoideae because of its raceme-like inflorescence, distinctive foliage, and fruit which contains a false partition (Jones, 1946).

The genus *Amelanchier* consist of slender often scaly-barked shrubs or small trees ranging in size from 0.5 to 15 meters. Their leaves are simple, deciduous, alternate, petioled, pinnately veined and usually serrate or sometimes entire. The perfect, entomophilous flowers, rarely solitary or paired, are borne in raceme-like structures that appear either before or as the leaves unfold. The calyx is five-lobed or five-cleft, persistent and may become reflexed or ascending on the fruit, but sometimes remains erect. The five white, or rarely pink, petals are oblanceolate to narrowly oval. The

stamens are short and can number from 10-20. Two to five styles may be present and are free or united at the base or to the middle. The ovaries are inferior, two to five loculed, and formed from two to five more or less united carpels. Each locule is two-ovuled, which in the fruit is nearly divided by a false partition which grows from the back of each carpel. This false partition forms an incompletely four to ten-loculed pome with the potential of one seed per locule at maturity. The pome is small, mealy or juicy and berry-like. The seeds are small, smooth, dark brown and contain no endosperm (Jones, 1946).

2.1 TAXONOMY OF THE NORTH AMERICAN *AMELANCHIER* SPECIES

At least one species of *Amelanchier* is found in every province or territory of Canada, and each state of the continental United States. The species are difficult to distinguish morphologically. Prior to the beginning of this century, the genus was thought to consist of one or only a few highly variable species (Jones, 1946). Early students of North American flora, including Michaux, Persoon, Pursh, Nuttall, Spach, Tausch, Britton and Brown recognized at most seven species (Jones, 1946; McKay, 1973).

Wiegand (1912) revised the treatment of eastern North American species by recognizing eight species. However, the great difficulty in drawing specific lines due to the variability within the genus was indicated. Wiegand (1912) suggested that "*Amelanchier*...is a group in which it will

never be possible to have the clearly cut condition found in so many genera where specimens will fall easily into one or another specific category". Three main problems in delimiting species within this genus were reported. These three problems were: (1) herbarium collections contained nearly as many transitional sheets (ie. putative hybrids) as those of supposed species; (2) the difficulty in application of specific names because of extensive synonymy; and (3) uncertainty with the identity of holotypes.

Early treatment of western North American species by Rydberg (1922) indicated 15 species. Two of these species, *A. canadensis* and *A. florida*, were also recognized by Wiegand (1912), which would bring the North American total to 21 species. However, the descriptions of *A. florida* by both authors suggests that they were not dealing with the same plant material.

The only treatment of all the North American *Amelanchier* was by Jones (1946). He believed that many species had been improperly typified and did not agree with the treatment of putative hybrids, which were generally not included in taxonomic keys (Fernald, 1950; Wiegand, 1912). As to the problem of synonymy, Jones (1946) reported nearly 200 binomials and trinomials representing the species of *Amelanchier* in America. Most of the taxonomic confusion was attributed to the extraordinary variation of the foliage that occurs within species, and which is manifested throughout all developmental stages and in various habitats.

Morphological peculiarities of the flowers and fruits were found to be the most useful taxonomic characters. Eighteen North American species of *Amelanchier* were recognized by Jones (1946). One of these species (*A. neglecta* Egglest.) was thought to be a natural hybrid between *A. bartramiana* (Tausch) M. Roemer and *A. laevis* Wieg., otherwise no other hybrids were given the status of species. Jones (1946) was criticized for recognizing too many western and too few eastern species (Robertson, 1974). The species recognized by Jones (1946) as well as other authors' interpretations of North American *Amelanchier* species are presented in Table 2.1.

Fernald (1946) did not agree with Jones' (1946) treatment of the genus for several reasons. Firstly, the use of characters, such as the pubescence of the top of the ovary, were considered variable and 'passing characters'. Secondly, the inclusion of *A. spicata* (Lam.) K. Koch as a North American species, which had previously been considered European, was questioned. Thirdly, the number of species recognized was considered too conservative.

In 1950, Fernald recognized 19 species in central and northeastern United States and adjacent Canada. If the six species Jones (1946) recognized in the western United States and Canada are included, then the total number of species in North America would be at least 25. Fernald

Table 2.1. A comparison of some interpretations of the North American *Amelanchier* species.

Jones 1946	Hitchcock & Cronquist 1961	Fernald 1950	Landry 1975	Scoggan 1978	Gleason & Cronquist 1993	Kartesz 1994
bartramiana		bartramiana	bartramiana	bartramiana	bartramiana	bartramiana
arborea		arborea	arborea	arborea	arborea	arborea
laevis		laevis	arborea subsp. laevis	laevis	laevis	laevis
x neglecta (bartramiana x laevis)				x neglecta (bartramiana x laevis)		x neglecta (bartramiana x laevis)
obovalis		obovalis	canadensis subsp. obovalis		obovalis	obovalis
canadensis		canadensis	canadensis	canadensis	canadensis	canadensis
canadensis		intermedia	arborea x canadensis	intermedia	arborea or laevis x canadensis	arborea x canadensis
canadensis		nantucketensis	canadensis		canadensis x spicata	canadensis x spicata
spicata		lucida		lucida	canadensis	canadensis
spicata		stolonifera	canadensis subsp. spicata var. stolonifera	stolonifera	spicata	stolonifera

Table 2.1. Continued.

Jones 1946	Hitchcock & Cronquist 1961	Fernald 1950	Landry 1975	Scoggan 1978	Gleason & Cronquist 1993	Kartesz 1994
spicata		mucronata	canadensis subsp. spicata var. stolonifera	mucronata	spicata	humilis
spicata		humilis	sanguinea var. sanguinea	humilis	sanguinea	humilis
interior		interior	canadensis subsp. spicata		hybrid swarm (laevis x sanguinea)	interior
interior		wiegandii	arborea x sanguinea	wiegandii	laevis or arborea x sanguinea	interior
fernaldii		fernaldii	canadensis subsp. spicata var. fernaldii	fernaldii	fernaldii	fernaldii
gaspensis		gaspensis	sanguinea var. gaspensis	gaspensis	fernaldii	sanguinea var. gaspensis
sanguinea		huronensis	sanguinea var. sanguinea	huronensis	sanguinea var. sanguinea	sanguinea var. sanguinea
sanguinea		amabilis	sanguinea var. sanguinea	sanguinea	sanguinea var. grandiflora	sanguinea var. sanguinea
sanguinea		sanguinea	sanguinea var. sanguinea	sanguinea	sanguinea	sanguinea

Table 2.1. Continued.

Jones 1946	Hitchcock & Cronquist 1961	Fernald 1950	Landry 1975	Scoggan 1978	Gleason & Cronquist 1993	Kartesz 1994
alnifolia	alnifolia var. alnifolia	alnifolia	sanguinea var. alnifolia	alnifolia var. alnifolia	alnifolia	alnifolia var. alnifolia
florida	alnifolia var. semiintegrifolia		sanguinea var. alnifolia	alnifolia var. semiintegrifolia		alnifolia var. semiintegrifolia
florida var. humptulipensis	alnifolia var. humptulipensis			alnifolia var. humptulipensis		alnifolia var. humptulipensis
cusickii	alnifolia var. cusickii		sanguinea var. pumila	alnifolia var. cusickii		alnifolia var. cusickii
basaltacola	alnifolia var. pumila		sanguinea var. pumila			pallida
pumila	alnifolia var. pumila		sanguinea var. pumila			pallida
pallida			sanguinea var. arguta			pallida
utahensis	utahensis		sanguinea var. arguta			utahensis

(1950) also suggested that hybridization between 10 species had resulted in at least 17 putative hybrids. These hybrids were considered 'perplexing' and the 'mongrel offspring' were not included in the taxonomic key (Fernald, 1950).

Cruise (1964) examined 442 plants from three species, *A. arborea* (Michx. f.) Fern., *A. laevis* Wieg., and *A. canadensis* (L.) Medic. These plants were obtained from eight populations growing in the Delaware River valley regions of New Jersey and Pennsylvania. Anderson's hybrid index was used to analyze numerically coded morphological data. Intermediate forms were found between *A. arborea* and *A. laevis*, and between *A. laevis* and *A. canadensis*, but not between *A. arborea* and *A. canadensis*. Cruise (1964) suggested *A. laevis* was the bridge or perhaps a hybrid between the other two species, and that all three should be treated as a variable species under the epithet *A. canadensis* (L.) Medic.

Morphological and chemotaxonomic characters were not found to clearly distinguish 10 *Amelanchier* species in Ontario (McKay, 1973). Although one species, *A. bartramiana*, could be distinguished, the other nine species had considerable overlap for the characters examined. Two species out of 10, *A. spicata* and *A. arborea*, developed a few seeds even though the flowers had been emasculated and pollination prevented. McKay (1973) suggested these seeds were apomictic. However, seeds were classified as appearing 'normal' and neither germination nor

morphological descriptions of the seeds or potential seedlings were reported.

Robinson and Partanen (1980) suggested recognition of two classes or complexes of the eastern North American *Amelanchier* based on cytological and morphological evidence. The 'canadensis' complex contained the diploid species *A. canadensis, A. arborea, A. intermedia, A. laevis,* and the hybrid *A. canadensis x A. laevis*. Triploids and tetraploids such as *A. sanguinea, A. humilis, A. obovalis, A. nantucketensis*, and *A. wiegandii*, made up the 'sanguinea' complex. Two exceptions to this division were *A. intermedia* and *A. obovalis*, which contained both diploids and tetraploids. However, other cytological studies (Table 2.2) report the presence of tetraploids among the 'canadensis' complex and diploids among the 'sanguinea' complex and are thus not in total agreement with this division.

The more recent treatments of *Amelanchier* indicate a reduction in the number of recognized species. In dealing with *Amelanchier* of the Pacific Northwest of Canada and the United States, Hitchcock and Cronquist (1961) tended to amalgamate species. Only two species, *A. alnifolia* Nutt. and *A. utahensis* Koehne. were recognized for this area. In particular, *A. alnifolia* was formed out of seven species recognized by Rydberg (1922) and four species recognized by Jones (1946). Treatment of these species as a single polymorphic species was due to the complete

Table 2.2. Chromosome counts for various *Amelanchier* species, synonyms (SY) and proposed species (pro sp.) following Kartesz (1994).

North American species			
alnifolia (Nutt.) Nutt. ex M. Roemer	17	2x	(Kim, N.-S., in Pruski *et al.*, 1991)
	34	4x	(Henderson *et al.*, 1975; Kim, N.-S, in Pruski *et al.*, 1991)
arborea (Michx. f.) Fern.	17	2x	(Robinson and Partanen, 1980)
	34	4x	(Cruise, 1964)
bartramiana (Tausch) M. Roemer	16	2x	(Robinson and Partanen, 1980)
	34	4x	(Love and Love, 1948, in Darrow, 1975)
canadensis (L.) Medic.	17	2x	(Robinson and Partanen, 1980)
	34	4x	(Cruise, 1964)
	34	4x	(Sax, 1931, in Darrow, 1975; Cruise, 1964)
	34	4x	(Darlington and Wylie, 1961, in Stushnoff, 1990)
fernaldii Wieg.	?		
humilis Wieg.	34	4x	(Robinson and Partanen, 1980)
	17	2x	(Sax, 1931, in Darrow, 1975)
	17	2x	(Darlington and Wylie, 1961, in Stushnoff, 1990)
interior Nielsen	36	4x	(Robinson and Partanen, 1980)
x intermedia Spach (pro sp.)	17	2x	(Robinson and Partanen, 1980)
	28	3x	(Robinson and Partanen, 1980)
laevis Wieg.	15,18	2x	(Robinson and Partanen, 1980)
	34	4x	(Cruise, 1964)
	34	4x	(Moffett, 1931, in Darrow, 1975; Cruise, 1964)

Table 2.2 Continued

laevis Wieg	34	4x	(Darlington and Wylie, 1961, in Stushnoff, 1990)
SY = *A. x grandiflora* Rehder	34	4x	(Sax, 1931, in Darrow, 1975)
nantucketensis Bickn.	34	4x	(Robinson and Partanen, 1980)
x neglecta Egglest. *ex* G.N. Jones	25	3x	(Robinson and Partanen, 1980)
obovalis (Michx.) Ashe	17	2x	(Robinson and Partanen, 1980)
	24,25	3x	(Robinson and Partanen, 1980)
pallida Greene	?		
x quiti-martii Louis-Marie (pro sp.)	?		
sanguinea (Pursh) DC.	20	3x	(Robinson and Partanen, 1980)
	17	2x	(Sax, 1931, in Darrow, 1975)
	34	4x	(Moffett, 1931, in Darrow, 1975)
stolonifera Wieg.	28	3x	(Robinson and Partanen, 1980)
	17	2x	(Sax, 1931, in Darrow, 1975)
	34	4x	(Moffett, 1931, in Darrow, 1975)
utahensis Koehne	?		
<u>Asian or European species</u>			
asiatica (Sieb. & Zucc.) Endl.	17	2x	(Sax, 1931, in Darrow, 1975)
	17	2x	(Darlington and Wylie, 1961, in Stushnoff, 1990)
ovalis Medicus	17	2x	(Favarger and Corrivon, 1967, in Darrow, 1975)
	34	4x	(Darlington and Wylie, 1961, in Stushnoff, 1990)

integration of the previously separate taxa, which apparently had no clear delimitation. Previous characters such as pubescence on certain flower parts, or petal and anther length were found to vary independently rather than in combination (Hitchcock and Cronquist, 1961). Similarly, Gleason and Cronquist (1993) recognized only nine species in the northeastern United States and adjacent Canada, including *A. alnifolia*, which was previously reported as a western species only.

A recent checklist of the vascular flora of North America recognizes 14 species and three interspecific hybrids for the genus *Amelanchier* (Kartesz, 1994), which seems to be consistent with the work of Hitchcock and Cronquist (1961) and Gleason and Cronquist (1993) discussed above. These species and hybrids are given in Table 2.1.

2.2 EVOLUTION OF *AMELANCHIER* SPECIES

Robinson (1982) suggested that polyploidy, hybridization and asexual reproduction were major evolutionary forces in *Amelanchier*. Polyploids have been reported in many *Amelanchier* species (Table 2.2). Interspecific hybrids have been produced (Harris, 1976; McKay, 1973; Robinson, 1982), and putative natural hybrids reported (Cruise, 1964; Fernald, 1950; Kartesz, 1994; Jones, 1946; Robertson, 1974; Wiegand, 1912). Apomixis was reported for the species *A. laevis* (Campbell *et al.*, 1985), *A. canadensis*, *A. stolonifera* (Campbell *et al.*, 1987), and *A. x neglecta*

(Weber and Campbell, 1989). Apomixis is associated with polyploidy, hybridization, self-compatibility, and colonizing ability, and these reproductive modes have played an important role in the evolution of other Maloidean genera (Campbell *et al.*, 1991).

Much of the intrageneric variation may be explained if the reproductive modes of *Amelanchier* are considered. Firstly, the discontinuities of the individual species would be obscured by interspecific hybrids and polyploids. Secondly, varying degrees of self-compatibility among species have been reported (Campbell *et al.*, 1987; Harris, 1970; Robinson, 1982), which along with vegetative reproduction and apomixis, could maintain unbalanced chromosomal forms resulting from hybridization. Vegetative reproduction by the formation of stoloniferous clumps and shoot development from the roots has been observed for many species (Robinson, 1982). Thirdly, hybridization has been observed in habitats disturbed by fire or deforestation (Cruise, 1964; McKay, 1973; Robinson, 1982; Wiegand, 1912) and hybrids such as *A. x neglecta* are know to persist over time (Weber and Campbell, 1989).

2.3 DESCRIPTION OF *AMELANCHIER ALNIFOLIA*

Amelanchier alnifolia Nutt. is native to western Canada, with a geographic distribution including the three prairie provinces, British Columbia, the Yukon, and the northern United States (Jones, 1946). *A.*

alnifolia produces edible fruit which are commonly referred to as 'saskatoons' or 'saskatoon berries' in western Canada. The name saskatoon is derived from the Cree word 'mis-sas-kwa-to-min' which means 'tree of much wood' (Harris, 1970). Fruit from related species are known by some eighteen different names such as 'serviceberry' and 'juneberry' (Harris, 1970). The term berry is a misnomer, for although it resembles a blueberry, it is actually a pome, and is more closely related to an apple or a pear. The saskatoon is a medium sized bush or small tree, ranging in size from about one to five meters. Its inflorescence is raceme-like and produces anywhere from one to 15 bright, white flowers in mid- to late-May (St. Pierre and Steeves, 1990). Fruit ripen to a dark-blue to purple colour in mid- to late-July. Generally, members of this species are very hardy to less than -50°C and will tolerate a wide range of soil types (Harris, 1970). Both diploid ($2n = 34$) and tetraploid ($2n = 68$) plants occur within *A. alnifolia* (Table 2.2).

The fruit of the saskatoon were important to many of the plains Indian tribes of North America. The Cree consumed it fresh or dried it in the sun for later use in such staples as pemmican. Pemmican was made by pounding lean, dried buffalo meat with stone hammers to a soft mass. Saskatoon berries were added and melted fat was poured over the mixture. Meat, fat and berries were mixed together, packed into buffalo hide bags (Mandelbaum, 1979), and could be stored over the entire winter (Fine Day,

1973). Fresh saskatoon berries were consumed as part of the sweat- lodge and purification ceremonies (Dusenberry, 1962). Other parts of the saskatoon plant were also used by the Cree. Arrows, pipestems, and digging sticks were made from saskatoon shoots and stems (Mandelbaum, 1979). In the sacred stories of the Cree, the spirit of the West told the Cree to utilize the saskatoon because it would help preserve the life of mortal man (Bloomfield, 1930). According to Cree mythology, walking sticks or canes made of saskatoon wood had magical powers that could cure illness, bring the dead back to life, or defeat evil (Bloomfield, 1930).

Early European settlers also consumed the fresh fruit or preserved it in jams and jellies. Development of processing methods and consumer evaluation of saskatoon berry products such as jellies (Mazza, 1979), and wine (Harris, 1976) have been done to a limited extent. Today, the fruit is eaten fresh or processed into a variety of products such as pies, jams, jellies, fruit leather, syrup, ice cream topping, wine, and chocolates.

Approximately 65 ha (160 acres) of saskatoon orchards were in production in Saskatchewan by 1993. In addition, an estimated total of 285 ha (700 acres) had been planted in the province by 1994 (SIAP, 1994). At present, there is good financial return for the crop, and demand for fruit exceeds production (St. Pierre, 1992). Production and economic feasibility studies have been conducted (Anonymous, 1990). It is anticipated that the industry will expand to about 4000 ha in Western

Canada (Pruski *et al.*, 1991). Recently, the city of Saskatoon's Economic Development Authority suggested that value-added food processing, such as saskatoon berry products, represented one of the greatest growth opportunities for the city (Robinson, 1993). St. Pierre (1993) and Williams (1994) have compiled the latest cultural information for saskatoon fruit production.

2.3.1 DEVELOPMENT OF SASKATOON CULTIVARS

Cultivation and domestication of the saskatoon began on the Canadian prairies in the early part of this century. Selection of superior genotypes began at the Beaverlodge Research Station in 1918 (Pruski *et al.*, 1991), and Harris (1976) and Wallace and Graham (1976) summarized much of this work. Harris (1972) recommended four cultivars: one ornamental, Altaglow; and three for fruit production, Smoky, Pembina and Forestburg. By 1976, the list of introduced saskatoon varieties had risen to thirteen, including three from the United States: Parkhill, Regent, and Success (Wallace and Graham, 1976). It is not clear from the literature if these three US cultivars belonged to the species *A. alnifolia*, nonetheless they will be referred to as saskatoons in this thesis. At least nineteen different cultivars have been described over the years by various authors (Darrow, 1975; McConkey, 1979; St. Pierre, 1991b; Stushnoff, 1990; Wallace and Graham, 1976). Recently, Davidson and St. Pierre (1994)

compiled a comprehensive and standardized description of fifteen, validly introduced cultivars, which included Altaglow, Bluff, Buffalo, Forestburg, Honeywood, Martin, Moonlake, Nelson, Northline, Parkhill, Pembina, Regent, Smoky, Success, and Thiessen. Another introduced cultivar, Pearson II, was inadvertently excluded from that list (R. St. Pierre, pers. comm.).

Although breeding work, including interspecific and intraspecific crosses, and selfing of cultivars has been conducted, Harris (1976), noted that the only progress made was through selecting within *A. alnifolia*. Thus, except for Martin, Pearson II, and Smoky 5-44, all the other cultivars listed above are superior selections from the wild. Martin is a seedling from self-pollinated Thiessen, and Pearson II (R. St. Pierre, pers. comm.) and Smoky 5-44 (Wallace and Graham, 1976) are seedlings from self-pollinated Smoky.

Cultivar differences are known to exist. Fruit characteristics, such as berry size, acidity, soluble solids and yields, and plant size and hardiness vary appreciably (Davidson and Mazza, 1991; Davidson and St. Pierre, 1994). However, cultivar evaluations under various climatic and soil conditions are just beginning, therefore, cultivar recommendations cannot be made (R. St. Pierre, pers. comm.). Despite these differences, distinguishing among cultivars, even mature cultivars, is very difficult based on morphological characters (St. Pierre, 1991b).

2.4 MOLECULAR CHARACTERIZATION OF PLANT GERMPLASM

Molecular markers such as isozymes, restriction fragment length polymorphisms (RFLP), and variable number of tandem repeats (VNTR) have been used to characterize germplasm at the intra- and interspecific levels. Isozymes have been used extensively for the characterization of particular plant genotypes and cultivars (Moore and Collins, 1983; Nielsen, 1985). Other uses include: (1) screening of germplasm for variation; (2) identification of hybrids; (3) as markers for commercially important traits; (4) population structure (eg. allele frequency and distribution); (5) paternity studies; and (6) origin of polyploid plants (Murphy *et al.*, 1990; Weeden, 1989). Isozyme analysis has been the most widely used approach in molecular systematics (Moritz and Hillis, 1990). RFLP markers have been generated for cultivar identification and have provided genetic markers for the analysis of variation within and among populations, levels of gene flow, effective population size, analysis of parentage and relatedness (Dowling *et al.*, 1990). VNTR analysis has been used to fingerprint apple (Nybom and Schaal, 1990a), blackberry, raspberry (Nybom and Schaal, 1990b), beet, barley (Beyermann *et al.*, 1992), and rice (Dallas, 1988) cultivars. These molecular methodologies are discussed below.

2.4.1 ISOZYME ANALYSIS

The combination of gel electrophoresis and specific stains for enzyme activity forms the basis for the study of protein polymorphism or isozyme analysis. Proteins catalyze biochemical reactions within plants, and any two distinguishable proteins which catalyze the same reaction are called isozymes or isoenzymes (Weeden, 1989). Generally three classes of isozymes are recognized including: (1) proteins arising from different loci (isozymes), (2) proteins arising from the same loci (allozymes), and (3) post-translational modifications of the proteins (secondary isozymes) (Kephart, 1990). However, secondary forms can also arise from assay conditions and are not treated as isozymes (Kephart, 1990). Genetic analysis is needed to determine if the enzymes arise from one locus and this can be complicated when different forms of an enzyme are coded by nuclear, mitochondrial, and chloroplast genomes (Kephart, 1990). The term isozyme is used unless allozymes have been revealed by genetic analysis.

Generally, the process of isozyme analysis begins with the homogenization of plant tissue in an extraction buffer to release enzymes (Kephart, 1990). Enzyme extract is loaded into a starch gel and a current is applied across the gel which separates proteins on the basis of charge and size. Starch gels are used because they can be sliced horizontally into a number of duplicate slabs and each slab can be assayed for a different enzyme system. In contrast, polyacrylamide gels, which offer greater

resolving power, cannot be sliced and are more expensive than starch gels (Weeden, 1989). Slabs are stained by a technique called enzyme activity staining (Vallejos, 1983). This technique uses catalytic activity specific to the enzyme being studied. Kephart (1990), Shields *et al.*, (1983), and Vallejos (1983) review techniques, apparatus requirements, and staining methodologies for plant isozyme analysis, and Murphy *et al.*, (1990) and Weeden (1989) provide an overall review of isozyme analysis.

2.4.2 RESTRICTION SITE ANALYSIS

Restriction site analysis is an assay for sequence variation involving the comparison of fragments resulting from DNA digestion with restriction endonucleases. Restriction endonucleases are enzymes that cleave DNA at a specific and consistently recognized sequence. Sequence mutations, additions, deletions, or sequence rearrangements cause variation in the length and number of fragments produced. The resulting variations are called restriction fragment length polymorphisms (RFLP). Fragments are separated by gel electrophoresis and detected by direct staining with ethidium bromide, end-labelling of fragments with radioactive nucleotides, or hybridization with a probe. The first two detection methods are applied to purified sequences such as chloroplast DNA. The reason for this is that restricted genomic DNA would produce a smear of indistinguishable fragments, whereas restricted chloroplast DNA would produce fewer and

more easily distinguished DNA fragments. Detection by hybridization involves two steps. First the gel is treated with a strong base to denature the fragments into single strands and these are transferred to a nitrocellulose membrane. Second, a single stranded probe is hybridized with complementary fragments bound to the membrane (Dowling *et al.*, 1990). Probes are usually radioactive, but recently non-radioactive probes have been reported (Parent and Page, 1992).

A technique related to RFLP, which also involves hybridization, is called variable number of tandem repeats (VNTR). Hybridization probes identify repeated DNA regions of different lengths that result from variable numbers of repeats of a DNA sequence. The repeated DNA sequences are called minisatellites or microsatellites (Newbury and Ford-Lloyd, 1993). For example, a human minisatellite consists of many dispersed arrays of 10- to 50-base-pair tandem direct repeats that contain a common "core" sequence. A high degree of length variability occurs in these arrays, probably due to changes in copy number of the tandem repeats (Dallas, 1988). Dallas (1988) used a human satellite probe, which will hybridize to the common core sequence, to detect restriction fragment length polymorphism in cultivars of rice.

2.4.3 LIMITATIONS OF ISOZYME, RFLP AND VNTR ANALYSIS

Isozyme, RFLP and VNTR analysis have certain limitations. Isozyme analysis often fails to uniquely characterize each cultivar (Bailey, 1983; Weeden, 1984; Byrne and Littleton, 1989) and can be influenced by environmental factors (Bailey, 1983). Intraspecific taxonomic studies require sufficient levels of isozyme variability, and this is often lacking (Murphy *et al.*, 1990). Nei (1987) suggested that the greatest utility for isozymes is at the level of species or closely related genera. Nonetheless, interspecific studies face limitations such as the number of loci resolved and the number of individuals required for population or phylogenetic studies (Murphy *et al.*, 1990). An overall limitation of this technique is that electromorphs (ie. enzymes which migrate the same distance) are considered homologous even though the amino acid sequence may not be identical (Murphy *et al.*, 1990). Thus, isozyme analysis tends to underestimate variability.

Developing RFLP or VNTR probes can be costly, radioactive materials are usually necessary (Salm, 1991), and Southern blot hybridizations are very laborious (Williams *et al.*, 1990). RFLP probes cannot always be used widely since the sequence similarity between the DNA probe(s) derived for one species may be insufficient for hybridization to another species' DNA (Newbury and Ford-Lloyd, 1993). A benefit of RFLP or VNTR analysis over isozyme analysis is that the bands on autoradiographs result from a close

homology between probe and target DNA, thus shared bands have closely related sequences.

Recently many of these limitations were overcome when RAPD markers were used to generate genomic fingerprints and distinguish between cultivars of rice (Welsh and McClelland 1990), and corn and soybean (Williams *et al.*, 1990).

2.4.4 RAPD ANALYSIS

Randomly amplified polymorphic DNA (RAPD) markers were first reported as a means of genetic fingerprinting by Williams *et al.* (1990) and Welsh and McClelland (1990). The technique is also known as AP-PCR, referring to arbitrarily primed polymerase chain reaction (Welsh and McClelland, 1990), and DAF, indicating DNA amplification fingerprinting (Caetano-Anolles *et al.*, 1991). Regardless of the acronym used, the techniques are similar in that single primers, of known sequence, are used in a polymerase chain reaction to amplify random segments of genomic DNA.

This technique has been used for the detection of genetic variation essential to a number of comparative genetic research endeavours. These include: cultivar or germplasm identification of rice (Welsh and McClelland, 1990), corn, soybean (Williams *et al.*, 1990), cocoa (Wilde *et al.*, 1992), cauliflower, broccoli (Hu and Quiros, 1991), and apples (Koller *et al.*,

1993); gene mapping in conifers (Carlson *et al.*, 1991), arabidopsis (Reiter *et al.*, 1992) and alfalfa (Echt *et al.*, 1993); parentage determination in iris (Arnold *et al.*, 1991), and *Stylosanthes* (Kazan *et al.*, 1993); population genetics of *Lycopersicon* (Williams and St. Clair, 1993); and molecular systematics in papaya (Stiles *et al.*, 1993), wheat (Joshi and Nguyen, 1993), *Allium* (Wilkie *et al.*, 1993), celery (Yang and Quiros, 1993), and *Brassica* (Demeke *et al.*, 1992). The technique has also proven sensitive enough to identify markers for disease resistance in near isogenic lines of several crops such as tomato (Carland and Staskawicz, 1993; Martin *et al.*, 1991), bean (Haley *et al.*, 1993), and lettuce (Paran *et al.*, 1991).

2.5 POLYMERASE CHAIN REACTION AMPLIFICATION OF DNA

The polymerase chain reaction (PCR) is a method of amplifying or copying segments of DNA *in vitro*. The basic steps are the denaturation of the template DNA, the annealing of oligonucleotide primers to the separated strands of template DNA, and the elongation (synthesis) of new strands of DNA starting from the primer ends by a DNA polymerase. Newly synthesized strands of DNA and the original DNA template can serve as templates in subsequent cycles of denaturation, annealing, and elongation, which results in an exponential growth of the targeted segment. However, after 15 to 20 cycles of amplification efficiency is reduced and amplification is no longer exponential (Linz, 1991). Nonetheless, after 25 cycles a

targeted segment can be amplified 300,000 to 500,000-fold (Linz, 1991). The three steps of a PCR cycle (denaturation, annealing, and elongation) are discussed below.

2.5.1 TEMPLATE DENATURATION

Denaturation of duplex DNA is achieved by heating the DNA to 94 - 98°C. (Erlich *et al.*, 1991). This temperature would degrade the Klenow fragment, a DNA polymerase of *Escherichia coli*, which was used to amplify DNA. Because it was not heat stable, and because of the denaturation step necessary to separate newly synthesized strands of DNA, the enzyme had to be added to each cycle (Saiki *et al.*, 1988). The Klenow fragment was replaced by a thermostable DNA polymerase from the bacterium *Thermus aquaticus* (Taq), which can survive extended incubation at 95°C (Saiki *et al.*, 1988). Addition of the thermostable Taq polymerase was not necessary at each cycle, which simplified the procedure and lead to the automation of PCR (Coen and Scharf, 1990).

Denaturation times are typically 95°C for 30 sec, or 97°C for 15 sec, but higher temperatures may be necessary for cytosine and guanine rich (C+G) segments (Innis and Gelfand, 1990). However, very high temperatures, or long times at high temperatures can lead to loss of enzyme activity. The half-life of Taq DNA polymerase is greater than two hours at 92.5° but only five min at 97.5°C (Innis and Gelfand, 1990). Although

Taq DNA polymerase has been the most commonly reported enzyme for PCR (Innis and Gelfand, 1990; Ling *et al.*, 1991), there are a number of other *Thermus aquaticus*-derived DNA polymerases, as well as *T. flavus*, *T. thermophilus*, *Thermococcus litoralis*, and *Pyrococcus* species-derived DNA polymerases available (Schierwater and Ender, 1993). Tolerance of these enzymes to high denaturation temperatures will depend upon the source. The KlenTaq 1 enzyme (AB Peptide, St. Louis), for example, is a truncated version of Taq DNA polymerase, that can tolerate repeated exposure to 98°C without loss of enzyme activity (Aldrich and Cullis, 1993). The information given below is applicable to Taq DNA polymerase, but optimum enzyme activity will vary according to the enzyme source.

2.5.2 PRIMER ANNEALING

Annealing of primers to the DNA template is facilitated by lowering the reaction temperature, and is necessary for amplification to occur. Choice of annealing temperature will vary according to primer length, which depends upon the main objective for amplification. If amplification of a specific DNA segment is desired, then some knowledge of the DNA sequence flanking each side of the segment is required. The flanking sequences are used to synthesize single stranded oligonucleotides which serve as primers. Each primer is complementary to one of the flanking regions. These primers are generally 18 to 24 nucleotides in length to

ensure that the sequence appears only once in the genome (Linz, 1991). The C+G content should be between 50 and 60% and the melting temperature (T_m) for the primer pair should be balanced (Innis and Gelfand, 1990). As a general guideline, the melting temperature can be calculated by allowing 2° for every A (adenine) or T (thymine) and 4°C for every C or G in the primer (Thein and Wallace, 1986 as cited in Innis and Gelfand, 1990). Annealing temperatures are set about 20°C below the T_m of the primers (Linz, 1991). Large differences between the T_ms of each primer can reduce the stringency of the reaction because of imperfect binding of the higher T_m primer due to low annealing temperature during the reaction. 'Stringency' refers to reaction conditions, such as annealing temperature and $MgCl_2$ concentration, which affect hybridization between primer and DNA template (Erlich *et al.*, 1991). Annealing temperature depends upon the base sequence, length, and concentration of the primers (Innis and Gelfand, 1990). Primers with less than 50% C+G content may require temperatures lower than 55°C for full annealing, whereas those with higher C+G content will require higher annealing temperatures (Coen and Scharf, 1990). Other general guidelines when choosing primers include avoiding primers with: (a) complementary 3' ends, as they could anneal together and promote the formation of "primer-dimer" artifacts; (b) runs (three or more) of Cs or Gs at the 3' end, which may misprime in C+G rich regions and generate non-specific products; and (c) palindromic sequences because they

may bind to themselves, thus reducing amplification of the target segment (Innis and Gelfand, 1990).

In contrast, only one primer is used to generate RAPD markers, its sequence is arbitrarily chosen, and sequence knowledge of the genomic DNA is not required. RAPD primers are shorter, typically around 10 nucleotides in length (Bowditch *et al.*, 1993). The shorter primer length, which requires a lower annealing temperature, coupled with the arbitrary primer sequence reduces the specificity of the reaction. This results in a number of anonymous (ie. not previously determined) but reproducibly amplified fragments (Bowditch *et al.*, 1993).

Williams *et al.* (1990) have established several guidelines for RAPD-primer composition, suggesting 9 nucleotides as a minimum length, a C+G content of 50 to 80%, and no palindromic sequences. A positive correlation between primer C+G content and amplification efficiency has been shown by Fritsch *et al.* (1993). Reports of primer length used for RAPD analysis varies from 5 (Caetano-Anolles, 1991) to 34 nucleotides (Welsh and McClelland, 1990), but 10 nucleotides is the most common length (Halward *et al.*, 1992; Hu and Quiros, 1991, Koller *et al.*, 1993; Levi *et al.*, 1993; Paran *et al.*, 1991; Torres *et al.*, 1993; Williams *et al.*, 1990; Yu and Pauls, 1992). Annealing temperatures and times range from 30°C for 1 sec (Caetano-Anolles, 1991) to 38°C for 2 min (Chapco *et al.*, 1992).

Only a few seconds are required for annealing at a primer concentration of 0.2 uM (Innis and Gelfand, 1990), and Taq DNA polymerase is active even at temperatures as low as 22°C (Gelfand and White, 1990). Thus, high annealing temperatures will increase the stringency of the reaction because mismatches will be less stably bound. Once extended from a mismatched primer, a non-target segment can become a template for subsequent cycles of PCR. The effect of a mismatch on PCR efficiency depends upon the location and nature of the mismatch. Mismatches such as A-G, G-A, and C-C at the 3' end of the primer reduce PCR efficiency to less than 1% (Gelfand and White, 1990). Huang *et al.* (1992) suggest that failure to extend mismatches appears to be an intrinsic property of the enzyme. Taq was observed to bind to matched or mismatched primers (at the 3'-terminal base) with the same affinity, and extension efficiencies were independent of temperature (Huang *et al.*, 1992). Kwok *et al.* (1990) also found reduced extension efficiencies for some mismatches at the 3'-terminal base, and found that double mismatches within the last four bases of the primer which included a mismatch at the 3' base, dramatically reduced extension of the primer. PCR is more sensitive to mismatches at the 3' end of the primer, in fact, short sequences which will not anneal to the template DNA can be added to the 5' end of the primer with little effect on elongation (Arnheim and Levenson, 1990).

2.5.4 PRIMER ELONGATION

Requirements for elongation of the primer are the presence of all four deoxynucleoside 5′-triphosphates (dATP, dCTP, dGTP, and dTTP), Mg^{+2}, and a primer with a free 3′-OH group. Taq DNA polymerase is a template directed enzyme, which recognizes the 3′-OH group of the primer and incorporates single nucleosides complementary to the template DNA in the 5′ to 3′ direction (Stryer, 1988). Elongation is carried out at 72°C, which is very near the optimal for Taq (T_{opt} = 75°C) (Coen and Scharf, 1990), but not hot enough to denature primers and template. Rates of incorporation of nucleotides vary from 35 to 100 per second at 72°C, depending upon the buffer, pH, and salt concentrations (Innis and Gelfand, 1990). Generally, one minute at 72°C is long enough to generate products up to 2 kb in length (Innis and Gelfand, 1990).

During elongation there can be misincorporation of nucleotides by the enzyme. Taq DNA polymerase has no exonuclease activity (ie. proofreading) in the 3′ to 5′ direction (Gelfand and White, 1990), but does have 5′ to 3′ exonuclease activity (Erlich *et al.*, 1991). Rates of misincorporation can be as high as 10^{-4} nucleotides per cycle, but by lowering concentrations of dNTP and $MgCl_2$, raising annealing temperatures, and shortening extension times, this rate can be reduced to less than 10^{-5} nucleotides per cycle (Erlich *et al.*, 1991). General recommendations are that fidelity can be increased by lowering the concentrations of dNTP and

$MgCl_2$, and lowering the pH (Eckert and Kunkel, 1991). However, raising dNTP can increase fidelity (error rate per nucleotide) when Mg^{+2} concentrations are high as long as the concentration of dNTP does not exceed that of the Mg^{+2} (Ling *et al.*, 1991). Misincorporation rates also depend upon the source of the DNA polymerase. The bacteriophage T4 DNA polymerase appears to have relatively low rate of misincorporation, as does the VENT DNA polymerase (isolated from *Themococcus litoralis*), which has 3′ to 5′ exonuclease activity (Erlich *et al.*, 1991).

2.5.5 PCR BUFFER

Components of the reaction buffer will vary for a given PCR application and should be optimized accordingly. Some of the components such as DNA template, dNTP, Taq DNA polymerase, and oligonucleotide primers were discussed above. In addition, the reaction buffer contains Tris-HCl, $MgCl_2$, KCl, and often an enzyme stabilizer such as gelatin or bovine serum albumin (Innis and Gelfand, 1990).

A general recommendation for PCR is 10 to 50 mM Tris-HCl at pH 8.3 to 8.8 when measured at 20°C (Innis and Gelfand, 1990). However, use of increased Tris-HCl concentrations (Collins and Symons, 1993; Echt *et al.*, 1992; Reiter *et al.*, 1992), and higher pH (Demeke *et al.*, 1992; Echt *et al.*, 1992) have been reported. Phosphate buffers inhibit Taq and should be avoided (Sambrook *et al.*, 1989).

Up to 50 mM potassium chloride (KCl) is included to facilitate primer annealing, but inhibition of Taq occurs at higher concentrations (Innis and Gelfand, 1990), with total inhibition above 100 mM (Eckert and Kunkel, 1991). Sixteen to 20 mM ammonium-sulfate has been used instead of the KCl, but this is usually in conjunction with increased concentrations of Tris-HCl and higher pH (Aldrich and Cullis, 1993; Collins and Symons, 1993; Echt *et al.*, 1992; Reiter *et al.*, 1992). Combinations of KCl and NH_4Cl have also been reported (Blanchard *et al.*, 1993). Relatively high levels of potassium lowers the stringency of the reaction by raising primer-template melting temperature, which in turn allows mismatches to bind more easily to the template and results in the appearance of more extraneous bands (Blanchard *et al.*, 1993). In contrast, relatively low levels of ammonium increase reaction stringency (Blanchard *et al.*, 1993; Eckert and Kunkel, 1991).

The magnesium ion is essential; without it the reaction will not proceed (Sambrook *et al.*, 1989). It is a divalent ion which activates the DNA polymerase, and also binds tightly to the phosphate sugar backbone of nucleic acids, primers and nucleotides (Eckert and Kunkel, 1991). The magnesium ion concentration may effect all of the following: primer annealing, strand dissociation temperatures of both template and amplification products, product specificity, formation of primer-dimer artifacts, and enzyme activity and fidelity (Innis and Gelfand 1990). As a

guideline, the magnesium concentration should be 0.5 to 2.5 mM greater than the total dNTP concentration (Innis and Gelfand, 1990). For a reaction containing a total of 0.4 mM dNTP, the magnesium concentration should fall in the range of 0.9 to 2.9 mM. Magnesium affects both DNA-DNA and DNA-protein interactions (Blanchard *et al.*, 1993), which will vary according to the species studied and choice of enzyme and primers. Therefore, it is important to optimize the magnesium concentration (Blanchard *et al.*, 1993; Innis and Gelfand, 1990; Sambrook *et al.*, 1989).

Blanchard *et al.* (1993) examined the effect of additives on the behaviour of the polymerase chain reaction. Additives such as bovine serum albumin, gelatin, glycerol, and polyethylene glycol may protect the Taq polymerase from premature decay, but no significant improvement was found by their inclusion. Other reports include the addition of Triton X-100 (Demeke *et al.*, 1992, Yu and Pauls, 1992), detergents such as NP40 and Tween 20 (Devos and Gale, 1992), EDTA, gelatin, and T4 gene 32 protein (gp32) (Collins and Symons, 1993), tetramethylammonium chloride (Vierling and Nguyen, 1992), dimethyl sulfoxide (DMSO) (Chester and Marshak, 1993), spermine, spermidine (Ahokas and Erkkila, 1993), and formamide (Sarkar *et al.*, 1990) to the reaction buffer to enhance reaction specificity. It is not clear from the literature which parameters these additives affect, except for DMSO (Chester and Marshak, 1993), nor was specificity always enhanced by their inclusion (Ahokas and Erkkila, 1993;

Blanchard *et al.*, 1993; Sarkar *et al.*, 1990).

2.5.6 OPTIMIZATION OF THE PCR

Munthali *et al.* (1992) suggested that there is no one optimum protocol for all species, and there is support for this statement in the literature cited above. Of the more than 20 citations discussed thus far in section 2.5, none of them have identical protocols for the amplification of DNA. Some of the differences in protocol may be due to variation in thermal cyclers or the source of Taq-polymerase, but the main difference is the species under study. From the previous discussion concerning PCR, it is clear that the polymerase chain reaction is composed of a number of interacting components which includes not only the reaction itself, but the sample preparation as well (Linz, 1991).

2.6 EXTRACTION OF DNA

According to Sytsma (1994) the need for extraction procedures yielding pure and high molecular weight DNA is three-fold. First, it is necessary for RFLP and RAPD analysis, construction of genomic libraries, and PCR amplification for sequencing. Second, these techniques are used in molecular systematics and evolutionary studies, which are the fastest growing areas of plant research. And, third, the loss of the world's plant diversity is leading to the development of genomic DNA-banks.

When I began this project in 1990, a number of methods for plant-DNA isolation had been reported which yielded DNA suitable for other enzymatic reactions such as RFLP analysis. Generally, these methods began with the grinding of plant tissues in liquid nitrogen followed by incubation in buffers containing detergents such as sodium dodecyl sulphate (SDS) (Dellaporta *et al.*, 1983; Varadarajan and Prakash, 1991), hexadecyltrimethyl-ammonium bromide (CTAB) (Doyle and Doyle, 1990; Murray and Thompson, 1980), or a combination of CTAB and SDS (Dellaporta *et al.*, 1983). The buffers also contained EDTA to chelate with Mg^{+2}, a cofactor for many nucleases. Precipitation of a DNA was possible due to different solubilities of DNA and contaminants such as polysaccharides. For example, DNA is soluble and most polysaccharides insoluble in a solution containing CTAB and approximately 1 M salt (Rogers, 1994). The DNA can be precipitated by adding alcohol (Murray and Thompson 1980). Baker *et al.* (1990) added the additional step of cesium chloride gradient centrifugation to further purify the DNA. Alternatively, separation of nuclei from other cellular components followed by lysing of the nuclei in an SDS buffer and centrifugation in cesium chloride has been reported (Couch and Fritz, 1990). In addition, polyvinylpyrrolidine (PVP) and purification columns such as Elutip-d have been used to remove phenolic compounds (Doyle and Doyle, 1990) and excess polysaccharides (Do and Adams, 1991), respectively.

Current literature indicates that extraction of DNA is not always simple or routine, and that published protocols are not necessarily reproducible for all species (Rogers, 1994; Stein, 1993; Sytsma, 1994). Rogers (1994) compared six different methods for extracting DNA from 20 plant and fungal species including fresh and herbarium material. In his study, methods utilizing the detergent CTAB were rated superior in terms of digestion with restriction enzymes and PCR amplification. Sytsma's (1994) review of current extraction procedures recommends researchers begin with a CTAB based protocol and he suggests potential solutions for problems such as low yield, purity, and contaminants (eg. proteins, polyphenols, mucilages, and polysaccharides). However, some recent protocols aim to reduce the number of steps in order to reduce cross contamination and extraction time, and use neither detergents nor cesium chloride gradient centrifugation (Luo *et al.*, 1992; Wang *et al.*, 1993).

With the exception of Couch and Fritz (1990), the above protocols were for the isolation of total DNA. However, Stein (1993) indicates that for many plant taxa comparison of nucleic acid is carried out separately for nuclear, chloroplast, and mitochondrial DNA. The initial step is to separate organelles from cell wall components. This can be achieved by degrading the plant cell wall using enzymes such as macerase and cellulysin (Deragon and Landry, 1992). The resulting protoplasts can be lysed and the organelles separated by centrifugation using such gradients as cesium

chloride, sucrose, or percoll (Stein, 1993). Alternatively, large amounts of plant material can be homogenized with buffer in a blender and the organelles separated by centrifugation. An enriched organelle preparation can then be lysed and its DNA extracted. Extraction protocols for organellar DNA have been reviewed by Stein (1990).

2.7 THE USE OF RAPD MARKERS IN SYSTEMATICS

One of the problems regarding the use of RAPD markers is that amplified bands are considered dominant because there is no way to distinguish the homozygote from the heterozygote for a given RAPD marker (Tingey *et al.*, 1992). However, segregation of bands in offspring or through a test cross can be used to determine this (Newbury and Ford-Lloyd, 1993). Another problem concerns homology of co-migrating bands. Only the primer sequence at each fragment end is known in RAPD analysis. There is no sequence information for the rest of the fragment and therefore homology is assumed for bands which migrate equal distances. Genetically speaking, this means that co-migrating fragments may not be allelic (Newbury and Ford-Lloyd, 1993).

The use of RAPD analysis in systematic (taxonomic) studies requires the assumption of homology between amplification products which migrate the same distance (electromorphs). Homologous characters are those characters which two species and their common ancestor share (Ridley,

1986). In contrast, analogous or convergent characters are those characters which are shared by two species but not shared with their common ancestor (Ridley, 1986). Homology among electromorphs has been determined by labelling of an amplified product and using it as a probe. Homologous electromorphs will hybridize to the probe. Using this technique with 15 different amplification products, Thormann and Osborn (1992) have shown that 3 products were not always homologous among different *Brassica* species, but all 15 products were homologous within species. Similarly, Wilkie *et al.* (1993) examined the homology among amplification products generated among species of *Allium*. Of the seven products used as probes, three hybridized to all electromorphs at both high and low stringency, while the other four probes hybridized at low stringency only. Another report indicated that within the genus *Glycine*, electromorphs were homologous as shown by hybridization (Tingey *et al.*, 1992). These results suggest some differences in degree of homology among electromorphs.

However, taxonomic studies indicate that RAPD data are consistent with other forms of molecular data. Demeke *et al.* (1992) demonstrated that RAPD markers could generate the classic U triangle among diploid and amphidiploid *Brassica* taxa. Phylogenetic trees were consistent whether generated from RAPD, VNTR or isozyme data for 10 strains of *Microseris elegans* (van Heusden and Bachmann, 1992). The consistency among

various molecular data including RAPD suggests that comigrating RAPD electromorphs must be homologous. Peltier *et al.* (1996) compared two data sets, one consisting of RAPD markers and the other consisting of RAPD markers for which homology of electromorphs were confirmed by hybridization, for *Petunia* species. Phenetic and phylogenetic inferences did not differ regardless of the data set used. It was suggested that confirmation of homology among electromorphs was unnecessary with respect to the gain in information in relationships among species (Peltier *et al.*, 1996).

2.8 CLADISTIC APPROACHES TO SYSTEMATICS

Cladistic methods based on the principle of maximum parsimony have been the most widely used as an approach to inferring phylogenies from character data (Swofford and Olsen, 1990). Parsimony methods operate by selecting trees that minimize the total tree length, ie. the number of evolutionary steps (transformation from one character to another) required to explain a given set of data. Often outgroup comparisons are used to infer the polarity of the characters, which helps to root the tree. The location at which the outgroup joins the tree implies a root with respect to the other taxa studied. Utilization of an outgroup requires the assumption that the remaining taxa are monophyletic, ie. that the group contains all descendants of the most recent common ancestor (Ashlock, 1984).

2.8.1 PARSIMONY ANALYSIS

Parsimony analysis of RAPD, using either the PAUP (Stiles *et al.*, 1993; van Heusden and Bachmann, 1992; Vierling and Nguyen, 1992; Williams and St. Clair, 1993; Yang and Quiros, 1993) or PHYLIP (Peltier *et al.*, 1996) software packages have been reported. Of the above reports, two specified using Wagner parsimony (Peltier *et al.*, 1996; van Heusden and Bachmann, 1992) and the others did not indicate their method. Several methods of parsimony are discussed below.

Wagner parsimony is one of the simplest parsimony methods available. The Wagner method assumes that characters are measured on an interval scale and is appropriate for binary characters such as RAPD markers. A second assumption of this method is that transformation from one character state to another implies a transformation through any intervening states. It also permits free reversibility, ie. the change from 0 to 1 is as probable as the change from 1 to 0 and that character states may transform from one state to another and back again (Swofford and Olsen, 1990). The assumption of free reversibility is perhaps unreasonable for RAPD. Substitution of a single nucleotide can create a mismatch between primer and priming site. Intuitively, the loss of a priming site would be more probable than the gain of a priming site. For similar reasons, this assumption of free reversibility is also considered unreasonable for restriction-site characters (Swofford and Olsen, 1990).

Unlike Wagner parsimony, Dollo parsimony does not assume free reversibility of characters and may be more appropriate for restriction-site (Swofford and Olsen, 1990) and RAPD data. Dollo parsimony is said to be based on Dollo's "law" that evolution is irreversible (Panchen, 1992). However, this method does not assume complete irreversibility, only that each character state may originate once on the tree. Parallel or convergent gains of the derived condition are not allowed (Swofford and Olsen, 1990). In the context of RAPD data, each priming site may be gained once and many parallel losses of the site may be necessary to explain the data. The drawback to use of Dollo parsimony for RAPD characters is that a particular site could originate independently in two lineages and this would lead to an overestimation of the number of evolutionary changes required to explain the data (Swofford and Olsen, 1990). Additionally, Dollo parsimony may not be an appropriate method of phylogenetic analysis because the independent gain of a character is effectively weighted to infinity, which is biologically unrealistic (Albert *et al.*, 1992). Instead, it has been argued that Wagner parsimony would produce more accurate topologies (trees) than Dollo (Albert *et al.*, 1992).

Camin-Sokal parsimony does not allow a derived character to return to the ancestral state, ie. evolution is irreversible. Swofford and Olsen (1990) suggest that molecular data could not support this highly unlikely assumption of irreversibility.

Regardless of the parsimony method employed, the straight-forward approach would be to evaluate all possible tree topologies one after another and pick the one which, according to the criterion used, is best. This is called an exhaustive search. However, an exhaustive search would not be possible for more than a small number of species, since the number of possible tree topologies can be enormous (ie. for 11 taxa there are over 34 million possible trees, Swofford and Olsen, 1990). Instead, all optimal trees can be found by an exact algorithm, called the 'branch and bound' method, that does not require an exhaustive search (Swofford and Olsen, 1990).

The branch and bound method begins by generating a random tree which minimizes the tree length under a parsimony criterion. This random tree would have a score (L) which becomes the upper limit or bound of the value of the criterion. The optimal tree(s) cannot exceed this score. New trees are examined along the path toward their tips. If a tree is encountered whose score exceeds L, then there is no need to continue along this path because connecting additional taxa cannot possibly decrease this score. The evaluation of all trees that descend from this node in the search tree can be dispensed with. The program will then backtrack and proceed down a different path. The elimination of portions of the search greatly reduces the number of trees that must actually be evaluated. If the end of a path on a search tree is reached and a tree is obtained with a score equal to the upper bound L, then it is a candidate for optimality. However,

if the score is less than L, then it is the best tree so far and a new, lower score L is established. An improvement in the score is important in that it may enable other search paths to be terminated sooner. All optimal trees are identified once the entire search tree has been traversed (Swofford and Olsen, 1990).

In contrast to the exact methods, heuristic approaches, which sacrifice the guarantee of optimality in favour of reduced computer time, are available. Heuristic approaches include 'stepwise addition' and 'branch swapping' methods. Stepwise addition begins with three taxa selected for the initial tree. A fourth taxon is added to the initial tree and all possible trees resulting from that addition are evaluated and the shortest tree is saved for the next round. The process continues, and with the addition of each taxon, the shortest tree is carried over to the next round until all taxa have joined the tree. There are a number of problems associated with stepwise addition. First, if the first three taxa in the input file are used to form the initial tree, then the shortest possible initial tree may not be found. Second, the shortest or optimal tree at an early stage may become suboptimal as more and more taxa are added, resulting in a local optimum (Swofford and Olsen, 1990).

The local-optima problem may be overcome by using a branch swapping technique such as 'global' optimization of the tree. This approach allows for each subtree to be removed from the tree and added

back in all possible places. This process continues until all subtrees can be removed and added again without an improvement in the tree. The purpose of this extra rearrangement is to make it less likely that one or more species gets 'stuck' in a suboptimal region of the space of all possible trees. In addition, Felsenstien (1993) suggests randomizing the input order of the taxa a number of times and determining the shortest tree for each randomization. This is important because the analysis is dependent on the order in which the species are present in the input file. The use of multiple runs can give an indication of the species that are likely to be the source of confusion in the topology. These confusing species can be placed last in the input order for the next analysis. This technique will allow the earlier species to have formed a stable topology around which the last few species can be fitted (Felsenstien, 1993).

2.8.2 CLIQUE ANALYSIS

Clique or character compatibility algorithms are the second most common cladistic approach (Stace, 1989). The aim of this method is to avoid homoplasy (ie. parallelism and convergence) by carefully choosing sets of mutually compatible characters which cannot be true all at once (Pankhurst, 1991). A set of mutually compatible characters is called a clique, and the largest clique contains a set of characters which is used to draw a cladogram. Stuessy (1990) indicates that characters are compatible

if their changes in evolutionary direction are correlated. In contrast, Pankhurst (1991) suggests that it is not necessary to know character polarity, instead a "network of relations" between taxa can be established and rooted later if desired. One problem related to compatibility analysis is that the largest clique may not be able to resolve the cladogram (Pankhurst, 1991).

2.9 PHENETIC APPROACHES TO SYSTEMATICS

Several software packages, such as NTSYS (Joshi and Nguyen, 1993; Stiles *et al.*, 1993; Vierling and Nguyen, 1992), SAS (Kazan et al., 1993; Peltier *et al.*, 1996) and others (van Heusden and Bachmann, 1992, Wilkie *et al.*, 1993; Williams and St. Clair, 1993) have been reported for the phenetic analysis of RAPD markers. Nearly all of these reports used cluster analysis, but there was one report of principal coordinate analysis (Demeke *et al.*, 1992).

2.9.1 CLUSTER ANALYSIS

Cluster analysis begins with pairwise comparisons between taxa which have been generated using Jaccard's coefficient of similarity (Joshi and Nguyen, 1993; Peltier *et al.*, 1996; Stiles *et al.*, 1993; Vierling and Nguyen, 1992), Roger's genetic distance (Wilkie *et al.*, 1993), Nei's genetic distance (Peltier *et al.*, 1996; Williams and St. Clair, 1993), Nei and Li's

distance (Kazan *et al.*, 1993), and the Simple matching coefficient (Peltier *et al.*, 1996; Stiles *et al.*, 1993). Clustering of taxa was almost always by the unweighted pair-group method using arithmetic averages (UPGMA) (Joshi and Nguyen, 1993; Kazan *et al.*, 1993; Peltier *et al.*, 1996; Stiles *et al.*, 1993; van Heusden and Bachmann, 1992; Vierling and Nguyen, 1992; Williams and St. Clair, 1993), with the Neighbour Joining method of Saitou and Nei (Peltier *et al.*, 1996), nearest neighbour and farthest neighbour clustering (Wilkie *et al.*, 1993) also reported.

Most clustering methods used in taxonomy are sequential, agglomerative, hierarchic, and nonoverlapping (SAHN) (Panchen, 1992). Sneath and Sokal (1973) indicate that most methods are sequential in that a recursive sequence of operations is applied to the set of taxa under study. Agglomerative techniques are also frequently used in conjunction with sequential, hierarchic and nonoverlapping procedures, because they are simple to program. Agglomerative methods successively group an initial set of (t) taxa into fewer than t sets until a single set containing all t taxa is generated. In contrast, divisive techniques begin with all t taxa in one set and subdivide this into subsets, which are further subdivided. Clustering methods which partition taxa such that there are successively fewer taxa in each cluster, but without limitation on the degree of overlap at any one level of clustering are called hierarchic. Thus, any system of things ranked one above another is called a hierarchy. Traditionally biological taxonomists

have used hierarchial clustering methods. Nonoverlapping methods generate taxa that at any one level are mutually exclusive, ie. the taxa contained within one cluster may not also be members of a second cluster of the same rank. Sneath and Sokal (1973) suggest that this may distort the phenetic relationship between taxa, but that overlapping techniques can become extremely difficult to draw and interpret.

In all SAHN clustering techniques there are two considerations which govern every clustering step. First, the similarity or dissimilarity coefficient is recalculated between an established cluster and a potential candidate for admission to that cluster, and this measure will differ depending on the SAHN method used. Second is the admission criteria for new members to an established cluster. Admission is based on a combination of three measures including: (1) a measure defining the extant cluster in terms of shape and dispersion; (2) the measure of similarity or dissimilarity between the taxa and the extant cluster; and (3) a measure describing the changes when the taxa and the extant cluster fuse (Sneath and Sokal, 1973).

It should be noted that within SAHN techniques there is an option of only one taxon (pair-group method) or simultaneously several taxa (variable-group method) being admitted to a cluster. Generally, there is not a great difference between these methods. Historically, the pair-group method has been used most frequently because it is easier to program. In addition, the number of taxa admitted to a cluster affects the criterion for admission to

that cluster as discussed above. For example, for all pair-group methods the criterion for admission of new members into a cluster is the same and is based only on the measure of similarity or dissimilarity between the taxon and the extant cluster (Sneath and Sokal, 1973).

Three hierarchal clustering methods are commonly used: single, complete, and average linkage (Pankhurst, 1991). The single linkage technique (also called the nearest neighbour or minimum method) links a taxon to an extant cluster through the most similar taxon within the cluster. Thus, connection between taxa and clusters or between two clusters are established by links between pairs of taxa (Sneath and Sokal, 1973). Single linkage tends to exaggerate similarities between groups, thus it may suggest some unrealistic groupings. Complete linkage (furthest neighbour technique, maximum method) joins the taxon to a cluster through similarity with the farthest taxon already in that cluster. Therefore, when two clusters join, their similarity is that existing between the furthest pair of members, one in each cluster (Sneath and Sokal, 1973). Complete linkage is a more conservative method, giving more cohesive clusters, but may not cluster less clearly defined groups (Stuessy, 1990). Average linkage relates a taxon to the average value of an existing group. The average linkage clustering method is further divided into arithmetic and centroid types, both of which can have weighted and unweighted approaches. The most frequently used clustering strategy is the unweighted pair-group method

using arithmetic averages (UPGMA) (Sneath and Sokal, 1973; Rohlf, 1993).

2.9.2 PRINCIPAL COORDINATE ANALYSIS

Principal coordinate analysis begins with the computation of a distance between taxa using a coefficient such as the average Manhattan distance, which is a dissimilarity measure. The resulting symmetrical (square) matrix is then transformed to a scalar product form so that its eigenvalues and eigenvectors can be computed (Rohlf, 1993).

Associated with any square matrix is a single number (scalar) that represents a unique function of the numbers in the matrix. This scalar function of a square matrix is called the determinant. Of particular interest in statistics is the determinant of a square symmetric matrix (D) whose diagonal elements are sample variances and off-diagonal elements are sample covariances (Cooley and Lohnes, 1971). A square dissimilarity matrix can be transformed into a D matrix by "double centering", which means the row and column means are subtracted from each element and the grand mean is added on (Rohlf, 1993). The diagonal elements of D are sample variances and the off-diagonal elements are sample covariances.

Besides a determinant, every square matrix has associated with it a characteristic equation. The characteristic equation of a matrix is formed by subtracting some one value (λ) from each of the diagonal elements of the matrix, where λ is selected so that the determinant of the resulting matrix is

zero. There will be many different values for λ, one for each diagonal element in the matrix that will satisfy the characteristic equation. These different values are called eigenvalues of the matrix. Associated with each eigenvalue is a vector called the eigenvector (Cooley and Lohnes, 1971). Eigenvalues and eigenvectors are then computed by finding the scalars and a new matrix (F) such that $Af_i = \lambda_i f_i$ where A is the symmetric matrix to be operated upon, λ_i is the i^{th} eigenvalue (the i^{th} diagonal element of matrix A) and f_i is the i^{th} eigenvector (i^{th} column of F) (Rohlf, 1993).

The eigenvectors describe a large portion of the dispersion (variance and covariance) created by the characters over the taxa (Sneath and Sokal, 1973). The matrix of normalized eigenvectors gives the coordinates of the taxa on their principal axes. Normally, taxa are plotted against the three axes which account for the greatest dispersion (Rohlf, 1993), and from this plot the relationships among taxa can be determined.

CHAPTER 3

EXTRACTION OF DNA FROM *AMELANCHIER ALNIFOLIA* LEAVES

3.0 ABSTRACT

Three DNA extraction procedures were examined to determine which might yield DNA suitable for randomly amplified polymorphic DNA (RAPD) analysis from *A. alnifolia* leaves. The methods examined were: the miniprep procedure, and the modified miniprep for difficult species, both described by Dellaporta *et al.* (1983), and a modified protocol of Doyle and Doyle (1990). Only the modified method of Doyle and Doyle (1990) consistently yielded DNA suitable for polymerase chain reaction (PCR) amplification, regardless of plant growing conditions or leaf age. This procedure yielded an average of 53 μg DNA per gram fresh weight of leaf material, with a molecular size of about 50 kb. Reproducible RAPD markers used to distinguish among saskatoon cultivars can be generated from DNA extracted by this procedure.

3.1 INTRODUCTION

RAPD analysis requires DNA of high molecular weight with suitable purity for the enzymatic polymerase chain reaction (PCR) (Sytsma, 1994).

However, it is often difficult to separate DNA from naturally occurring plant cell contaminants. In particular, polysaccharides (Do and Adams, 1991) and phenolic compounds (Newbury and Possingham, 1977) can form a complex with, and become irreversibly bound to nucleic acids during extraction (Varadarajan and Prakash, 1991). Some of these contaminants can inhibit the activity of DNA-modifying enzymes (Draper and Scott, 1988).

The main objective of this study was to examine three common, and relatively quick, DNA extraction methods for their ability to yield DNA suitable for PCR-amplification, from saskatoon leaves. The need for polyvinylpyrrolidone (PVP) or the Elutip-d (a purification column) in the extraction process was also evaluated. Two requirements of the extraction method were that the DNA be extracted from some convenient tissue such as the leaf, and that it not be influenced by tissue age or growing conditions. The rational behind these requirements were two-fold: (1) the DNA fingerprints generated from the DNA would be used for diagnostic purpose and (2) material for diagnosis may come from a variety of sources (eg. field, greenhouse, or tissue culture).

Upon successful extraction of suitable DNA, the final objectives were to screen saskatoon cultivars using a number of different primers to determine which may be useful for cultivar identification, and to examine the reproducibility of the amplification products.

3.2 MATERIALS AND METHODS

3.2.1 PLANT MATERIAL

The plant material used in this study included the saskatoon cultivars Altaglow, Honeywood, Nelson, Pembina, Smoky, SX86-57, and Thiessen. Leaf material of these cultivars was available from 6-7 year-old field-grown material, 1-2 year-old rooted greenhouse cuttings, and micropropagated material that was subcultured on a regular basis.

Leaf material included the blade and petiole from field- and greenhouse-grown material, and stems and leaves of micropropagated shoots. The leaf material was washed to remove any source of foreign DNA, such as insects or insect eggs, on the field- or greenhouse-grown leaves. Between 0.1 and 0.5 g of leaf material was then frozen in liquid nitrogen, ground with a pre-chilled mortar and pestle, and subjected to one of the following extraction procedures.

3.2.2 DNA EXTRACTION

Three DNA extraction methods were examined, referred to here as the SDS, SDS-CTAB, and CTAB methods because they utilize the detergents sodium dodecyl sulfate (SDS), hexadecyltrimethylammonium bromide (CTAB), or both (SDS-CTAB). The first method (SDS) was the *'miniprep' procedure described by Dellaporta et al.* (1983). The second method (SDS-CTAB) was the modified miniprep also described by Dellaporta

et al. (1983) for difficult species. The third method (CTAB) was a modified version of the protocol suggested by Doyle and Doyle (1990) (Appendix A). In all cases, extracted nucleic acids were treated with RNase (10 μg/ml) and incubated for 30 min at 37°C to degrade co-precipitated RNA. Three replications of each extraction method for each of three tissue growing conditions were conducted.

In a separate study, the effects of insoluble PVP (Sigma P-6755) and the Elutip-d (Schleicher & Schuell) on DNA yields and purity were analyzed using three replications of a randomized complete block design. One percent (w:v) insoluble PVP was added to the extraction buffer. The Elutip-d was used according to the manufacturer's directions.

3.2.3 DNA QUANTIFICATION

Two DNA quantification methods were examined. The first method was by spectrophotometric measurement of UV absorption at 260 nm. This was determined using either a Shimadzu UV-260 or Beckman DU 7400 spectrophotometer. The second method was by the minigel method using ethidium bromide fluorescence and a lambda DNA standard, as described by Sambrook *et al.* (1989).

Measures of DNA purity were determined by the A_{260}:A_{280} and A_{260}:A_{230} ratios. These ratios provide indications of protein, and polyphenol and carbohydrate contamination, respectively (Manning, 1991). Absorption

ratios were determined using the UV spectrophotometers listed above.

3.2.4 PCR CONDITIONS

Generation of RAPD markers was done according to the procedure of Williams *et al.* (1990). Reaction volumes of 25 μl contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 μM each of dATP, dCTP, dGTP, and dTTP, 0.2 μM primer, 2.5 ng of genomic DNA, and 1.0 unit of Taq DNA polymerase (BRL). The reaction mixture was overlaid with 25 μl of light mineral oil. However, prior to the addition of Taq and the mineral oil, the reactions were heated to 97°C for 5 min. Amplification was performed in a Hybaid DNA Thermal Reactor (Bio/Can Scientific) programmed for 1 cycle of 2 min at 94°C followed by 45 cycles of 15 sec at 94°C, 1 min at 36°C, and 2 min at 72°C. Temperature profiles were regulated via the Hybaid's control tube. The thermal reactor was placed in a 4°C refrigerator during amplification to speed cooling time. Amplified DNA was electrophoresed in a 1.4% agarose gel containing 0.5 μg/ml ethidium bromide, and photographed on a UV transilluminator.

The primers and corresponding sequences used in this study appear in Table 3.1. Primers were synthesized and supplied by the Plant Biotechnology Institute, NRC, Saskatoon.

3.3 RESULTS AND DISCUSSION

3.3.1 EXTRACTION AND AMPLIFICATION OF DNA

The number of PCR-amplifiable DNA samples resulting from the SDS, CTAB, and SDS-CTAB extraction methods are given in Table 3.2. Only the CTAB method consistently yielded DNA suitable for PCR-amplification from all growing conditions.

DNA extracted by the SDS and SDS-CTAB methods was considered unsuitable because: (1) DNA yields were extremely low; and (2) only a few of these DNA samples could be amplified by the PCR using ten single primers. Fourteen of eighteen DNA samples obtained using these two methods could not be quantified using the minigel method because of extremely low yields. In these cases, quantification was by UV absorption, and some of these samples were amplified by the PCR. The other four DNA samples were quantified using the minigel method, but not all four were PCR-amplifiable. Non-amplification of these DNA samples could be due to the extremely low yields, residual contaminants which interfere with the PCR, or both. Experiments using PVP and Elutip-d in conjunction with the SDS extraction method did not increase the number of DNA samples which could be amplified by the PCR.

Similarly, other researchers (Kanazawa and Tsutsumi, 1992; Webb and Knapp, 1990) were unable to extract PCR-amplifiable DNA from plant tissues using the Dellaporta *et al.* (1983) method. However, Guillemaut and

Table 3.1. Base sequence of primers scored for ability to generate amplification products and detect polymorphism.

CODE	SEQUENCE	AMPLIFICATION
RC-2	TCA ACC CGT	•
RC-3	CAG TCG CTT	•
RC-4	GCC CTA AAG	
RC-5	CTC AGT CAC	•
RC-6	TCA ACC CGG	•
RC-7	CAG TCG CTT	•
RC-8	CTC AGT CAC	•
RC-9	TCC GAT CCA	•
RC-10	AGG CGG TCT	• •
RC-11	TGA CAC CTC	•
RC-12	GGG CAA TGA	• •
RC-13	CCA AGC AGT	•
RC-14	GTG CTG AAG	•
RC-15	CGG CTA GGT	• •
RC-16	GAG CGT TGT	• •
RC-17	CCC AGC GTT	•
RC-18	GCT CAC ATC	•
RC-19	TAC GCA CGG	• •
RC-20	ACC CGG ACA	•
RC-21	CAA ACG CCA	• •
RC-22	TGG AGC AAG	•
RC-23	GGT GCC ATC	•
RC-24	GAC CTC TCC	•
RC-25	CTT GCC AAG	•
RC-26	CCG ACC ATC	•
RC-27	GCT GCT TCA	•
RC-28	CAC GGC AGT	•
RC-29	TAG CAG CGG	•
RC-30	ACC GCT GTG	• •
RC-31	CGA GGA GTT	•
RC-32	GTC ACC GGA	•
RC-33	ACC CTG GTG	•
RC-34	CAA CCA CGA	•
RC-35	CAA GTC CAG	•
RC-36	TGC GTG AGA	•
RC-37	ACC GCC GTT	•
RC-38	GTA CGA ACC	•
RC-39	GCA TCA GGT	
RC-40	CAT CCG CTC	•
RC-41	ACA GCA CCC	•

• primer generated amplification products for cultivars tested.
• • primer detected polymorphism among cultivars tested.

Table 3.2. Number of samples, out of 3 replications, amplified by PCR for DNA extracted by the SDS, CTAB, and SDS-CTAB methods from field, greenhouse, and tissue culture plant material.

Extraction Method	Source of leaf material		
	Field	Greenhouse	Micropropagated
SDS	1	2	3
CTAB	3	3	3
SDS-CTAB	2	0	3

Marechal-Drouard (1992) used an extraction procedure similar to that of Dellaporta *et al.* (1983), and found that the use of a purification column was necessary to yield DNA suitable for PCR. Their protocol was further refined by Rether *et al.* (1993), who added a treatment using cayalase and RNases, and extractions with phenol, phenol-choloroform-isoamyl alcohol, and choloroform-isoamyl alcohol, to yield DNA from cell cultures, callus, and leaf tissue suitable for PCR. The results in Table 3.2 agree with those of Kochko and Hamon (1990), which show that the SDS method does not work well with older leaf material.

In contrast, yields for all CTAB extracted samples could be quantified using the minigel method and amplified by PCR. Extraction protocols similar to the CTAB method presented here have since been reported for the extraction of PCR-amplifiable DNA (Demeke *et al.*, 1992; Echt *et al.*, 1992; Rogers, 1994; Sytsma, 1994).

The addition of PVP and Elutip-d on CTAB-extracted DNA did not interfere with amplification (Table 3.3 and Figure 3.1). However, the use of Elutip-d significantly reduced yields, which may be due to an unknown anionic contaminant that had greater affinity than DNA for the exchange resin.

The average yield for all CTAB-extractions in this study was 53 μg of DNA per gram fresh weight of leaf material. Yields were determined using the minigel method and are similar to those obtained using a variety of DNA extraction procedures found in the literature (Collins and Symons, 1992; Dellaporta *et al.*, 1983; Oard and Dronavalli, 1992; Rogers and Bendich, 1985), including cesium chloride gradient ultracentrifugation (Murray and Thompson, 1980). The literature also indicates the recovery of high molecular weight DNA ranging in size from just over 25 kb (Deragon and Landry, 1992), up to 70 kb (Rogers and Bendich, 1985), with several reports of 50 kb or longer (Dellaporta *et al.*, 1983; Murray and Thompson, 1980). Saskatoon DNA extracted using the CTAB method co-migrated with linear lambda DNA, which indicates a molecular weight of about 50 kb.

3.3.2 DNA QUANTIFICATION

There are a number of reports of quantification of DNA by UV absorption following extraction by CTAB or SDS methods (Gawel and Jarret, 1991; Honeycutt *et al.*, 1992; Moller *et al.*, 1992; Pich and

Table 3.3. Mean DNA yield (μg DNA per gram fresh weight leaf material) and number of samples amplified by PCR for CTAB extraction methods, with and without the inclusion of PVP and the Elutip-d purification column. Results are based on three replications for each extraction method.

Extraction Method	Yield (μg/g)	Number of samples amplified by PCR
CTAB	35	3
CTAB + 1% PVP	37	3
CTAB + Elutip-d	<1	3
CTAB + 1% PVP + Elutip-d	<1	3
LSD (0.05)	11	

Schubert, 1993). Doyle and Doyle (1990) suggested that CTAB may interfere with UV absorption at 260 nm. Comparison of the two methods of quantifying DNA indicates that yields estimated by UV absorption were up to 500-times greater than those estimated by the minigel method. This difference was found for CTAB and SDS-extracted samples. Interference by RNA is possible, but agarose gel electrophoresis of extracted DNA samples did not reveal contamination by RNA. Another possible source of interference could be cellular compounds not removed during the extraction process. Both polysaccharide (Draper and Scott, 1988) and phenolic compounds (Manning, 1991) can interfere with DNA extraction and quantification by UV absorption. Many polyphenolic compounds absorb in

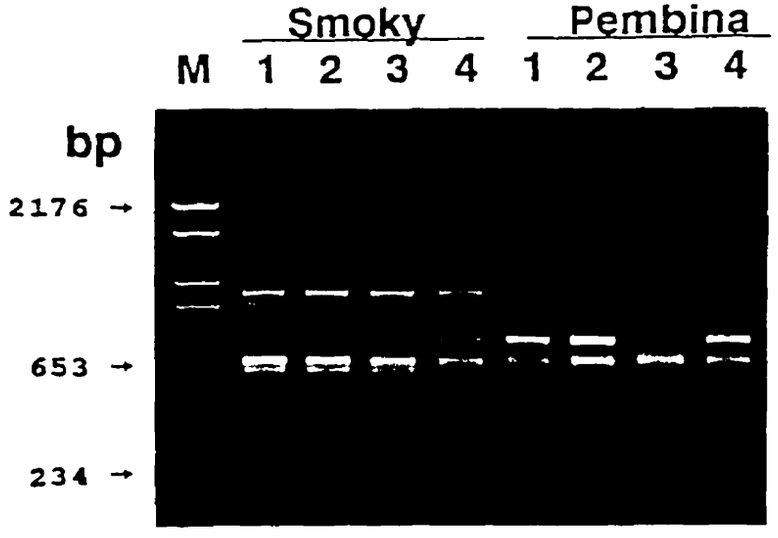


Figure 3.1. Effect of PVP and Elutip-d on amplification products for saskatoon cvs. Smoky and Pembina. Standard PCR conditions and primer RC-16 were used. Lane 1, CTAB extraction method only; lane 2, CTAB plus 1% PVP; lane 3, CTAB plus Elutip-d; lane 4, CTAB plus 1% PVP and Elutip-d. Lane M contains molecular weight marker VI (Boehringer) with fragment size in base pairs (bp) indicated.

the 260 to 280 nm range (Harborne, 1963). The use of PVP or Elutip-d, discussed above, did not reduce the absorption-interfering contaminants. Consequently, because of UV interference by an unknown contaminant, the validity of absorption ratios was questioned, and DNA yield was determined by the minigel method.

3.3.3 SCREENING OF PRIMERS

DNA was extracted using the CTAB method from seven saskatoon cultivars. These cultivars were screened using 40 random primers, of which 38 primers generated amplification products (Table 3.1). However, not all of these primers generated useful amplification products. Some of the primers generated complex banding patterns, which made differentiation of cultivars difficult. Other primers gave false products (primer artifacts) in control lanes containing no DNA. It has often been necessary to screen primers to determine their usefulness for RAPD analysis. Primers generating inconsistent amplifications, no amplifications, complex patterns that were difficult to interpret, and primer artifacts, have been found by other researchers (Demeke *et al.*, 1992; Echt *et al.*, 1992; Wilde *et al.*, 1992). In this preliminary study, seven primers were identified as potentially useful in distinguishing saskatoon cultivars (Table 3.1). As few as two of these primers could be used to completely distinguish the seven cultivars used in this study (Figure 3.2).

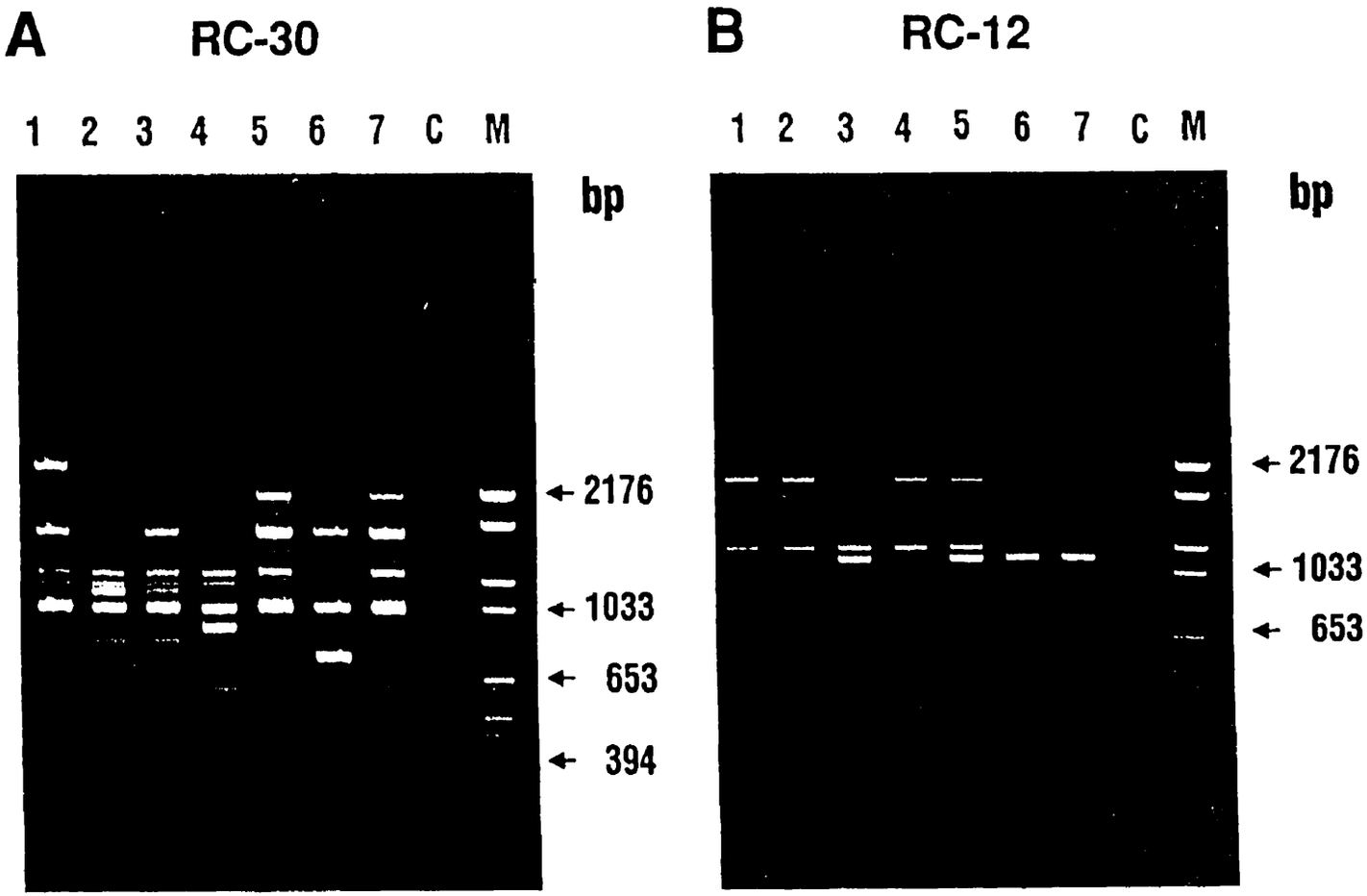


Figure 3.2. Random amplification of DNA segments from seven cultivars generated using standard PCR conditions and primers RC-30 (A) and RC-12 (B). Lanes 1 through 7 correspond to the cultivars Thiessen, SX86-57, Nelson, Pembina, Honeywood, Altaglow, and Smoky, respectively. Genomic DNA was omitted in reactions (lane C) to indicate the presence of primer artifacts. Lane M contains molecular weight marker VI (Boehringer) with fragment size in base pairs (bp) indicated.

3.3.4 REPRODUCIBILITY OF AMPLIFICATION PRODUCTS

The reproducibility of amplification products was examined for CTAB-extracted DNA from the cultivars Smoky and Pembina. Amplification products from five replications of a single DNA sample, and from leaf samples that differ in age and source are shown in Figures 3.3 and 3.4, respectively. Note that amplification products below the 653 bp marker are not amplified consistently, even on multiple runs from a single extraction. This suggests that inconsistencies may be due to amplification conditions and not the DNA extraction procedure.

Nonetheless, unique bands generated using RC-30 (Figure 3.2a, lanes 4 and 7) that differentiate Smoky and Pembina are reproducible. Amplification products generated using RC-30 from four separate DNA extractions of Smoky and Pembina are shown in Figure 3.5. These results indicate that reproducible RAPD markers can be generated for saskatoon cultivar identification.

The effects of PVP and the Elutip-d on the reproducibility of amplification products were examined. Figure 3.1 shows some minor differences in band intensity and number of products among the extraction methods. However, these differences were not consistent from run to run, which suggests that the addition of PVP and Elutip-d, or both, did not enhance the reproducibility of the amplification products over the CTAB method alone.

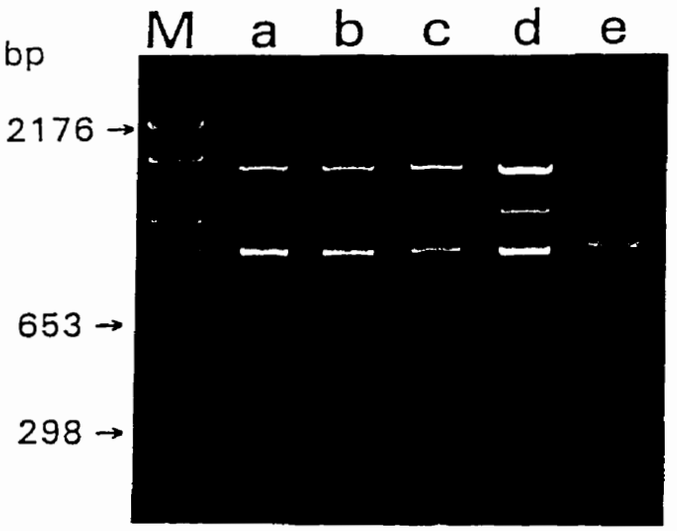


Figure 3.3. Effect of leaf age and source on amplification products for the saskatoon cv. Smoky. Standard PCR conditions and primer RC-30 were used. Lanes a,b and c were collected from field-grown material at leaf unfolding, post-harvest, and beginning of leaf senescence respectively; lane d was collected from micropropagated shoots; and lane e from greenhouse-grown material. Lane M contains molecular weight marker VI (Boehringer) with fragment size in base pairs (bp) indicated.

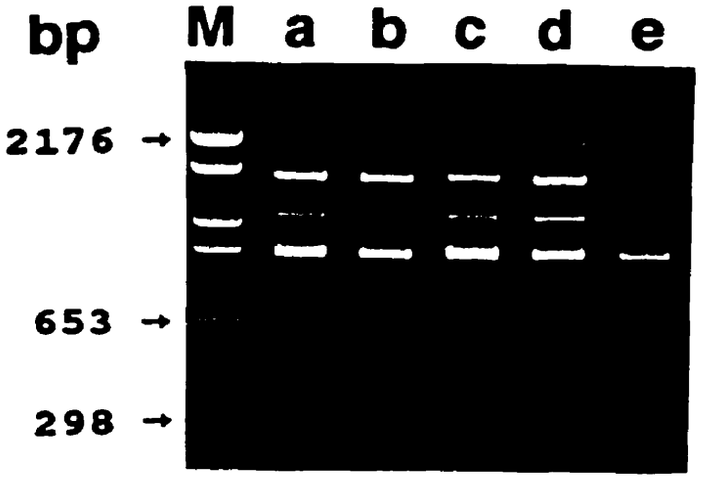


Figure 3.4. Amplification products generated from saskatoon cv. Smoky using standard PCR conditions and primer RC-30. Lanes a through e represent five replications of PCR from a single DNA extraction. Lane M contains molecular weight marker VI (Boehringer) with fragment size in base pairs (bp) indicated.

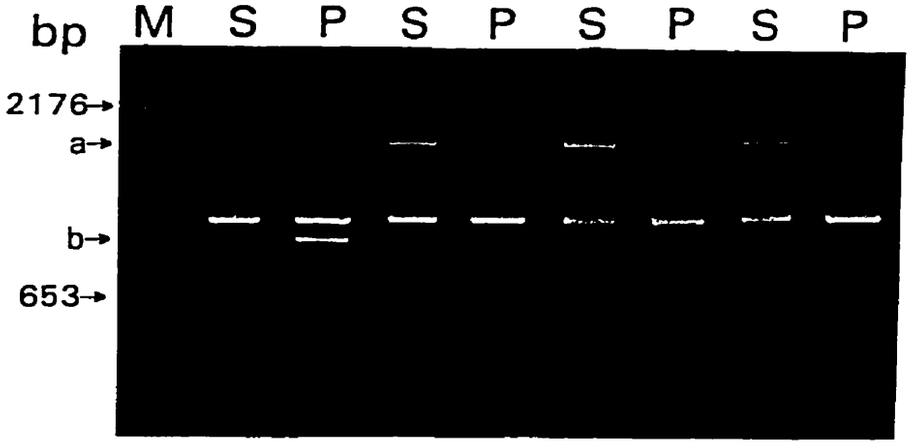


Figure 3.5. Amplification products generated from four separate extractions of saskatoon cvs. Smoky (S) and Pembina (P), using standard PCR conditions and primer RC-30. Arrows (a) and (b) indicate reproducible RAPD markers that differentiate these cultivars. Lane M contains molecular weight marker VI (Boehringer) with fragment size in base pairs (bp) indicated.

In contrast to the above DNA-extraction modifications, other researchers extensively modified their extraction protocol and were able to reduce, but not totally eliminate, amplification product variability within single lines (Aldrich and Cullis, 1993). However, this problem may not be entirely due to the extraction procedure. Generation of reproducible and non-reproducible products by a single primer has been reported (Aldrich and Cullis, 1993; Devos and Gale 1992; Echt *et al.*, 1992; Hu and Quiros, 1991; Koller *et al.*, 1993; Penner *et al.*, 1993; Torres *et al.*, 1993: Weeden *et al.*, 1992), and it is not uncommon to use multiple runs for selection of reproducible RAPD markers (Demeke *et al.*, 1992; Devos and Gale, 1992; Hu and Quiros, 1991; Koller *et al.*, 1993; Weeden *et al.*, 1992). Williams *et al.* (1990) have suggested that these non-reproducible products or 'ambiguous polymorphisms' are the result of primer-template mismatches. The specificity of the primer-template interaction is dependent upon many variables of the PCR, such as $MgCl_2$ concentration, annealing temperatures and source of Taq polymerase (Innis and Gelfand, 1990). Optimization of the PCR for the saskatoon is examined in Chapter 4 of this thesis.

3.4 CONCLUSIONS

Only the modified CTAB method used in this study consistently yielded DNA suitable for PCR-amplification from saskatoon leaves, regardless of plant growing conditions or leaf age. Yield and molecular

weight of the CTAB-extracted DNA are comparable with DNA extracted using other more complex procedures. The generation of reproducible RAPD markers to distinguish among saskatoon cultivars is possible using the CTAB-extraction and amplification protocols presented in this study.

CHAPTER 4

OPTIMIZATION OF THE POLYMERASE CHAIN REACTION FOR *AMELANCHIER ALNIFOLIA*

4.0 ABSTRACT

A number of components of the polymerase chain reaction were optimized for the amplification of saskatoon (*Amelanchier alnifolia* Nutt.) DNA. These components included: (a) buffer composition and pH; (b) concentrations of magnesium chloride, template DNA, and primer; (c) the source and concentration of Taq DNA polymerase; (d) annealing temperature; (e) number of amplification cycles; and (f) pre-amplification heat treatments of the reaction buffer. The optimal reaction conditions were determined to be a reaction volume of 25 μl containing: 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.0 mM $MgCl_2$, 0.001% gelatin, 2 units Taq DNA polymerase (Boehringer), 0.2 μM primer, 100 μM each of dATP, dCTP, dGTP, and dTTP, and 2.5 ng genomic DNA template. Amplifications were performed on a Hybaid Thermal Reactor programmed for 1 cycle at 95°C for 1.5 min, followed by 35 cycles at 95°C for 1 min, 36°C for 1.5 minute, and 73°C for 2.25 minutes, using the fastest possible transition between temperatures.

Variation in any of the above reaction components could affect the reproducibility of amplification products. Some of the components, such as DNA template concentration, could be varied by a 100-fold range without altering the amplification products. In contrast, variation in the magnesium concentration by as little as 0.5 mM had a dramatic effect on the intensity and number of amplification products. The results of this study suggest that lower levels of magnesium may enhance specificity. The optimized reaction conditions reported here generated clear, easily distinguished amplification products from *A. alnifolia* as well as a number of other plant species.

4.1 INTRODUCTION

The polymerase chain reaction is composed of a number of interacting components (Section 2.5). Several observations from Chapter 3 suggested that these components needed to be optimized for the PCR amplification of saskatoon DNA. Firstly, saskatoon DNA was not amplified by the conditions described by Williams *et al.* (1990). The amplification protocol given in Chapter 3 differed from Williams *et al.* (1990) in the following ways: the reaction buffer had a higher pH and was heated to 97°C for 5 min prior to the addition of Taq DNA polymerase, and the thermal cycler was placed in a 4°C refrigerator. Secondly, multiple reactions from single DNA extractions generated a mixture of reproducible

and inconsistently produced products.

Many studies (Aldrich and Cullis, 1993; Caetano-Anolles *et al.*, 1991; Devos and Gale, 1992; Munthali *et al.*, 1992; Weeden *et al.*, 1992; Yu and Pauls, 1992) have shown the need for optimization of the PCR for a given species. The objective of this study was to determine the optimal reaction conditions for the amplification of saskatoon DNA. A number of variables were examined including: use of two different thermal cycling machines; three pre-amplification treatments; buffer composition and pH levels; magnesium chloride, Taq DNA polymerase, DNA template, and primer concentrations; additives such as formamide; and cycling parameters such as annealing temperature and number of amplification cycles.

4.2 MATERIALS AND METHODS

4.2.0 EXTRACTION AND AMPLIFICATION OF DNA

DNA was extracted from fresh, field-grown leaf material of *A. alnifolia* cv. Pembina, using the CTAB-extraction method presented in Chapter 3. Quantification of DNA was by the minigel method using ethidium bromide fluorescence and a lambda DNA standard, as described by Sambrook *et al.* (1989).

The protocol of Williams *et al.* (1990) was used as an initial starting point for the optimization of the polymerase chain reaction (PCR) for the amplification of saskatoon DNA. Amplification conditions according to

Williams *et al.* (1990) included the following: reactions were performed in a volume of 25 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.001% gelatin, 0.5 units Taq DNA polymerase, 0.2 μM primer; 100 μM each of dATP, dCTP, dGTP, and dTTP; and 25 ng genomic DNA template. The reaction mixture was overlaid with 25 μl mineral oil. Amplifications were performed in a Perkin Elmer Cetus DNA Thermal Cycler programmed for 45 cycles. Each cycle consisted of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C. Transition times between temperatures were the fastest available. Base sequences for the primers used in this study (RC-10, RC-16, and RC-30) were previously given in Table 3.1.

4.2.1 THERMAL CYCLING MACHINES

Two thermal cycling machines were compared for the amplification of saskatoon DNA. The two machines were: the Hybaid Thermal Reactor (Bio/Can), referred to as the Hybaid; and the Perkin Elmer Cetus DNA Thermal Cycler (model 480), referred to as the Cetus. Temperature profiles of each machine were monitored using a thermocouple connected to a Bromma 1-channel recorder (LKB). The thermocouple was immersed in 50 μl mineral oil in a 500 μl Eppendorf tube to measure temperatures within a reaction tube. Additionally, the Bromma 1-channel recorder could be directly connected to the Hybaid to monitor the temperature of the plate, which holds the reaction tubes.

4.2.2 PRE-AMPLIFICATION HEAT TREATMENTS

Four pre-amplification heat treatments were examined. These treatments were: (1) heating of the reaction mixture for 5 minutes at 97°C before the addition of Taq DNA polymerase and the mineral oil over-lay, (2) heating of the reaction mixture including Taq and mineral oil to 95°C for 1 minute, (3) heating of the reaction mixture including Taq and mineral oil to 95°C for 2 minutes, and (4) no pre-amplification heating as suggested by Williams *et al.* (1990).

4.2.3 BUFFER COMPOSITION

Four buffers were compared to determine their effect on amplification products. The four buffers were: (1) the standard buffer, pH 9.0; (2) standard buffer, pH 8.7; (3) standard buffer, pH 8.3; and (4) the ammonium-sulfate buffer, pH 9.0. The standard buffer refers to the buffer of Williams *et al.* (1990), [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, and 0.001% gelatin], and the ammonium-sulfate buffer was described by Echt *et al.* (1992), [80 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$]. In addition, reactions were performed on both thermal cycling machines using either the standard (pH 9.0) or the ammonium sulfate (pH 9.0) buffers.

4.2.4 MAGNESIUM CHLORIDE

Magnesium chloride concentrations in the range from 0 to 7.0 mM were examined for their effect on amplification. In addition, magnesium chloride concentrations of 2, 3, and 4 mM, combined with standard buffer at pH 8.3, 8.7, and 9.0 were examined for effect on amplification products.

4.2.5 Taq DNA POLYMERASE

Taq DNA polymerase (Boehringer) concentrations ranging from 0.5 to 2.0 units per reaction were examined for their effect on amplification products. Pre-amplification times of 1 and 2 min, standard buffer pH of 8.3, 8.7, or 9.0, and $MgCl_2$ levels of 2 or 3 mM were also examined for their effect on amplification products. Two other sources of Taq (BRL and Promega) were examined for their effect on amplification products.

4.2.6 OTHER REACTION COMPONENTS

Three other reaction components were examined for their effect on amplification products. These components included the concentrations of template DNA, ranging from 6.25 pg to 20 ng; primer, ranging from 0.0125 to 1.0 μM, and formamide (Fisher, F84-1), ranging from 0.1 to 1.0 %.

4.2.7 OTHER THERMAL CYCLER VARIABLES

Two other thermal cycler variables, annealing temperature (ranging

from 30° to 60°C) and number of amplification cycles (ranging from 25 to 45) were examined for their effect on amplification products.

4.2.8 APPLICATION TO OTHER SPECIES

Amplification conditions optimized for the saskatoon were then applied to a number of other species. DNA was extracted using the CTAB-extraction method described in Chapter 3 from fresh leaf material of the following species: kiwi (*Actinidia kolomikta*), strawberry (*Fragaria sp.*), blueberry (*Vaccinium sp.*), peach (*Prunus persica*), clematis (*Clematis X jackmanii*), banana (*Musa sp.*), crabapple (*Malus sp.*), and cherry (*Prunus cerasis*).

4.3 RESULTS AND DISCUSSION

4.3.0 THERMAL CYCLING MACHINES

Preliminary attempts at DNA amplification did not result in detectable products until the air-cooled Hybaid was placed in a 4°C refrigerator, which decreased the cooling transition time (Figure 4.1a). Temperatures on the Hybaid can be controlled by a thermocouple within either a control tube or the plate. The plate holds and heats or cools the reaction tubes. Initially, temperatures were controlled by using the control tube. However, temperatures within the tube do not rise or fall as fast as those of the plate. Thus, the plate temperature may exceed the control tube temperatures by

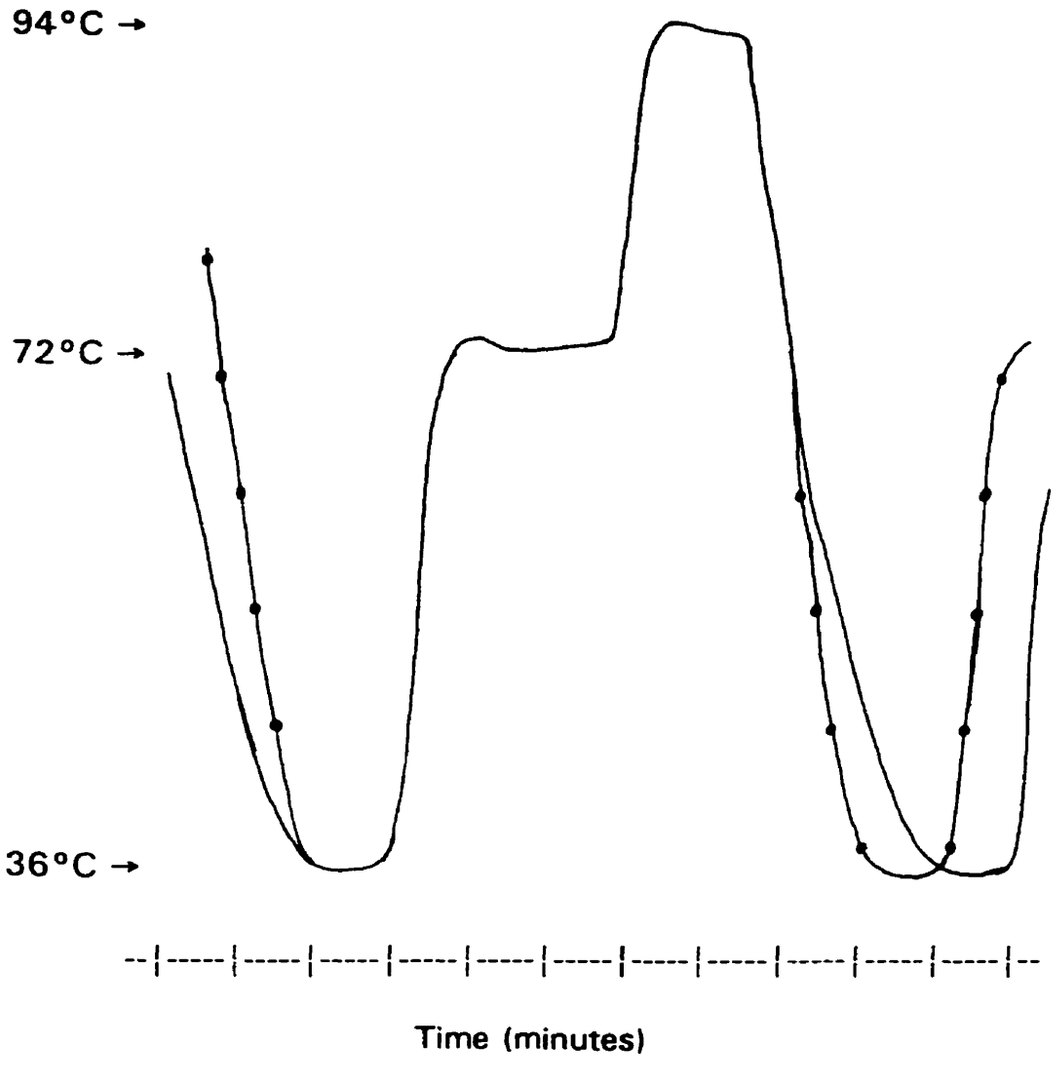


Figure 4.1 (a). Thermal profiles of the Hybaid control tube with ambient air temperature at 4°C (—●—) or 21°C (——). The Hybaid was set for one cycle of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. One minute increments are indicated along bottom of figure.

up to 15°C during denaturation (Figure 4.1b). In conjunction with these temperature differences, slight variation among tube wall thickness and individual well temperature differences could result in reactions which reach higher or lower than desired temperatures. This is important because changes in annealing temperature as small as 1°C have been shown to affect the amplification of DNA (Tingey *et al.*, 1992). As a result, the tube-controlled temperatures used in Chapter 3 were converted to plate-controlled temperatures.

There was a relative temperature difference between the Hybaid and Cetus machines of 2°, 1° and 1°C at settings of 94°, 36° and 72°C respectively (Figure 4.1c). Temperature differences among machines have been reported (Devos and Gale, 1992; Klein-Lankhorst *et al.* 1991) and these differences can affect amplification products (Penner *et al.*, 1993). Consequently, for remaining reactions which compare results from either machine, the cycling temperatures of the Cetus were set to mimic those of the Hybaid. Similar, but not identical, amplification products were obtained from both machines for the primers RC-10, RC-16, and RC-30 (Figure 4.2). Unless stated otherwise, the remaining reactions were performed on the Hybaid.

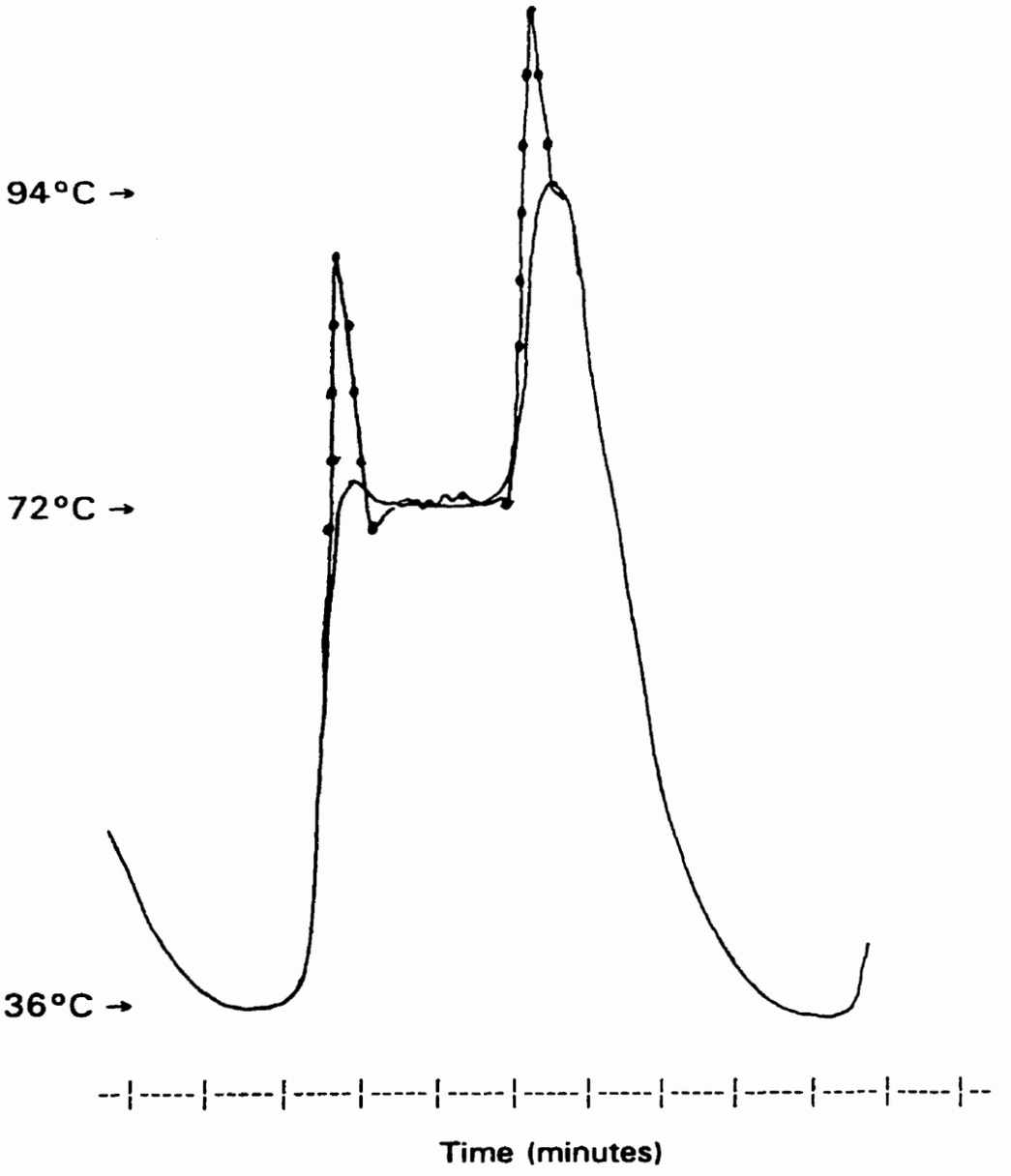


Figure 4.1 (b). Thermal profiles of the Hybaid control tube (——) and plate (—●—) temperatures with ambient air temperature at 4°C. The Hybaid was set for one cycle of 94°C for 15 sec, 36°C for 1 min, and 72°C for 2 min, with temperatures controlled by the control tube. One minute increments are indicated along bottom of figure.

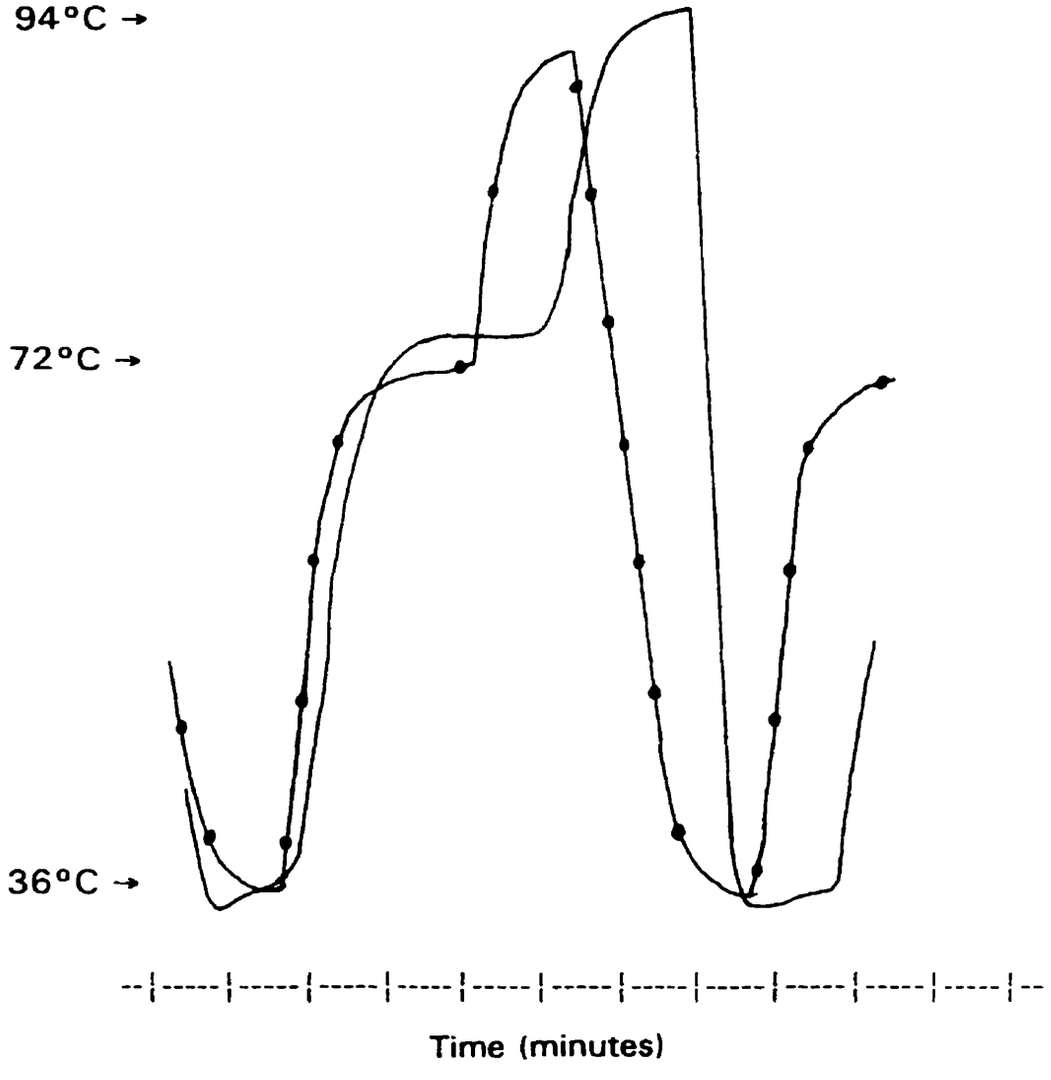


Figure 4.1 (c). Thermal profiles of the Hybaid at 4°C (—●—) and the Cetus at 21°C (——) machines for identical program settings. Machines were set for one cycle of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. One minute increments are indicated along bottom of figure.

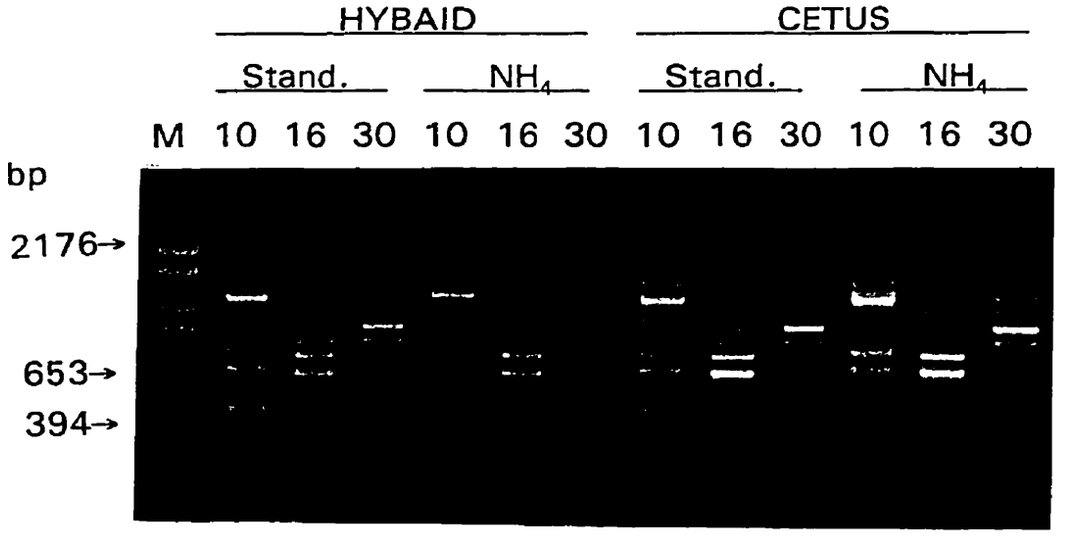


Figure 4.2. Effect of thermal cycling machine and buffer on the amplification of *A. alnifolia* cv. Pembina DNA using primers RC-10, RC-16, RC-30. Reactions were run on either a Hybaid or Cetus thermal cycler with either the standard buffer (Stand.) or the ammonium-sulfate buffer (NH_4) both at pH 9.0. Numbers above each lane correspond to each of the three primers used in this study (eg. lanes marked '10' indicate the use of primer RC-10). Lane M is molecular weight marker VI (Boehringer) with base pairs (bp) indicated.

4.3.1 PRE-AMPLIFICATION TREATMENTS

Several reports suggest using a pre-amplification heat treatment before the addition of some key reagent to the reaction (Carlson *et al.*, 1991; Aldrich and Cullis, 1993; Williams *et al.*, 1991). This has been termed a 'Hot Start' (Chou *et al.*, 1992). A hot start should allow the DNA to fully denature, and would prolong the life of the Taq polymerase if it is added after the heat treatment. The hot start technique is reported to increase specificity and yield of amplification products (Chou *et al.*, 1992). However, in my study the pre-amplification heating of the reaction mixture prior to the addition of Taq DNA polymerase often generated low-intensity or no detectable products (Figure 4.3).

There are numerous published protocols which do not employ any special pre-amplification heat treatment (Devos and Gale, 1992; Quiros *et al.*, 1991; Torres *et al.*, 1993; Vierling and Nguyen, 1992; Wilde *et al.*, 1992; Williams *et al.*, 1990; Yu an Pauls, 1992). However, this method frequently did not generate any detectable products in my study (Figure 4.3).

Reproducible results were obtained by heating the entire reaction mixture for 1 or 2 min at 95°C for one cycle prior to amplification. This type of pre-amplification treatment has been reported (Chapco *et al.*, 1992; Collins and Symons, 1993; Demeke *et al.*, 1992; Echt *et al.*, 1992; Paran *et al.*, 1992; Reiter *et al.*, 1992). An additional benefit of this technique

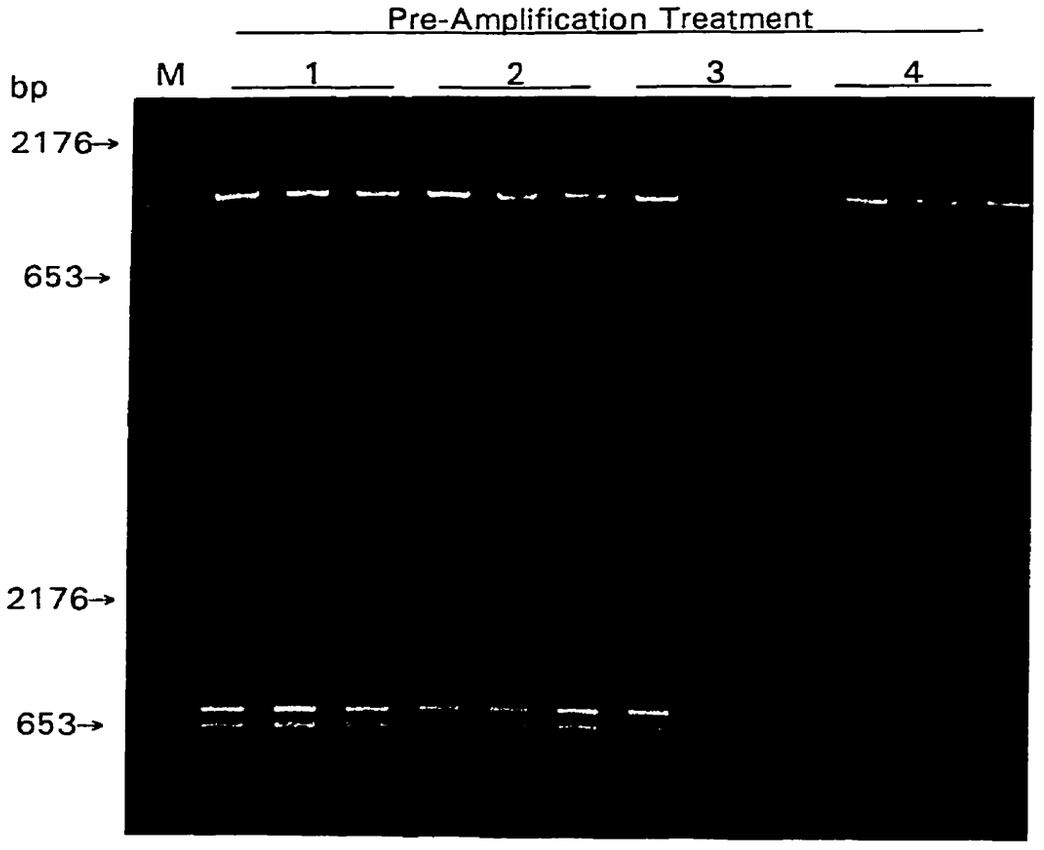


Figure 4.3 . Effect of pre-amplification treatment on the amplification of *A. alnifolia* cv. Pembina DNA using standard PCR conditions and primers RC-10 (top) and RC-16 (bottom). Pre-amplification treatments were: (1) heating of the reaction mixture including Taq and mineral oil to 95°C for 2 minutes, (2) heating of the reaction mixture including Taq and mineral oil to 95°C for 1 minute, (3) no pre-amplification heating, and (4) heating of the reaction mixture for 5 minutes at 97°C before the addition of Taq DNA polymerase and the mineral oil over-lay. Lane M is molecular weight marker VI (Boehringer) with base pairs (bp) indicated.

over the hot start is the reduced handling of reaction mixtures, which should help reduce contamination.

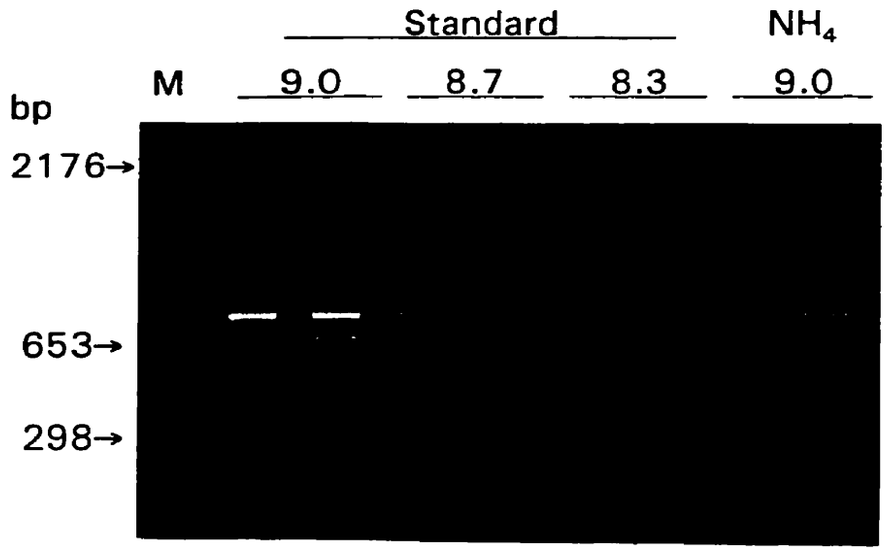
4.3.2 BUFFER COMPOSITION

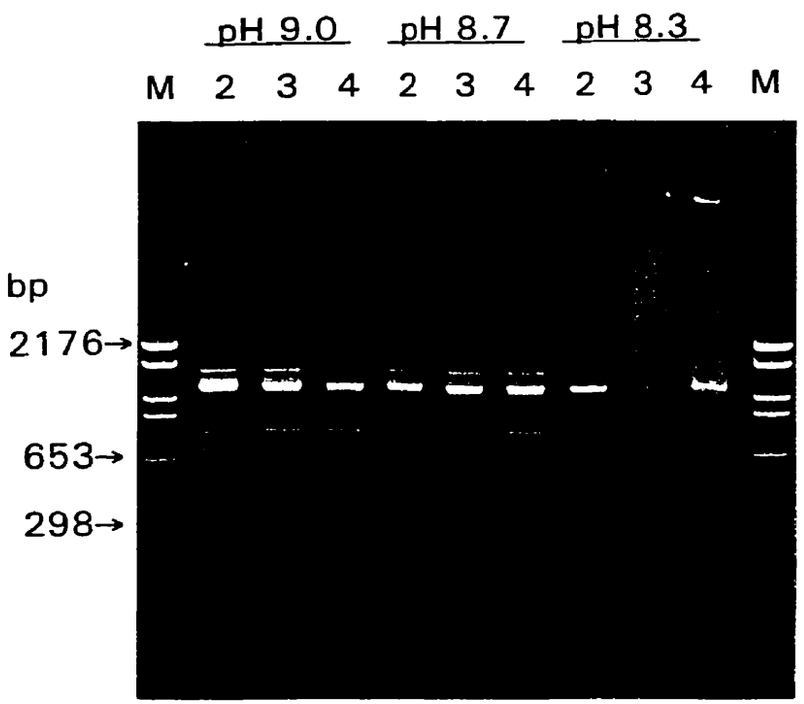
Clear, easy-to-distinguish and reproducible amplification products were obtained using the standard buffers at pH 8.7 and 9.0 (Figure 4.4). Generally the intensity of products was greater at pH 9.0. The standard buffer at pH 8.3 often generated fewer products with reduced intensity and background smearing (Figures 4.4 and 4.5). Amplification products were not consistently generated using the ammonium-sulfate buffer.

However, consistent results have been reported using the ammonium-sulfate buffer with reactions performed on the Cetus (Echt *et al.*, 1992). Differences between the Hybaid and Cetus thermal cycling machines were reported earlier in this study. Therefore, reactions using either the standard (pH 9.0) or ammonium-sulfate (pH 9.0) buffers were performed on either the Hybaid or Cetus machines to examine the combined effect on amplification products. Reactions containing the ammonium-sulfate buffer did not consistently amplify saskatoon DNA on either machine. In contrast, reactions containing the standard buffer (pH 9.0) consistently amplified saskatoon DNA on either machine. A number of differences in buffer composition could account for these results. For example, the standard buffer contains gelatin and KCl, whereas the

Figure 4.4. Effect of different buffers and primer RC-16 on the amplification of *A. alnifolia* cv. Pembina DNA. Pairs of lanes have an upper label indicating the type of buffer, with the buffer pH indicated below. Lanes (Standard), indicate reactions using the standard buffer with pH of 9.0, 8.7 or 8.3. Lane (NH_4) indicates reactions using the ammonium-sulfate buffer with pH 9.0. Lane M is molecular weight marker VI (Boehringer) with base pairs (bp) indicated.

Figure 4.5. Effect of varying magnesium concentration and buffer pH on the amplification of *A. alnifolia* cv. Pembina DNA. Standard PCR buffers at pH 9.0, 8.7 and 8.3, magnesium chloride concentrations of 2, 3, and 4 mM, and primer RC-10 were used to generated amplification products. Lane M is molecular weight marker VI (Boehringer) with base pairs (bp) indicated.


Standard
NH_4
M
9.0
8.7
8.3
9.0
bp
2176→
653→
298→


pH 9.0
pH 8.7
pH 8.3
M 2 3 4 2 3 4 2 3 4 M
bp
2176→
653→
298→

ammonium-sulfate buffer does not. Gelatin and KCl are included to help stabilize the enzyme and facilitate primer annealing, respectively (Innis and Gelfand, 1990). The standard buffer (pH 9.0) was used in all remaining reactions unless otherwise stated.

4.3.3 MAGNESIUM CHLORIDE CONCENTRATION

Magnesium chloride levels were examined in the range from 0 to 7.0 mM, with consistent amplification occurring in the range of 1.5 to 4.0 mM (Figure 4.6). Variation in magnesium chloride concentration appears to have the most dramatic effect on the polymorphism generated. In these experiments, there were no amplification products without $MgCl_2$ in the reaction buffer, and the number of products increased with increasing concentrations of $MgCl_2$, to a maximum of 4.0 mM. Munthali *et al.* (1992) reported similar results for concentrations between 0 and 4.0 mM. At concentrations above 4.0 mM my results became variable and often no amplification products were observed.

Devos and Gale (1992) suggest that the stringency of primer annealing decreases at higher magnesium levels, yet Welsh and McClelland (1990) suggest that higher levels actually stabilize primer and DNA-template interaction. Results from this study and those of Munthali *et al.* (1992) show that as magnesium levels increase the number of amplification products increase, which may be due to lower stringency.

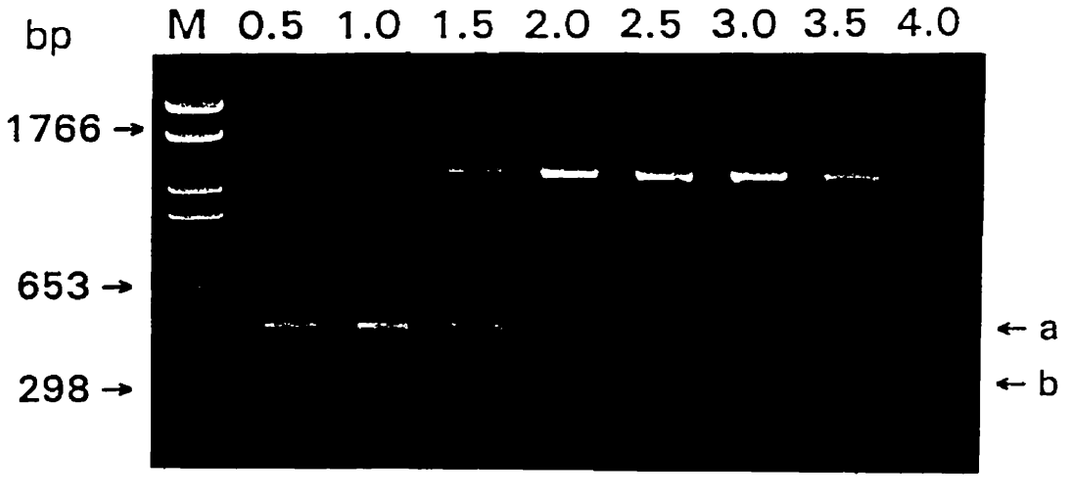


Figure 4.6. Effect of magnesium concentration on the amplification of *A. alnifolia* cv. Pembina DNA. Standard PCR conditions and primer RC-10 were used. Concentration of magnesium (mM) is indicated above each lane. Presence and intensity of certain products are indicated by arrows a and b. Lane M is molecular weight marker VI (Boehringer) with base pairs (bp) indicated.

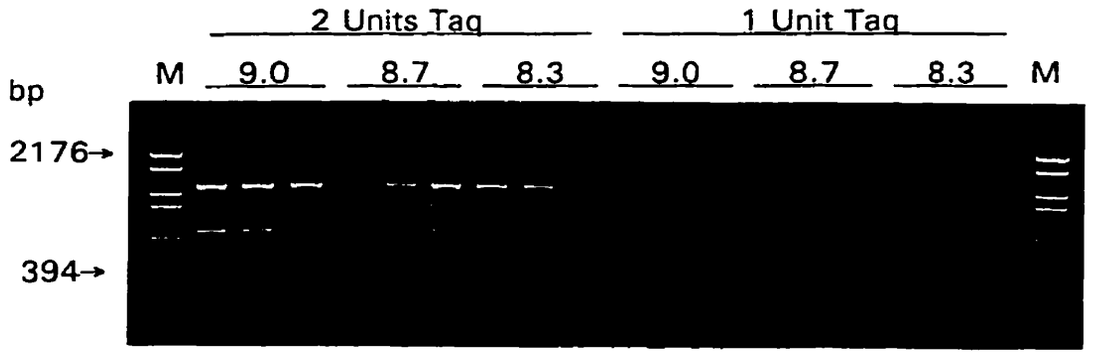
Reactions performed with magnesium chloride concentrations of 2, 3, or 4 mM, and standard buffer at pH 8.3, 8.7, or 9.0 were examined for their combined effect on amplification products (Figure 4.5). More amplification products were generated at 3 and 4 mM magnesium for pH 8.7 and 9.0 buffers, which may suggest reduced stringency at higher magnesium concentrations. Background smearing is evident in all three magnesium levels at pH 8.3, however, at 2 mM magnesium the background smearing is reduced and products are more clearly defined. These results suggest that lower magnesium levels increase the specificity of the reaction. All remaining reactions were performed using 2.0 mM magnesium chloride in a standard buffer (pH 9.0), unless stated otherwise.

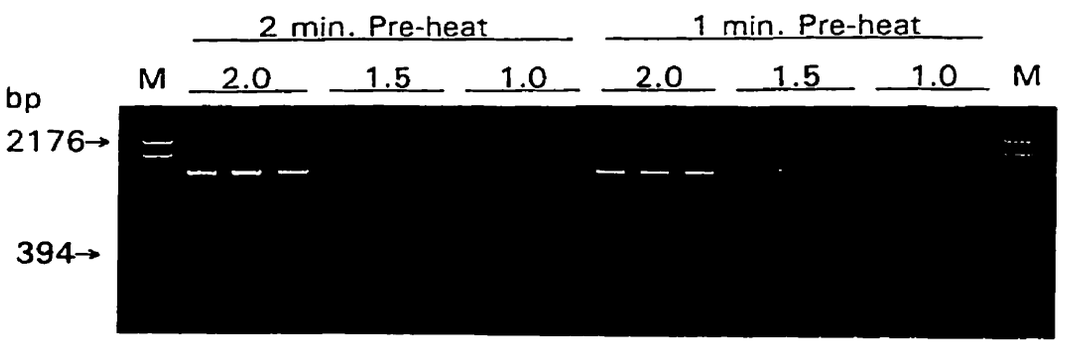
4.3.4 TAQ DNA POLYMERASE

Two units of Taq DNA polymerase (Boehringer) were required to generate reproducible amplification products. Lower concentrations resulted in decreased intensity or no amplification products. Reducing the buffer pH (Figure 4.7), decreasing the pre-amplification time from 2 to 1 min (Figure 4.8), and increasing $MgCl_2$ levels to 3 mM did not reduce the requirement for Taq. Current literature indicates a range of 0.5 (Williams *et al.* 1990 and others) to 7.5 (Caetano-Anolles *et al.* 1992) units of Taq polymerase. The quality and quantity of the DNA, cycling times and temperatures, and polymerase source may account for this wide range.

Figure 4.7. Effect of varying standard buffer pH and units of Taq DNA polymerase on the amplification of *A. alnifolia* cv. Pembina DNA. Standard PCR conditions and primer RC-10 were used. Standard buffer pH (9.0, 8.7, and 8.3) and units of Taq DNA polymerase are indicated above appropriate lanes. Lane M is molecular weight marker VI (Boehringer) with base pairs (bp) indicated.

Figure 4.8. Effect of varying pre-amplification heating time and units of Taq DNA polymerase on the amplification of *A. alnifolia* cv. Pembina DNA. Standard PCR conditions and primer RC-10 were used. Pre-amplification heating was for 1 or 2 minutes 95°C. Lane M is molecular weight marker VI (Boehringer) with base pairs (bp) indicated.


2 Units Taq
1 Unit Taq
M
9.0
8.7
8.3
9.0
8.7
8.3
M
bp
2176→
394→


2 min. Pre-heat
1 min. Pre-heat
M
2.0
1.5
1.0
2.0
1.5
1.0
M
bp
2176→
394→

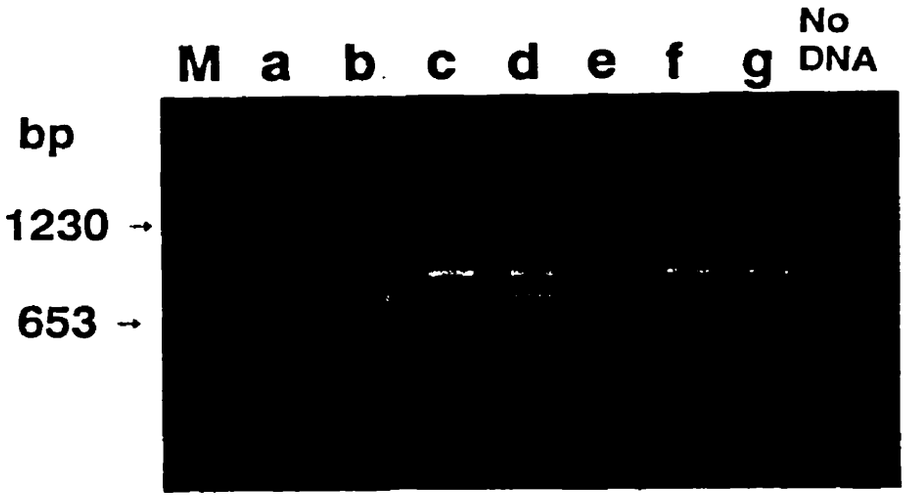
The BRL-Taq generated products were identical to those generated by Boehringer-Taq for a number of primer and template combinations tested. In contrast, the Promega-Taq did not generate any detectable products using either our standard buffer or the Promega buffer supplied with their enzyme. Weeden *et al.* (1992) obtained identical results using three polymerases from different sources. However, Carlson *et al.* (1991) indicate that enzyme source is critical to obtaining consistent results, and other reports (Aldrich and Cullis, 1993; Caetano-Anolles *et al.*, 1992; Schierwater and Ender, 1993) have demonstrated that different Taq sources generate different amplification products. Williams *et al.* (1991) suggest that different species of Taq will have different sensitivities to mismatches between primer and template, and therefore could give different amplification products. A concentration of 2 units of Taq (Boehringer) was used in all remaining experiments unless otherwise stated.

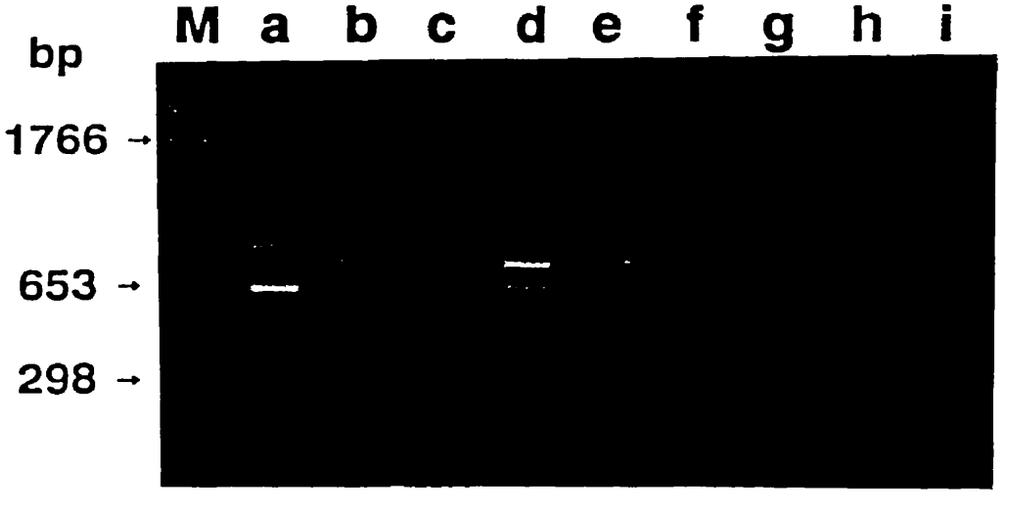
4.3.5 OTHER REACTION COMPONENTS

DNA concentrations were examined in the range from 6.25 pg to 20 ng, with reproducible amplification generated from concentrations ranging from 62.5 pg to 5 ng. At 10 ng some background smearing was generated, while above 10 ng amplification was inconsistent (Figure 4.9). This may be due to contaminants not removed during the DNA extraction process, which at high levels may inhibit Taq polymerase. Inconsistent

Figure 4.9. Effect of *A. alnifolia* cv. Pembina DNA concentration on amplification products generated using standard PCR and primer RC-16. DNA concentrations for lanes (a) 20 ng, (b) 10 ng, (c) 5 ng, (d) 2.5 ng, (e) 1.25 ng, (f) 625 pg, and (g) 62.5 pg. The last lane did not contain genomic DNA. Lane M is molecular weight marker VI (Boehringer) with base pairs (bp) indicated.

Figure 4.10. Effect of primer concentration on the amplification of *A. alnifolia* cv. Pembina DNA using standard PCR conditions and primer RC-16. Primer concentrations (μM) for each lane are (a) 1.0, (b) 0.5, (c) 0.25, (d) 0.2, (e) 0.1, (f) 0.05, (g) 0.025, (h) 0.0125, and (i) no primer added. Lane M is molecular weight marker VI (Boehringer) with base pairs (bp) indicated.


M a b c d e f g No DNA
bp
1230 →
653 →


M a b c d e f g h i
bp
1766 →
653 →
298 →

amplification occurred at template levels below 62.5 pg. These results were consistent for three primers tested: RC-10, RC-16, and RC-30.

Reported DNA concentrations generating reproducible results varies greatly from 10 pg (Caetano-Anolles *et al.* 1991) to 400 ng (Klein-Lankhorst *et al.*, 1991). These differences in DNA concentration may reflect differences in DNA quality resulting from the extraction procedures employed for the different species under investigation. Alternatively, one report has shown that the optimal range for DNA concentration varied considerably depending on the Taq DNA polymerase source (Aldrich and Cullis, 1993).

Primer concentrations ranging from 0.0125 to 1.0 μM were examined (Figure 4.10). Primers RC-10 and RC-16 generated consistent results over ranges of 0.1 to 0.4 and 0.2 to 0.4 μM respectively. Generally, more background smearing occurred at all concentrations of RC-10 than for RC-16. For both primers, concentrations of 0.5 and 1.0 μM generally resulted in an increased number of amplification products and background smearing. At primer concentrations below 0.5 μM, background smearing was reduced and amplification products were more clearly distinguished. The number of amplification products decreased at primer concentrations of 0.05 μM and lower, with no detectable products at 0.0125 μM. Similar observations were reported by Munthali *et al.* (1992).

Formamide has been added to the buffer to enhance the specificity of

the reaction (Sarkar *et al.*, 1990). In this study, formamide concentrations ranging from 0 to 1.0% formamide were added to both the standard buffer (pH 9.0) and the ammonium-sulfate buffer (pH 9.0), and reactions were run on both thermal cycling machines. Some individual reactions with formamide had reduced background smearing and fewer of the faint bands which are often not reproducible from run to run. However, the more intense, reproducible bands in the non-formamide reactions appeared less intense with the addition of formamide. Similar results were obtained regardless of machine or buffer. The use of formamide in the reaction buffer was not warranted by the above results.

4.3.6 OTHER THERMAL CYCLER VARIABLES

The effect of annealing temperatures, ranging from 30° to 60°C, for the primers RC-16 and RC-10 were examined (Figure 4.11). The number of amplification products and intensity of products was not consistent across this temperature range. For example, at 36°C and above, a lower weight band generated by RC-10, increased in intensity and was consistently amplified at the higher temperatures (Figure 4.11A, arrow (a)). Amplification products were not detected at 50° for primer RC-16 and 55°C for RC-10.

Reports regarding the effect of annealing temperatures on amplification products are conflicting. Other studies (Devos and Gale,

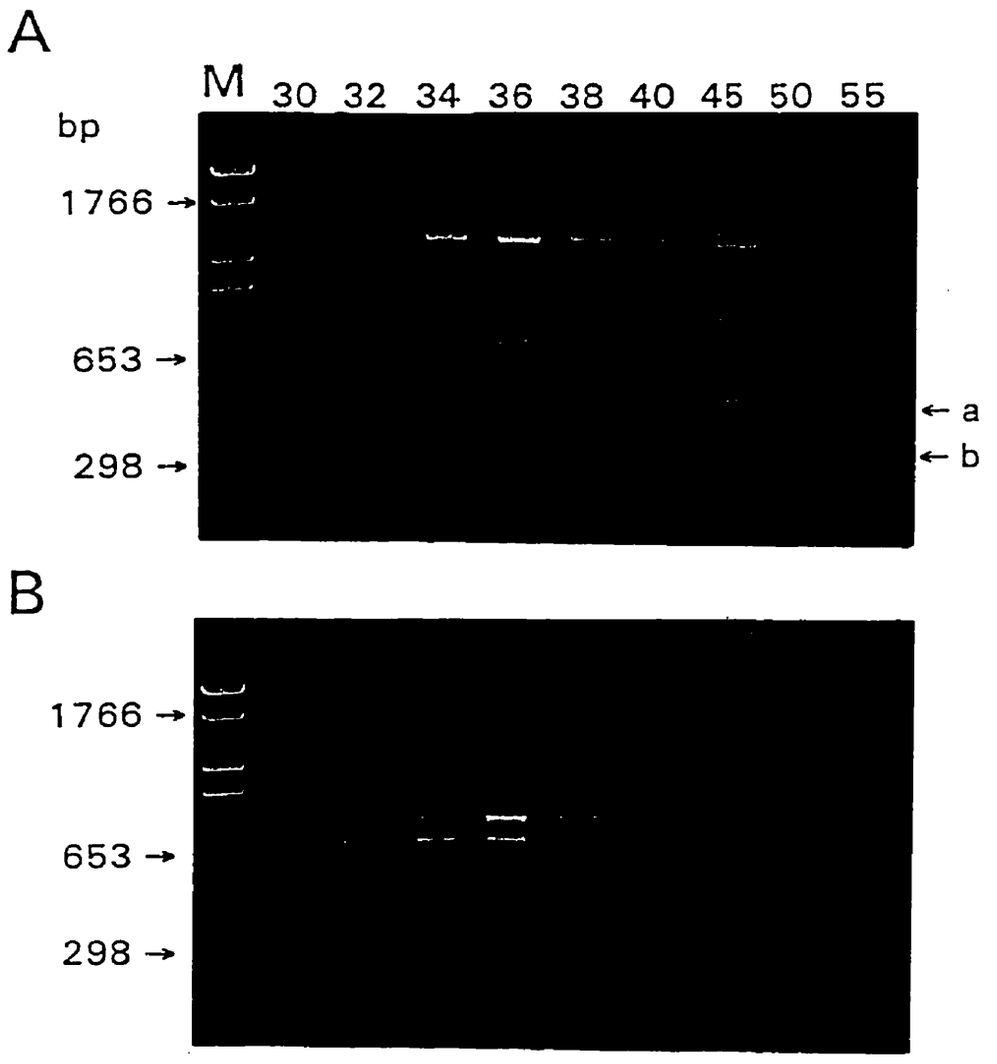


Figure 4.11. Effect of annealing temperature on the amplification of *A. alnifolia* cv. Pembina DNA using standard PCR conditions and primers RC-10 (A) and RC-16 (B). Temperatures (°C) are given above each lane. Arrows indicate effects of annealing temperature on product generation. Lane M is molecular weight marker VI (Boehringer) with base pairs (bp) indicated.

1992; Weeden *et al.*, 1992) have suggested that minimal changes in amplification products occur over a 5° to 7°C range of annealing temperatures. In contrast, Tingey *et al.* (1992) have generated different amplification products by changing the annealing temperature by only 1°C. The results of this study support both observations. At temperatures of 34°, 36°, and 38°C the amplification products only differ in intensity for both primers. In contrast, differences in product number and intensity occur for both primers at temperatures of 32° and 34°C.

Similarly, Devos and Gale (1992) reported no priming by 10-mers at 42°C, and the results of this study report no priming of 9-mers at 50° to 55°C. However, Caetano-Anolles *et al.* (1992), have demonstrated that 8- and 10-mers generated products at an annealing temperature of 65°C. Differences in the upper limit for annealing temperatures may be due to: (1) primer sequence - a perfectly matched primer should anneal at higher temperatures than a mismatched primer, (2) $MgCl_2$ levels can affect the stringency of primer annealing (Devos and Gale, 1992; Welsh and McClelland, 1990), (3) the source of Taq polymerase - different species of the polymerase may have different sensitivities to primer\template mismatches (Williams *et al.* 1991), and (4) a combination of the above factors.

Amplification products generated using 25 to 45 amplification cycles were examined (Figure 4.12). Clear, easily distinguished bands were

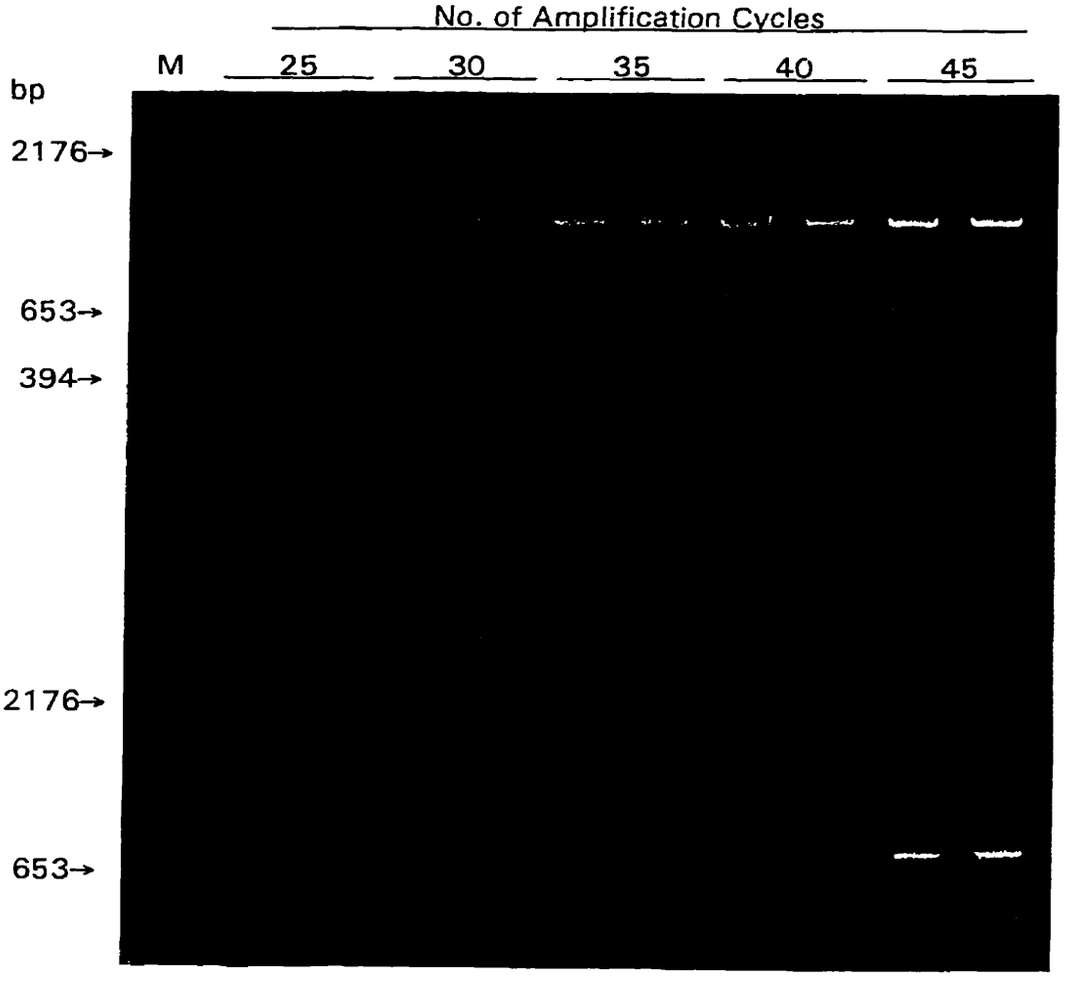


Figure 4.12 . Effect of the number of amplification cycles on the amplification of *A. alnifolia* cv. Pembina DNA using standard PCR conditions and primers RC-10 (top) and RC-16 (bottom). Lane M is molecular weight marker VI (Boehringer) with base pairs (bp) indicated.

obtained at 35, 40 and 45 cycles, although there was a tendency for increased background smearing and non-specific products at 45 cycles. Reduced intensity of amplification products occurred at 25 and 30 cycles. These results concur with those of Munthali *et al.* (1992) and Yu and Pauls (1992), who suggested there was no benefit in using more than 35 cycles. Decreasing the number of cycles from 45 to 35 represents a one hour reduction in total cycling time. A total of 35 cycles were used in all reactions unless otherwise stated.

4.3.7 APPLICATION TO OTHER SPECIES

Standard amplification conditions were used to generate products for a number of other species (Figure 4.13). Clear, easily distinguished amplification products were generated for all species in this study. These results suggests that both the DNA extraction and amplification protocols standardized for the saskatoon may be useful for RAPD analysis in many other species.

4.4 CONCLUSIONS

Based on the above testing, the following protocol for the amplification of saskatoon DNA was determined. The reaction volume of 25 μl contains: 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.0 mM $MgCl_2$, 0.001% gelatin, 2 units Taq DNA polymerase (Boehringer), 0.2 μM of a

Figure 4.13. Amplification of CTAB-extracted DNA from various plant species. Standard PCR conditions and primer RC-16 were used to generate products from: (a) saskatoon, (b) kiwi, (c) strawberry, (d) blueberry, (e) peach, (f) clematis, (g) banana, (h) crabapple, and (i) cherry. Lane M is molecular weight marker VI (Boehringer) with base pairs (bp) indicated.

single 9-mer primer, 100 μM each of dATP, dCTP, dGTP, and dTTP, and 2.5 ng genomic DNA template. The reaction mixture was overlaid with 25 μl mineral oil. Reactions were performed in 0.5 ml microfuge tubes and were kept on ice during manipulation. Amplifications were performed on a Hybaid Thermal Reactor programmed for 1 cycle at 95°C for 1.5 min, followed by 35 cycles at 95°C for 1 min, 36°C for 1.5 minute, and 73°C for 2.25 minutes, using the fastest possible transition between temperatures. Temperatures were controlled by the plate temperature. The Hybaid thermal cycler was placed in a 4°C refrigerator to reduce the cooling transition time.

Variation in the any of the above reaction components could affect the reproducibility of amplification products. Some of the components, such as DNA template concentration, could be varied by a 100-fold without altering the amplification products. In contrast, only a 2 to 4-fold range in primer concentration generated consistent amplification products. Variation in the magnesium concentration had a dramatic effect on the intensity and numbers of amplification products. The results of this study suggest that lower levels of magnesium may enhance specificity. Magnesium levels must be kept constant from reaction to reaction to ensure reproducibility. The optimized reaction conditions reported here were also used to generate clear, easily distinguished amplification products from a number of other plant species.

CHAPTER 5

GENETIC VARIATION AMONG SASKATOON CULTIVARS, CLONES, AND SEEDLINGS

5.0 ABSTRACT

Randomly amplified polymorphic DNA (RAPD) markers were used to distinguish among 16 cultivars of saskatoon (*Amelanchier* spp.). Eight 9-base primers were chosen that revealed polymorphism among the cultivars. These eight primers generated a total of 98 bands. Among these 98 bands, 29 were useful as reproducible polymorphic markers. Twelve cultivars and two pairs of cultivars were uniquely characterized by these 29 markers. Polymorphism was not detected among five sources of the cultivar Thiessen, whereas variability was found among seedlings from self-pollinated Thiessen. Samples of the cultivars Regent and Parkhill could be distinguished from one source but were indistinguishable from another source, suggesting the latter source had mislabelled their samples.

5.1 INTRODUCTION

Almost all of the available saskatoon cultivars are unimproved selections from the wild. These cultivars are generally maintained asexually

by cuttings and micropropagation. Descriptions of at least 19 different cultivars have appeared over the years (Darrow, 1975; McConkey, 1979; St. Pierre, 1991; Stushnoff, 1990; Wallace and Graham, 1976; Williams, 1991). Recently, Davidson and St. Pierre (1994) compiled the first comprehensive and standardized descriptions of 15 validly introduced saskatoon cultivars. Characteristics such as size, yield, and acidity of the fruit, plant size and flowering dates vary among these cultivars (Davidson and St. Pierre, 1994). Despite these differences, distinguishing among cultivars, even mature cultivars, is nearly impossible (St. Pierre, 1991), thus there can be some confusion regarding the true identity of planted material.

This confusion originates from the fact that vegetatively propagated material can become mislabelled in the nursery or tissue culture laboratory, and seedlings are not always identical to parents. A single study, limited to the cultivar Smoky, which only compared bush type and fruit quality, suggested that seedlings may be as much as 85% true to type (Wallace and Graham, 1976). Advertisements from several nurseries use the 85% figure in their literature and sell seedlings under the same name as clonally propagated material.

The main objective of this study was to characterize 16 saskatoon cultivars at the molecular level using RAPD analysis. The second objective

was to monitor for genetic variability among five sources of Thiessen, two sources of Parkhill and Regent, and among seedlings of self-pollinated Thiessen.

5.2 MATERIALS AND METHODS

The 16 saskatoon cultivars characterized using RAPD analysis are listed in Table 5.1. The five sources of Thiessen and eight seedlings from self-pollinated Thiessen are listed in Table 5.5. Martin is included in Table 5.5 because it is reported to be a seedling from self-pollinated Thiessen (Davidson and St. Pierre, 1994). Sources of the cultivars Parkhill and Regent are listed in Table 5.6. Leaf samples were collected from all cultivars and seedlings in 1992. Parkhill and Regent were re-sampled from the Alberta Tree Nursery and Horticulture Centre (ATN) in 1993. Fresh leaf material was collected from all cultivars and seedlings in this study. Leaves were washed to remove any source of foreign DNA (such as insects or insect eggs), frozen in liquid nitrogen, and stored at -70°C until required.

DNA was extracted from the frozen leaf material using the modified method of Doyle and Doyle (1990) reported in Chapter 3. RAPD markers were generated according to the protocol described in Chapter 4, using a Hybaid DNA Thermal Cycler (Bio\Can). Results were replicated at least three times for each primer-cultivar combination. Primers used for RAPD

Table 5.1* Saskatoon cultivars fingerprinted using randomly amplified polymorphic DNA (RAPD) markers.

Cultivar # and Name	*Amelanchier* Species	Cultivar Origin	Year Released	Leaf Source
1 Thiessen	alnifolia	Waldheim, SK	1976	ATN
2 Pembina	alnifolia	Barrhead, AB	1952	ATN
3 Moonlake	alnifolia	Moon Lake, SK	1974	ATN
4 Northline	alnifolia	Beaverlodge, AB	1960	ATN
5 Honeywood	alnifolia	Parkside, SK	1973	ATN
6 Smoky	alnifolia	Peace River, AB	1952	BRS
7 Success	sanguinea ?	Pennsylvania	1878	ATN
8 Parkhill	sanguinea ?	Michigan	1974	MRS
9 Regent	alnifolia ?	Regent, ND	1977	MRS
10 Forestburg	alnifolia	Forestburg, AB	1958	MRS
11 Pearson II	alnifolia	Bowden, AB[1]	-	ATN
12 Altaglow	alnifolia	Lacombe, AB	1958	MRS
13 Bluff	alnifolia	Buffalo Lake, AB	1990	PBI
14 Nelson	alnifolia	Bradwell, SK	1992	UofS
15 Buffalo	alnifolia	Buffalo Lake, AB	1990	PBI
16 Martin	alnifolia	Langham, SK[2]	1990	PPS

* Information compiled from: Davidson and St. Pierre (1994).

? Species designation uncertain in literature.

[1] Pearson II is a selection from self-pollinated Smoky

[2] Martin is a selection from self-pollinated Thiessen

ATN Alberta Tree Nursery and Horticulture Centre, Edmonton, AB

PBI Pacific Biotechnologies Inc., Vedder Crossing, BC

UofS Dept. of Horticulture Science, University of Saskatchewan, Saskatoon, SK

MRS Agriculture Canada Research Station, Morden, MB

BRS Agriculture Canada Research Station, Beaverlodge, AB

PPS Prairie Plant Systems, Saskatoon, SK

analysis were RC-12, -16, -19, -22, -28, -30, -36, and -37. Sequences for these primers were previously given in Table 3.1. Amplification products were separated by electrophoresis on a 1.4% agarose gel containing 0.5 ug/ml ethidium bromide, and photographed on a UV transilluminator.

Each amplified product was named according to the primer used and its size in base pairs (bp). For example, RC16-500 refers to a 500 bp fragment amplified by primer RC-16. Amplification products (bands) were scored as present (1) or absent (0) for each replication. Only consistently amplified bands were compiled into a diagnostic fingerprint for the cultivars. If, for example, RC16-500 was reproducibly amplified for several cultivars, but inconsistently amplified for another, it was excluded as a marker for all cultivars.

Molecular weights for amplification products were determined from the graph of the linear relationship between the log of molecular weight of the standards and their migration distances. Marker VI (Boehringer) was used as the standard.

5.3 RESULTS AND DISCUSSION

5.3.1 MOLECULAR CHARACTERIZATION OF CULTIVARS

Amplification products generated using eight primers for the sixteen cultivars are shown in Figures 5.1 and 5.2. A total of 98 bands were generated (Table 5.2). There was variation in the number and size of bands

Figure 5.1. Amplification products for sixteen saskatoon cultivars generated using standard PCR conditions and primers RC-12, RC-16, RC-19, and RC-22. Numbers above each lane correspond to the following cultivars: (1) Thiessen, (2) Pembina, (3) Moonlake, (4) Northline, (5) Honeywood, (6) Smoky, (7) Success, (8) Parkhill, (9) Regent, (10) Forestburg, (11) Pearson II, (12) Altaglow, (13) Bluff, (14) Nelson, (15) Buffalo, and (16) Martin. Lane M contains molecular weight marker VI (Boehringer) with fragment size in base pairs (bp) indicated. (These figures are reproductions and may not clearly show all of the amplification products which were reproducible and clearly distinguishable on the original agarose gels and photographs. Thus, the cultivars Smoky and Pearson II (lanes 6 and 11, respectively) which could not be distinguished based on the reproducible amplification products scored in Table 5.3, do not appear to have identical amplification products in these figures).

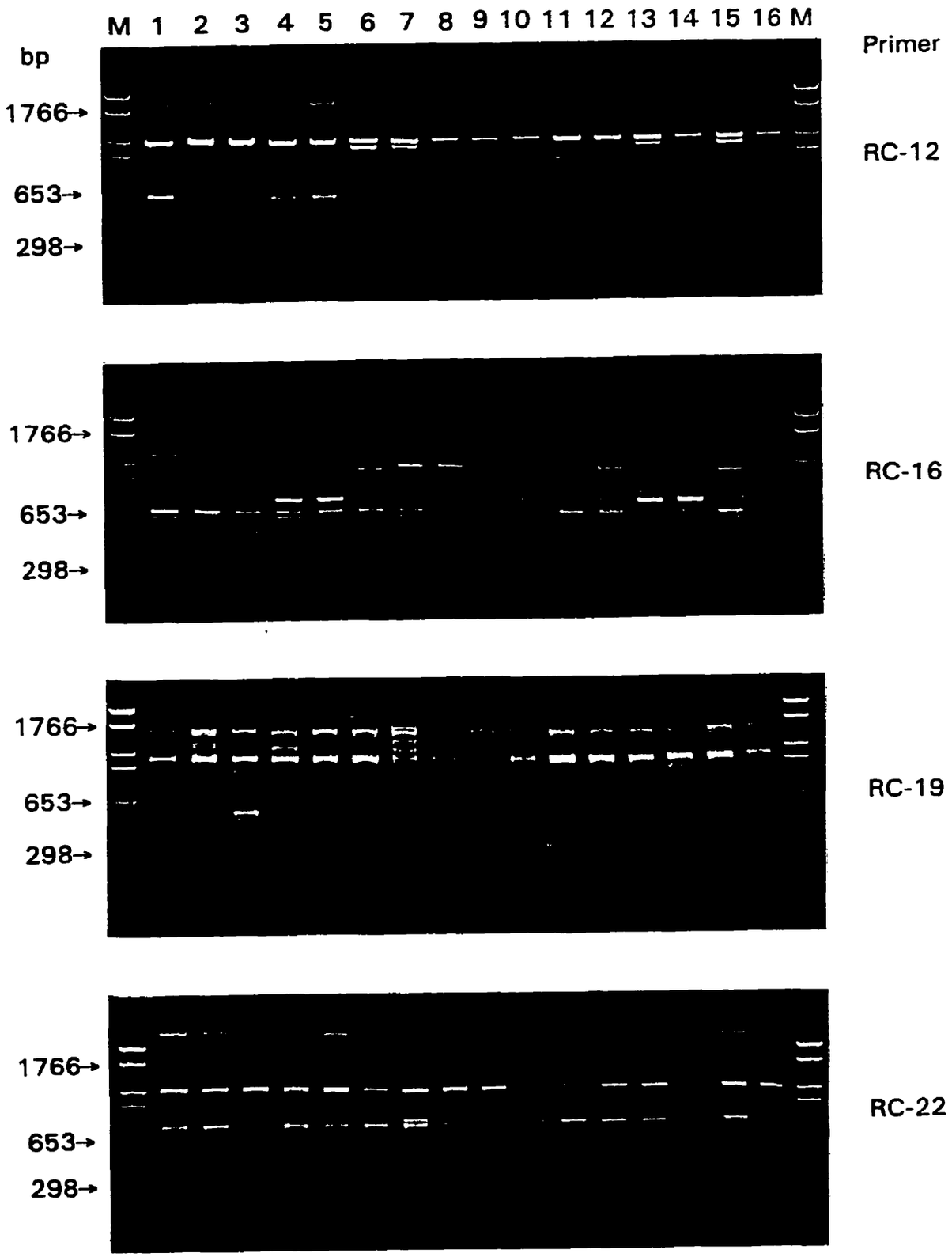

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M
bp
Primer
1766
653
298
RC-12
RC-16
RC-19
RC-22

Figure 5.2. Amplification products for sixteen saskatoon cultivars generated using standard PCR conditions and primers RC-28, RC-30, RC-36, and RC-37. Numbers above each lane correspond to the following cultivars: (1) Thiessen, (2) Pembina, (3) Moonlake, (4) Northline, (5) Honeywood, (6) Smoky, (7) Success, (8) Parkhill, (9) Regent, (10) Forestburg, (11) Pearson II, (12) Altaglow, (13) Bluff, (14) Nelson, (15) Buffalo, and (16) Martin. Lane M contains molecular weight marker VI (Boehringer) with fragment size in base pairs (bp) indicated. (These figures are reproductions and may not clearly show all of the amplification products which were reproducible and clearly distinguishable on the original agarose gels and photographs. Thus, the cultivars Smoky and Pearson II (lanes 6 and 11, respectively) which could not be distinguished based on the reproducible amplification products scored in Table 5.3, do not appear to have identical amplification products in these figures).

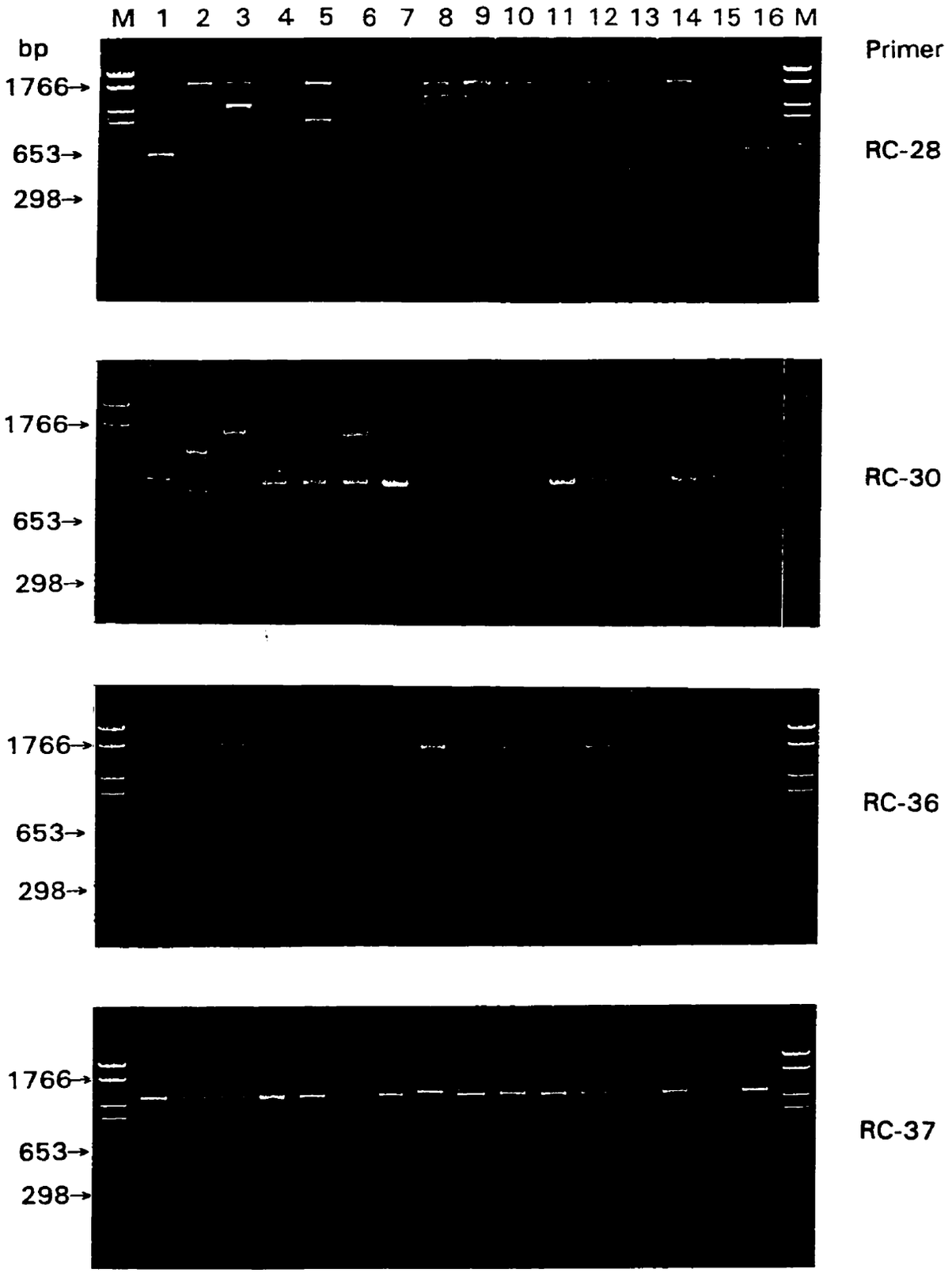

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M
bp
Primer
1766→
653→
298→
RC-28
1766→
653→
298→
RC-30
1766→
653→
298→
RC-36
1766→
653→
298→
RC-37

Table 5.2. Total number of bands, minimum and maximum band size (bp), and number of stable and unstable bands generated by the primers used to characterize the sixteen saskatoon cultivars.

Primer	No. band	Band Size (bp) min	max	Stable bands poly[1]	mono[2]	Unstable bands
RC-12	14	400	2200	4	1	9
RC-16	13	300	1800	3	3	7
RC-19	13	450	1750	4	3	6
RC-22	10	650	2300	1	4	5
RC-28	11	400	2300	5	2	4
RC-30	17	300	2700	8	2	7
RC-36	12	500	2600	2	7	3
RC-37	8	450	2300	2	3	3
Total	98			29	25	44

[1] Polymorphic bands
[2] Monomorphic bands

generated by each primer. The number of bands per primer ranged from 8 (RC-37) to 17 (RC-30), with an average of 12.25 bands per primer. Lower (6.6), similar (11) and higher (15.5) average bands per primer have been reported for other plant species (Kazan *et al.*, 1993; Yang and Quiros, 1993; Demeke *et al.*, 1992; respectively). These differences in number of bands generated per primer are likely due to differences in primer length, primer sequence, and primer-DNA template interaction among the various reports. Rafalski *et al.* (1991) observed that the number of bands in RAPD profiles is independent of genome complexity.

Among the 98 bands generated in this study, 29 (29.6%) were useful as reproducible polymorphic markers, 25 (25.5%) were reproducible but showed no variation among the cultivars, and 44 (44.9%) were considered unstable and therefore excluded (Table 5.2). In contrast, to identify celery cultivars, Yang and Quiros (1993) used 28 primers, which generated 309 bands, of which only 9.3% were useful as polymorphic markers, 68% were reproducible but uninformative (monomorphic), and 22.7% were unstable. The three-fold difference in polymorphic markers for saskatoon cultivars suggests a broader genetic base than for celery cultivars. Nearly 45% of the bands generated in the current study were considered unstable, which was twice the value found by Yang and Quiros (1993). This difference in band stability may be due to differences in the stringency of the PCR in each study. Factors such as source of polymerase, DNA quality, cycling

temperatures, primer sequence, and magnesium concentration are known to affect PCR stringency (Innis and Gelfand, 1991). It is also possible that the longer (10-base) primers used in the celery study were generally more stable than the 9-base primers used in this study, but there is no indication of this in the literature. Overall, these results indicate the importance of replication to identify reproducible RAPD markers.

Table 5.3 illustrates the DNA polymorphism detectable among saskatoon cultivars. It can be used as a reference to determine the more informative RAPD markers which can distinguish among the saskatoon cultivars examined. Twelve saskatoon cultivars and two pairs of cultivars were uniquely characterized by the amplification products of as few as two primers. The cultivars Pembina, Moonlake, Northline, Success, Parkhill, Forestburg, Altaglow, Buffalo, and Bluff were distinguished by primer RC-30, and primer RC-12 distinguished Regent, Honeywood, and Nelson. The remaining four cultivars form two pairs: Smoky and Pearson II, and Thiessen and Martin. Amplification products from the other six primers did not resolve either of these two pairs (Table 5.3).

The number of primers needed to distinguish among cultivars in other species has been variable. Two primers were sufficient to distinguish 14 cultivars of broccoli, three primers for 12 cultivars of cauliflower (Hu and Quiros, 1991), one primer for 13 genotypes of cocoa (Wilde *et al.*, 1992), 12 primers for 21 celery cultivars (Yang and Quiros, 1993) and 11 primers

Table 5.3. Scoring of RAPD markers for 16 saskatoon cultivars. Markers were scored as present (1) or absent (0).

RAPD Marker	Cultivar #															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
RC12-1190	0	0	0	0	0	1	1	1	1	0	1	0	0	1	1	0
RC12-1100	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
RC12-670	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
RC12-640	1	0	0	1	1	1	1	0	0	1	1	0	1	1	0	1
RC16-650	1	0	0	1	0	1	1	0	1	0	1	0	0	1	0	1
RC16-600	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
RC16-380	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
RC19-1760	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
RC19-1450	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
RC19-1390	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
RC19-540	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
RC22-800	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0
RC28-1500	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
RC28-1325	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0
RC28-1030	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
RC28-640	1	1	0	0	1	1	1	0	0	1	1	1	1	1	1	1
RC28-450	1	0	1	1	0	1	1	0	1	0	1	1	1	1	1	1
RC30-2000	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
RC30-1700	1	0	1	0	1	1	1	1	1	1	1	0	0	1	1	1
RC30-1100	0	1	1	1	1	0	0	1	0	0	0	1	0	1	1	0
RC30-1030	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
RC30-990	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
RC30-950	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
RC30-920	0	1	1	1	1	0	0	0	0	0	0	0	0	1	1	0
RC30-550	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1
RC36-1100	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0
RC36-800	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0
RC37-1050	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
RC37-970	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0

Cultivar numbers correspond to the following cultivars: (1) Thiessen, (2) Pembina, (3) Moonlake, (4) Northline, (5) Honeywood, (6) Smoky, (7) Success, (8) Parkhill, (9) Regent, (10) Forestburg, (11) Pearson II, (12) Altaglow, (13) Bluff, (14) Nelson, (15) Buffalo, and (16) Martin.

to distinguish 11 apple cultivars (Koller *et al.*, 1993). In contrast, Williams and St.Clair (1993) could only distinguish 9 of 19 tomato cultivars using 24 primers, and Halward *et al.* (1992) were unable to detect any polymorphism among 25 unadapted germplasm lines of cultivated peanut using 10 primers. Halward *et al.* (1992) reported that the RAPD results for peanut were consistent with RFLP studies, which indicated a narrow genetic base of the cultivated peanut.

The pairs of saskatoon cultivars, Smoky and Pearson II, and Thiessen and Martin, were not uniquely characterized by the primers used in this study. A possible reason is that they originate from a narrower genetic base relative to the other cultivars. Pearson II originated from self-pollinated Smoky, and Martin from self-pollinated Thiessen. If Smoky and Thiessen were highly homozygous, then more primers may need to be examined to allow them to be distinguished from their respective offspring. Near isogenic lines of other crop species have been distinguished by screening hundreds of primers (Carland and Staskawicz, 1993; Haley *et al.*, 1993; Paran *et al.*, 1991).

Another possible explanation for the identical fingerprints for these pairs of cultivars is that Pearson II and Martin were the products of apomixis. Two separate reports lend some support to this hypothesis. Firstly, studies conducted with *A. alnifolia* indicated that 20% of the flowers set fruit even when pollination was completely prevented (St.

Pierre, 1991a). Secondly, apomictic individuals have been found in the related species *A.laevis* (Campbell *et al.*, 1985), *A.canadensis*, *A.stolonifera* (Campbell *et al.*, 1987) and *A.* x *neglecta* (Weber and Campbell, 1989). Also, it is not uncommon for apomixis to occur among other genera of the Maloideae, such as *Cotoneaster, Crataegus, Pyrus* (Campbell *et al.*, 1985), *Sorbus* and *Malus* (Robinson, 1982).

This is the first report of molecular characterization of the saskatoon cultivars examined. Growers and propagators now have a reliable method to distinguish among most of the available commercial cultivars. As new cultivars are developed, their DNA fingerprints can be added to those reported here. This technique could be also be used for germplasm collection and preservation, or to identify specific RAPD markers linked to desirable traits, such as disease resistance (Paran *et al.* 1991).

5.3.2 MONITORING CLONES AND SEEDLINGS FOR GENETIC VARIABILITY

5.3.2.1 THIESSEN CLONES AND SEEDLINGS

A total of 51 bands were generated by the eight primers used in this study (Table 5.4). Of these 51 bands, 37 (72.5%) reproducible RAPD markers were scored for five sources of Thiessen, a single source of Martin, and eight seedlings from self-pollinated Thiessen (Table 5.5). These 37 bands were not able to distinguish among Martin and the five Thiessen clones. Segregation of amplification products was detected among the

Table 5.4. Number of stable and unstable bands generated by each primer used to detect genetic variability among Thiessen clones and seedlings.

Primer	Stable	Unstable	Total
RC-12	5	3	8
RC-16	3	3	6
RC-19	4	1	5
RC-22	4	2	6
RC-28	5	2	7
RC-30	4	2	6
RC-36	8	0	8
RC-37	4	1	5
Total	37	14	51

Table 5.5. Scoring of reproducible RAPD markers for five sources of Thiessen, Martin, and eight Thiessen seedlings. Markers were scored as present (1) or absent (0).

RAPD	Thiessen					Martin	Thiessen seedlings from LSN							
Marker	ATN	LSN	PBI	PPS	PAR	PPS	1	2	3	4	5	6	7	8
RC12-1900	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC12-1230	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC12-1030	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC12-800	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC12-640	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC16-850	1	1	1	1	1	1	1	0	1	0	0	0	0	0
RC16-700	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC16-650	1	1	1	1	1	1	0	1	0	1	0	0	1	1
RC19-1660	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC19-1100	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC19-1000	1	1	1	1	1	1	1	1	1	0	1	1	1	0
RC19-500	1	1	1	1	1	1	1	1	1	0	1	1	1	0
RC22-2400	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC22-2140	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC22-1250	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC22-750	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC28-1800	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC28-1030	1	1	1	1	1	1	1	1	1	1	1	0	1	1
RC28-900	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC28-640	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC28-450	1	1	1	1	1	1	0	1	0	0	0	1	1	0
RC30-1700	1	1	1	1	1	1	1	1	1	1	1	0	1	0
RC30-1400	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC30-1030	1	1	1	1	1	1	1	1	1	1	0	1	1	1
RC30-550	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC36-2600	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC36-2280	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC36-1950	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC36-1500	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC36-1230	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC36-1030	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC36-775	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC36-570	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC37-2700	1	1	1	1	1	1	0	1	0	0	0	1	1	0
RC37-1440	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC37-1290	1	1	1	1	1	1	1	1	1	1	1	1	1	1
RC37-820	1	1	1	1	1	1	1	1	1	1	1	1	1	1

ATN Alberta Tree Nursery and Horticulture Centre, Edmonton, AB
LSN Lakeshore Nursery, Saskatoon, SK
PBI Pacific Biotechnologies INC., Vedder Crossing, BC
PPS Prairie Plant Systems, Saskatoon, SK
PAR R. Parenteau, Langham, SK

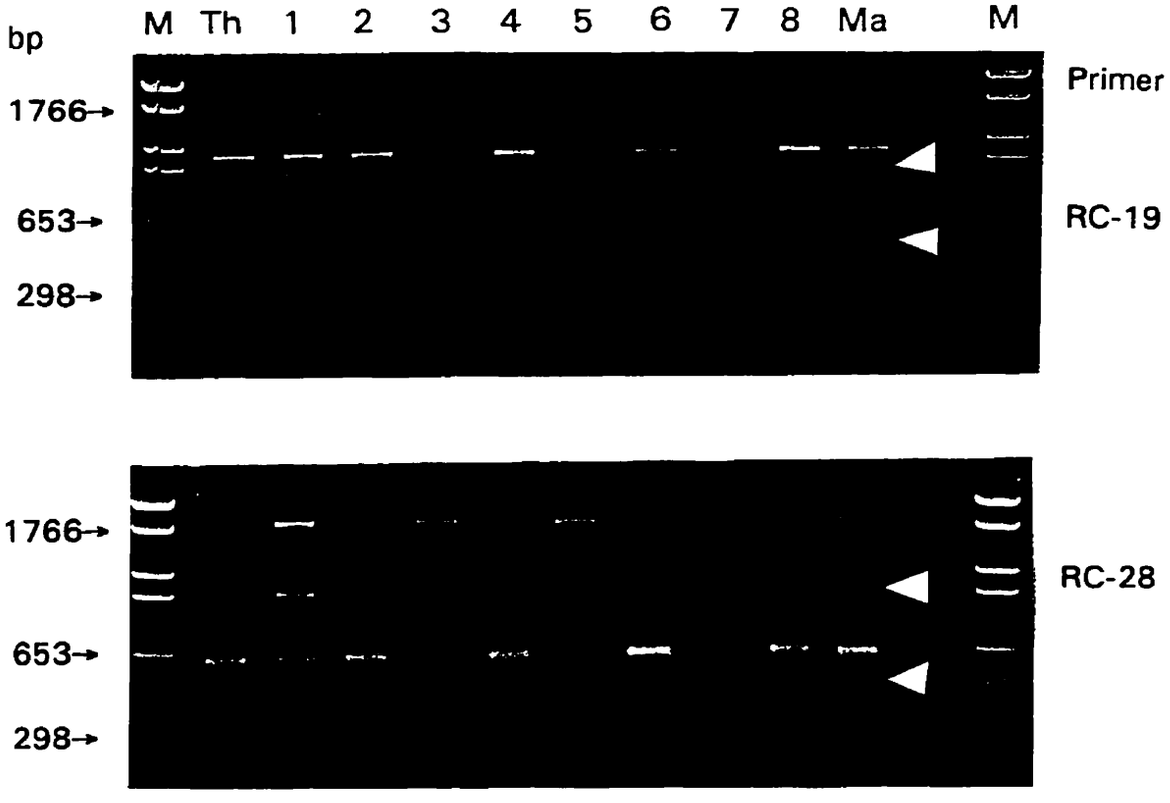
Thiessen seedlings using primers such as RC-19 and RC-28 (Figure 5.3). A total of nine bands (24.3%) segregated among the seedlings including RC16-850, RC16-650, RC19-1000, RC19-500, RC28-1030, RC28-450, RC30-1700, RC30-1030, and RC37-2700. Unlike the other Thiessen seedlings examined here, segregation of RAPD markers was not detected for Martin. The possibilities of screening more primers to detect polymorphism between Thiessen and Martin, or of Martin being an apomictic seedling were discussed above.

5.3.2.2 GENETIC VARIABILITY AMONG REGENT AND PARKHILL

Reproducible RAPD markers generated by eight primers for the cultivars Regent and Parkhill are given in Table 5.6. In total, 16 out of 47 reproducible bands generated could be used to distinguish between Regent (MRS) and Parkhill (MRS). However, reactions were not run for all primer and source combinations. Amplification products were generated for all sources using the five primers: RC-12, RC-16, RC-19, RC-22, and RC-30. These five primers generated 29 reproducible markers. Of these 29 markers, there was no detectable polymorphism among any of the Parkhill sources and the Regent from ATN. In contrast, these five primers detected 10 polymorphic bands which distinguished Regent (MRS) from all Parkhill

Figure 5.3. Amplification products generated using standard PCR conditions and primers RC-19 and RC-28 for Thiessen (Th), Martin (Ma), and eight Thiessen seedlings (1-8). White arrows indicate segregating bands. Lane M contains molecular weight marker VI (Boehringer) with fragment size in base pairs (bp) indicated.

Figure 5.4. Amplification products generated using standard PCR conditions and primers RC-19 and RC-30 for Parkhill (P) and Regent (R). Sources of Parkhill and Regent were the Alberta Tree Nursery and Horticulture Centre (ATN) and Agriculture Canada Research Station, Morden (MRS), as indicated. Black arrows indicate polymorphic bands. Lane M contains molecular weight marker VI (Boehringer) with fragment size in base pairs (bp) indicated.


bp
M Th 1 2 3 4 5 6 7 8 Ma M
1766
653
298
Primer
RC-19
1766
653
298
RC-28

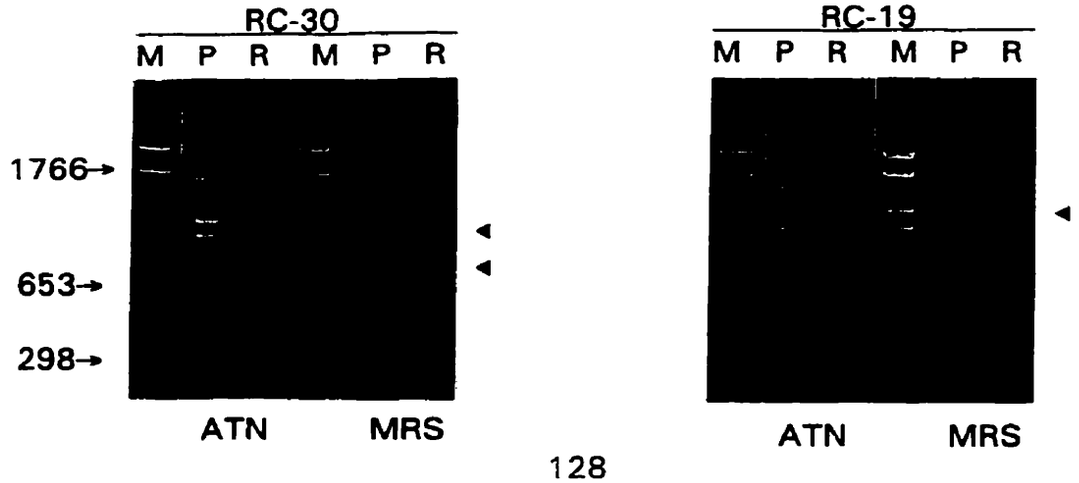

RC-30
M P R M P R
1766
653
298
ATN MRS
RC-19
M P R M P R
ATN MRS

Table 5.6. Scoring of reproducible RAPD markers for two sources of Parkhill and Regent. Markers scored as present (1) or absent (0).

RAPD Marker	Parkhill			Regent			RAPD Marker	Parkhill			Regent		
	ATN[1]	ATN[2]	MRS	ATN[1]	ATN[2]	MRS		ATN[1]	ATN[2]	MRS	ATN[1]	ATN[2]	MRS
RC12-1230	1	1	1	1	1	1	RC28-1030	ns	ns	1	ns	ns	1
RC12-1190	1	1	1	1	1	1	RC28-450	ns	ns	0	ns	ns	1
RC12-1100	1	1	1	1	1	0	RC28-425	ns	ns	1	ns	ns	0
RC16-1230	1	1	1	1	1	1	RC30-2170	0	0	0	0	0	1
RC16-1090	1	1	1	1	1	1	RC30-2000	1	1	1	1	1	0
RC16-850	1	1	1	1	1	1	RC30-1700	1	1	1	1	1	1
RC16-700	1	1	1	1	1	1	RC30-1450	1	1	1	1	1	0
RC16-650	0	0	0	0	0	1	RC30-1400	1	1	1	1	1	1
RC16-330	1	1	1	1	1	1	RC30-1200	1	1	1	1	1	0
RC19-1760	1	1	1	1	1	1	RC30-1180	1	1	1	1	1	0
RC19-1660	1	1	1	1	1	1	RC30-1030	1	1	1	1	1	1
RC19-1450	1	1	1	1	1	1	RC30-950	1	1	1	1	1	0
RC19-1230	0	0	0	0	0	1	RC36-2600	ns	1	1	ns	1	1
RC22-2400	1	1	1	1	1	1	RC36-2280	ns	1	1	ns	1	1
RC22-2140	1	1	1	1	1	1	RC36-1950	ns	1	1	ns	1	1
RC22-1760	1	1	1	1	1	1	RC36-1230	ns	1	1	ns	1	1
RC22-1230	1	1	1	1	1	1	RC36-1150	ns	1	1	ns	1	0
RC22-1170	1	1	1	1	1	1	RC36-1030	ns	1	1	ns	1	1
RC22-800	0	0	0	0	0	1	RC36-775	ns	1	1	ns	1	1
RC22-750	1	1	1	1	1	1	RC37-1440	ns	1	1	ns	1	1
RC22-700	1	1	1	1	1	1	RC37-1290	ns	0	0	ns	0	1
RC28-1800	ns	ns	1	ns	ns	1	RC37-1030	ns	1	1	ns	1	0
RC28-1500	ns	ns	1	ns	ns	1	RC37-970	ns	1	1	ns	1	0
RC28-1375	ns	ns	1	ns	ns	1							

ATN[1] Alberta Tree Nursery and Horticulture Centre, Edmonton, AB. Sampled in 1992.
ATN[2] Alberta Tree Nursery and Horticulture Centre, Edmonton, AB. Sampled in 1993.
MRS Agriculture Canada Research Station, Morden, MB. Sampled in 1992.
ns Not screened for this primer.

sources and Regent (ATN). Figure 5.4 illustrates some of the polymorphism detected between Regent (MRS) and Parkhill (MRS) which was not detected between Regent (ATN) and Parkhill (ATN).

These results suggest that the Alberta Tree Nursery and Horticulture Centre (ATN) may have the cultivar Parkhill incorrectly identified as both Parkhill and Regent. Both Parkhill and Regent were obtained from ATN as part of the recently established saskatoon cultivar trials being conducted by the Department of Horticulture, University of Saskatchewan. The practical application of RAPD analysis to screen for duplications among germplasm is illustrated by these results.

5.4 CONCLUSIONS

Reproducible RAPD markers were generated using eight, nine-base primers. These primers generated 98 different bands, of which 29 (29.6%) were useful as reproducible polymorphic markers, 25 (25.5%) were reproducible but showed no variation among the cultivars, and 44 (44.9%) were unstable and therefore not useful as polymorphic markers. Twelve of sixteen saskatoon cultivars (Pembina, Moonlake, Northline, Honeywood, Success, Parkhill, Regent, Forestburg, Altaglow, Bluff, Nelson, and Buffalo) were uniquely characterized by these polymorphic markers. The remaining four cultivars formed indistinguishable pairs: Smoky and Pearson II, and Thiessen and Martin. The cultivars within each pair are genetically related

in that Pearson II and Martin were selected from self-pollinated Smoky and Thiessen respectively. Thus, similar, but not necessarily identical, amplification products would be expected within each group. Possible explanations for the lack of detected polymorphism were that (1) not enough primers were screened to reveal within-pair polymorphism, and (2) the offspring are apomictic seedlings and therefore are genetically identical to their respective parents.

Genetic variation was not detected among five sources of the cultivar Thiessen, but was detected among seedlings from a self-pollinated Thiessen plant. Genetic variation was demonstrated between two sources of Regent. One source of Regent and two sources of Parkhill were indistinguishable for the primers used in this study. These results suggest a possible mislabelling of Regent as Parkhill originating from the Alberta Tree Nursery and Horticulture Centre.

CHAPTER 6

GENETIC VARIATION AMONG *AMELANCHIER* SPECIES

6.0 ABSTRACT

Randomly amplified polymorphic DNA (RAPD) markers were used to assess the genetic relationships among 56 taxa and individuals representing 16 species within the genus *Amelanchier*. The majority of these species are native to North America, but a European and Asian species were also included. The Asian species, *A. asiatica*, was used for outgroup comparison. Eight arbitrary 9 base-pair primers generated 107 bands, of which 69 were used in this study. These 69 bands were analyzed using three different methods: Jaccard's coefficient and UPGMA clustering, principal coordinates analysis, and Wagner parsimony. Based on these analyses, the bulk of the taxa were divided into three groups. Within the first group (*A. arborea, A. laevis, A. canadensis, A. intermedia*, and *A. x grandiflora*) there was discordance between morphological and genetic delimitation. In the second group (*A. alnifolia, A. florida, A. cusickii, A. oxyodon*, and *A. gaspensis*), taxa generally clustered according to morphological delimitation. The third group (*A. spicata, A. sanguinea*, and *A. stolonifera*) appears to be intermediate to the first two groups suggesting

it resulted from hybridization between them. Generally, the genetic relationships presented here can be supported by morphological observations and the breeding systems reported for this genus.

6.1 INTRODUCTION

In North America, the genus *Amelanchier* (Rosaceae, subfamily Maloideae) has been estimated to contain anywhere from one or two highly variable species (Jones, 1946) to as many as 25 species (Fernald, 1950; Jones 1946) with an additional 17 natural hybrids reported (Fernald, 1950). As many as 200 binomials and trinomials representing these species have been reported (Jones, 1946). Hybridization, synonymy, and uncertainty concerning the identity of holotypes have plagued the taxonomy of *Amelanchier* (Wiegand, 1912). Most of the taxonomic confusion has been attributed to the extraordinary variation of the foliage that occurs within species. This variation is manifested throughout all developmental stages and from various habitats (Jones, 1946). Currently, 17 species, including 3 natural hybrids, are recognized in North America, as well as one European and one Asian species (Kartesz, 1994). Although this treatment (Kartesz, 1994) is consistent with the amalgamation of many western North American species as suggested by Hitchcock and Cronquist (1961), it maintains a number of eastern species which are totally integrated and difficult to separate morphologically (Cruise, 1964; Landry, 1975).

Taxonomic confusion may also be due to the evolutionary forces shaping this genus (Robinson, 1982).

The genus *Amelanchier*, and other Maloid genera, could be identified by the Miocene if not Oligocene in North America (Hickey, 1984, in Phipps *et al.*, 1991). Phipps *et al.* (1991) suggest that following an alloploid origin of the subfamily Maloideae, possibly in the late Eocene, the genera arose through a polychotomous evolution. Morphological diversification of these genera was so rapid that resolution using parsimony methods may not be possible (Dickinson *et al.*, 1991). Apomixis (Weber and Campbell, 1991), polyploidy (Table 2.1), natural hybridization (Cruise, 1964; Fernald, 1950, Jones, 1946), varying degrees of self-compatibility (Campbell *et al.*, 1987; Harris, 1970; Robinson, 1982), and colonizing ability (Robinson, 1982) have been reported among the species of *Amelanchier*, and have played an important role in the evolution of other Maloid genera (Campbell *et al.*, 1991). These reproductive mechanisms will influence phylogenetic analysis, which is supposed to reveal evolutionary patterns among individuals.

Randomly amplified polymorphic DNA (RAPD) markers have recently been used in taxonomic studies to assess the genetic relationships among various species (Joshi and Nguyen, 1993; Kazan *et al.*, 1993; Williams and St. Clair, 1993; van Heusden and Bachmann, 1992). The use of RAPD markers in taxonomic studies requires the assumption that co-migrating

bands (electromorphs) represent homologous characters. Homologous characters are those characters which two species and their common ancestor share (Ridley, 1986). Homology among electromorphs has been determined by labelling of a band and using it as a probe. Using this technique, differences in the degree of homology among electromorphs has been reported. Within species of *Brassica* (Thormann and Osborn, 1992) and *Glycine* (Tingey *et al.*, 1992) electromorphs were shown to be homologous, whereas among species of *Brassica* (Thormann and Osborn, 1992) and *Allium* (Wilkie *et al.*, 1993) not all electromorphs were homologous. Nonetheless, taxonomic studies indicate that RAPD data are consistent with other forms of molecular data. Demeke *et al.* (1992) demonstrated that RAPD markers could generate the classic U triangle among diploid and amphiploid *Brassica* taxa. Phylogenetic trees were consistent whether generated from RAPD, VNTR or isozyme data for 10 strains of *Microseris elegans* (van Heusden and Bachmann, 1992). The consistency among various molecular data including RAPD suggests that electromorphs must be homologous. Peltier *et al.* (1996) compared two data sets for species within the genus *Petunia*. One set consisted of RAPD markers and the other consisted of RAPD markers for which homology of electromorphs was confirmed by hybridization. Phenetic and phylogenetic inferences did not differ regardless of the data set used, suggesting that confirmation of homology among electromorphs was unnecessary (Peltier *et*

al., 1996).

Phylogenetic (cladistic) and phenetic approaches have been used to analyze RAPD data for systematic studies. Cladistic methods based on the principle of maximum parsimony have been the most widely used as an approach to inferring phylogenies from character data (Swofford and Olsen, 1990). Parsimony methods operate by selecting trees that minimize the total tree length, ie. the number of evolutionary steps (transformation from one character to another) required to explain a given set of data. Often outgroup comparisons are used to infer the polarity of the characters which helps to root the tree. Utilization of an outgroup requires the assumption that the remaining taxa are monophyletic, ie. that they have arisen by the diversification of a single ancestor.

Cladistic analysis of RAPD markers using Wagner parsimony has been reported (Peltier *et al.*, 1996; van Heusden and Bachmann, 1992). Wagner parsimony is one of the simplest parsimony methods available. The Wagner method assumes that transformation from one character state to another implies a transformation through any intervening states. It also permits free reversibility, ie. the change from 0 to 1 is as probable as the change from 1 to 0, and that character states may transform from one state to another and back again (Swofford and Olsen, 1990). The assumption of free reversibility is perhaps unreasonable for RAPD data. Substitution of a single nucleotide can create a mismatch between primer and priming site.

Intuitively, the loss of a priming site would be more probable than the gain of a priming site. For similar reasons, this assumption of free reversibility is also considered unreasonable for restriction-site characters (Swofford and Olsen, 1990).

Phenetic analyses of RAPD markers have also been reported (Joshi and Nguyen, 1993; Kazan *et al.*, 1993; Peltier *et al.*, 1996; Stiles *et al.*, 1993; van Heusden and Bachmann, 1992; Vierling and Nguyen, 1992; Wilkie *et al.*, 1993; Williams and St. Clair, 1993). Nearly all of these reports used cluster analysis but there has been one report of principal coordinate analysis (PCO) (Demeke *et al.*, 1992). Cluster analysis begins with pairwise comparisons between taxa which have been generated using Jaccard's coefficient of similarity (Joshi and Nguyen, 1993; Peltier *et al.*, 1996; Stiles *et al.*, 1993; Vierling and Nguyen, 1992), Roger's genetic distance (Wilkie *et al.*, 1993), Nei's genetic distance (Peltier *et al.*, 1996; Williams and St. Clair, 1993), Nei and Li's distance (Kazan *et al.*, 1993), and the Simple matching coefficient (Peltier *et al.*, 1996; Stiles *et al.*, 1993). Clustering of taxa was almost always by the unweighted pair-group method using arithmetic averages (UPGMA) (Joshi and Nguyen, 1993; Kazan *et al.*, 1993; Peltier *et al.*, 1996; Stiles *et al.*, 1993; van Heusden and Bachmann, 1992; Vierling and Nguyen, 1992; Williams and St. Clair, 1993), with the Neighbour Joining method of Saitou and Nei (Peltier *et al.*, 1996), nearest neighbour and farthest neighbour clustering (Wilkie et al,

1993) also reported. The main assumption of the phenetic approach is that overall similarity represents evolutionary relationships.

The terminology regarding individuals in a taxonomic study can become cumbersome. In the phenetic approach to classification, the individuals, species, genera or any other taxonomic unit under study is called an 'operational taxonomic unit' or 'OTU' (Stace, 1989), which is equivalent to the 'evolutionary unit' or 'EU' of the cladistic approach (Stuessy, 1990). In this study, both phenetic and cladistic approaches are used in the analysis, so instead of switching back and forth between the above terms, the term 'OTU' will be used in reference to an individual in the analysis.

The main objective of this study was to determine the phylogenetic relationship among species of the genus *Amelanchier* based on RAPD markers (a molecular character), and then compare the molecular-based phylogeny to the morphological-based relationships suggested in the literature. In addition, the clustering of the American cultivars (Regent, Parkhill, and Success) with the various species in this study is of interest because their species delimitation is not clear in the literature. Similarly, the literature has suggested several parents for the putative hybrid *A. x grandiflora* and the analyses presented here may suggest the origin of this putative hybrid.

6.2 MATERIALS AND METHODS

Forty-one OTU representing as many as sixteen species of *Amelanchier* were characterized using RAPD markers (Table 6.1). Fresh leaf material of some of these OTU was collected by the author from the Agriculture Canada Research Station, Morden (MRS), packed on ice, and transported to Saskatoon. All other leaf material was placed in small plastic sample bags and mailed or sent by courier from the sources listed in Table 6.1. Leaves were washed to remove any source of foreign DNA (such as insects or insect eggs), frozen in liquid nitrogen, and stored at -70°C until required.

DNA was extracted from the frozen leaf material using the modified method of Doyle and Doyle (1990) reported in Chapter 3. RAPD markers were generated according to the protocol described in Chapter 4, using a Hybaid DNA Thermal Cycler (Bio\Can). Primers used for RAPD analysis included RC-12, -16, -19, -22, -28, -30, -36, and -37. Sequences for these primers were previously given in Table 3.1. Amplification products were separated by electrophoresis on a 1.4% agarose gel containing 0.5 ug/ml ethidium bromide, and photographed on a UV transilluminator. Both a positive (containing *A.alnifolia* cv. Pembina DNA) and a negative (containing no DNA) control reaction were run for each primer.

Each amplified band was named according to the primer used and its size in base pairs (bp). For example, RC16-500 refers to a 500 bp fragment

Table 6.1. Sources of *Amelanchier* species used in this study.

No.	Species	Leaf Source	Origin
1	A. alnifolia cv. Pembina	ATN	Barrhead, AB
2	A. gaspensis	MRD #255	CEF, Ottawa
3	A. gaspensis	MRD #255	CEF, Ottawa
4	A. ovalis	MRD #259	NDSU
5	A. ovalis	MRD #260	CEF, Ottawa
6	A. spicata	MRD #270	Guelph, ON
7	A. spicata	MRD #272	CEF, Ottawa
8	A. stolonifera	MRD #274	BRS
9	A. florida	MRD # 13	BRS
10	A. florida	MRD # 13	BRS
11	A. alnifolia	KAM	Tranquille, BC
12	A. alnifolia	KAM	Kamloops, BC
13	A. cusickii	KAM	Empire, BC
14	A. cusickii	KAM	Kamloops, BC
15	A. laevis	KRS #8401	Nova Scotia
16	A. cusickii	NCGR #51	Vaseux Lake, BC
17	A. canadensis	NCGR #6	Oregon, USA
18	A. ovalis	NCGR #64	Uzbekistan
19	A. asiatica	NCGR #63	Uzbekistan
20	A. laevis	MUB #8431	Newfoundland
21	A. canadensis	MUB	Newfoundland
22	A. intermedia	MUB #8443	Newfoundland
23	A. x grandiflora	MRS #277	Bailey Nurs., St. Paul, Min.
24	A. arborea	WAR #R93	Key's Gap, W.Va.
25	A. arborea	WAR #R108	Elliottsville, Pa.
26	A. intermedia	WAR #R119	Pike Co., Pa.
27	A. bartramiana	WAR #R168	Kane, Pa.
28	A. cusickii	BRS #8712	Kamloops, BC
29	A. cusickii	BRS #8719	Kamloops, BC
30	A. sanguinea	BRS #6121	Goluchow, Poland
31	A. x grandiflora	BRS #5901	McConnell Nurseries
32	A. florida	BRS #6112	Leningrad, Russia
33	A. oxyodon	BRS #6601	Kirov, Murmansk
34	A. canadensis	MRS #252	R. Simonet, Edmonton, AB
35	A. canadensis	MRS #253	BRS

Table 6.1. Continued.

No.	Species	Leaf Source	Origin
36	A. intermedia	KRS #8421	Nova Scotia
37	A. intermedia	KRS #8413	Nova Scotia
38	A. laevis	KRS #8414	Nova Scotia
39	A. laevis	MUB #8442	Newfoundland
40	A. canadensis	WAR R115a	Ocean Co., N.J.
41	A. intermedia	WAR #R144	Rutland Co., Vt.
42	A. alnifolia cv. Thiessen	ATN	Waldheim, SK
43	A. alnifolia cv. Moonlake	ATN	Moon Lake, SK
44	A. alnifolia cv. Northline	ATN	Beaverlodge, AB
45	A. alnifolia cv. Honeywood	ATN	Parkside, SK
46	A. alnifolia cv. Smoky	BRS	Peace River, AB
47	A. sanguinea ? cv. Success	ATN	Pennsylvania
48	A. sanguinea ? cv. Parkhill	MRS	Michigan
49	A. alnifolia ? cv. Regent	MRS	Regent, ND
50	A. alnifolia cv. Forestburg	MRS	Forestburg, AB
51	A. alnifolia cv. Pearson II	ATN	Bowden, AB
52	A. alnifolia cv. Altaglow	MRS	Lacombe, AB
53	A. alnifolia cv. Bluff	PBI	Buffalo Lake, AB
54	A. alnifolia cv. Nelson	UofS	Bradwell, SK
55	A. alnifolia cv. Buffalo	PBI	Buffalo Lake, AB
56	A. alnifolia cv. Martin	PPS	Langham, SK

?	Species designation uncertain in literature.
ATN	Alberta Tree Nursery and Horticulture Centre, Edmonton, AB
PBI	Pacific Biotechnologies Inc., Vedder Crossing, BC
UofS	Dept. of Horticulture, University of Saskatchewan, Saskatoon, SK
MRS	Agriculture Canada Research Station, Morden, MB
BRS	Agriculture Canada Research Station, Beaverlodge, AB
KRS	Agriculture Canada Research Station, Kentville, NS
KAM	Agriculture Canada Research Station, Kamloops, BC
PPS	Prairie Plant Systems, Saskatoon, SK
MUB	Memorial University Botanical Gardens, St. Johns, NF
WAR	W.A. Robinson Collection, Monroeville, PA
NCGR	National Clonal Germplasm Repository, Corvalis, OR

amplified by primer RC-16. Molecular weights for amplification products were determined from the graph of the linear relationship between the log of molecular weight of the standards and their migration distances. Marker VI (Boehringer) was used as the standard.

6.2.1 ANALYSIS OF RAPD MARKERS

Amplification products (bands) for the 41 OTU were scored as present (1) or absent (0). In addition, amplification products for the saskatoon cultivars presented in Chapter 5 were included in this study. Thus, a total of 56 OTU were analyzed. Two computer software packages were used to analyze the data. The first package, NTSYS (Numerical Taxonomy and Multivariate Analysis System, version 1.80, Exeter Software, Setauket, NY), developed by F. James Rohlf, was used for all the phenetic analyses. Two methods of phenetic analysis, cluster and ordination, were used. The second software package, PHYLIP (Phylogeny Inference Package, version 3.5), developed by Joseph Felsenstein, was used for the cladistic analysis. Cladistic methods used included Wagner parsimony and clique analysis.

6.2.2 PHENETIC ANALYSIS

Cluster analysis begins with the generation of a matrix using a distance coefficient. Three matrices were generated using the Jaccard,

Dice, and Nei coefficients, which are given below.

Jaccard's (1908) coefficient:

$$d_{ij} = a/(a+b+c)$$

Dice's (1945) coefficient:

$$d_{ij} = 2a/(2a+b+c)$$

Nei's (1972) distance:

$$d_{ij} = -\ln a/\sqrt{(a+b)(a+c)}]$$

For the above coefficients, a is equal to the number of bands shared by two taxa (i and j), b is the number of bands present in (i) but not in (j), and c is the number of bands present in (j) but not in (i). Distance matrices were computed using the SIMGEND and SIMQUAL programs. These matrices were then clustered using the SAHN program and the UPGMA (unweighted pair group method using arithmetic averages), complete linkage and single linkage clustering methods to generate phenograms. Consensus trees and comparison between trees was carried out using the MXCOMP program.

Principal coordinates analysis began with the generation of a dissimilarity matrix using the Manhattan distance coefficient by the SIMMIT program. The average Manhattan distance is defined as $M_{ij} = 1/n\ (b+c)$, where n is the total number of bands, and b and c are as given above. Transformation (double centering) of Manhattan distances by the DCENTER program allowed for computation of eigenvectors and eigenvalues by the EIGEN program. Three dimensional plotting of the principal coordinates was

accomplished using the MOD3D program. Cophenetic matrices were generated using COPH program, and matrices were compared using the MXCOMP program.

6.2.3 CLADISTIC ANALYSIS

Wagner parsimony analysis was calculated using the MIX program from the PHYLIP software package. The MIX program uses a branch swapping or 'global' approach to find the optimal tree(s), and is sensitive to the input order of the taxa (Felsenstein, 1993). Taxon order was randomized 20 times and the shortest tree(s) generated. The CLIQUE program was used for clique analysis and it utilizes a branch and bound search method (Felsenstein, 1993). The out group for all cladistic analysis was *A. asiatica*.

6.3 RESULTS

6.3.0 GENERATION OF RAPD MARKERS

Amplification products generated using standard PCR and eight different primers for the 41 OTU are shown in Appendix B. Several reactions resulted in faint bands, so these reactions were repeated to enable accurate scoring of the bands. Bands were scored as present (1) or absent (0) (Table 6.2). Included in Table 6.2 are amplification products for the sixteen saskatoon cultivars examined in chapter 5. A total of 107 bands

Table 6.2. Bands scored as present (1) or absent (0) for 69 randomly amplified polymorphic DNA (RAPD) markers. RAPD markers are designated by primer number and size in base pairs along top of table (ie. RC16-500 indicates a 500 base pair amplification product generated using primer RC16). Names of OTU are given along left side.

alnifolia-11
alnifolia-12
Altaglow
Buffalo
Bluff
Forestburg
Honeywood
Martin
Moonlake
Nelson
Northline
Pearsonii
Pembina
Parkhill
Regent
Success
Smoky
Thiessen
arborea-24
arborea-25
aslatica-19
bartramiana-27
canadensis-17
canadensis-21
canadensis-34
canadensis-35
canadensis-40
cuslckii-13
cuslckii-14
cuslckii-16
cuslckii-28
cuslckii-29
florida-9
florida-10
florida-32
gaspensis-2
gaspensis-3
grandiflora-23
grandiflora-31
intermedia-22
intermedia-26
intermedia-36
intermedia-37
intermedia-41
laevis-15
laevis-20
laevis-38
laevis-39
ovalis-4
ovalis-5
ovalis-18
oxyodon-33
sanguinea-30
spicata-6
spicata-7
stolonifera-8

were generated, of which 69 distinct bands were used in the analyses. The remaining bands were not analyzed because they were monomorphic or not clearly distinguishable across all 56 OTU.

6.3.1 CLUSTER ANALYSIS

Phenograms depicting genetic relationships among the 56 OTU are presented in Figures 6.1 to 6.9. Cophenetic correlations for each of the nine phenograms is shown in Table 6.3. To facilitate comparisons among phenograms, individual OTU and clusters of OTU have been labelled with lower case letters along the right hand side of each phenogram. The phenogram based on Jaccard's coefficient and clustered using the UPGMA method (Jaccard/UPGMA)(Figure 6.1), for example, has the following individuals and clusters: (a) asiatica; (b) bartramiana; (c) canadensis-17,-21,-35,-40, arborea-24,-25; laevis-15,-20,-38,-39, intermedia-22,-26,-36,-37,-41, grandiflora-23, and -31; (d) cusickii-13,-14,-16,-28,-29, florida-9 and -10; (e) Moonlake, Pembina, Bluff, Honeywood, Altaglow, Northline, Buffalo, Forestburg, Nelson, Pearson II, Smoky, Martin and Thiessen; (f) Parkhill, Success, Regent, stolonifera-8, sanguinea-30, ovalis-5, spicata-6 and -7; (g) florida-32, ovalis-4, ovalis-18, alnifolia-11, alnifolia-12; canadensis-34 and oxyodon-33; and (h) gaspensis-2 and -3.

Interestingly, for each particular clustering method (ie. UPGMA, single and complete linkage) there are only minor differences among

Figure 6.1. Phylogenetic tree for 56 OTU of *Amelanchier* based on RAPD data analyzed by UPGMA clustering of Jaccard's coefficient of similarity. The coefficient of similarity is given above the phenogram and clusters (a) through (h) are indicated along the right.

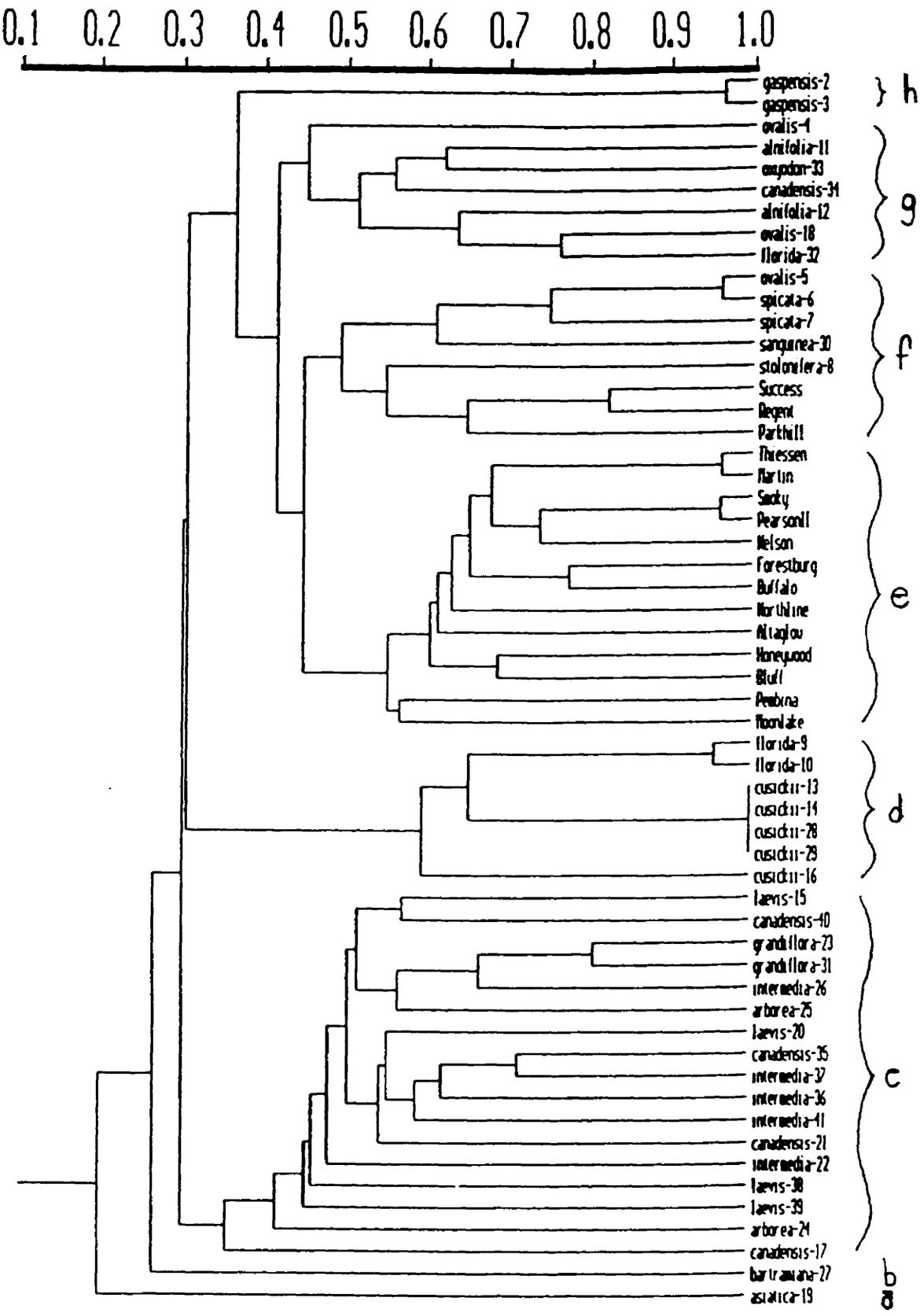

0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
gaspensis-2
gaspensis-3
ovalis-4
alnifolia-11
eupatorium-33
canadensis-34
alnifolia-12
ovalis-18
Florida-32
ovalis-5
spicata-6
spicata-7
sanguinea-30
stolonifera-8
Success
Regent
Parkhill
Thiessen
Martin
Saucy
Pearsonii
Nelson
Forestburg
Buffalo
Northline
Altaglow
Honeywood
Bluff
Penbina
Floralate
Florida-9
Florida-10
cusickii-13
cusickii-14
cusickii-28
cusickii-29
cusickii-16
laevis-15
canadensis-40
grandiflora-23
grandiflora-31
intermedia-26
arborea-25
laevis-20
canadensis-35
intermedia-37
intermedia-36
intermedia-41
canadensis-21
intermedia-22
laevis-38
laevis-39
arborea-24
canadensis-17
bartramiana-27
asiatica-19
h
g
f
e
d
c
b
a

Figure 6.2. Phylogenetic tree for 56 OTU of *Amelanchier* based on RAPD data analyzed by UPGMA clustering of Dice's coefficient of similarity. The coefficient of similarity is given above the phenogram and clusters (a) through (h) are indicated along the right.


0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
gaspensis-2
gaspensis-3
ovalis-4
alnifolia-11
coupdori-33
canadensis-34
alnifolia-12
ovalis-18
florida-32
ovalis-5
spicata-6
spicata-7
sanguinea-30
stolonifera-8
Success
Regent
Parkhill
Thiessen
Martin
Saxby
Pearsonii
Nelson
Forestburg
Buffalo
Northline
Honeywood
Bluff
Pembina
Altaglow
Moonlake
florida-9
florida-10
cusickii-13
cusickii-14
cusickii-28
cusickii-29
cusickii-16
laevis-15
canadensis-40
grandiflora-23
grandiflora-31
intermedia-26
arborea-25
laevis-20
canadensis-35
intermedia-37
intermedia-36
intermedia-41
canadensis-21
intermedia-22
laevis-38
laevis-39
arborea-24
canadensis-17
bartramiana-27
asiatica-19
g
f
e
d
c
b
a

Figure 6.3. Phylogenetic tree for 56 OTU of *Amelanchier* based on RAPD data analyzed by UPGMA clustering of Nei's coefficient of genetic distance. The coefficient of genetic distance is given above the phenogram and clusters (a) through (h) are indicated along the right.

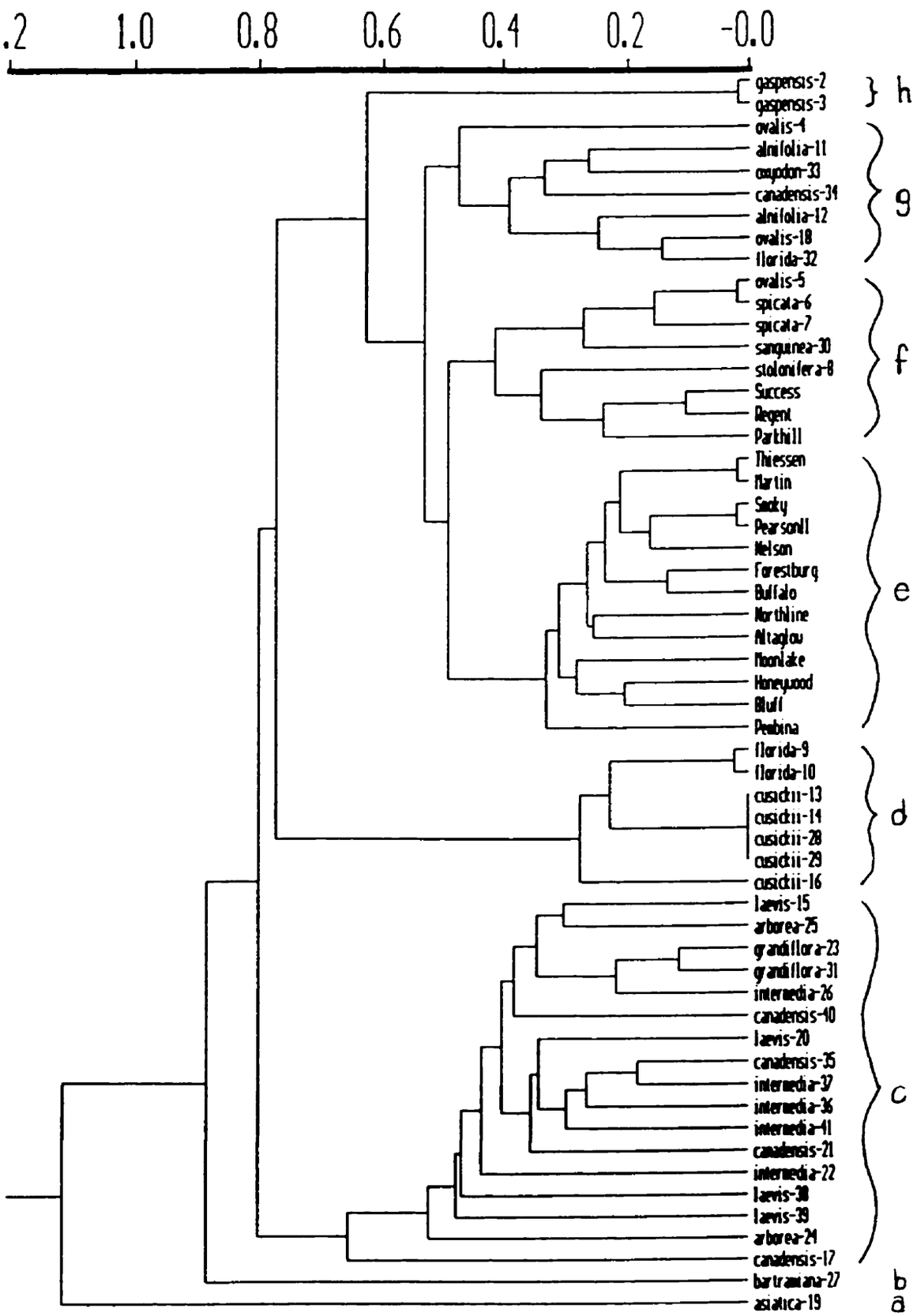

1.2
1.0
0.8
0.6
0.4
0.2
-0.0
gaspensis-2
gaspensis-3
h
ovalis-4
alnifolia-11
occidentalis-33
canadensis-34
alnifolia-12
ovalis-18
florida-32
g
ovalis-5
spicata-6
spicata-7
sanguinea-30
stolonifera-8
Success
Regent
Parthill
f
Thiessen
Martin
Sacky
Pearsonii
Nelson
Forestburg
Buffalo
Northline
Altaglow
Moonlake
Honeywood
Bluff
Peubina
e
florida-9
florida-10
cusickii-13
cusickii-14
cusickii-28
cusickii-29
cusickii-16
d
laevis-15
arborea-25
grandiflora-23
grandiflora-31
intermedia-26
canadensis-40
laevis-20
canadensis-35
intermedia-37
intermedia-36
intermedia-41
canadensis-21
intermedia-22
laevis-38
laevis-39
arborea-24
canadensis-17
c
bartramiana-27
b
asiatica-19
a

Figure 6.4. Phylogenetic tree for 56 OTU of *Amelanchier* based on RAPD data analyzed by complete linkage clustering of Jaccard's coefficient of similarity. The coefficient of similarity is given above the phenogram and clusters (a) through (h) are indicated along the right.

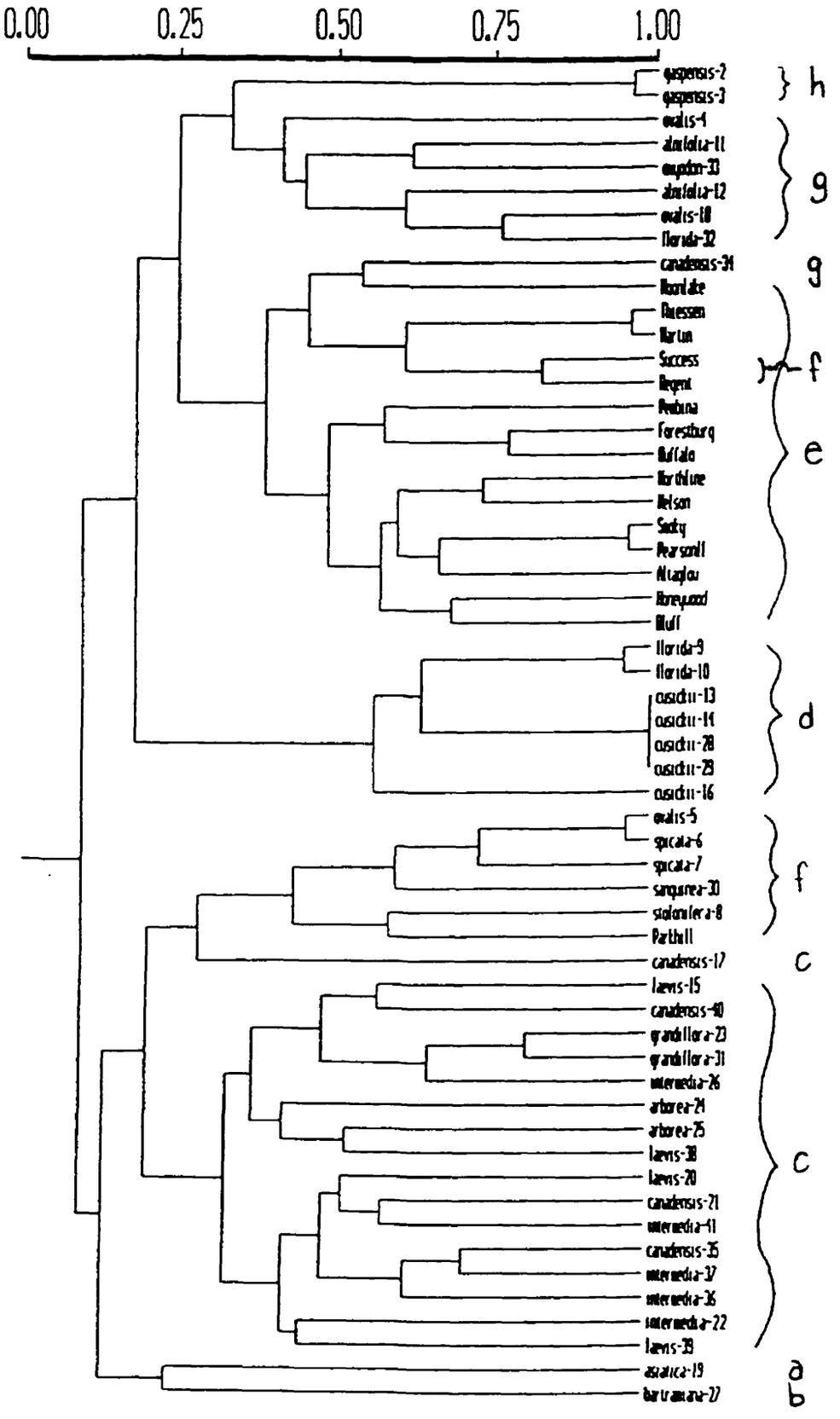

0.00
0.25
0.50
0.75
1.00
h
g
g
f
e
d
f
c
c
a
b

Figure 6.5. Phylogenetic tree for 56 OTU of *Amelanchier* based on RAPD data analyzed by complete linkage clustering of Dice's coefficient of similarity. The coefficient of similarity is given above the phenogram and clusters (a) through (h) are indicated along the right.

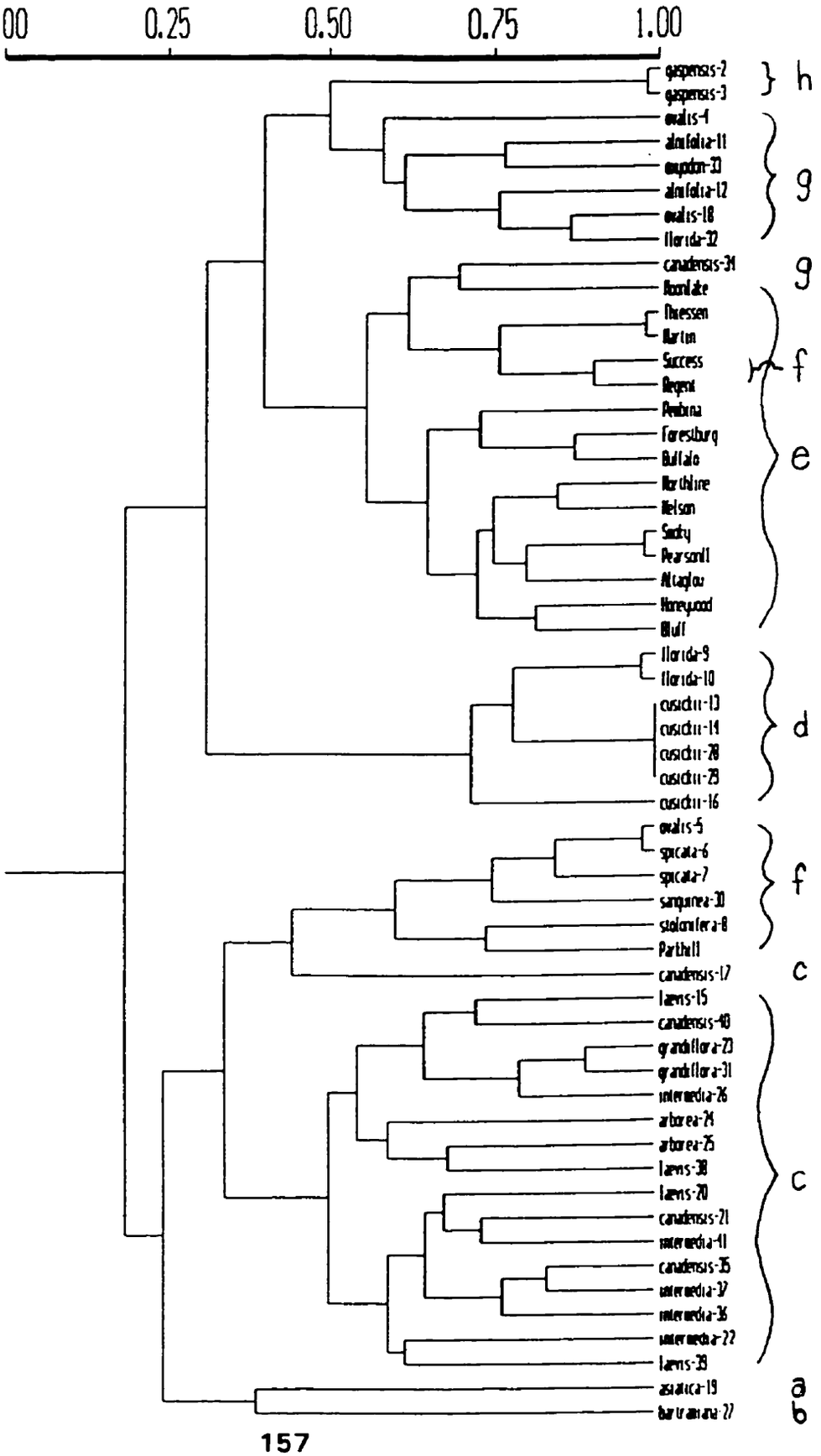

0.00
0.25
0.50
0.75
1.00
gaspensis-2
gaspensis-3
ovalis-4
alnifolia-11
europaea-23
alnifolia-12
ovalis-18
florida-22
canadensis-24
Woodlake
Thiessen
Martin
Success
Regent
Pembina
Forestburg
Buffalo
Northline
Nelson
Sudsy
Pearsall
Attaglow
Honeywood
Bluff
florida-9
florida-10
cusickii-13
cusickii-14
cusickii-28
cusickii-29
cusickii-16
ovalis-5
spicata-6
spicata-7
sanguinea-30
stolonifera-8
Parthill
canadensis-17
laevis-15
canadensis-40
grandiflora-23
grandiflora-31
intermedia-26
arborea-24
arborea-25
laevis-38
laevis-20
canadensis-21
intermedia-41
canadensis-35
intermedia-37
intermedia-36
intermedia-22
laevis-39
asiatica-19
barbarorum-27
h
g
g
f
e
d
f
c
c
a
b

Figure 6.6. Phylogenetic tree for 56 OTU of *Amelanchier* based on RAPD data analyzed by complete linkage clustering of Nei's coefficient of genetic distance. The coefficient of genetic distance is given above the phenogram and clusters (a) through (h) are indicated along the right.

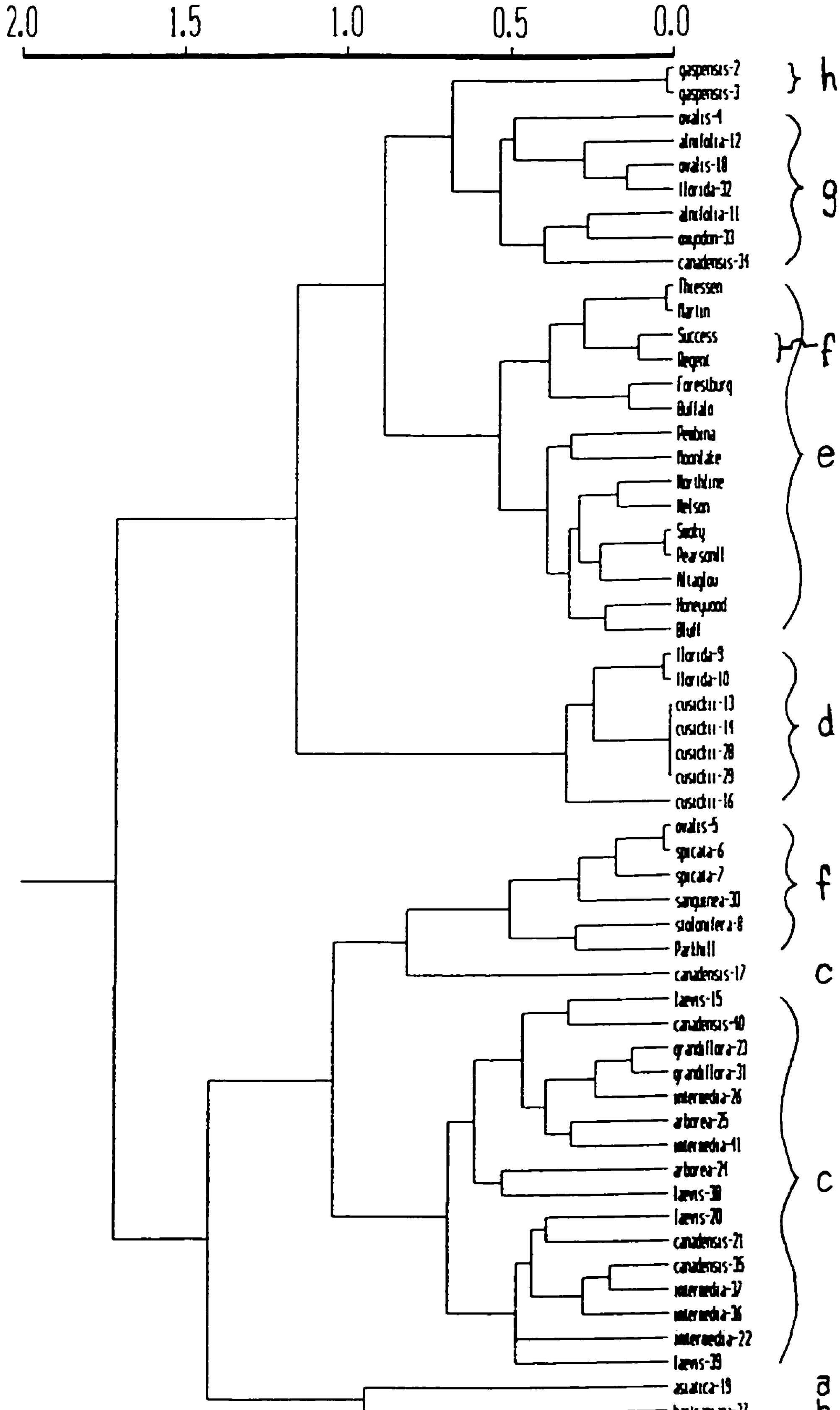

2.0
1.5
1.0
0.5
0.0
gaspensis-2
gaspensis-3
h
ovalis-4
alnifolia-12
ovalis-18
florida-32
alnifolia-11
oxyodon-33
canadensis-34
g
Thiessen
Martin
Success
Regent
f
Forestburg
Buffalo
Pembina
Moonlake
Northline
Nelson
Smoky
Pearsonll
Altaglow
Honeywood
Bluff
e
florida-9
florida-10
cusickii-13
cusickii-14
cusickii-28
cusickii-29
cusickii-16
d
ovalis-5
spicata-6
spicata-7
sanguinea-30
stolonifera-8
Parkhill
f
canadensis-17
c
laevis-15
canadensis-40
grandiflora-23
grandiflora-31
intermedia-26
arborea-25
intermedia-41
arborea-24
laevis-38
laevis-20
canadensis-21
canadensis-35
intermedia-37
intermedia-36
intermedia-22
laevis-39
c
asiatica-19
a
bartramiana-27
b

Figure 6.7. Phylogenetic tree for 56 OTU of *Amelanchier* based on RAPD data analyzed by single linkage clustering of Jaccard's coefficient of similarity. The coefficient of similarity is given above the phenogram and clusters (a) through (h) are indicated along the right.

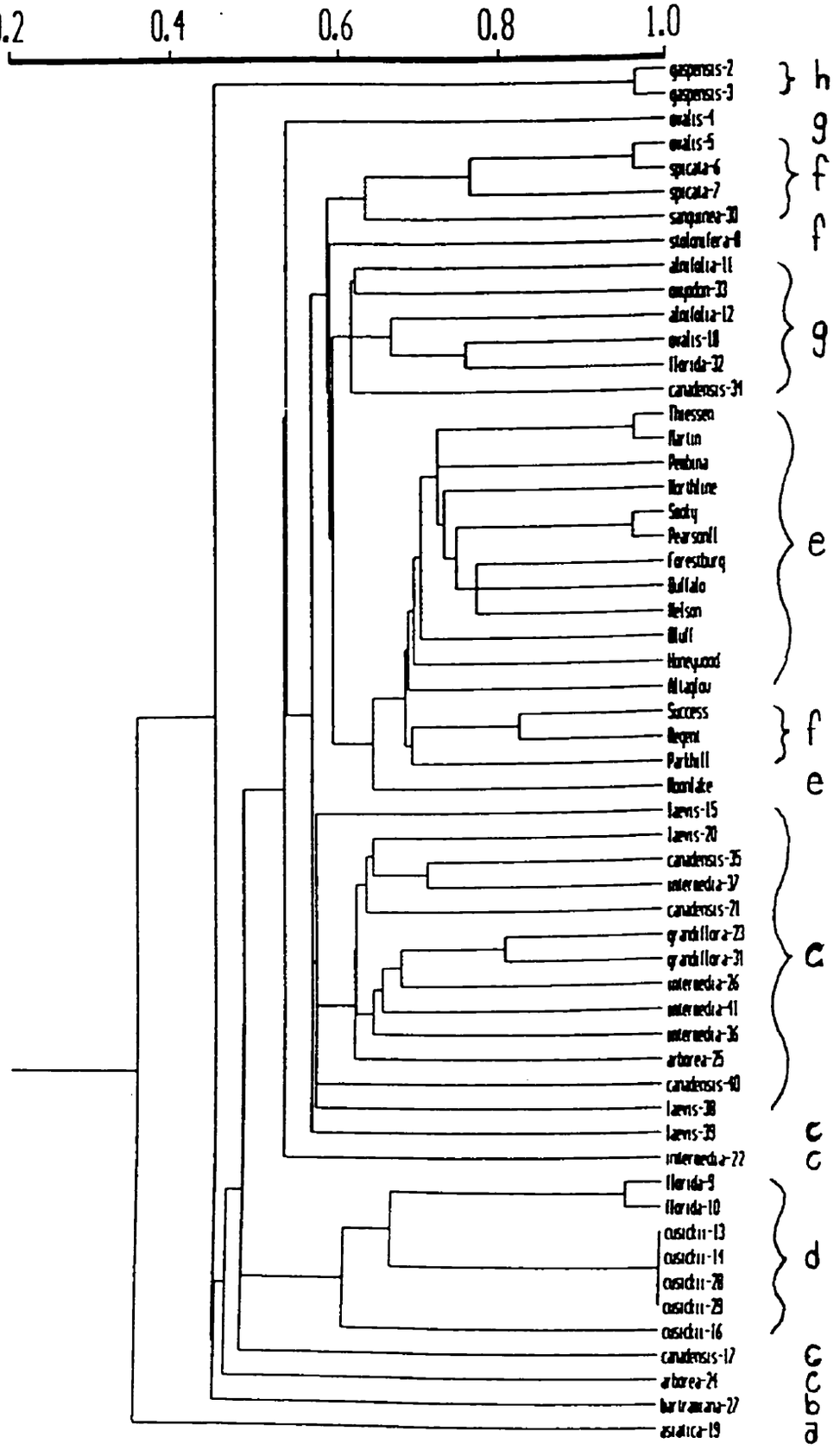

0.2
0.4
0.6
0.8
1.0
gaspensis-2
gaspensis-3
ovalis-4
ovalis-5
spicata-6
spicata-7
sanguinea-30
stolonifera-8
aboriginum-33
ovalis-18
canadensis-34
Thuessen
Martin
Pembina
Northline
Sandy
Pearsonii
Forestburg
Buffalo
Nelson
Bluff
Honeywood
Success
Regent
Parthill
Laevis-15
Laevis-20
canadensis-35
intermedia-37
canadensis-21
grandiflora-23
grandiflora-31
intermedia-26
intermedia-41
intermedia-36
arborea-25
canadensis-40
Laevis-38
Laevis-39
intermedia-22
florida-9
florida-10
ossicula-13
ossicula-14
ossicula-28
ossicula-29
ossicula-16
canadensis-17
arborea-24
hartwegiana-27
asiatica-19
h
g
f
f
g
e
f
e
c
c
d
c
c
b
a

Figure 6.8. Phylogenetic tree for 56 OTU of *Amelanchier* based on RAPD data analyzed by single linkage clustering of Dice's coefficient of similarity. The coefficient of similarity is given above the phenogram and clusters (a) through (h) are indicated along the right.

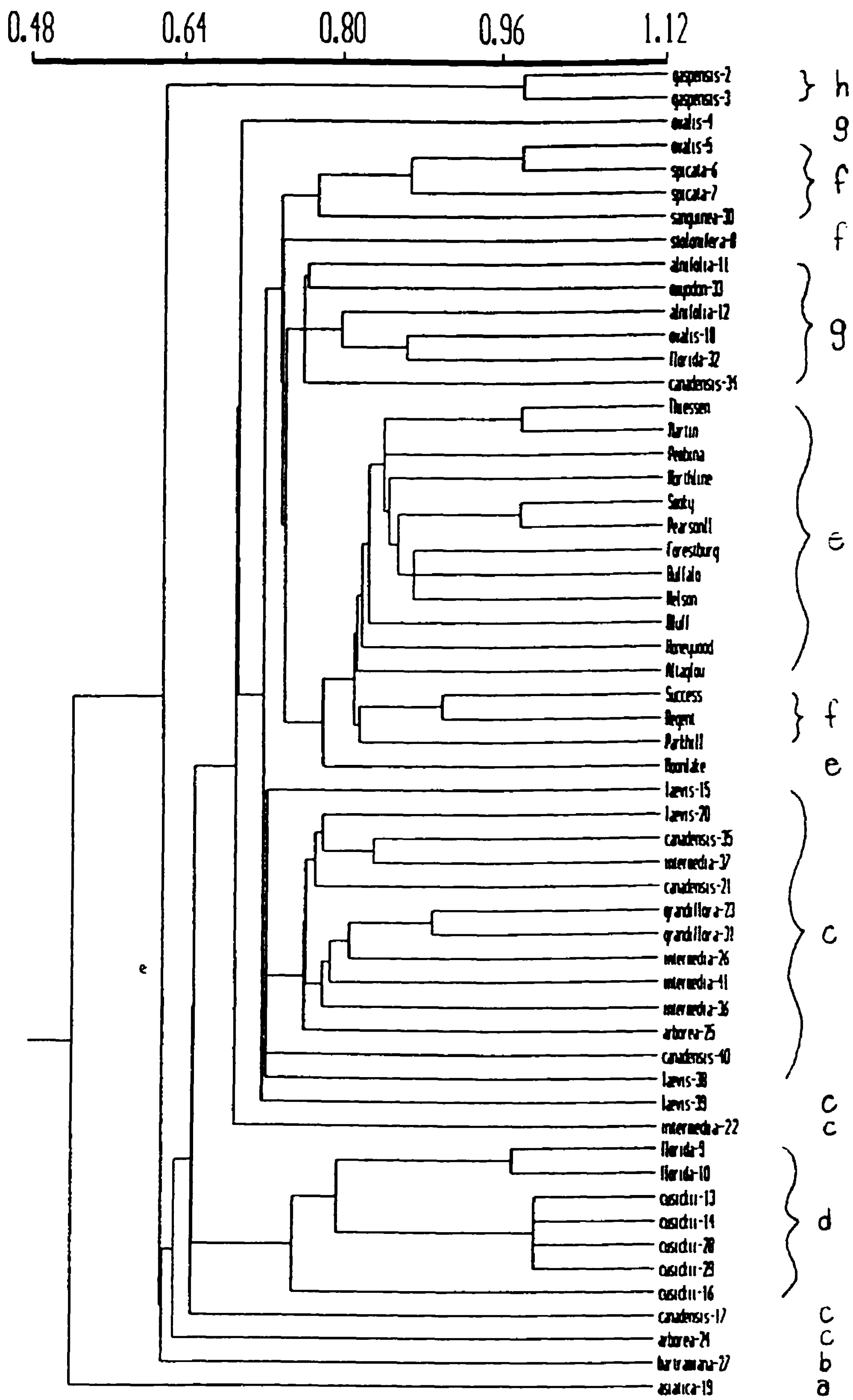
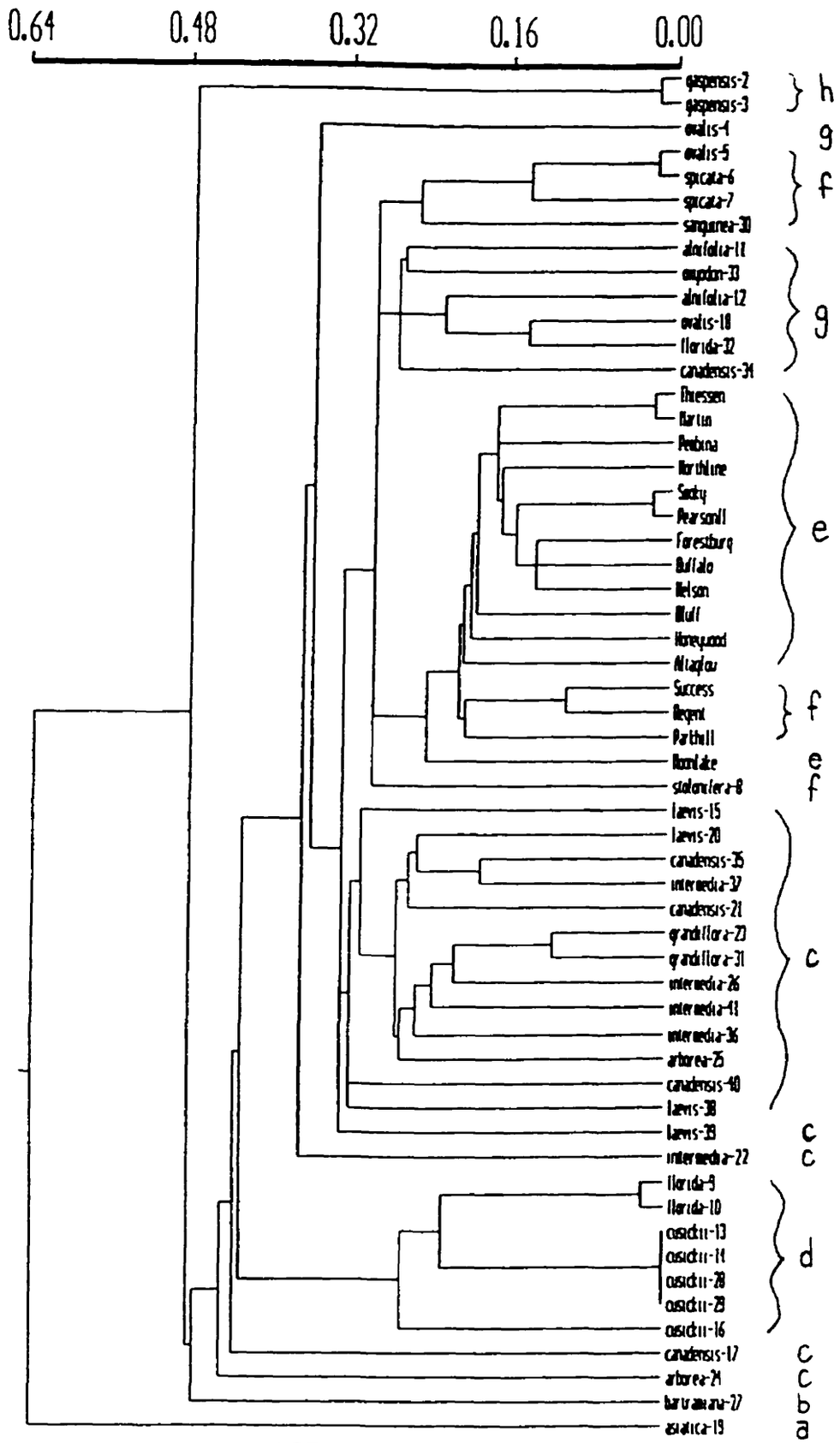

0.48
0.64
0.80
0.96
1.12
gaspensis-2
gaspensis-3
h
ovalis-4
g
ovalis-5
spicata-6
spicata-7
f
sanguinea-30
stolonifera-8
f
alnifolia-11
alnifolia-12
ovalis-18
florida-32
canadensis-34
g
Thiessen
Martin
Pembina
Northline
Smoky
Pearson II
Forestburg
Buffalo
Nelson
Bluff
Honeywood
Success
Regent
Parkhill
f
e
laevis-15
laevis-20
canadensis-35
intermedia-37
canadensis-21
grandiflora-23
intermedia-26
intermedia-41
intermedia-36
arborea-25
canadensis-40
laevis-38
c
laevis-39
c
intermedia-22
c
florida-9
florida-10
cusickii-13
cusickii-14
cusickii-16
d
canadensis-17
c
arborea-24
c
bartramiana-27
b
asiatica-19
a
e

Figure 6.9. Phylogenetic tree for 56 OTU of *Amelanchier* based on RAPD data analyzed by single linkage clustering of Nei's coefficient of genetic distance. The coefficient of genetic distance is given above the phenogram and clusters (a) through (h) are indicated along the right.


0.64
0.48
0.32
0.16
0.00
gaspensis-2
gaspensis-3
ovalis-4
ovalis-5
spicata-6
spicata-7
sanguinea-30
dentata-11
europaea-33
dentata-12
ovalis-18
floridana-32
canadensis-34
Thiessen
Martin
Penbina
Northline
Sacky
Pearsall
Forestburg
Buffalo
Nelson
Bluff
Honeywood
Altaglow
Success
Regent
Parshall
Hamlake
stolonifera-8
laevis-15
laevis-20
canadensis-35
intermedia-37
canadensis-21
grandiflora-23
grandiflora-31
intermedia-26
intermedia-41
intermedia-36
arborea-25
canadensis-40
laevis-38
laevis-39
intermedia-22
floridana-9
floridana-10
occidentalis-13
occidentalis-14
occidentalis-28
occidentalis-29
occidentalis-16
canadensis-17
arborea-24
buergeriana-27
asiatica-19
h
g
f
g
e
f
e
f
c
c
d
c
c
b
a

phenograms regardless of the coefficient used. For example, all three UPGMA-generated phenograms contain identical OTU within clusters (a) through (h), with only minor rearrangements of OTU within some clusters (Figure 6.1, 6.2, and 6.3). In contrast, the relationships among clusters and OTU within clusters differs according to the clustering method employed. For example, complete linkage of Jaccard's coefficient matrix (Figure 6.4) results in the splitting of cluster (f) such that six of its OTU display a greater similarity to the (c) cluster, and two other OTU cluster in the (e) cluster.

Rohlf (1993) indicates a method for selecting the best tree based on the goodness-of-fit of each cluster analysis, which can be determined by computing the correlation between the cophenetic value of each matrix and the original matrix being clustered. Since the comparison is between a cophenetic value matrix and the matrix upon which the clustering is based, the correlation is called the "cophenetic correlation". The cophenetic correlation is used as a measure of goodness-of-fit. The significance of these correlations cannot be tested using usual procedures such as comparing the Z-value with its permutational distribution and is interpreted subjectively as follows: $0.9 \leq r$ = very good fit

$0.8 \leq r < 0.9$ = good fit

$0.7 \leq r < 0.8$ = poor fit

$0.6 \leq r < 0.7$ = very poor fit

Table 6.3. Matrix correlation (r) for nine phenograms generated using each of three coefficients (Jaccard, Dice, and Nei) and each of three clustering methods (UPGMA, complete- and single-linkage).

Coefficient	Clustering method		
	UPGMA	Complete	Single
Jaccard	0.85573	0.78845	0.72727
Dice	0.82169	0.73720	0.67454
Nei	0.77739	0.65687	0.62830

Clustering by UPGMA had the highest correlation among clustering methods regardless of the coefficient used (Table 6.3). Phenograms generated using Jaccard's coefficient had the highest correlation among the coefficients examined. Thus, the phenogram generated using Jaccard's coefficient and UPGMA clustering (Figure 6.1), which had the highest cophenetic correlation, will be used in comparisons with other systematic approaches.

6.3.2 PRINCIPAL COORDINATE ANALYSIS

Principal coordinate (PCO) analysis gave 8 eigenvalues that accounted for 97.22 % of the variance among the 56 OTU (Table 6.4). Each of the eight eigenvalues explain more variance than would be expected by chance alone under the broken stick model (Rohlf, 1993). Eigenvalues 4, 5, 6, 7, and 8 appeared to asymptote in value and were not examined further as suggested by Demeke *et al.* (1992). The first three eigenroots contained 36.47, 21.05, and 9.48 %, respectively, for a total of 67.0 % of the variance. A plot of the 56 OTU onto the first two principal coordinates is shown in Figure 6.10, with a minimum spanning network superimposed. Because of the large number of OTU examined, an additional view of the same plot is provided for clarity (Figure 6.11). Note that all label numbers, which correspond to the numbers given in Table 6.1, cannot be seen in each figure because they may be hidden by other labels.

Table 6.4. Top eight eigenvalues accounting for over 97% of the variance in principal coordinates analysis. Expected variance is the portion of variance expected using the broken-stick model (Rohlf, 1993).

Eigenvalue	Percent Variance	Cumulative Percentage	Expected Variance
1.114890	36.4720	36.4720	8.2348
0.643372	21.0470	57.5189	6.4491
0.289809	9.4807	66.9996	5.5562
0.260044	8.5070	75.5066	4.9610
0.197086	6.4474	81.9539	4.5145
0.193147	6.3185	88.2725	4.1574
0.148945	4.8725	93.1450	3.8598
0.124640	4.0774	97.2224	3.6047

Figure 6.10. Principal coordinate analysis of the similarity matrix of 56 *Amelanchier* OTU. The OTU are plotted against the first two principal coordinates. Solid lines are the minimum spanning network for the *Amelanchier* OTU and dashed lines indicate the clusters (a) through (h).

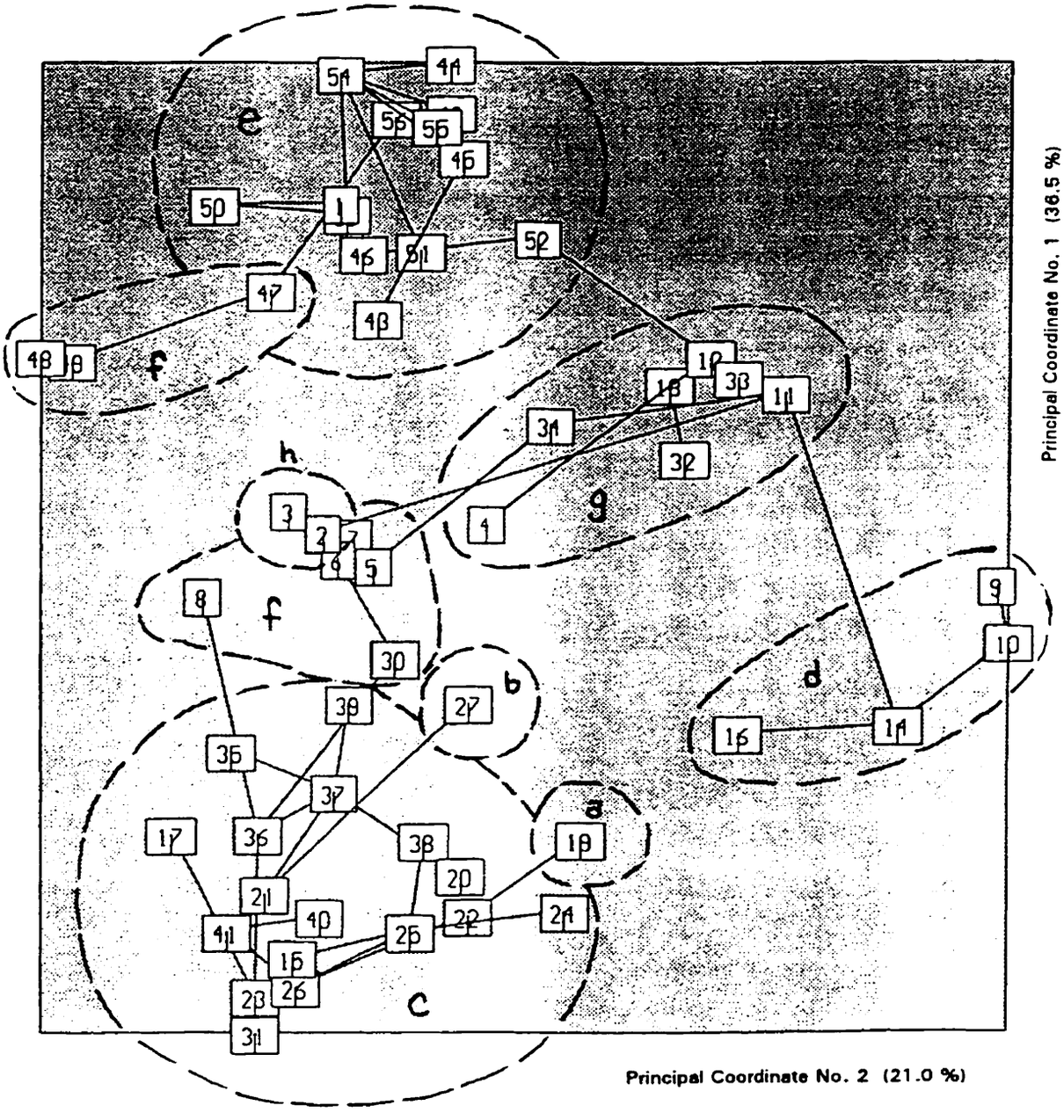
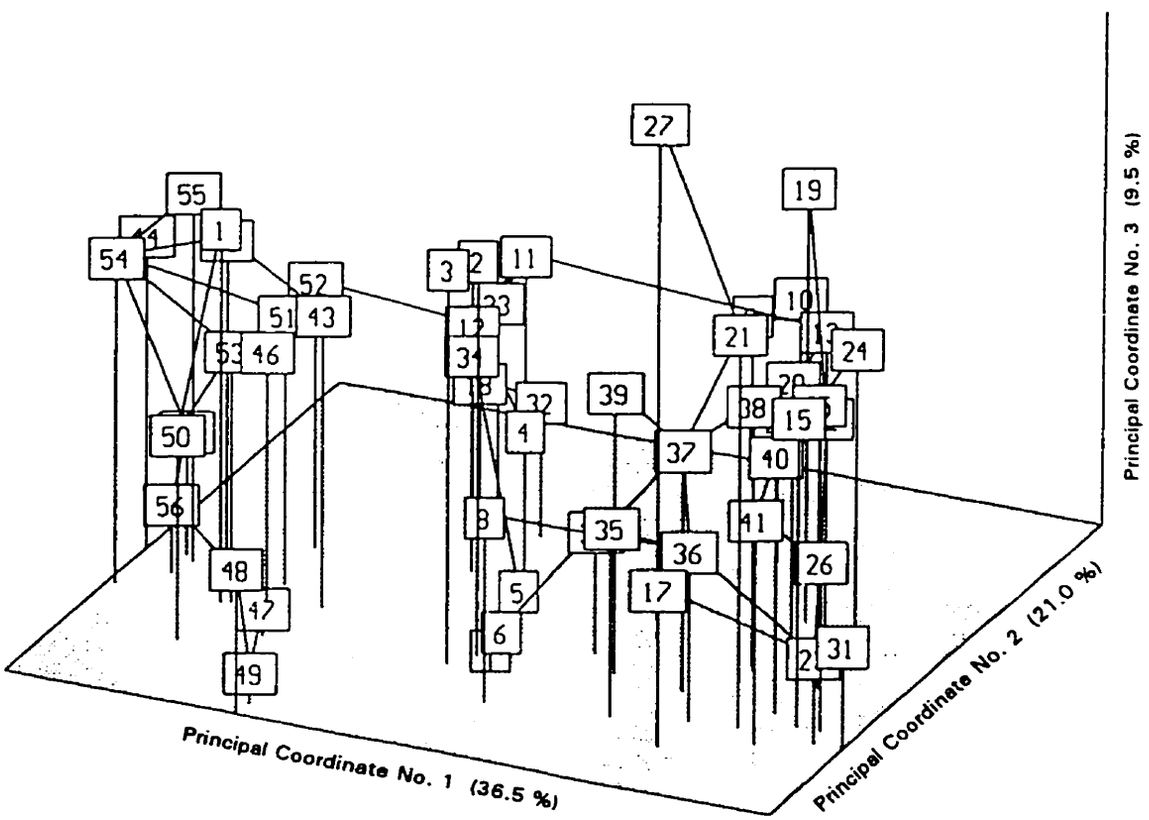

Principal Coordinate No. 1 (36.5 %)
Principal Coordinate No. 2 (21.0 %)

Figure 6.11. Principal coordinate analysis of the similarity matrix of 56 *Amelanchier* OTU. The OTU are plotted against the first three principal coordinates. Solid lines are the minimum spanning network for the *Amelanchier* OTU.


Principal Coordinate No. 3 (9.5 %)
Principal Coordinate No. 2 (21.0 %)
Principal Coordinate No. 1 (36.5 %)

Principal coordinates analysis of the 56 OTU is consistent with the Jaccard/UPGMA cluster analysis. The eight Jaccard/UPGMA clusters have been circled and identified in the PCO plot (Figure 6.11). Clusters (a), (b), (c), (d), (e), and (h) are separate from each other, although examination of the other view (Figure 6.11) is necessary to see the spatial separation of (a), (b), and (h) from the other clusters. In contrast to the Jaccard/UPGMA analysis, PCO analysis fragments cluster (f), placing the saskatoon cultivars of American origin (numbers 47, 48 and 49) closer to the Canadian cultivars in cluster (e), and placing the other OTU of (f) between (g) and (c).

6.3.3 CLADISTIC ANALYSIS

Cladograms generated using Clique and Wagner parsimony analysis are shown in Figures 6.12 to 6.14. Clique analysis generated two cliques of 13 characters each, and the cladogram in Figure 6.12 is a consensus tree derived from these two largest cliques. Neither clique nor the consensus of the two was able to resolve the cladogram (Figure 6.12). Failure of the largest clique to resolve the cladogram is a problem of compatibility analysis (Pankhurst, 1991) and, thus, this analysis was deemed unsuitable.

Wagner parsimony analysis generated sixteen trees, each containing 377 steps. The consensus of these trees is shown in Figures 6.13 and 6.14, the former tree is rooted while the later is an unrooted tree. There

Figure 6.12. Consensus tree generated from clique analysis for 56 OTU of *Amelanchier*. The OTU belonging to clusters (a) through (h) are indicated along right side of cladogram.

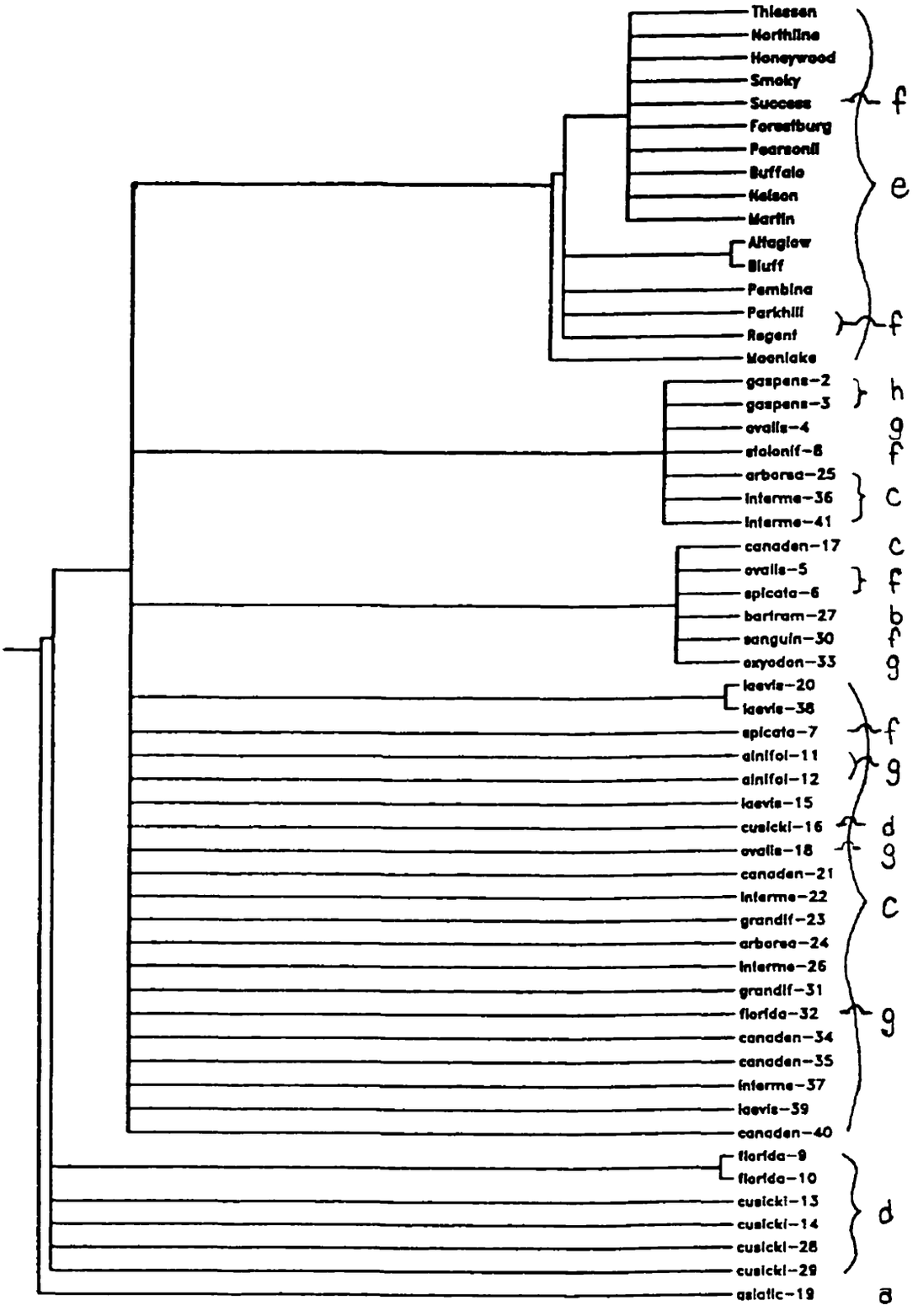

Thiessen
Northline
Honeywood
Smoky
Success
Forestburg
Pearsonii
Buffalo
Nelson
Martin
Altaglow
Bluff
Pembina
Parkhill
Regent
Moonlake
gaspens-2
gaspens-3
ovalis-4
stolonif-8
arborea-25
interme-36
interme-41
canaden-17
ovalis-5
spicata-6
bartram-27
sanguin-30
oxyodon-33
laevis-20
laevis-38
spicata-7
sinifol-11
sinifol-12
laevis-15
cusicki-16
ovalis-18
canaden-21
interme-22
grandif-23
arborea-24
interme-26
grandif-31
florida-32
canaden-34
canaden-35
interme-37
laevis-39
canaden-40
florida-9
florida-10
cusicki-13
cusicki-14
cusicki-28
cusicki-29
asiatic-19
f
e
f
h
g
f
c
c
f
f
b
g
f
g
d
g
c
g
d
a

Figure 6.13. Consensus tree generated from Wagner parsimony analysis for 56 OTU of *Amelanchier*. Out-group for this analysis was asiatica-19. Clusters (a) through (h) are indicated on the right side of the cladogram.

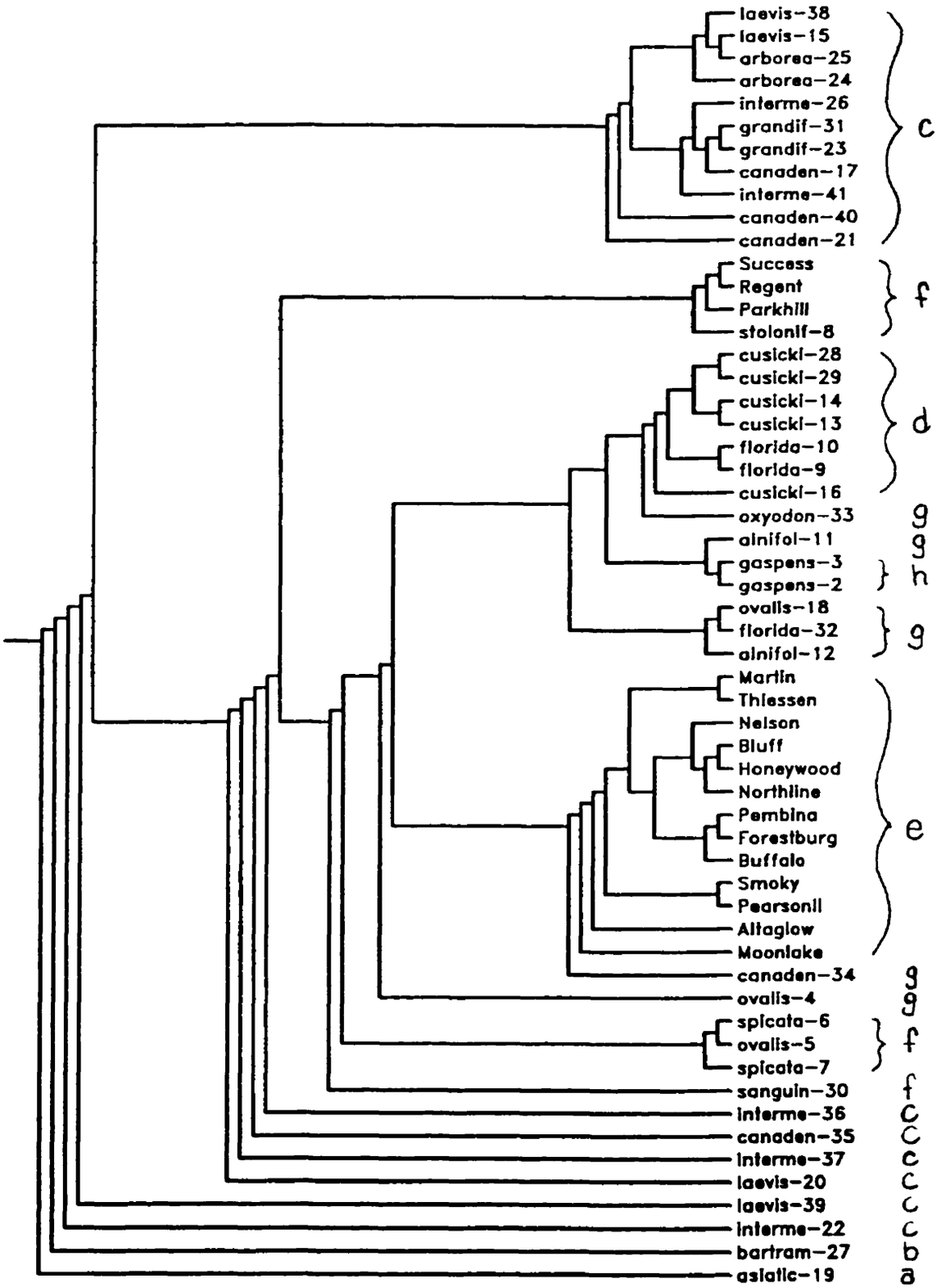
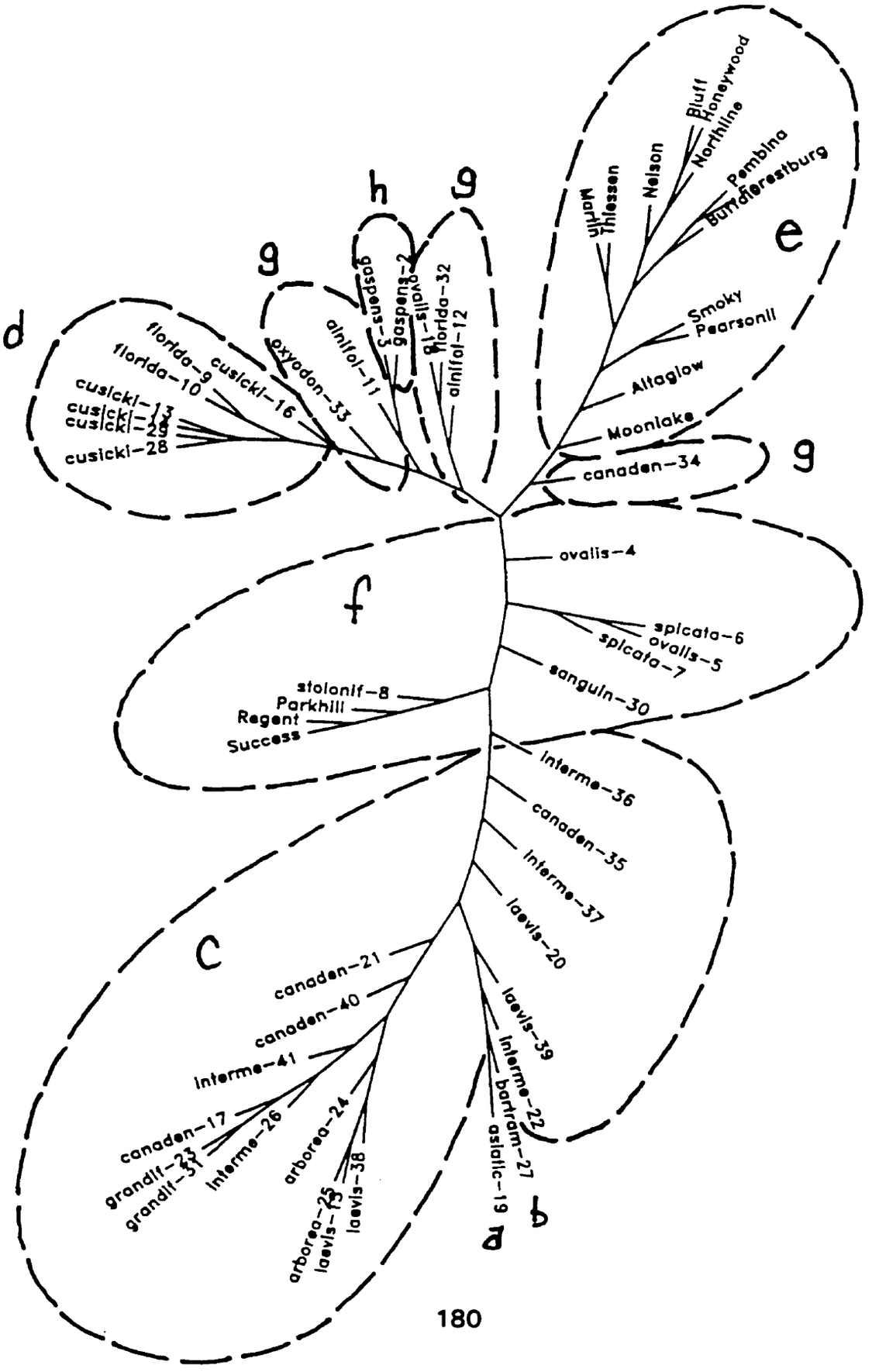

laevis-38
laevis-15
arborea-25
arborea-24
interme-26
grandif-31
grandif-23
canaden-17
interme-41
canaden-40
canaden-21
c
Success
Regent
Parkhill
stolonif-8
f
cusicki-28
cusicki-29
cusicki-14
cusicki-13
florida-10
florida-9
cusicki-16
d
oxyodon-33
g
alnifol-11
g
gaspens-3
gaspens-2
h
ovalis-18
florida-32
alnifol-12
g
Martin
Thiessen
Nelson
Bluff
Honeywood
Northline
Pembina
Forestburg
Buffalo
Smoky
Pearsonii
Altaglow
Moonlake
e
canaden-34
g
ovalis-4
g
spicata-6
ovalis-5
spicata-7
f
sanguin-30
f
interme-36
c
canaden-35
c
interme-37
c
laevis-20
c
laevis-39
c
interme-22
c
bartram-27
b
asiatic-19
a

Figure 6.14. Consensus tree generated from Wagner parsimony analysis for 56 OTU of *Amelanchier*. There was no out-group comparison in this analysis. Dashed lines indicate clusters (a) through (h).


d
florida-9
florida-10
cusicki-16
cusicki-13
cusicki-29
cusicki-28
oxyodon-33
g
gaspens-3
gaspens-2
ovalis-18
h
g
alnifol-11
florida-32
alnifol-12
e
Bluff
Honeywood
Nelson
Northline
Pembina
Burnt Forest
Thlesson
Smoky
Pearsonii
Altaglow
Moonlake
canaden-34
g
ovalis-4
f
spicata-6
ovalis-5
spicata-7
sanguin-30
stolonif-8
Parkhill
Regent
Success
interme-36
canaden-35
interme-37
laevis-20
laevis-39
interme-22
bartram-27
asialic-19
b
a
C
canaden-21
canaden-40
interme-41
canaden-17
grandif-23
grandif-31
interme-26
arborea-24
arborea-25
laevis-38

are differences between the Jaccard/UPGMA phenogram and these cladograms with regard to clustering of individuals and relationships among clusters. Some clusters, such as (e) and (d), are found in both analyses, while others, such as (c) and (f) have been fragmented into smaller clusters or individuals. In the unrooted Wagner parsimony analysis (also called a network), the characters are not polarized in relationship to the outgroup *A. asiatica*. The network topology places the OTU of (f) intermediate to the two ends of the network, with (d,e,g,h) and (a,b,c) comprising each end.

6.4 DISCUSSION

Three topologies were chosen as the most likely representatives of phylogeny in *Amelanchier* (Jaccard/UPGMA, PCO, and Wagner parsimony). Reasons for discarding the other cluster and clique analyses were given above. Does any one of these three analyses depict the true phylogeny of *Amelanchier*? This is difficult to answer considering the true phylogeny is not known and we can only use the tools of phylogenetic reconstruction to estimate the evolutionary relationships among these OTU. Generally, all of the analyses used in this study can contribute to our understanding of the relationships among *Amelanchier* species. For that reason, it is necessary to examine the OTU within each cluster as well as the differences and similarities between the morphological-based relationships suggested in the literature and the genetic relationships suggested in this study.

6.4.1 CLUSTERS (a) AND (b)

The taxon *A. asiatica* (a), which is native to Japan, Korea, and China (Ohwi, 1965; Robertson, 1974), clustered separately from the remaining, mainly North American OTU. There was only 20% similarity between *A. asiatica* and the remaining OTU (Figure 6.1). This lack of similarity supports the use of *A. asiatica* for out-group comparison in parsimony analysis. While Robertson (1974) suggested *A. asiatica* is closely related to *A. arborea*, none of these analyses support this association. Similarly, *A. bartramiana* (b), a morphologically distinct taxon, displayed little genetic similarity to the other North American OTU and did not cluster with other OTU.

6.4.2 CLUSTER (c)

Cluster (c) contains the taxa *A. arborea*, *A. canadensis*, *A. laevis*, *A. intermedia*, and *A. x grandiflora*. Of the these taxa, only the *A. x grandiflora* OTU clustered according to morphological designation. Many authors have had difficulties maintaining the treatment of these as separate delimitable taxa. Cruise (1964) demonstrated that *A. arborea*, *A. canadensis* and *A. laevis* form a highly variable and integrated group which should fall under the binomial *A. canadensis* (L.) Medic. Landry (1975) and McKay (1973) agreed partially by treating *A. laevis* as a subspecies of *A. arborea*. Robinson and Partanen (1980) included four species, *A.*

canadensis, *A. arborea*, *A. laevis* and *A. intermedia*, in the 'canadensis complex', a grouping which Blanchard suggested in 1907. The most recent taxonomic treatments suggest *A. intermedia* is a hybrid between *A. arborea* or *A. laevis* and *A. canadensis* (Gleason and Cronquist, 1993; Kartesz, 1994). Similarly, *A. x grandiflora* is believed to be a natural hybrid between *A. arborea* and *A. laevis* (Dirr, 1987; Jones, 1946) or *A. laevis* and *A. intermedia* (Hilton, 1988). Thus, it is not surprising for these taxa and putative hybrids to be clustered together. Blanchard's (1907) term 'canadensis complex' will be used in reference to this large diverse cluster containing the taxa *A. arborea*, *A. canadensis*, *A. laevis*, *A. intermedia*, and *A. x grandiflora*.

Discordance between morphological and genetic delimitation has been reported in other plant species using both RAPD (Campos *et al.*, 1993; Joshi and Nguyen, 1993) and RFLP data (Jung *et al.*, 1993, van de Ven *et al.*, 1993). As well, analyses of morphologically similar taxa have revealed a complete lack of specificity of their cpDNA genotypes in several genera (Laurent *et al.*, 1993; Petit *et al.*, 1993; Soltis *et al.*,1991).

The discordance between morphological and genetic delimitation of the taxa in the canadensis complex may be explained by the major evolutionary forces which have shaped this genus. Robinson (1982) argued that the genus represented an agamic complex with polyploidy, hybridization, and asexual vegetative reproduction being major forces in its

evolution. Since then, considerable evidence in support of the agamic complex has been presented. Chromosome counts have revealed more polyploid taxa (Table 2.1). Morphological studies continue to confirm the presence of long-lived hybrids such as *A. x neglecta* (Weber and Campbell, 1989), and apomixis has been reported in four taxa, *A. laevis* (Campbell *et al.*, 1985), *A. canadensis*, *A. stolonifera* (Campbell *et al.*, 1987), and *A. x neglecta* (Weber and Campbell, 1989). The effect of these evolutionary forces on morphological and genetic classification is discussed below.

Polyploidy plays a major role in plant evolution (Soltis, P. *et al.*, 1992). It is estimated that 47 to 52% of all angiosperms are polyploid (Grant, 1981 in Soltis, P. *et al.*, 1992). Autopolyploids become genetically isolated from their diploid parent yet remain very similar to them phenetically (Stace, 1989). However, allopolyploidy, ie. hybridization between two taxa followed by chromosome doubling, is more widespread in plants than autopolyploidy (Soltis, P. *et al.*, 1992). Stace (1989) suggests that 30 to 70% of all vascular plant species arose through allopolyploidy, but also indicates that fertile diploid hybrids can result from interspecific crosses among diploid taxa. In the canadensis complex, diploids, triploids, and tetraploids have been reported for the species and putative hybrids (Table 2.1) and could account for some of the discordance between morphological and genetic classification.

Polyploid species are typically polythetic with two or more

independent origins, and there is evidence that in some plant groups polyploidy recurs. The polyphyletic nature of many polyploid species implies that most polyploids are not genetically uniform (Soltis, P. *et al.*, 1992). In the past, systematists perceived that there was little within-species variation, but this has been disproven by molecular analysis of flavonoids, isozymes and cpDNA (Soltis, D. *et al.*, 1992). Polymorphism within diploid and polyploid species has also been revealed using RAPD analysis (Joshi and Nguyen, 1993; Kazan *et al.*, 1992; Vierling and Nguyen, 1992). In this study, Wagner parsimony analysis (Figure 6.13 and 6.14) suggests that two putative hybrids (*A. laevis* and *A. intermedia*), appear to have arisen independently on several occasions, and the Jaccard/UPGMA analysis indicates (6.1) that individuals within these two taxa are not genetically uniform. Thus, these results may indicate a polyphyletic origin for *A. laevis* and *A. intermedia*.

Interspecific hybrids also cause taxonomic problems because they are not identifiable with any one species. Parsimony analysis, for example, is unable to deal with introgression and reticulation because that would require the recognition of paraphyletic or polyphyletic groups (Buck, 1986). Integration between two taxa blurs the distinction between the parental taxa. Cruise (1964) suggested that *A. laevis* was of hybrid origin, with *A. canadensis* and *A. arborea* as the parents. He demonstrated that intermediate forms linking *A. canadensis* to *A. laevis* and *A. laevis* to *A.*

arborea blurred the distinction between these taxa such that they should all fall under one binomial, *A. canadensis*. A consequence of hybridization among taxa is that they tend to exhibit genetic limits that are wider than their morphological limits (Stace, 1989), and this appears to be the situation in the canadensis complex.

Wiegand (1935) recognized hybrids among *Amelanchier* taxa, but suggested they were localized and like bees were "buzzing around for a time, only to disappear, leaving the fundamental species to continue throughout the ages." However, the hybrid *A. x neglecta* has been shown to persist over time (Weber and Campbell, 1989) and has a distribution including five states and three provinces in eastern North America (Jones, 1946, map 2, page 26). Similarly, the putative hybrids *A. laevis* and *A. intermedia*, have wide distributions (Fernald, 1970; Scoggan, 1978). Stace (1989) indicates that the formation of polyploids and asexual reproduction, both of which are reported in the canadensis complex, can stabilize interspecific hybrids.

Apomixis has been reported within the canadensis complex for *A. laevis* (Campbell *et al.*, 1985) and *A. canadensis* (Campbell *et al.*, 1987). Apomixis results in the preservation and propagation of pure lines by essentially vegetative propagation. These apomictic lines are often highly heterozygous especially if they resulted from hybrid origin. Facultative apomicts in the genus *Rubus* cause great taxonomic difficulty because they

can give rise to plants which are variously intermediate between previously distinct but closely related species (Stace, 1989). The idea that agamospecies arise from a single hybridization event followed by the onset of apomixis has been abandoned (Stace, 1989), and there is evidence that the apomictic *A. x neglecta* arose more than once (Weber and Campbell, 1989). Facultative apomixis in conjunction with cross- and self-pollination will significantly slow the rate at which population structure equilibrium is reached (Marshall and Weir, 1979). Apomixis also allows for the rapid and indefinite production of successful gene combinations, permitting the rapid colonization and exploitation of available habitats. Coexistence of sexual and asexual reproduction within the facultatively apomictic species facilitates introgressive hybridization (Clausen, 1954, in Marshall and Weir, 1979). Both *A. laevis*, a facultative apomict (Campbell and Dickinson, 1991) and *A. canadensis*, an apomict (Campbell *et al.*, 1987) are implicated as parents of putative hybrids within the canadensis complex (Dirr, 1987; Gleason and Cronquist, 1993; Hilton, 1988; Jones, 1946; Kartesz, 1994), suggesting that apomixis plays a major role in maintaining these genetically diverse but morphologically similar taxa.

Fossil records identify *Amelanchier* by the Miocene and possibly as long ago as the Oligocene (36.5 million years) in North America (Hickey, 1984, in Phipps *et al.*, 1991). If the effects of polyploidy, hybridization and apomixis over long periods of time are considered, then we may develop a

better understanding of the canadensis complex today. Repeated advance and retreat of glaciers during the pleistocene (Cox and Moore, 1993) could have brought previously separated species in close contact, allowing for recurring hybridization and integration among species. As the glaciers retreated a spectrum of ecological niches would become available to satisfy the requirements of a wide range of hybrid offspring (Stace, 1989). Maintenance of these older and newer hybrid forms could occur through apomixis and polyploidy, as discussed above. Polyploidy within a species genetically isolates the polyploid while maintaining phenetic similarity (Stace, 1989). During the last glaciation, areas such as Newfoundland and Nova Scotia were not covered by ice (Cox and Moore, 1993), and could have harboured a number of species and putative hybrids, beginning the process of allopatric speciation. Recent disturbances by European settlers in eastern North America have opened large areas in the east to colonization by *Amelanchier* hybrids. The presence of facultative apomicts within this complex will slow down the rate at which this complex will reach equilibrium (Marshall and Weir, 1979).

One of the objectives of this thesis was the application of RAPD markers in the determination of parentage for the hybrid *A. x grandiflora*. Unfortunately, within the canadensis complex there were no bands specific to any one taxon, and therefore it is difficult to determine the possible parents of *A. x grandiflora*. Suggested parentage has been between *A.*

arborea and *A. laevis* (Dirr, 1987; Jones, 1946) or *A. laevis* and *A. intermedia* (Hilton, 1988). However, it is possible to rule out hybridization between *A. arborea* and *A. laevis* as the parents and thereby suggest *A. laevis* and *A. intermedia* by default. If we examine the scored bands for each of these taxa, we see that a band is present in either or both of the *A. x grandiflora* OTU for markers RC16-1140, RC19-1450, and RC22-1760. These three bands are not present in any of the *A. arborea* or *A. laevis* OTU and therefore it is unlikely a hybrid between these two taxa would possess these markers. These three bands are present among the *A. intermedia* OTU examined. Thus, of the combinations suggested above, *A. laevis* and *A. intermedia* are the most probable parents of *A. x grandiflora*.

6.4.3 CLUSTERS (d), (e) AND (g)

Of the remaining clusters, (d), (e) and (g) will be discussed because the close relationship among the taxa in these clusters can be supported by the literature. The major taxa within these three clusters are *A. cusickii*, *A. florida*, and the saskatoon cultivars originating from the prairies which are selections from *A. alnifolia*. Although Jones (1946) described these taxa as separate but closely related species, they are more recently treated as varieties within the species *A. alnifolia* (Table 2.2). In addition, *A. oxyodon* is synonymous with *A. a.* var. *semiintegrifolia* (Hitchcock and Cronquist, 1961; Jones, 1946), and should cluster within this group. The two *A.*

ovalis and single *A. canadensis* (-34) which have clustered in these groups will be discussed later.

Jaccard/UPGMA (Figure 6.1) and PCO (Figure 6.10) analyses divided these OTU into three clusters (d, e, and g) based on overall similarity. Treatment of the OTU in cluster (e) as *A. alnifolia* var. *alnifolia* is suggested here for several reasons. Firstly, they originate from an area that is physically isolated by the Rocky Mountains or through great distances from the taxa of (d) and (g). Secondly, these other taxa are generally not reported from the Canadian prairies (Boivin, 1966; Jones, 1946; Rydberg, 1922, 1932; Scoggan, 1978; but see Looman and Best, 1979). Cluster (d) contains all of the cusickii and two of the florida OTU in this study, and may represent *A. alnifolia* var. *cusickii*. The *A. florida* OTU are now treated as *A. a.* var. *semiintegrifolia* (Kartesz, 1994), but the varieties *cusickii* and *semiintegrifolia* are known to be completely integrated (Hitchcock and Cronquist, 1961), which may explain the clustering of *A. florida* with *A. cusickii*. PCO analysis (Figure 6.10) clearly places (g) between (e) and (d), which may suggest that it is of hybrid origin (ie. *A.a.* var. *alnifolia x A.a.* var. *cusickii*), and these varieties are known to be completely integrated West of the Rockies. In contrast, Wagner parsimony (Figure 6.14) generated two large clusters and separated these OTU to either side of the Rocky Mountains. This treatment suggests evolution due to allopatric speciation. Thus, all three analyses can be supported by morphological

evidence from the literature and enhance our understanding of the relationships among these taxa.

According to Landry (1975) clusters (d), (e), and (g) belong to the species *A. sanguinea* varieties *alnifolia* and *pumila*. Following his treatment (Landry, 1975) cluster (d) could represent *A. sanguinea* var. *pumila*, cluster (e) *A. sanguinea* var. *alnifolia*, and (g) may be of hybrid origin with var. *pumila* and *alnifolia* as the parents. Thus, Landry (1975) is in agreement with the close relationship among the taxa *A. alnifolia*, *A. cusickii*, and *A. florida*, but disagrees with Kartesz (1994) and others above, on the species name and the recognition of varieties. The use of *A. sanguinea* as the species name for this group appears to be incorrect in that 'sanguinea' was published in 1835, whereas 'alnifolia' was published in 1818 and should take precedence. Moreover, if 'ovalis' is recognized as a member of this group then its name would take precedence as it was published in 1793 (Blanchard, 1907; Jones, 1946).

6.4.4 CLUSTER (h)

The two *A. gaspensis* OTU (h) have consistently clustered with the (d), (e) and (g) groups (Figures 6.1, 6.10, and 6.14). The treatment of *A. gaspensis* in the literature has varied from recognition as a separate species under the names *A. gaspensis* (Jones, 1946), *A. fernaldii* (Gleason and Cronquist, 1993) to a variety of *A. sanguinea* (Kartesz, 1994; McKay,

1973; Wiegand, 1912). A close relationship has been suggested between *A. gaspensis* and *A. alnifolia* (Jones, 1946) and *A. sanguinea* (Boivin, 1967; Kartesz, 1994; Wiegand, 1912). Landry (1975) suggested all three were closely related and placed them in the species *A. sanguinea*. Although this study does not entirely support Landry's (1975) treatment, it does support his treatment of *A. gaspensis* and *A. cusickii* as varieties within the same species.

The close relationship between *A. gaspensis* and *A. cusickii* may be explained in several ways. Firstly, both taxa may represent glacial relics of an ancestral species which once covered or extended throughout all or parts of North America and then was isolated due to recent glaciation (Cox and Moore, 1993). Secondly, an ancestor of these two taxa may have been an arctic species which has migrated both southwestward and southeastward to their current habitats in western and eastern North America (Marie-Victorin, 1938). Thirdly, as the glaciers melted, an ancestral form related to *A. cusickii* or *A. alnifolia* may have migrated following the glacial run-off from west-central Canada through Ontario along what is now the Great Lakes and the St Lawrence Seaway (Cox and Moore, 1993; Marie-Victorin, 1938), to the current habitat of *A. gaspensis* (Jones, 1946). Regardless of how this close relationship came about, the results of this study suggest that *A. gaspensis* is closely related to *A.*

alnifolia and should be treated as a variety of this species in the same manner as varieties *alnifolia* and *cusickii*.

6.4.5 CLUSTER (f)

In the Jaccard/UPGMA analysis (Figure 6.1), cluster (f) contains the three American cultivars (Success, Regent and Parkhill), stolonifera-8, sanguinea-30, spicata-6 and -7, and ovalis-5. There is little support in the literature for these taxa to cluster among *A. alnifolia* (d, e, g, and h). Two authors have proposed links between some members of (f) and *A. alnifolia*. McKay (1973) suggested the designation *A. alnifolia* var. *compacta* for some of holotypes placed under various labels such as *A. spicata* (Jones, 1946), *A. humilis* (Fernald, 1970; Scoggan, 1978; Kartesz, 1994), and *A. sanguinea* (Gleason and Cronquist, 1993). And, Landry (1975) placed the taxa *A. alnifolia*, *A. cusickii*, *A. florida*, *A. sanguinea*, and *A. gaspensis* all within the species *A. sanguinea*. Although Landry (1975) seems to be in error regarding the correct species name (see above), he is the only author to suggest a close relationship between *A. ovalis* Medicus and *A. pallida* Greene (a close relative of *A. alnifolia*) by describing them as vicariant species. However, Landry (1975) also suggested species such as *A. spicata* and *A. stolonifera* were varieties of *A. canadensis*, a view not supported by this analysis.

In contrast, PCO analysis (Figure 6.10) suggests that most of (f) are

intermediate between (g) and (c) and that the three American cultivars are more closely related to the OTU in cluster (e). In fact, OTU such as stolonifera-8 are equidistant between intermedia-36, a member of (c), and ovalis-4, a member of (g) (Appendix C). Wagner parsimony analysis (Figure 6.14) also suggests that members of (f) are intermediate to *A. alnifolia* (d, e, g, and h) and the canadensis complex (c), and that ovalis-4 and intermedia-36 may be integrated OTU linking (f) with its putative ancestral parents.

In addition, cluster (f) appears to have arisen through hybridization because it has no cluster-specific RAPD markers (Table 6.2); all of its bands are shared with one or more of the other OTU. Moreover, many of the bands in the spicata complex occur at higher frequency in the canadensis complex and at a lower frequency among *A. alnifolia* varieties or vice versa (Table 6.5). This suggests a possible hybrid origin between ancestors of *A. alnifolia* and the canadensis complex, as introgression can lead to the infiltration of germplasm into that of another (Stace, 1989).

Although limited in the number of specimens examined, these analyses suggest a close relationship among *A. sanguinea*, *A. stolonifera*, *A. spicata*, and possibly *A. ovalis*. Jones (1946) suggested that *A. stolonifera*, *A. spicata*, and *A. humilis* were closely related, and the later species has often been treated as *A. sanguinea* (Table 2.2). Considering the prior treatment of these taxa (Table 2.2) it appears that these taxa have

Table 6.5. RAPD markers which may indicate gene flow between *A. alnifolia* varieties (d,e,g,h) and the canadensis complex (c) through the putative hybrid spicata complex (f). The upper table indicates RAPD markers with relatively high frequency in (d,e,g,h) and low frequency in (c), and vice versa in lower table. The OTU in each cluster are as indicated by Jaccard/UPGMA analysis. The number of OTU scored for the presence of each RAPD marker and the frequency ( ) of each marker are indicated.

RAPD Marker	Cluster (d,e,g,h) (29 OTU)	Cluster (f) (8 OTU)	Cluster (c) (17 OTU)
RC16-850	29 (100)	8 (100)	8 (47.1)
RC36-775	29 (100)	8 (100)	8 (47.1)
RC28-640	24 (82.8)	6 (75)	6 (35.3)
RC36-2280	22 (75.9)	8 (100)	2 (11.8)
RC22-2140	20 (70)	4 (50)	1 (5.9)
RC28-450	18 (62.1)	5 (62.5)	1 (5.9)
RC30-1400	17 (58.6)	7 (87.5)	2 (11.8)
RC36-1150	13 (44.8)	4 (50)	3 (17.6)
RC19-1450	2 (6.9)	5 (62.5)	9 (52.9)
RC28-1500	3 (10.3)	4 (50)	14 (82.3)
RC28-1325	2 (6.9)	8 (100)	14 (82.3)
RC37-1440	15 (51.7)	8 (100)	15 (88.2)
RC19-390	7 (24.1)	5 (62.5)	16 (94.1)
RC30-2170	8 (27.6)	4 (50)	17 (100)

presented problems to many taxonomists. The taxon which Wiegand (1912) designated as *A. stolonifera*, for example, has been treated as *A. stolonifera* and *A. spicata*, the binomial alternating with each new published treatment (Table 2.2). Similarly, some of these taxa have been treated as closely related to *A. canadensis* (Gleason and Cronquist, 1993; Landry, 1975) or *A. alnifolia* (Landry, 1975; McKay, 1973). If, as discussed above, the taxa in (f) are of hybrid origin, then it is understandable that taxonomists might suggest a close relationship with either putative parent.

Within (f) there was often only one individual representing a given taxon. Thus, neither the discordance of the canadensis complex, nor the relative agreement between morphology and genetic delimitation among the *A. alnifolia* varieties were revealed among the OTU of (f). Nonetheless, the term 'spicata complex' will be used to refer to the (f) cluster because the polyploidy, apomixis and hybridization which have shaped the canadensis complex have also been reported for taxa in the spicata complex. Apomixis has been reported for *A. stolonifera* (Campbell *et al.*, 1987) and both *A. stolonifera* and *A. spicata* are known to form stoloniferous clumps (Robinson, 1982). Polyploidy for all the taxa in this cluster (Table 2.1), and hybridization among these and other taxa have been reported (Fernald, 1970; Scoggan, 1978). If the spicata complex was formed through hybridization between or among ancestors of the canadensis complex and the *A. alnifolia* varieties, then polyploidy and vegetative reproduction could

maintain these taxa, as argued for the canadensis complex. Although members of the spicata complex have fallen under a variety of names (Table 2.2), given the close genetic relationship indicated by this study, I would suggest placing *A. sanguinea*, *A. spicata*, and *A. stolonifera* as varieties under the name *A. spicata*. Although ovalis-5 consistently clustered with the spicata complex, it and the other *A. ovalis* in this study will be discussed later.

There has been some speculation in the literature as to the species designation of the American cultivars (Table 6.6), and both Jaccard/UPGMA (Figure 6.1) and Wagner parsimony (Figure 6.14) analyses suggest that they are phenetically and phylogenetically most closely related to *A. stolonifera*. However, examination of the PCO analysis (Figure 6.10) reveals that the minimum spanning distance (Appendix C) between stolonifera-8 and Parkhill-48 and between Parkhill-48 and the closest *A. alnifolia* (cv. Honeywood-45) is equidistant at 0.202. This means that Parkhill is approx. 80% similar to both stolonifera and Honeywood. Regent-49 is also 80% similar to the alnifolia and only 78% similar to stolonifera-8, whereas Success is 83% and 73% similar, respectively. These results may indicate that the American cultivars are hybrids between ancestors of *A. alnifolia* and *A. stolonifera*, and tend to support the observation of Wallace and Graham (1976) that the American cultivars may belong to either *A. alnifolia* or *A. stolonifera*. In addition, there are three RAPD markers (RC12-

640, RC19-1660, and RC28-1030) that the prairie *A. alnifolia* share with the American cultivars and are not found in any other taxa. These results suggest a member or ancestor of the prairie *A. alnifolia* as a potential parent of the American cultivars.

Examination of the RAPD markers (Table 6.2) can eliminate potential parents. If a RAPD marker is present in any of the American cultivars, which is not present in two other taxa or complexes, then the combination of those two could not have contributed that particular marker to their potential offspring. In this manner, the parental combinations of clusters involving the prairie *A. alnifolia* (e) and any other cluster have been examined. The markers indicating which cluster combinations could not have hybridized to form the american cultivars are: (a) x (e), markers RC12-1110, RC19-1760, RC19-1450, and RC28-1500; (b) or (g) x (e), markers RC12-1110, RC19-1450, and RC28-1500; (e) x (d), markers RC12-1110 and RC19-1450; and (e) x (h), marker RC12-1110. Thus, hybridization between either (e) and (c) or (e) and (f) could generate the banding pattern found among the American cultivars. However, given that the three analyses discussed earlier suggested a close relationship among the American cultivars, *A. alnifolia* and *A. stolonifera*, then *A. alnifolia* and *A. stolonifera* are most probably the parents. None of the analyses supported the designation of any of the American cvs. as *A. oblongifolia* (McConkey, 1979) or *A. canadensis* (Stushnoff, 1990).

Table 6.6 . Proposed species designation for the cultivars Parkhill, Regent and Success.

	Literature Citation			
Cultivar	Wallace & Graham (1976)	McConkey (1979)	Stushnoff (1990)	Davidson & St. Pierre (1994)
Parkhill	alnifolia or stolonifera	?	alnifolia	sanguinea
Regent	alnifolia or stolonifera	alnifolia	alnifolia	alnifolia
Success	alnifolia or stolonifera	oblongifolia[1]	oblongifolia or canadensis	sanguinea

[1] oblongifolia is a synonym for arborea (Kartesz, 1994).

6.4.6 Ovalis and Canadensis-34

The three ovalis (-4, -5, and -18) in this study did not cluster together, and it is difficult to explain the presence of this European species among *A. alnifolia* and the spicata complex. There is a slim chance that inclusion of ovalis-5 with the spicata complex may be due to the misuse of the binomial *A. ovalis* by many American authors. Jones (1946) indicates that *A. ovalis* sensu Borkhausen, and many other authors, but not Medikus, is synonymous with *A. spicata*, whereas *A. ovalis* Medikus is a European species (Poyarkova, 1939; Tutin *et al.*, 1968). Ovalis-18 was received by the National Plant Germplasm Repository (Corvallis, Oregon) from a botanical garden in Uzbeckistan as open-pollinated seed, but its identity had not been verified by the Repository. Poyarkova (1939) notes that *A. florida* Lindl. (= *A.a.* var. *semiintegrifolia*) can be found in gardens of the former Soviet Union, so possibly this was a mislabelled specimen. Both ovalis-5 and ovalis-18 consistently clustered with *A. spicata* and *A. alnifolia*, respectively, regardless of the method of analysis. On the other hand, ovalis-4, which came from North Dakota State University, appeared to be intermediate between the spicata complex and the varieties of *A. alnifolia* in both the PCO and Wagner parsimony analyses.

Whether or not *A. ovalis* is closely related to *A. alnifolia* or *A. spicata* is interesting for several reasons. If *A. ovalis* is conspecific with *A. spicata* then we might assume that the existence of ovalis/spicata occurred before

the land connections between eastern North America and Europe disappeared some 40 million years ago (Cox and Moore, 1993). On the other hand, if *A. ovalis* is conspecific with *A. alnifolia* then it would support Landry's (1975) suggestion that *A. ovalis* and *A. alnifolia* var. pallida are vicariant species. Numerous species are common to Europe and eastern North America that differ only slightly on each continent (Marie-Victorin, 1938). It has also been suggested that many circumpolar species are polythetic in origin and that many polyploid species have circumboreal or circumpolar distributions (Soltis, P. *et al.*, 1992). Polyploidy has been reported among *A. alnifolia*, *A. spicata*, and *A. ovalis* (Table 2.2). If *A. ovalis* is conspecific with either *A. spicata* or *A. alnifolia* then they would fall under the name 'ovalis' because it was published prior to either of the other two species (Blanchard, 1907; Jones, 1946). Examination of more *A. ovalis* individuals will be necessary to determine their relationship among the other *Amelanchier.*

Similar to ovalis-4, canadensis-34 has clustered among the varieties of *A. alnifolia* or intermediate to *A. alnifolia* and the spicata complex. This specimen was obtained from Robert Simonet (Fort Saskatchewan, AB) via the Morden Research Station. An avid plant breeder and collector, Simonet was known to collect saskatoons (*A. alnifolia*) throughout Western Canada in hopes of improving berry size and quality through interbreeding (Wallace and Graham, 1976). Wallace and Graham (1976) also suggest that

Simonet may have collected *A. florida*, but made no mention of *A. canadensis*. Rydberg (1922, 1932) indicates the presence of *A. canadensis* in Alberta, but not the rest of Western Canada. Most other authors have not reported *A. canadensis* in Western Canada (Boivin, 1967; Looman and Best, 1979; Skoggan, 1957; Taylor, 1973). Possibly canadensis-34 may have been mislabelled in the field, or may be a hybrid between *A. alnifolia* and *A. canadensis*. In either case the morphological characters should be re-examined for verification.

6.5 CONCLUSIONS

The main objective of this study was to use a number of analytical methods to determine the phylogenetic relationship among species of the genus *Amelanchier* based on randomly amplified polymorphic DNA (RAPD) markers. Based on the methods examined in this study the bulk of the species divided into two distinct groups with a third group not clearly associated with either of the other two. The first group, the canadensis complex, consists of the taxa *A. canadensis*, *A. laevis*, *A. arborea*, *A. intermedia*, and *A. x grandiflora*. Morphologically these taxa are considered closely related (Blanchard, 1907; Cruise, 1964; Landry, 1975; McKay, 1973; Robinson and Partanen, 1980; Wiegand, 1912), but the treatment of each as separate species has been questioned (Cruise, 1964; Landry, 1975; Mckay, 1973). In this study, there was little genetic similarity among the

OTU representing these taxa and the OTU did not cluster according to morphological designation. The discordance between morphological and genetic delimitation was attributed to the occurrence of reproductive modes such as hybridization, polyploidy and apomixis among these taxa. The combination of these reproductive modes suggests these taxa form an agamic complex (Robinson, 1982), which is capable of maintaining genetic limits that are wider than the morphological ones (Soltis, P. *et al.*, 1992; Stace, 1989). With regard to the taxa in the canadensis complex, the results presented in this study are best reconciled with the treatment of Cruise (1964) and all of these taxa should be placed as varieties or forms under binomial *A. canadensis*. The results of this study supported Hilton's (1988) suggestion that A. x grandiflora is a hybrid between *A. laevis* and *A. intermedia*.

The second group consisted of the taxa *A. alnifolia*, *A. florida*, *A. cusickii*, *A. oxyodon*, and *A. gaspensis*. Generally, there was more agreement between morphological and genetic delimitation within these taxa relative to those in the canadensis complex. In the past, all of these taxa, except *A. gaspensis*, were considered closely related based on morphological evidence (Hitchcock and Cronquist, 1961; Jones, 1946; Landry, 1975; Scoggan, 1978) and are currently treated as varieties of *A. alnifolia* (Kartesz, 1994). Only Landry (1975) suggested *A. gaspensis*

belonged to this group. The results of this study suggest that *A. gaspensis* be treated as a variety of *A. alnifolia*.

The third group, the spicata complex, consists of the taxa *A. spicata*, *A. sanguinea*, *A. stolonifera*, and three American cultivars. Results from this study suggest that the spicata complex is intermediate to the canadensis complex and the varieties of *A. alnifolia*, and may indicate a hybrid origin for the taxa in the spicata complex. The members of the spicata complex could be maintained as hybrids through polyploidy, apomixis and vegetative reproduction. Based on the results of this study, these three taxa should be considered varieties under the binomial *A. spicata*.

There has been confusion regarding the species designation for American cultivars (Success, Parkhill, and Regent). The results of this study suggest that all three are hybrids between *A. stolonifera* and *A. alnifolia*, and should be designated as such. Morphologically, Wallace and Graham (1976) designated these cultivars as either *A. stolonifera* or *A. alnifolia*, which tends to agree with the genetic analyses presented here.

The relationship of *A. ovalis*, a European species, to the North American species was not clearly established because individuals representing *A. ovalis* clustered among *A. alnifolia* and *A. spicata*, or intermediate to these two.

CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

The main objective of this thesis was to develop and apply RAPD technology for intra- and interspecific characterization within the genus *Amelanchier*. To achieve this objective, protocols were established for the extraction and reliable amplification of *Amelanchier* DNA by the polymerase chain reaction. These protocols were used to generate RAPD markers to distinguish among saskatoon cultivars and to survey genetic relationships among species within the genus *Amelanchier*.

The results of this study indicate that reliable RAPD markers can be developed for the identification of saskatoon cultivars. The majority of saskatoon cultivars were selected from the wild and are maintained vegetatively. The ability to distinguish among these cultivars using RAPD markers is important in terms of maintenance, distribution and identification of genetic variability, which in turn facilitates germplasm evaluation. Saskatoon cultivars are only now being evaluated under different soil and environmental conditions. In the present study, twelve cultivars and two pairs of two cultivars were uniquely characterized by RAPD markers. This is the first report of molecular characterization of saskatoon cultivars.

An immediate application of RAPD technology was the comparison of cultivars from a number of sources. The present study suggests that one source had distributed the cultivar Parkhill under two different names, Parkhill and Regent. Duplication of cultivars could have resulted in years of redundant evaluation in the cultivar trial. In contrast, monitoring of cv. Thiessen clones from five different sources did not reveal any polymorphism.

In the present study, two pair of cultivars were not uniquely characterized. The cultivars Thiessen and Martin were indistinguishable, as were Smoky and Pearson II. One of the limitations of RAPD analysis has been the inability to uniquely characterize cultivars or genotypes in some studies (Halward *et al.*, 1992; Williams and St. Clair, 1993). However, this limitation may be due to the small number of primers examined in each study rather than the technique itself. In other studies RAPD analysis has revealed polymorphism in near isogenic lines by utilizing hundreds of primers (Carland and Staskawicz, 1993; Paran *et al.*, 1991). Although there is no reason to suspect that these two pairs of cultivars are near isogenic lines, they are closely related. Martin and Pearson II were selected from self-pollinated Thiessen and Smoky, respectively. Thus, the examination of more primers should overcome this limitation. Nevertheless, Martin and Pearson II may simply be duplications of their parents. It is possible that each cultivar arose through apomixis, which has

been reported in *Amelanchier*. Examination of additional primers as well as controlled breeding experiments may lend support to this hypothesis.

Future application of RAPD technology could benefit the saskatoon industry in a number of ways such as the identification and isolation of desired genetic traits. For example, a hybrid between the cultivars Nelson and Thiessen has been produced. Subsequently this hybrid could be self-pollinated and the F2 generation monitored for segregation of RAPD markers associated with late versus early flowering times. Nelson flowers later than Thiessen, a trait which may prevent fruit loss due to late spring frosts. Other applications of RAPD technology include the confirmation of hybridization, patenting and registration of new cultivars, and monitoring for crop quality.

Genetic relationships among species within the genus *Amelanchier* were also examined using RAPD analysis. Fifty-six OTU, including 16 saskatoon cultivars, and representing 16 species, were included in this study. Polymorphism was detected and analyzed using both cladistic and phenetic approaches. There was some discordance between morphological and genetic delimitation, but generally, the genetic relationships reported in this study could be supported by morphological observations and the breeding systems reported in the literature for this genus.

There were a number of logistical limitations encountered in this study. Firstly, some researchers and taxonomists were reluctant to

contribute leaf material to this study. This was due mainly to the lack of experience and lack of unique morphological characters to distinguish among local species of *Amelanchier*. Secondly, this study was dependent on contributors to correctly identify submitted samples. As discussed earlier, I was particularly concerned about the origin and identification of the *A. ovalis* samples in this study. Thirdly, a number of North American taxa were not included in this study. Additional taxa from across North America and more samples of the taxa in the study could only enhance our understanding of the phylogeny of this genus. Generally these limitations could be overcome with further studies, which could include more taxa and additional sources of taxa such as *A. ovalis*. Using the protocols established in this study, RAPD markers could be generated for any number of additional taxa and included with the data presented here for re-analysis.

Additionally, there were a number of limitations regarding the suitability of RAPD markers as molecular characters in systematic studies. There was concern that bands migrating the same distance may not be homologous, especially among species (Thormann and Osborn, 1992; Wilkie *et al.*, 1993). However, agreement among phylogenies generated by RAPD analysis and other molecular or morphological data indirectly indicated that the majority of co-migrating bands must be homologous (Demeke *et al.*, 1992; Peltier *et al.*, 1996; van Heusden and Bachmann, 1992), and therefore were suitable for systematic studies. Ideally, the

homology of co-migrating bands should be demonstrated for phylogenetic studies. Homology could be determined using Southern blotting or DNA sequencing, but the cost may be prohibitive considering the 69 RAPD markers and 56 OTU examined in this study.

Genetic relationships among the OTU were determined using phenetic and cladistic approaches. Choosing one approach over another is controversial regardless of character type (eg. molecular, morphological) and all approaches may be limited by the assumptions under which they operate. Phenetic approaches assume that overall similarity represents evolutionary relationships, while cladistic approaches assume that the shortest tree best represents evolution. Specific cladistic methods, such as Wagner parsimony, operate under the assumption of free reversibility of character states, and this may be unreasonable for RAPD data. Also, cladistic methods cannot deal with introgression and reticulation which are commonly occurring phenomenon in the evolution of plants. Nonetheless, three methods of inferring phylogenies (clustering of Jaccard's coefficient of similarity by UPGMA, principal coordinates analysis, and Wagner parsimony) were discussed extensively in this study.

The results of this study suggest that the North American species studied here should be considered varieties within three groups or complexes. The first group, the canadensis complex, consists of the taxa *A. canadensis, A. laevis, A. arborea, A. intermedia*, and *A. x grandiflora*.

The second group consists of the taxa *A. alnifolia, A. florida, A. cusickii, A. oxyodon*, and *A. gaspensis*. The third group, the spicata complex, consists of the taxa *A. spicata, A. sanguinea*, and *A. stolonifera*. Total agreement between this study and any one morphological treatment in the literature was not possible. However, as discussed earlier, there was considerable agreement between Landry's (1975) treatment of *Amelanchier* and this study. Further examination of Landry's (1975) treatment may reveal better morphological characters for the basis of species delimitation.

There has been some confusion regarding the species delimitation for the American cultivars of *A. alnifolia*, Success, Parkhill, and Regent. The results of this study suggest that all three are hybrids between *A. stolonifera* and *A. alnifolia*, and should be designated as such. Morphologically, Wallace and Graham (1976) designated these cultivars as either *A. stolonifera* or *A. alnifolia*, which tends to agree with the genetic analysis presented here.

An understanding of the taxonomy of *Amelanchier* is important with regard to the domestication of the saskatoon. Primarily, plant taxonomy can offer a system of nomenclature which allows for comparative references for names of plants (Jones and Luchsinger, 1986). Additionally, an understanding of taxonomy is useful for the enhancement and development of germplasm. Wild relatives of cultivated plants often provide desirable qualities needed by the plant breeder for crop

improvement (Jones and Luchsinger, 1986). As the development of the saskatoon as a horticultural crop continues, it is conceivable that breeders may look to its wild relatives for various traits such as: (1) insect and disease resistance, (2) fruit characteristics such as increased acidity and fruit size, (3) and reduced plant height and suckering to facilitate cultural mechanization including harvest. In addition, screening for diversity within breeding populations might keep the total population low while maintaining high diversity; this is especially important for perennial crops like the saskatoon, which has a three year period of juvenility and requires large space allocations.

CHAPTER 8

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APPENDIX A

Protocol for the extraction of DNA from *Amelanchier* and other woody plant species.

1. Preheat 10 ml of CTAB buffer [2% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0] in a 50 ml Oakridge centrifuge tube to 65°C. Note: CTAB and 2-mercaptoethanol are added just prior to use.

2. Grind frozen leaf tissue (0.1 to 1.0 g) to a fine powder in liquid nitrogen using a pre-chilled mortar and pestle.

3. Keep powder frozen until transferred into the preheated buffer then shake to mix thoroughly.

4. Incubate at 65°C for 30 min on shaking water bath or shake occasionally during incubation.

5. Remove from water bath and add 1 vol (10 ml) chloroform/isoamylalcohol (24:1;v/v) mixing gently by inverting tube.

6. Centrifuge at 4000 rpm on a Centra 8R centrifuge (swinging bucket rotor) for 5 min at room temperature.

7. Use a pipette to transfer the upper aqueous layer to a clean centrifuge tube. Note: do not get any of the interface layer; if necessary leave behind 10% of the upper layer.

8. Add 2/3 vol (6 ml) cold isopropanol and mix gently to precipitate the nucleic acids. Note: this is often a convenient stopping point. I generally store samples at -20°C for a few hours to overnight. Doyle and Doyle (1990) indicate that samples can be left at room temperature overnight and that this allows further precipitation of nucleic acids.

9a. Generally the nucleic acids can be hooked and spun using a glass pipette or plastic pipette tip, and transferred to a clean tube.
9b. If the nucleic acids are not visible or cannot be hooked with a pipette, then spin the samples for 30 min on a Beckman J2-21 centrifuge using the JA-20 rotor at 10,000 rpm and 4°C. Decant liquid and leave nucleic acid pellet in tube.

10. Wash nucleic acids twice with wash buffer [70% ethanol, 10 mM sodium acetate]. Note: leave nucleic acids in wash buffer as possible stopping point.

11. Decant wash buffer and transfer nucleic acids to 1.5 ml Eppendorf tube, allow nucleic acids to air dry lightly (5 min).

12. Redissolve nucleic acids in 200 ul RNase buffer [10 mM Tris-HCl (pH 8.0), 15 mM NaCl]

13. Add 2 ul RNase (1 ug/ul stock) and incubate at 37°C for 30 min. (I have increased this to 4 ul RNase).

14. Add 100 ul 7.5 M Ammonium acetate and 750 ul ethanol to precipitate the DNA. Note: it may be necessary to add 1 ul 0.25% linear polyacrylamide to aid in DNA precipitation.

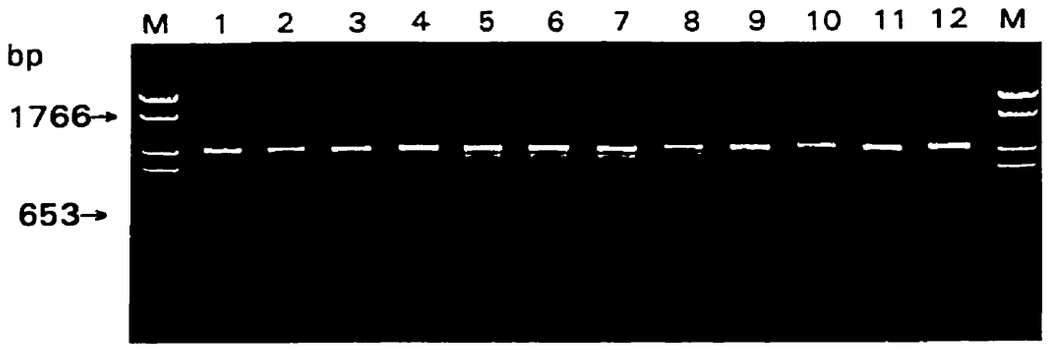
15. Generally easy to decant or pipette off the liquid phase at this stage. However, it may be necessary to spin samples at 13,000 rpm on a refrigerated (4°C) minicentrifuge for 15 min and then decant the liquid phase.

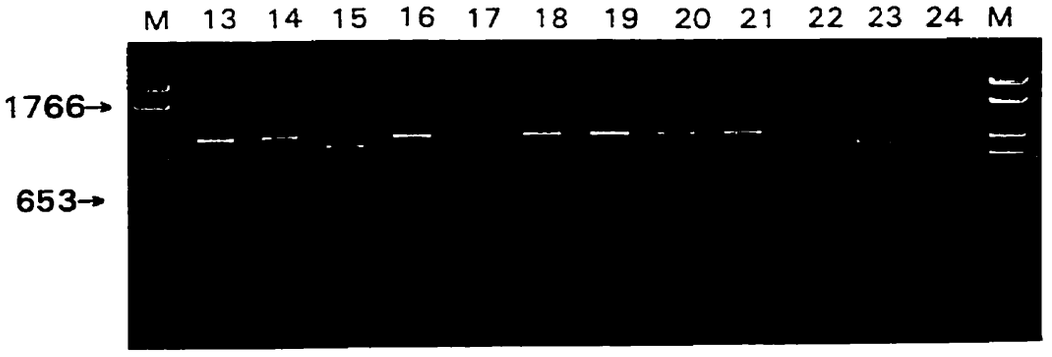
16. Wash 1X in 200 ul wash buffer.

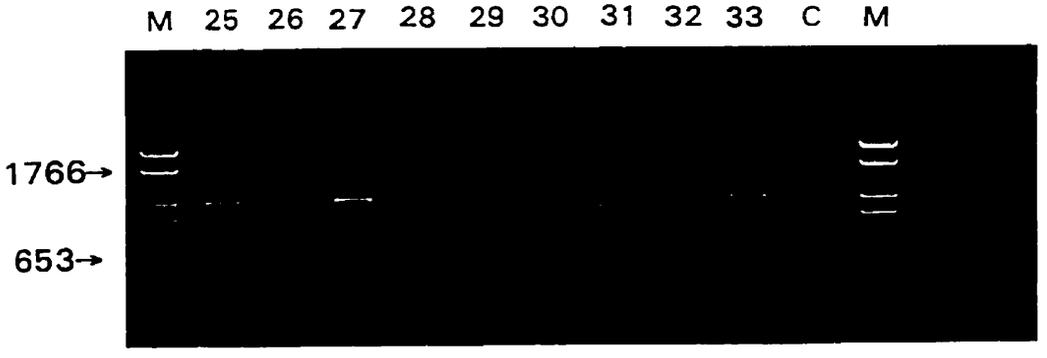
17. Air dry sample for about 10-15 min and then dissolve in sterile water or TE buffer.

APPENDIX B

Figure 1. Randomly amplified polymorphic DNA generated using standard PCR and primer RC-12. Lanes 1 through 41 correspond to the following species: (1) *A. alnifolia* cv. Pembina, (2) *A. gaspensis*, (3) *A. gaspensis*, (4) *A. ovalis*, (5) *A. ovalis*, (6) *A. spicata*, (7) *A. spicata*, (8) *A. stolonifera*, (9) *A. florida*, (10) *A. florida*, (11) *A. alnifolia*, (12) *A. alnifolia*, (13) *A. cusickii*, (14) *A. cusickii*, (15) *A. laevis*, (16) *A. cusickii*, (17) *A. canadensis*, (18) *A. ovalis*, (19) *A. asiatica*, (20) *A. laevis*, (21) *A. canadensis*, (22) *A. intermedia*, (23) *A. x grandiflora*, (24) *A. arborea*, (25) *A. arborea*, (26) *A. intermedia*, (27) *A. bartramiana*, (28) *A. cusickii*, (29) *A. cusickii*, (30) *A. sanguinea*, (31) *A. x grandiflora*, (32) *A. florida*, (33) *A. oxyodon*, (34) *A. canadensis*, (35) *A. canadensis*, (36) *A. intermedia*, (37) *A. intermedia*, (38) *A. laevis*, (39) *A. laevis*, (40) *A. canadensis*, (41) *A. intermedia*. Genomic DNA was omitted in a reaction (lane C) to indicate the presence of primer artifacts. Lane M contains molecular weight marker IV (Boehringer) with fragment size in base pairs (bp) indicated.


M 1 2 3 4 5 6 7 8 9 10 11 12 M
bp
1766
653


M 13 14 15 16 17 18 19 20 21 22 23 24 M
1766
653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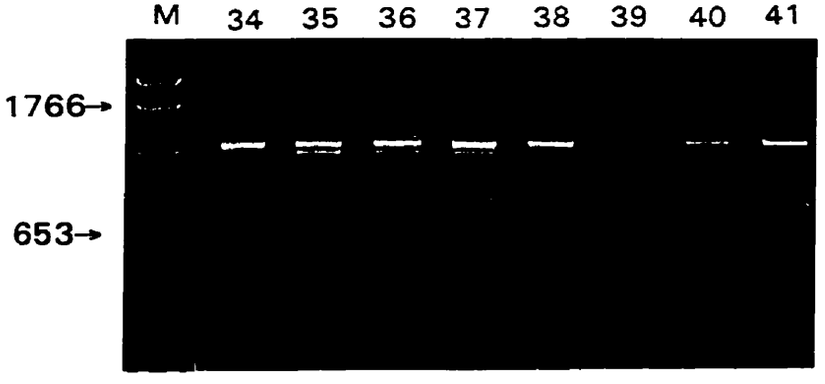
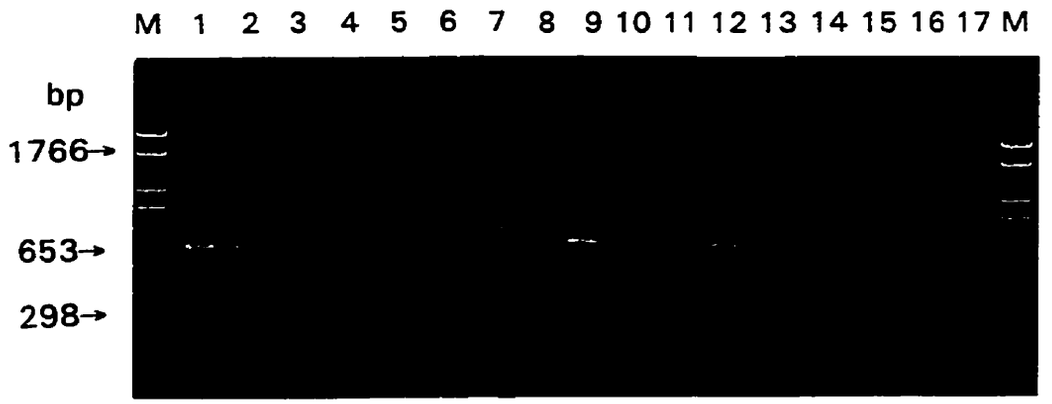
1766
653


M 34 35 36 37 38 39 40 41
1766
653

APPENDIX B

Figure 2. Randomly amplified polymorphic DNA generated using standard PCR and primer RC-16. Lanes 1 through 41 correspond to the following species: (1) *A. alnifolia* cv. Pembina, (2) *A. gaspensis*, (3) *A. gaspensis*, (4) *A. ovalis*, (5) *A. ovalis*, (6) *A. spicata*, (7) *A. spicata*, (8) *A. stolonifera*, (9) *A. florida*, (10) *A. florida*, (11) *A. alnifolia*, (12) *A. alnifolia*, (13) *A. cusickii*, (14) *A. cusickii*, (15) *A. laevis*, (16) *A. cusickii*, (17) *A. canadensis*, (18) *A. ovalis*, (19) *A. asiatica*, (20) *A. laevis*, (21) *A. canadensis*, (22) *A. intermedia*, (23) *A. x grandiflora*, (24) *A. arborea*, (25) *A. arborea*, (26) *A. intermedia*, (27) *A. bartramiana*, (28) *A. cusickii*, (29) *A. cusickii*, (30) *A. sanguinea*, (31) *A. x grandiflora*, (32) *A. florida*, (33) *A. oxyodon*, (34) *A. canadensis*, (35) *A. canadensis*, (36) *A. intermedia*, (37) *A. intermedia*, (38) *A. laevis*, (39) *A. laevis*, (40) *A. canadensis*, (41) *A. intermedia*. Genomic DNA was omitted in a reaction (lane C) to indicate the presence of primer artifacts. Lane M contains molecular weight marker IV (Boehringer) with fragment size in base pairs (bp) indicated.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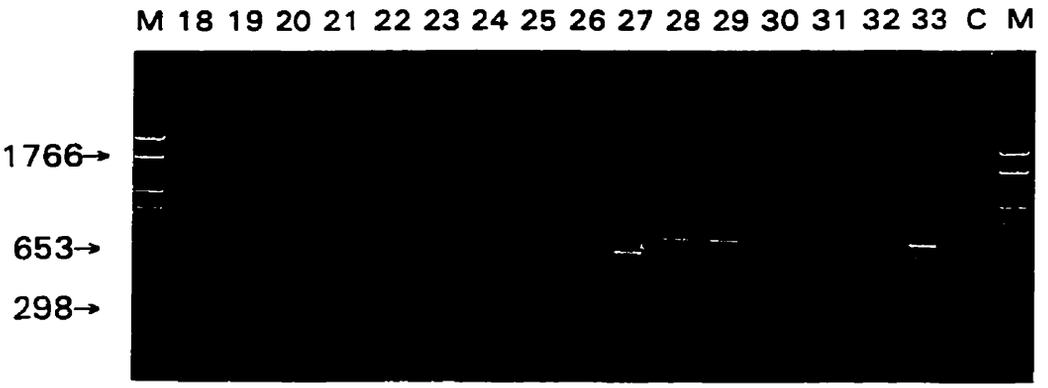
bp
1766
653
298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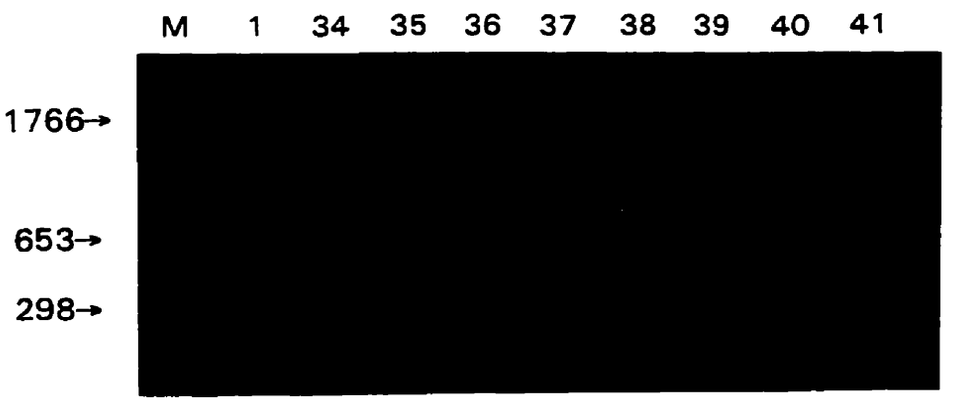
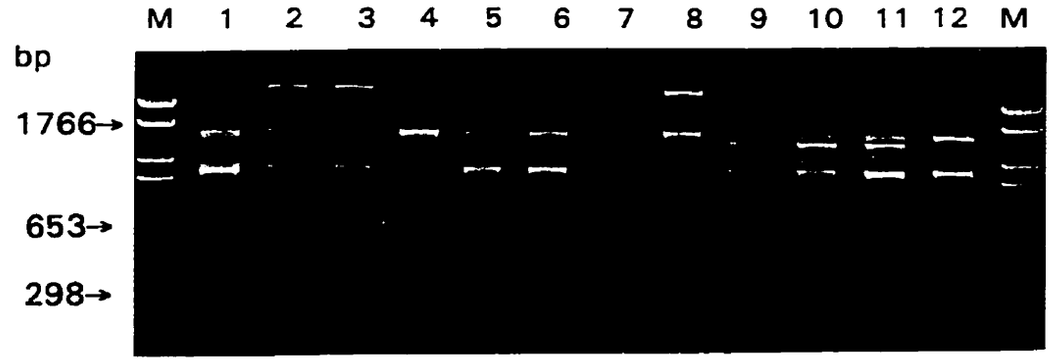
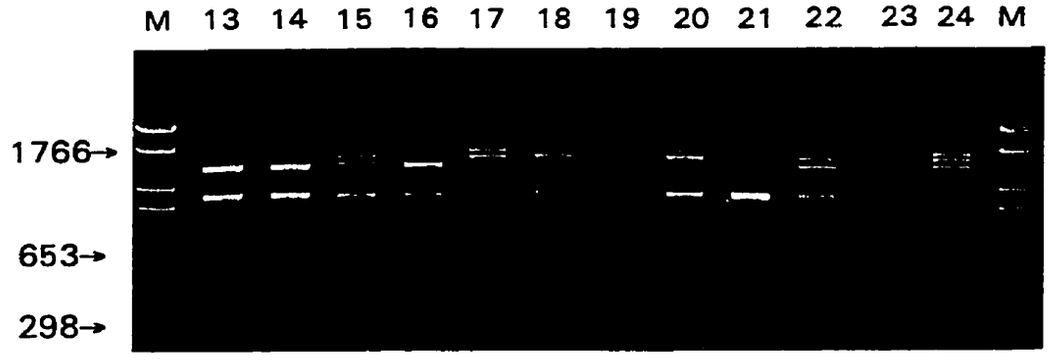
1766
653
298

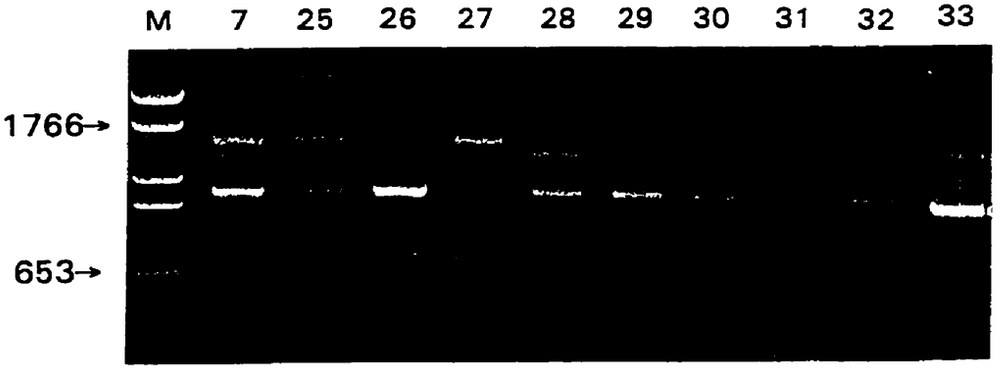

M 1 34 35 36 37 38 39 40 41
1766
653
298

APPENDIX B

Figure 3. Randomly amplified polymorphic DNA generated using standard PCR and primer RC-19. Lanes 1 through 41 correspond to the following species: (1) *A. alnifolia* cv. Pembina, (2) *A. gaspensis*, (3) *A. gaspensis*, (4) *A. ovalis*, (5) *A. ovalis*, (6) *A. spicata*, (7) *A. spicata*, (8) *A. stolonifera*, (9) *A. florida*, (10) *A. florida*, (11) *A. alnifolia*, (12) *A. alnifolia*, (13) *A. cusickii*, (14) *A. cusickii*, (15) *A. laevis*, (16) *A. cusickii*, (17) *A. canadensis*, (18) *A. ovalis*, (19) *A. asiatica*, (20) *A. laevis*, (21) *A. canadensis*, (22) *A. intermedia*, (23) *A. x grandiflora*, (24) *A. arborea*, (25) *A. arborea*, (26) *A. intermedia*, (27) *A. bartramiana*, (28) *A. cusickii*, (29) *A. cusickii*, (30) *A. sanguinea*, (31) *A. x grandiflora*, (32) *A. florida*, (33) *A. oxyodon*, (34) *A. canadensis*, (35) *A. canadensis*, (36) *A. intermedia*, (37) *A. intermedia*, (38) *A. laevis*, (39) *A. laevis*, (40) *A. canadensis*, (41) *A. intermedia*. Genomic DNA was omitted in a reaction (lane C) to indicate the presence of primer artifacts. Lane M contains molecular weight marker IV (Boehringer) with fragment size in base pairs (bp) indicated.


M 1 2 3 4 5 6 7 8 9 10 11 12 M
bp
1766
653
298


M 13 14 15 16 17 18 19 20 21 22 23 24 M
1766
653
298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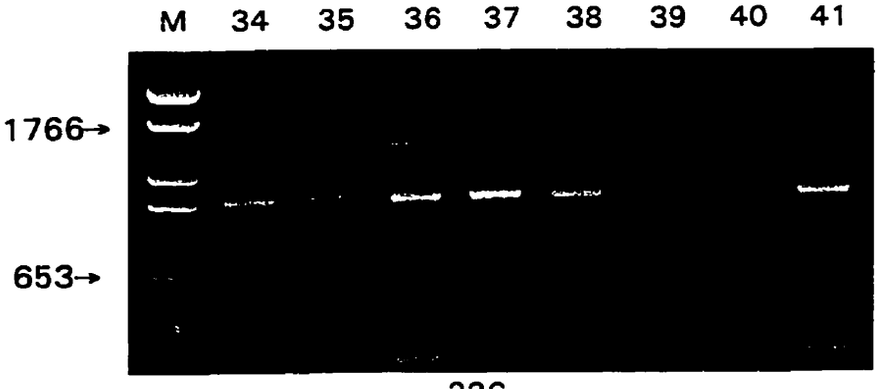
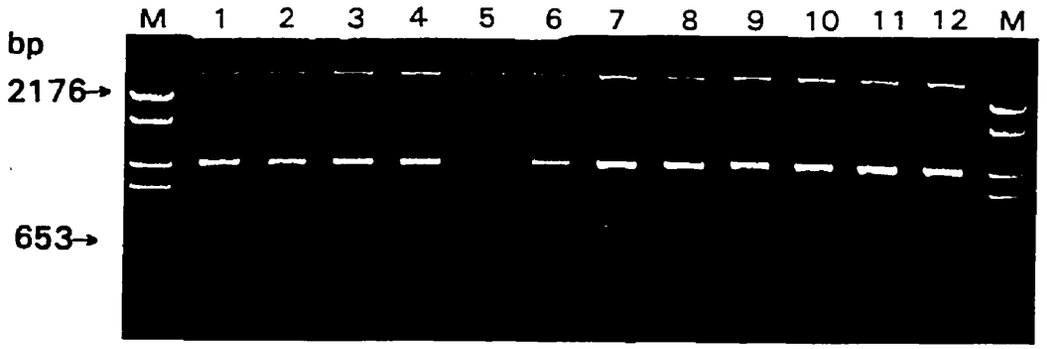
1766
653


M 34 35 36 37 38 39 40 41
1766
653

APPENDIX B

Figure 4. Randomly amplified polymorphic DNA generated using standard PCR and primer RC-22. Lanes 1 through 41 correspond to the following species: (1) *A. alnifolia* cv. Pembina, (2) *A. gaspensis*, (3) *A. gaspensis*, (4) *A. ovalis*, (5) *A. ovalis*, (6) *A. spicata*, (7) *A. spicata*, (8) *A. stolonifera*, (9) *A. florida*, (10) *A. florida*, (11) *A. alnifolia*, (12) *A. alnifolia*, (13) *A. cusickii*, (14) *A. cusickii*, (15) *A. laevis*, (16) *A. cusickii*, (17) *A. canadensis*, (18) *A. ovalis*, (19) *A. asiatica*, (20) *A. laevis*, (21) *A. canadensis*, (22) *A. intermedia*, (23) *A. x grandiflora*, (24) *A. arborea*, (25) *A. arborea*, (26) *A. intermedia*, (27) *A. bartramiana*, (28) *A. cusickii*, (29) *A. cusickii*, (30) *A. sanguinea*, (31) *A. x grandiflora*, (32) *A. florida*, (33) *A. oxyodon*, (34) *A. canadensis*, (35) *A. canadensis*, (36) *A. intermedia*, (37) *A. intermedia*, (38) *A. laevis*, (39) *A. laevis*, (40) *A. canadensis*, (41) *A. intermedia*. Genomic DNA was omitted in a reaction (lane C) to indicate the presence of primer artifacts. Lane M contains molecular weight marker IV (Boehringer) with fragment size in base pairs (bp) indicated.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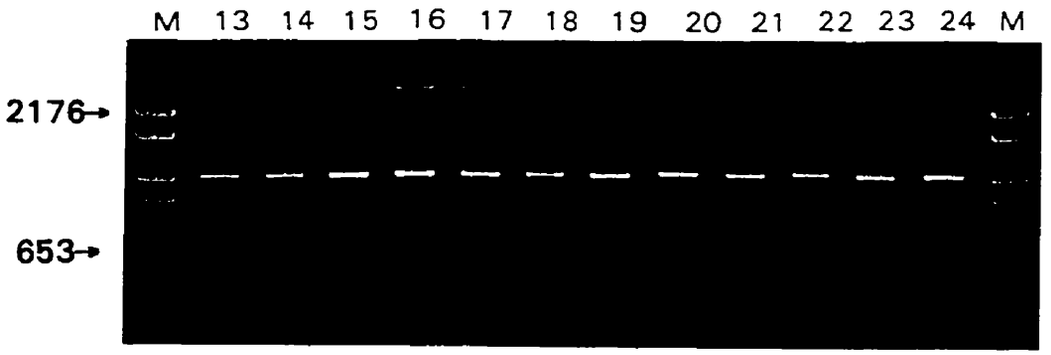
bp
2176
653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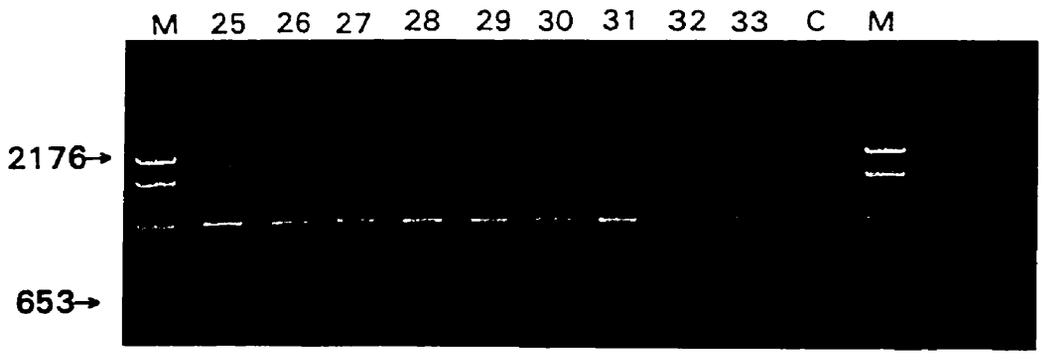
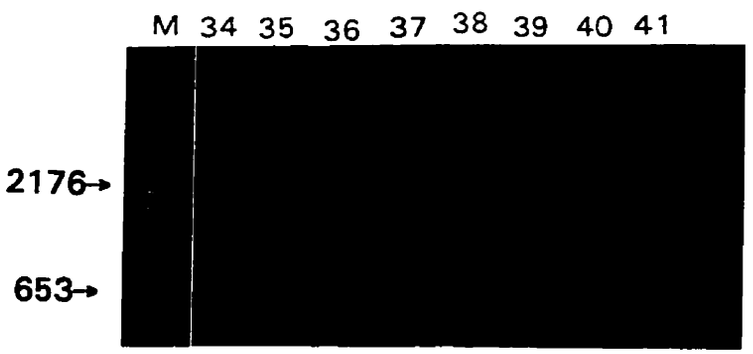
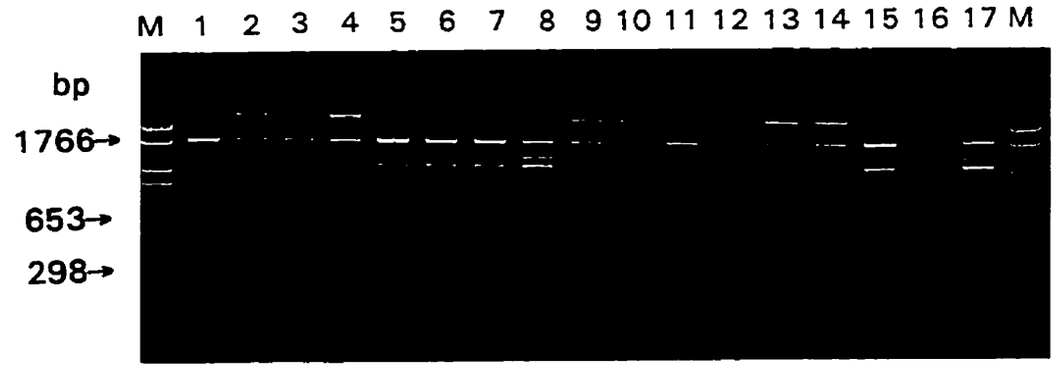
2176
653

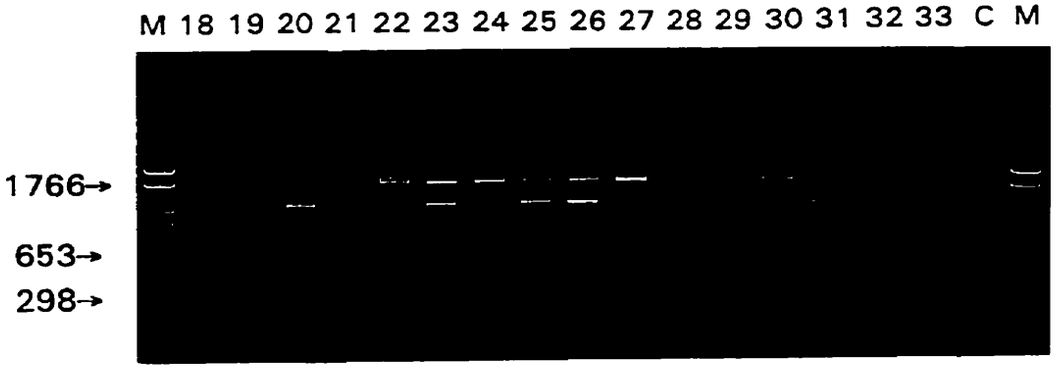

M 25 26 27 28 29 30 31 32 33 C M
2176
653

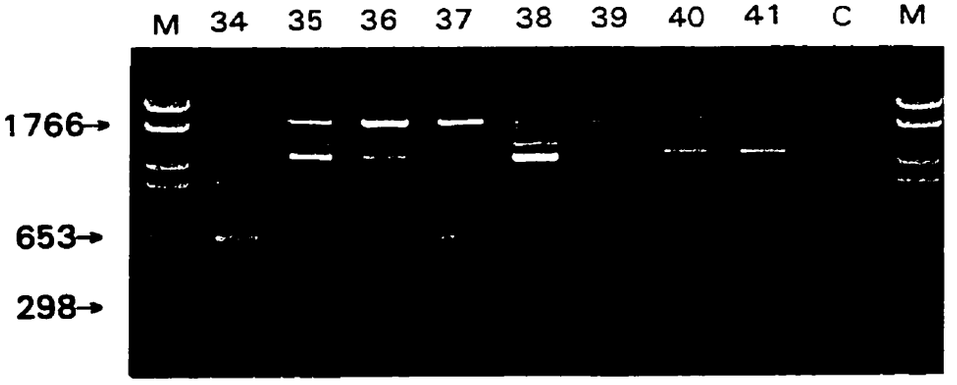

M 34 35 36 37 38 39 40 41
2176
653

APPENDIX B

Figure 5. Randomly amplified polymorphic DNA generated using standard PCR and primer RC-28. Lanes 1 through 41 correspond to the following species: (1) *A. alnifolia* cv. Pembina, (2) *A. gaspensis*, (3) *A. gaspensis*, (4) *A. ovalis*, (5) *A. ovalis*, (6) *A. spicata*, (7) *A. spicata*, (8) *A. stolonifera*, (9) *A. florida*, (10) *A. florida*, (11) *A. alnifolia*, (12) *A. alnifolia*, (13) *A. cusickii*, (14) *A. cusickii*, (15) *A. laevis*, (16) *A. cusickii*, (17) *A. canadensis*, (18) *A. ovalis*, (19) *A. asiatica*, (20) *A. laevis*, (21) *A. canadensis*, (22) *A. intermedia*, (23) *A. x grandiflora*, (24) *A. arborea*, (25) *A. arborea*, (26) *A. intermedia*, (27) *A. bartramiana*, (28) *A. cusickii*, (29) *A. cusickii*, (30) *A. sanguinea*, (31) *A. x grandiflora*, (32) *A. florida*, (33) *A. oxyodon*, (34) *A. canadensis*, (35) *A. canadensis*, (36) *A. intermedia*, (37) *A. intermedia*, (38) *A. laevis*, (39) *A. laevis*, (40) *A. canadensis*, (41) *A. intermedia*. Genomic DNA was omitted in a reaction (lane C) to indicate the presence of primer artifacts. Lane M contains molecular weight marker IV (Boehringer) with fragment size in base pairs (bp) indicated.

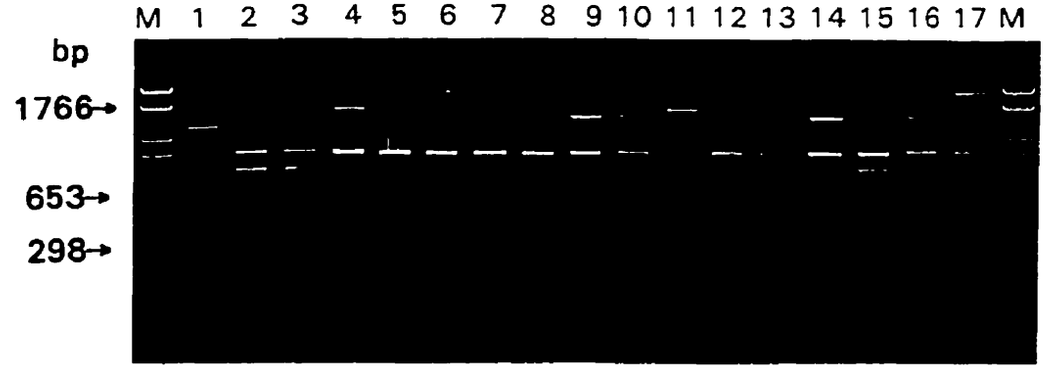

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 M
bp
1766
653
298

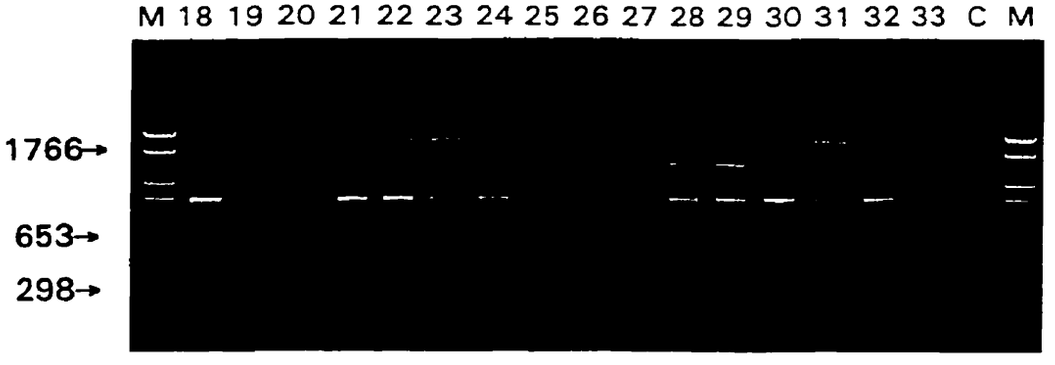

M 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 C M
1766
653
298

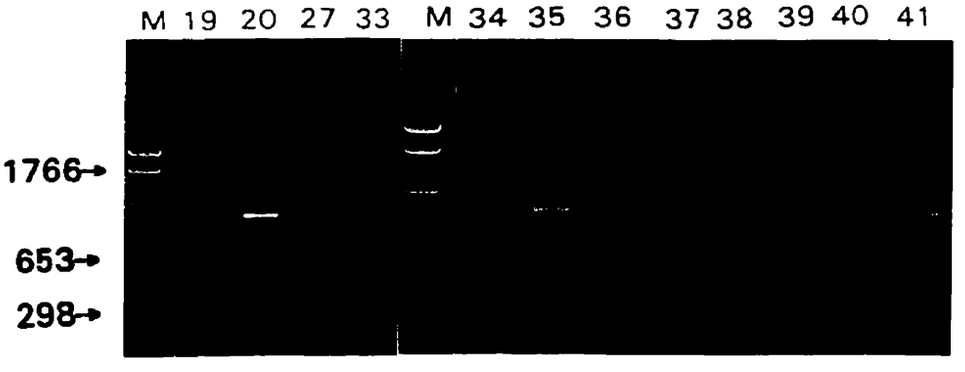

M 34 35 36 37 38 39 40 41 C M
1766
653
298

APPENDIX B

Figure 6. Randomly amplified polymorphic DNA generated using standard PCR and primer RC-30. Lanes 1 through 41 correspond to the following species: (1) *A. alnifolia* cv. Pembina, (2) *A. gaspensis*, (3) *A. gaspensis*, (4) *A. ovalis*, (5) *A. ovalis*, (6) *A. spicata*, (7) *A. spicata*, (8) *A. stolonifera*, (9) *A. florida*, (10) *A. florida*, (11) *A. alnifolia*, (12) *A. alnifolia*, (13) *A. cusickii*, (14) *A. cusickii*, (15) *A. laevis*, (16) *A. cusickii*, (17) *A. canadensis*, (18) *A. ovalis*, (19) *A. asiatica*, (20) *A. laevis*, (21) *A. canadensis*, (22) *A. intermedia*, (23) *A. x grandiflora*, (24) *A. arborea*, (25) *A. arborea*, (26) *A. intermedia*, (27) *A. bartramiana*, (28) *A. cusickii*, (29) *A. cusickii*, (30) *A. sanguinea*, (31) *A. x grandiflora*, (32) *A. florida*, (33) *A. oxyodon*, (34) *A. canadensis*, (35) *A. canadensis*, (36) *A. intermedia*, (37) *A. intermedia*, (38) *A. laevis*, (39) *A. laevis*, (40) *A. canadensis*, (41) *A. intermedia*. Genomic DNA was omitted in a reaction (lane C) to indicate the presence of primer artifacts. Lane M contains molecular weight marker IV (Boehringer) with fragment size in base pairs (bp) indicated.

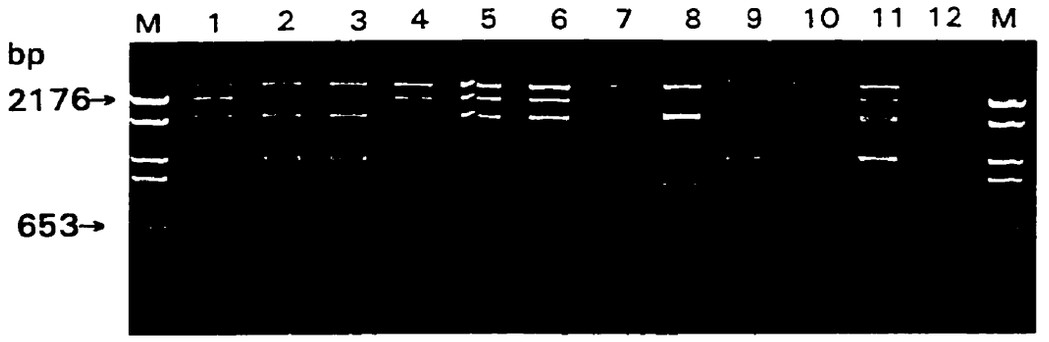

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 M
bp
1766
653
298

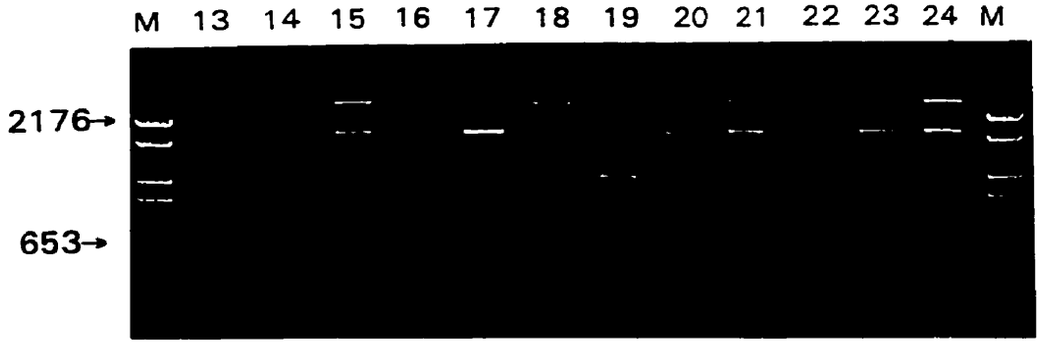

M 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 C M
1766
653
298

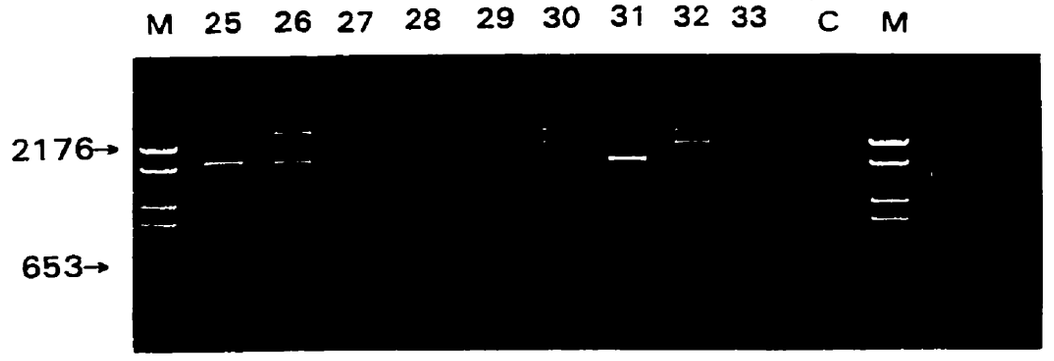

M 19 20 27 33 M 34 35 36 37 38 39 40 41
1766
653
298

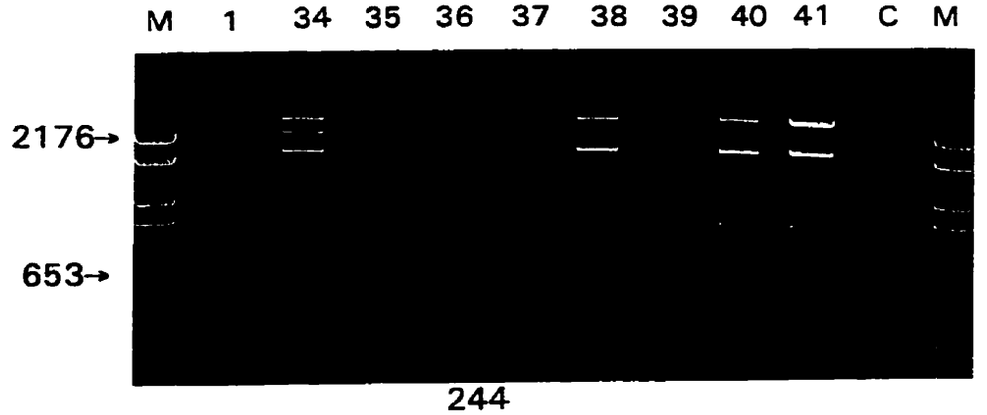
APPENDIX B

Figure 7. Randomly amplified polymorphic DNA generated using standard PCR and primer RC-36. Lanes 1 through 41 correspond to the following species: (1) *A. alnifolia* cv. Pembina, (2) *A. gaspensis*, (3) *A. gaspensis*, (4) *A. ovalis*, (5) *A. ovalis*, (6) *A. spicata*, (7) *A. spicata*, (8) *A. stolonifera*, (9) *A. florida*, (10) *A. florida*, (11) *A. alnifolia*, (12) *A. alnifolia*, (13) *A. cusickii*, (14) *A. cusickii*, (15) *A. laevis*, (16) *A. cusickii*, (17) *A. canadensis*, (18) *A. ovalis*, (19) *A. asiatica*, (20) *A. laevis*, (21) *A. canadensis*, (22) *A. intermedia*, (23) *A. x grandiflora*, (24) *A. arborea*, (25) *A. arborea*, (26) *A. intermedia*, (27) *A. bartramiana*, (28) *A. cusickii*, (29) *A. cusickii*, (30) *A. sanguinea*, (31) *A. x grandiflora*, (32) *A. florida*, (33) *A. oxyodon*, (34) *A. canadensis*, (35) *A. canadensis*, (36) *A. intermedia*, (37) *A. intermedia*, (38) *A. laevis*, (39) *A. laevis*, (40) *A. canadensis*, (41) *A. intermedia*. Genomic DNA was omitted in a reaction (lane C) to indicate the presence of primer artifacts. Lane M contains molecular weight marker IV (Boehringer) with fragment size in base pairs (bp) indicated.


M 1 2 3 4 5 6 7 8 9 10 11 12 M
bp
2176→
653→

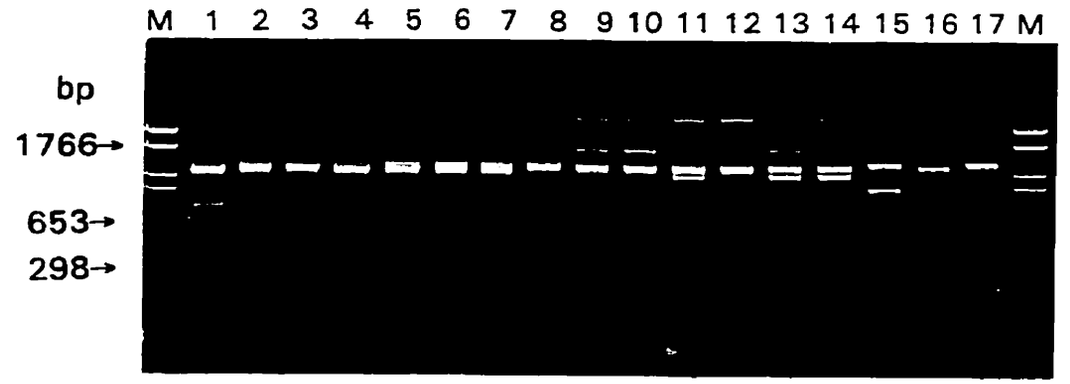

M 13 14 15 16 17 18 19 20 21 22 23 24 M
2176→
653→

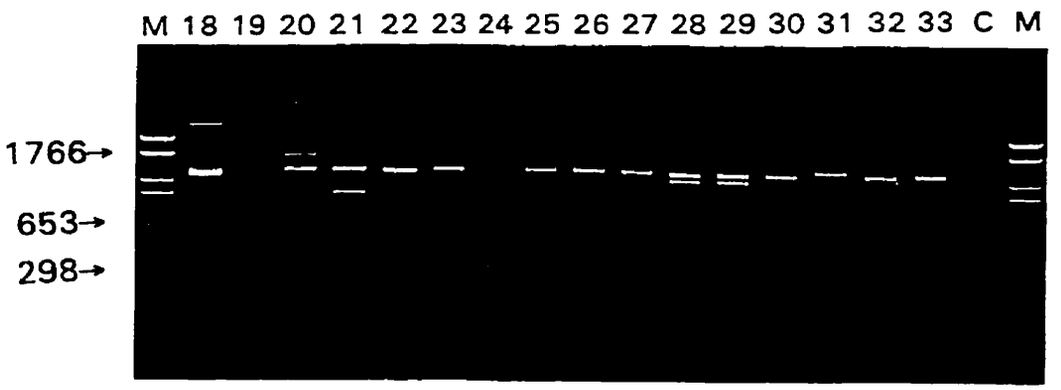

M 25 26 27 28 29 30 31 32 33 C M
2176→
653→

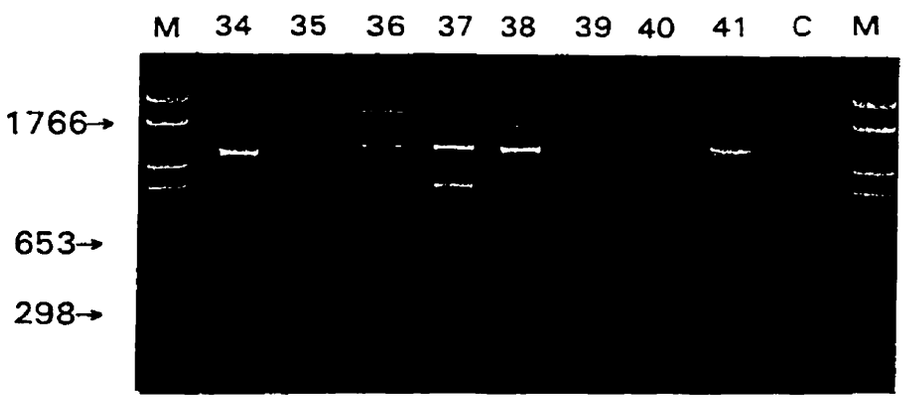

M 1 34 35 36 37 38 39 40 41 C M
2176→
653→

APPENDIX B

Figure 8. Randomly amplified polymorphic DNA generated using standard PCR and primer RC-37. Lanes 1 through 41 correspond to the following species: (1) *A. alnifolia* cv. Pembina, (2) *A. gaspensis*, (3) *A. gaspensis*, (4) *A. ovalis*, (5) *A. ovalis*, (6) *A. spicata*, (7) *A. spicata*, (8) *A. stolonifera*, (9) *A. florida*, (10) *A. florida*, (11) *A. alnifolia*, (12) *A. alnifolia*, (13) *A. cusickii*, (14) *A. cusickii*, (15) *A. laevis*, (16) *A. cusickii*, (17) *A. canadensis*, (18) *A. ovalis*, (19) *A. asiatica*, (20) *A. laevis*, (21) *A. canadensis*, (22) *A. intermedia*, (23) *A. x grandiflora*, (24) *A. arborea*, (25) *A. arborea*, (26) *A. intermedia*, (27) *A. bartramiana*, (28) *A. cusickii*, (29) *A. cusickii*, (30) *A. sanguinea*, (31) *A. x grandiflora*, (32) *A. florida*, (33) *A. oxyodon*, (34) *A. canadensis*, (35) *A. canadensis*, (36) *A. intermedia*, (37) *A. intermedia*, (38) *A. laevis*, (39) *A. laevis*, (40) *A. canadensis*, (41) *A. intermedia*. Genomic DNA was omitted in a reaction (lane C) to indicate the presence of primer artifacts. Lane M contains molecular weight marker IV (Boehringer) with fragment size in base pairs (bp) indicated.


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 M
bp
1766
653
298


M 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 C M
1766
653
298


M 34 35 36 37 38 39 40 41 C M
1766
653
298

APPENDIX C

Manhattan distance coefficient for the 56 *Amelanchier* OTU. The OTU were input in the following order: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 1, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56. Numbers correspond to those given in Table 6.1 of this thesis. Distance is given between OTU such that the first line indicates a distance of 0.0000000 between OTU 2 and itself. The second line indicates a distance of 0.144928 between OTU 3 and OTU 2, and a distance of 0.0000000 between OTU 3 and itself, etc.

0.0000000
0.0144928 0.0000000
0.3043478 0.3188406 0.0000000
0.4057971 0.3913043 0.2173913 0.0000000
0.4202899 0.4057971 0.2318841 0.0144928 0.0000000
0.4347826 0.4202899 0.2753623 0.1159420 0.1014493
0.0000000
0.3188406 0.3043478 0.1884058 0.2318841 0.2173913
0.2608696 0.0000000
0.4492754 0.4347826 0.3188406 0.3333333 0.3478261
0.3333333 0.4202899 0.0000000
0.4347826 0.4202899 0.3333333 0.3478261 0.3623188
0.3478261 0.4347826 0.0144928 0.0000000
0.2898551 0.3043478 0.2463768 0.2898551 0.3043478
0.3188406 0.3478261 0.2463768 0.2318841 0.0000000
0.3478261 0.3333333 0.1884058 0.2898551 0.3043478
0.2898551 0.2608696 0.2463768 0.2318841 0.1449275
0.0000000
0.4347826 0.4492754 0.2753623 0.3478261 0.3623188
0.3478261 0.3768116 0.1304348 0.1159420 0.2028986
0.2318841 0.0000000
0.4347826 0.4492754 0.2753623 0.3478261 0.3623188
0.3478261 0.3768116 0.1304348 0.1159420 0.2028986
0.2318841 0.0000000 0.0000000
0.4347826 0.4202899 0.3623188 0.3188406 0.3333333
0.3768116 0.3478261 0.4202899 0.4057971 0.4057971
0.4347826 0.3478261 0.3478261 0.0000000
0.3623188 0.3768116 0.3478261 0.3043478 0.3188406
0.3043478 0.3333333 0.2028986 0.1884058 0.2463768
0.3043478 0.1594203 0.1594203 0.3623188 0.0000000
0.3913043 0.3768116 0.3478261 0.3623188 0.3768116
0.3333333 0.3623188 0.4927536 0.4782609 0.4492754

0.4202899 0.4202899 0.4202899 0.3043478 0.4347826
0.0000000
0.3188406 0.3043478 0.2173913 0.2898551 0.2753623
0.2898551 0.2898551 0.2463768 0.2318841 0.1449275
0.1159420 0.2608696 0.2608696 0.4637681 0.2463768
0.4202899 0.0000000
0.4347826 0.4492754 0.3333333 0.4057971 0.4202899
0.4057971 0.3768116 0.4202899 0.4057971 0.3478261
0.4057971 0.3188406 0.3188406 0.3768116 0.3913043
0.4202899 0.3768116 0.0000000
0.3768116 0.3623188 0.3333333 0.2898551 0.3043478
0.3188406 0.2898551 0.3333333 0.3188406 0.3478261
0.3478261 0.2898551 0.2898551 0.2608696 0.2753623
0.3623188 0.3188406 0.3188406 0.0000000
0.3913043 0.3768116 0.3768116 0.3623188 0.3478261
0.3913043 0.2463768 0.4347826 0.4202899 0.4492754
0.3913043 0.3623188 0.3623188 0.2173913 0.3188406
0.4057971 0.3913043 0.3043478 0.2173913 0.0000000
0.3768116 0.3913043 0.3333333 0.3478261 0.3623188
0.3188406 0.2898551 0.3333333 0.3188406 0.3478261
0.3478261 0.2608696 0.2608696 0.2608696 0.2463768
0.3043478 0.3768116 0.2608696 0.2318841 0.2173913
0.0000000
0.3913043 0.4057971 0.2898551 0.2753623 0.2608696
0.2753623 0.2753623 0.4347826 0.4202899 0.3913043
0.4202899 0.3333333 0.3333333 0.2173913 0.3478261
0.2318841 0.3913043 0.3623188 0.2463768 0.2898551
0.2463768 0.0000000
0.3333333 0.3188406 0.3188406 0.3333333 0.3478261
0.3333333 0.3043478 0.2898551 0.2753623 0.3333333
0.3043478 0.2463768 0.2463768 0.2463768 0.2898551
0.2898551 0.3623188 0.3623188 0.2173913 0.2608696
0.2173913 0.2608696 0.0000000
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0.3623188 0.2463768 0.3478261 0.3333333 0.3623188
0.3333333 0.2753623 0.2753623 0.1594203 0.3188406
0.2608696 0.3333333 0.2753623 0.1884058 0.2028986
0.2173913 0.1739130 0.1739130 0.0000000
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0.2753623 0.2463768 0.4057971 0.3913043 0.3913043
0.3913043 0.3043478 0.3043478 0.2173913 0.3188406
0.2608696 0.3623188 0.3043478 0.1884058 0.2028986
0.1884058 0.1159420 0.2028986 0.1159420 0.0000000
0.3768116 0.3623188 0.3623188 0.3478261 0.3623188

0.4057971 0.3188406 0.4202899 0.4057971 0.3768116
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0.3913043 0.3478261 0.3188406 0.2608696 0.2463768
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0.0000000
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0.2318841 0.0000000 0.0000000 0.3478261 0.1594203
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0.2608696 0.3333333 0.2463768 0.2753623 0.3043478
0.3768116 0.0000000
0.4347826 0.4492754 0.2753623 0.3478261 0.3623188
0.3478261 0.3768116 0.1304348 0.1159420 0.2028986
0.2318841 0.0000000 0.0000000 0.3478261 0.1594203
0.4202899 0.2608696 0.3188406 0.2898551 0.3623188
0.2608696 0.3333333 0.2463768 0.2753623 0.3043478
0.3768116 0.0000000 0.0000000
0.3478261 0.3333333 0.2463768 0.1739130 0.1594203
0.1739130 0.2028986 0.3333333 0.3188406 0.2898551
0.2898551 0.2898551 0.2898551 0.3478261 0.2753623
0.3623188 0.2318841 0.3188406 0.2898551 0.2753623
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0.4347826 0.3478261 0.3478261 0.2028986 0.3623188
0.2463768 0.4057971 0.3478261 0.2318841 0.2463768
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0.3333333 0.3333333 0.2608696 0.2753623 0.1594203
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0.0000000
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0.1884058 0.2463768 0.0000000

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0.2028986 0.2318841 0.2463768 0.0000000
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0.4202899 0.4637681 0.4637681 0.4347826 0.4637681
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0.2608696 0.3188406 0.2463768 0.3913043 0.4057971
0.3623188 0.4202899 0.3478261 0.4202899 0.4492754
0.0000000
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0.4492754 0.4347826 0.4637681 0.4347826 0.4637681
0.3623188 0.4202899 0.4202899 0.3333333 0.4492754
0.3188406 0.2898551 0.2753623 0.3623188 0.4057971
0.3913043 0.4202899 0.3478261 0.3913043 0.4492754
0.2898551 0.0000000
0.3478261 0.3333333 0.2173913 0.2608696 0.2753623
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0.3478261 0.3478261 0.3478261 0.2898551 0.4057971
0.3043478 0.3043478 0.1739130 0.2608696 0.3623188
0.2898551 0.3188406 0.2753623 0.3478261 0.3768116
0.2173913 0.1884058 0.0000000
0.3913043 0.3768116 0.3188406 0.3623188 0.3768116

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0.4637681 0.3043478 0.4782609 0.4782609 0.4927536
0.4782609 0.4927536 0.4057971 0.4347826 0.4927536
0.4202899 0.4202899 0.4202899 0.4202899 0.5072464
0.3768116 0.3188406 0.2753623 0.3913043 0.4927536
0.4492754 0.4202899 0.4347826 0.4782609 0.5072464
0.2028986 0.2028986 0.2173913 0.0000000
0.3768116 0.3623188 0.2753623 0.3478261 0.3623188
0.3478261 0.2898551 0.3623188 0.3768116 0.2898551
0.2608696 0.3478261 0.3478261 0.4347826 0.3623188
0.4782609 0.2898551 0.4057971 0.4057971 0.3913043
0.3768116 0.4782609 0.4782609 0.4202899 0.4782609
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