Chapter 2
Petunia as a Model System for the Genetics and Evolution of Pollination Syndromes

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Abstract In recent years Petunia has become a promising model system for studying the genetics and evolution of pollination syndromes. Here we provide a brief introduction to the issue of pollination syndromes, explain why Petunia is a suitable model for its study, present useful background information about pollinators and plants, review recent studies, and discuss questions related to the genetics and evolution of Petunia pollination syndromes.

2.1 Petunia as a Model for the Evolution of Pollination Syndromes

Flowering plants often feature pollination syndromes, sets of traits such as flower color, morphology, scent and nectar production, which appear to fit the morphology and behavior of specific pollinator types (Faegri and van der Pijl 1979). The general correspondence of floral traits and pollinators makes it possible to predict which pollinators are attracted to a given flower phenotype. The genus *Petunia* features three pollination syndromes: bee, hawkmoth, and hummingbird pollination. The bee-pollinated Petunia species, such as *Petunia integrifolia*, feature purple flowers with a wide floral tube into which bees crawl in order to reach pollen and nectar. These bee-pollinated flowers produce little nectar and hardly any scent. The hawkmoth-pollinated *Petunia axillaris* flowers feature white corollas with narrow tubes, allowing only nectar feeders with long probosces to reach the abundant nectar. Characteristic for hawkmoth-pollinated flowers, *P. axillaris* flowers produce large amounts of odor after dusk. *Petunia exserta* flowers bear the hallmarks of hummingbird pollination: the flower corolla is red, the petal limbs reflexed, anthers and style exserted, and the flowers emit no detectable scent. Similar to hawkmoth-pollinated flowers, *P. exserta* flowers have a long tube carrying copious amounts of nectar.

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Published phylogenies (Ando et al. 2005; Kulcheski et al. 2006) suggest that bee pollination in Petunia species such as *P. integrifolia* represents the ancestral state in the genus and that hawkmoth pollination in *P. axillaris* evolved from bee pollination in the *P. axillaris* ancestor. Nuclear DNA sequences suggest that hummingbird pollination in *P. exserta* has evolved from hawkmoth pollination (unpublished data).

2.1.1 The Complexity of Pollination Syndromes

It is generally thought that selection by pollinators drives floral evolution and also explains evolutionary shifts in pollination syndromes. The underlying idea is that plants increase their fitness by adapting to the most efficient (and abundant) pollinator, that is, the pollinator that transfers the most pollen to conspecific flowers. Despite the frequently observed match of pollinators and flower types, there are some controversies surrounding pollination syndromes. First, the association of a single plant species and a single pollinator is rarely as exclusive as is found in some orchid species. In fact, flowers corresponding to a particular pollination syndrome are sometimes found to be visited by other (non-specialist) pollinators. In some cases the expected specialist pollinator, deduced from a pollination syndrome, may be rare or even absent and other (non-specialist) pollinators may be more important for pollination (Herrera 1996). Besides such empirical criticism, evolutionary theory predicts that extreme dependence of a plant species on one or a few pollinators always carries a risk of extinction, because if pollinators decline, so will the adapted plant species (Waser, Chittka, Price, Williams, and Ollerton 1996; Johnson and Steiner 2000; Fenster, Armbruster, Wilson, Dudash, and Thompson 2004). Furthermore, in addition to pollinators, other agents such as nectar robbers, herbivores, or pathogens may interact with floral traits and affect the evolution of these traits (Fenster et al. 2004). Despite these caveats, the pollination syndrome concept is useful for generating hypotheses about likely pollinators and the adaptive value of floral traits in relation to these pollinators.

2.1.2 Phenotypic and Genetic Approaches to Pollination Syndromes

Major aims for the study of pollination syndromes are to demonstrate the adaptive value of floral traits and to elucidate how these traits have come about. The adaptive value of pollination traits can be tested in two ways: by phenotypic manipulation or by genetic approaches. For decades ecologists have manipulated natural or artificial flowers in order to test the attractiveness of particular floral features to pollinators, that is, altering the size of attractive petals or bracts (Fenster et al. 2004). An alternative approach to produce phenotypes for testing is to cross taxa displaying divergent pollination traits. This approach allows for the establishment of lines carrying chromosomal fragments determining the phenotype of one taxon in the

genetic background of another. Usually near-homogeneous lines are obtained from F2 or backcross (BC) populations by selfing individual lines for several generations (usually $n > 4$) generating recombinant inbred lines (RIL) or backcross inbred lines (BIL).

Genetic compatibility of plant taxa also allows for the genetic dissection of intra- or interspecific quantitative differences by quantitative trait locus (QTL) mapping. For this, a segregating population derived from an initial cross of the two parental lines of interest is phenotypically and genetically characterized. First, a genetic linkage map is established from the genotypic marker data, followed by a test of association between genetic markers and phenotypic values. The QTL approach takes the linkage of markers into account in order to estimate the contribution of chromosomal regions to the phenotypic variation in the segregating population. QTL analysis can also provide estimates of the size effect and the number of loci contributing to the variation in a trait. This is of particular interest for the genetic dissection of pollination syndromes. Determining the number of genes involved and their quantitative effects is critical for understanding the evolutionary transition from one pollination syndrome to another. Initial QTL analysis can be followed by positional cloning of genes underlying trait differences. Cloning of these genes will allow for unraveling of the molecular basis and the evolution of phenotypic variation. Finally, transgenic experiments may allow for the manipulation of single genes to test their phenotypic effects.

2.1.3 Petunia as a Model System for Studying Natural Variation

Among other emerging model systems for the study of pollination syndromes, such as *Ipomoea, Aquilegia, Penstemon* and *Mimulus*, *Petunia* occupies a special role, as it is, at present, the only genus that is genetically accessible, with several available genetic tools (Galliot, Stuurman, and Kuhlemeier 2006a). Among other traits, a relatively short generation time, ease of culturing, and particularly, the availability of transposon tagging (see Chapter 17) and genetic transformation (see Chapter 19), make Petunia an excellent system for studying the genetics of phenotypic traits. Transposon tagging is usually performed in a *Petunia hybrida* background (W138) that has about 200 active copies of the transposon *dTph1* (Gerats et al. 1990). QTL from wild species can be tagged with this system by introgression into this *P. hybrida* background. Hence, BIL produced from introgressing *P. axillaris parodii* S7 or *P. integrifolia inflata* S6 chromosomal fragments into a W138 *P. hybrida* background provide a resource for gene isolation (Stuurman et al. 2004). Further, *dTph1* activity can be almost entirely shut down by introgression of a *P. integrifolia inflata* chromosomal fragment on chromosome I, allowing for the stabilization of tagged mutants (Stuurman and Kuhlemeier 2005).

The possibility of testing the preference of pollinators for specific flower traits is essential for establishing the adaptiveness of these traits. In Petunia at least one natural pollinator, the hawkmoth *Manduca sext*a, can be reared in the laboratory and is commercially available.

2.2 Evolution of Hawkmoth from Bee Pollination Syndrome

Flowers representing bee and hawkmoth pollination syndromes differ markedly in flower morphology, color, scent, and nectar production. Bee-pollinated flowers tend to have shorter and wider corolla openings than hawkmoth-pollinated flowers, which often feature long corolla tubes. Many bee-pollinated flowers appear morphologically less specialized than most hawkmoth-pollinated flowers; that is, their morphology allows a broader spectrum of pollinators to access pollen and nectar.

P. integrifolia and *P. axillaris* correspond to this pattern of trait differences in bee and hawkmoth pollination syndromes. Here, we focus on *P. integrifolia* (subspp. *integrifolia* and *inflata*) representing bee pollination and *P. axillaris* (subspp. *axillaris and parodii*) representing hawkmoth pollination. Although *P. integrifolia* and *P. axillaris* may occur in sympatry, and cross-pollination by hand produces viable seed, the failure to observe natural hybrids suggests that one or more prezygotic isolation barriers exist (Ando et al. 2001). Ethological isolation (due to visitation by different pollinators) is one likely factor contributing to this isolation barrier.

P. integrifolia is primarily visited by solitary bee species, in particular by species of *Callonychium*, *Calliopsis,* and *Leioproctus* (Figs. 2.1 and 2.2). The *Calliopsis*

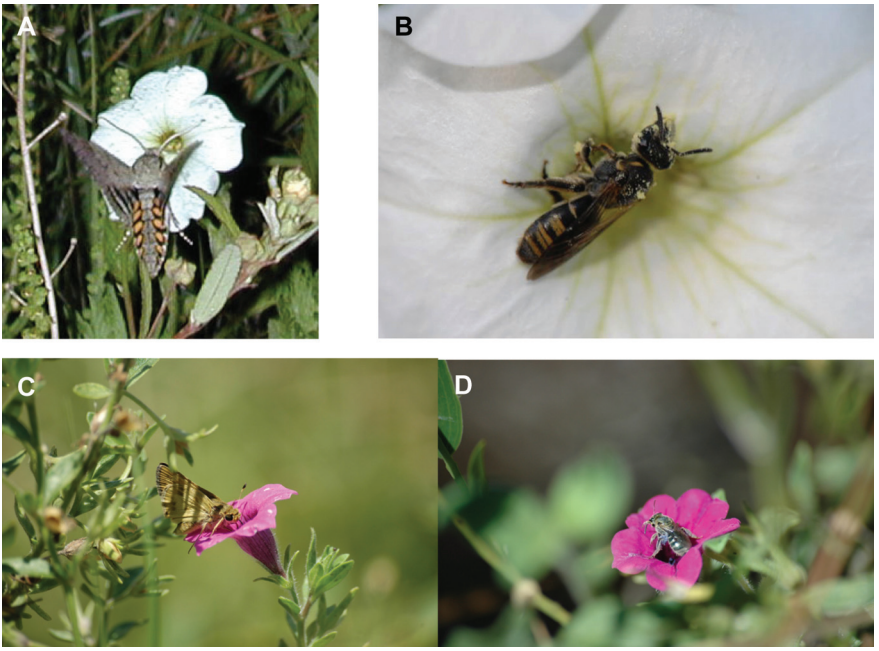

Fig. 2.1 Pollinators of *P. axillaris* and *P. integrifolia* in the wild in Uruguay. (**A**) A nocturnal sphinx moth (*Manduca* spp.) nectar feeding on *P. axillaris*: (**B**) An unidentified solitary bee collecting pollen on *P. axillaris*: (**C**) A diurnal butterfly (*Hylephila phyleus*) probing for nectar on *P. integrifolia*, (**D**) A solitary bee collecting pollen on *P. integrifolia*

Fig. 2.2 *P. integrifolia* flowers are used by some solitary bees as mating sites. Here a male and female *Calliopsis* sp. are seen *in copula*. Note the color match between eyes of the bees and anthers of the flower

species observed on *P. integrifolia* in Uruguay is a small solitary bee that uses Petunia flowers not only to feed on nectar and pollen but also as mating places (Fig. 2.2). We often observed males waiting inside the floral tube and then attempting to mate with a female as soon as she landed, suggesting that the Petunia flowers function not only as a feeding resource but also as a rendézvous site for the bees. Similar behavior was observed in *Callonychium* species on purple-flowering *Petunia* species in Brazil (Wittmann, Radtke, Cure, and Schifino-Wittmann 1990).

These observations indicate a strong mutualism between these solitary bee species and some purple Petunia species. *P. axillaris* is nocturnally visited by sphingid moths, in Uruguay, mainly *M. sexta* and *Eumorpha vitis* (Table 2.1), and diurnally by solitary bees (mainly *Halictus* spp.). Day and night pollinator exclusion experiments have shown that *P. axillaris* is effectively pollinated by both diurnal and nocturnal pollinators (Hoballah et al. 2007), suggesting that at least in some habitats bee pollinators can be also effective pollinators of *P. axillaris*. However, the sets of bee species observed on *P. axillaris* and *P. integrifolia* differed markedly (Hoballah et al. 2007), suggesting the species are visited by different bee species. In this context it is worth noting that the abundance of pollinators may vary spatially and temporally. Observation of pollinators in an artificial population of both Petunia species in Uruguay in 2007 showed that some bee species visit both flower types (unpublished results). More observational data, in particular from natural sympatric populations, will be required to determine the extent to which both Petunia species are visited by the same sets of bee species.

Whether bees and hawkmoths have an innate preference for either flower type was tested under controlled conditions in the greenhouse. Naïve *Bombus terrestris* (although not a natural pollinator of Petunia) and naïve *M. sexta* were given the choice of *P. axillaris* or *P. integrifolia* in a greenhouse. The bumblebees clearly preferred to feed on flowers of *P. integrifolia* species and the hawkmoth *M. sexta* clearly preferred *P. axillaris* flowers (Hoballah et al. 2007).

Table 2.1 Species and locations of flower visitors collected from *P. axillaris* and *P. integrifolia* flowers in the natural habitat in Uruguay. Abbreviations for the collection locations: R: Rivera; LC: Las Cañas; PV: Puerto Viejo; C: Carmelo; PF: Playa Fomento; M: Minas; PA: Playa Agraciada;?: precise location unknown

Petunia species	Visitor species (family, subfamily)	Location, month, and year of collection
P. integrifolia	*Calliopsis* sp. (Apidae, Colletinae)	R 01.05, LC 02.07
P. integrifolia	*Calliopsis* sp. (Apidae, Colletinae)	PV 01.05
P. integrifolia	*Halictus* sp. (Apidae, Halictinae)	R 01.05
P. integrifolia	*Lasioglossum* sp. (Apidae, Halictinae)	R 01.05
P. integrifolia	*Lasioglossum* sp. (Apidae, Halictinae)	PV 01.05
P. integrifolia	*Leioproctus* sp. subgen. Hexantheda (Apidae, Colletinae)	? 11.02
P. integrifolia	*Leioproctus enneomera* (Apidae, Colletinae)	R 01.05
P. integrifolia	*Leioproctus enneomera* (Apidae, Colletinae)	PV 01.05
P. integrifolia	*Hylephila phyleus* (Hesperiidae, Hesperiinae)	JI 02.05
P. axillaris	*Halictus* sp. (Apidae, Halictinae)	? 11.02
P. axillaris	indet. panurgine genus (Apidae)	JI 01.04
P. axillaris	*Halictus* sp. (Apidae, Halictinae)	C 02.04
P. axillaris	*Lasioglossum* sp. (Apidae, Halictinae)	C 02.04
P. axillaris	*Manduca diffissa* (Sphingidae, Sphinginae)	PF 11.02, C 02.07
P. axillaris	*M. sexta* (Sphingidae, Sphinginae)	R 02.05, JI 02.07, C 02.07
P. axillaris	*Eumorpha vitis* (Sphingidae, Macroglossinae)	C 04, JI 02.06, 02.07
P. axillaris	*Eumorpha labruscae* (Sphingidae, Macroglossinae)	C 2007,
P. axillaris	*Agrius cingulata* (Sphingidae, Sphinginae)	M 02.05
P. axillaris	*Erinnyis ello* (Sphingidae, Macroglossinae)	C 02.06
P. axillaris	*Hyles lineata* (Sphingidae, Macroglossinae)	C 02.07
P. axilaris	*Diabrotica emorsitans* (Chrysomelidae, Galerucinae)	C 02.04
P. axillaris	*Chrysodina cupricollis* (Chrysomelidae, Eumolpinae)	C 02.04
P. axillaris	*Dahlibruchus* sp. (Chrysomelidae, Bruchinae)	JI 01.04
P. axillaris	harvester ants (unidentified genus)	PA 02.06
P. axillaris	Crabspider (unidentified genus)	

In addition to more or less efficient pollinators, the evolution of floral traits may be affected by nectar robbers, herbivores, pathogens, and pollinator predators. Apart from bees and hawkmoths, we observed occasional visits by hummingbirds, Lepidoptera, and Coleoptera to *P. integrifolia* or *P. axillaris* flowers in Uruguay (Table 2.1). So far no pathogens affecting *P. integrifolia* or *P. axillaris* flowers in the wild have been described. *Xylocopa* bees are occasional nectar robbers of *P. axillaris* but have not been observed on *P. integrifolia*. The most commonly observed herbivore of floral tissue in Uruguay was the chrysomelid beetle *Diabrotica*, which can be found on both Petunia species but more often on *P. axillaris* (Fig. 2.3). *Diabrotica* specimens were also observed to carry Petunia pollen and hence may play a minor role as pollinators. On one occasion *P. axillaris* flower petals were observed being harvested by ants. The only predators of pollinators occasionally observed

Fig. 2.3 Herbivores and pollinator predators on Petunia flowers. (**A**) unidentified cricket and (**B**) *Diabrotica* sp. beetle feeding on *P. axillaris* flowers. (**C**) crabspider waiting for prey on a *P. integrifolia* flower and (**D**) crabspider waiting for prey on a *P. axillaris* flower

mainly on *P. axillaris* flowers were crabspiders preying on bees and diurnal butterflies (Fig. 2.3). The observed crabspider species are most likely too small to overpower hawkmoths; however, a potentially deterring effect on hawkmoths has not been tested. An overview of flower visitors observed in field sites in Uruguay is given in Table 2.1. One may conclude that bees and hawkmoths are the main visitors of Petunia flowers and that other flower visitors are likely to play only a minor role in the pollination ecology of Petunia species in Uruguay. In the following paragraphs we will treat the four floral traits in which bee- and hawkmoth-pollinated Petunia species differ.

2.2.1 Color

Color is perhaps the most obvious difference between flowers of *P. integrifolia* and *P. axillaris*, and it is also genetically and biochemically the best characterized trait in Petunia. According to Wijsman's classical studies (Wijsman 1983) and more recent work, the floral color differences between *P. integrifolia* and *P. axillaris* can be largely accounted for by differences at six loci. In the flower limb the regulatory locus *AN2* (Quattrocchio et al. 1999) and the *hydroxylation-at-five*

(*HF1* and *HF2*) loci, encoding flavonol 3′5′-hydroxylases (Holton et al. 1993b), regulate anthocyanin amount and quality (see Chapter 13). Important differences can also be observed at shorter wavelengths invisible to the human eye. In contrast to flowers of *P. integrifolia*, those of *P. axillaris* (with the exception of *P. axillaris parodii* S7) express ultraviolet (UV)-absorbing flavonols at high levels in the adaxial epidermis of the flower limb. This difference can be explained by variation at the *FL* locus, encoding flavonol synthase (FLS) and controlling the production of flavonols (Holton, Brugliera, and Tanaka 1993a). The floral color phenotypes in Petunia species are in accordance with the bee and hawkmoth pollination syndromes. Hawkmoths have a preference for white flowers, which do not reflect UV light (White, Stevenson, Bennett, Cutler, and Haber 1994), while bees prefer UV-reflecting flowers. In the flower tube and anthers, the *AN2* homolog *AN4* (Kroon 2004) and *HF1* account for anthocyanin production. Variations in the promoter of *chalcone isomerase A* (*CHI-A* also called *Po*) (van Tunen, Mur, Recourt, Gerats, and Mol 1991) determine the accumulation of chalcone in the pollen (see below).

Choice experiments using naïve insects under controlled greenhouse conditions show that bees have an innate preference for purple-flowered accessions and hawkmoths for white-flowered ones (Hoballah et al. 2007). The MYB transcription factor AN2 controls most of the anthocyanin variation between *P. integrifolia* and *P. axillaris* (Quattrocchio et al. 1999). It had been shown that lack of anthocyanins in *P. axillaris* flower limbs involves loss-of-function mutations in *AN2* (Quattrocchio et al. 1999). Interestingly, loss of function has occurred at least five times in *P. axillaris* (Hoballah et al. 2007). Choice experiments using genetic introgression lines differing in flower color showed that the color change can significantly shift the innate preferences of hawkmoths and bees (Fig. 2.4; Hoballah et al. 2007). Choice experiments with wild-type and transgenic *P. axillaris* plants expressing *AN2* showed that *AN2* alone could confer a major shift in pollinator attractions (Fig. 2.5). Changes in flower color have been implicated in early speciation in plant taxa with different pollination syndromes (Bradshaw, Wilbert, Otto, and Schemske 1995). However, sequence analysis of *AN2* suggests that loss-of-function mutations occurred relatively recently and might have been preceded by changes in other traits (Quattrocchio et al. 1999; Hoballah et al. 2007). It can be speculated that *P. axillaris* was preceded by an ancestor with pink flowers that may have been attractive to butterflies and diurnal hawkmoths.

Anther coloration also differs strikingly between *P. axillaris* and *P. integrifolia*, with *P. axillaris* having yellow pollen and *P. integrifolia* blue pollen. *Callonychium petuniae* bees appear to mate in purple Petunia flowers (mainly *P. integrifolia* but also in *Calibrachoa* species with either blue or yellow pollen) in southern Brazil (Wittmann et al. 1990). The males wait for foraging females in or near the flowers, mating takes place on or in the flowers, and the couple go on to visit other flowers *in copula* (Wittmann et al. 1990). We observed the same behavior in the bees belonging to the genus *Calliopsis* on *P. integrifolia* in Uruguay. Eyes of the bees match *P. integrifolia* anthers in color and shape (see Fig. 2.2), leading to the intriguing speculation that male bee behavior may have been involved in the evolution of pollen color phenotype. Moreover, *C. petuniae* is thought to have tetrachromatic

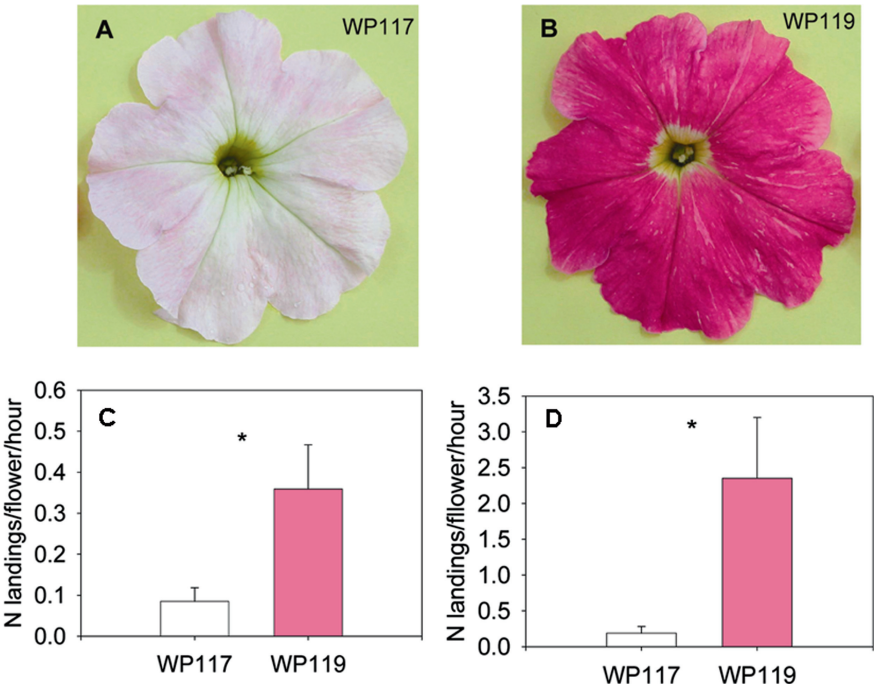

Fig. 2.4 Petunia introgression lines WP117 and WP119 are polymorphic for the *AN2* locus and affect pollinator behavior. Floral phenotypes of (**A**) line WP117, which carries a nonfunctional *AN2* allele, and (**B**) WP119, which carries an active *AN2* allele (Stuurman et al. 2004). (**C**) Mean (±SE) number of visits of diurnal butterflies in the wild habitat in José Ignacio (Uruguay) is higher for the line WP119 than for the white-flowered WP117. (**D**) Mean (±SE) number of visits of hymenopterans (Minusio, Switzerland) is also higher for line WP119 than for WP117. *Asterisks over bars* indicate the significance level of the statistical test $P = 0.01^*$. (Figures modified from Hoballah et al. 2007, ©The Plant Cell, ASBP)

vision, with receptors sensitive to red light and a strong preference for dark purple flowers, while other *Callonychium* species are much less specific in their color choice, suggesting that *C. petuniae* may have co-evolved with purple Petunia species (Wittmann et al. 1990).

2.2.2 Scent

The hawkmoth-pollinated *P. axillaris* flowers differ remarkably from bee-pollinated *P. integrifolia* flowers in the amount, quality, and timing of scent production. *P. axilllaris* flowers emit a blend of several compounds, dominated by methylbenzoate, benzaldehyde, and benzyl alcohol, during the night (Hoballah et al. 2005; see Chapter 3). The emission peak coincides with the nocturnal activity of the hawkmoth pollinators. Electroantennogram (EAG) recordings of female *M. sexta* antennae show that the three major volatiles methylbenzoate, benzaldehyde, and benzyl

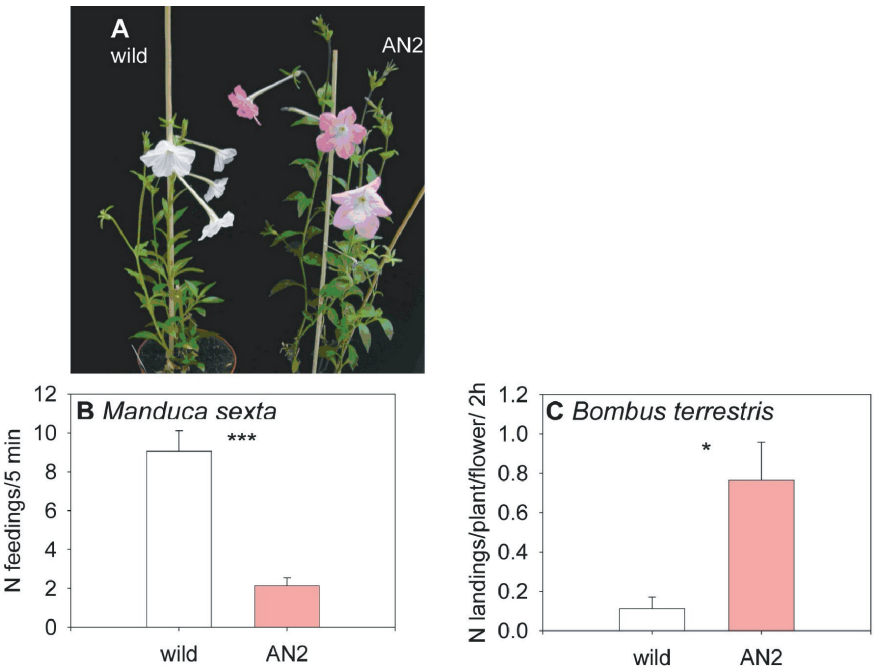

Fig. 2.5 Effect of the *AN2* gene on pollinator preference. Comparison of (**A**) wild-type and *AN2*-transformed *P. axillaris* flowers. (**B**) Mean (±SE) number of visits for feeding by *M. Sexta* is significantly higher for wild *P. axillaris* than for *AN2*-transformed flowers. (**C**) Mean (±SE) number of landings over 2 h per flower per plant of *B. terrestris* is higher on *AN2*-transformed plants than on wild-type ones. Asterisks over bars indicate significance of the statistical tests ($P = 0.0001$ ***, $P = 0.01$ *). (**B** and **C** modified from Hoballah et al. 2007, ©The Plant Cell, ASBP)

alcohol elicit the highest response (Hoballah et al. 2005), suggesting that these three compounds may be the most important for *Manduca* attraction. Floral scent is an essential cue for eliciting feeding behavior in *M. sexta* (Raguso and Willis 2002, 2005). This would suggest that the gain of scent attractive to hawkmoths must be an early step in the process of recruiting hawkmoth as pollinators. Whether single-scent components can elicit feeding behavior in hawkmoths or whether they interact synergistically has not been tested. Bees also use scent as a cue in their choice of flowers (Dobson 1994). However, bee-pollinated *P. integrifolia* flowers produce comparatively low amounts of benzaldehyde and only traces of a few other compounds (Hoballah et al. 2005).

Notably there is intraspecific variation in quality and quantity of floral odors in wild accessions of *P. axillaris* (Hoballah et al. 2005; Kondo et al. 2006). However, there was no clear association of flower volatile composition with the subspecific classification of these accessions (Kondo et al. 2006). Similarly, multiple *P. axillaris axillaris* accessions from different locations in Uruguay did not differ significantly in the total amount or composition of floral odor components (Fig. 2.6). Kondo

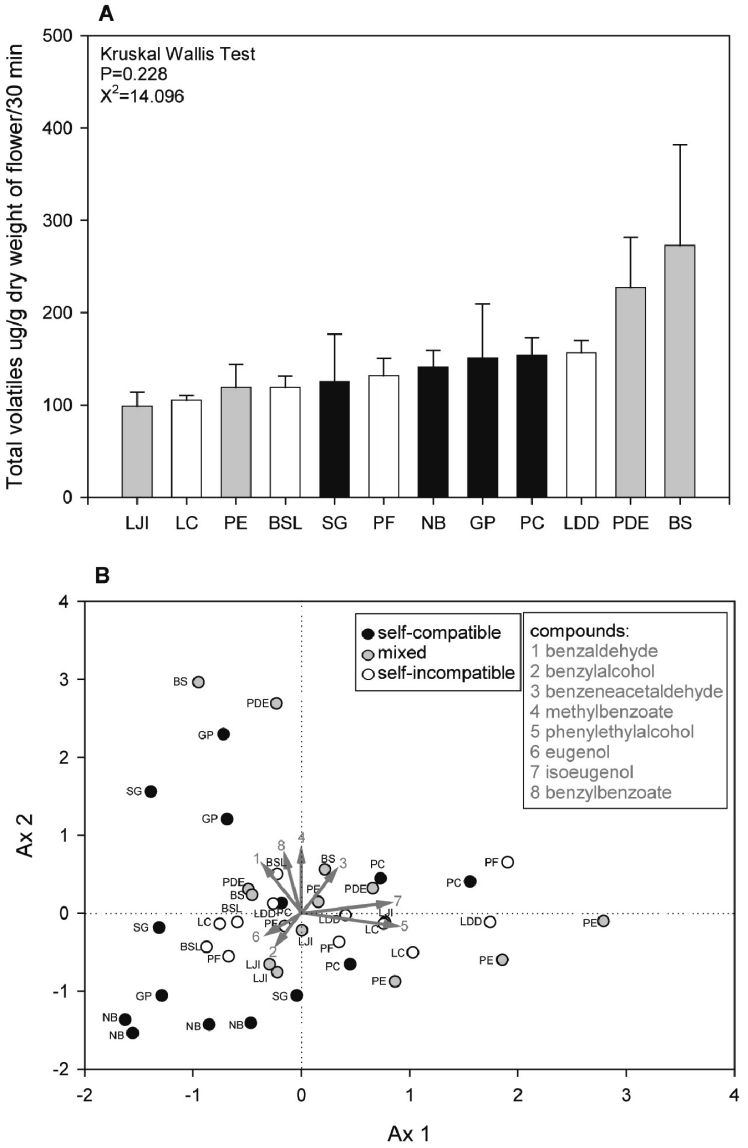

Fig. 2.6 *P. axillaris* wild accessions from Uruguay emit odor compounds that are similar quantitatively and quantitatively. (**A**) Total amounts of odor volatiles. The breeding system in each locality does not correlate with total odor production (bar fillings: black = self-compatible, gray = mixes, white = self-incompatible). (**B**) Plot of the first two principal components derived from multivariate analysis of the eight most abundant odor components. Locality labels are **LJI** = Laguna José Ignacio, **LC** = Las Canas, **PE** = Punta Espinillo, **BSL** = Barra Santa Lucia, **SG** = San Gregorio, **PF** = Playa Fomento, **NB** = Nuevo Berlin, **GP** = Gruta del Palacio, **PC** = Punta Colorada, **LDD** = Laguna del Diario, **PDE** = Punta dell'Este, **BS** = Balnearis Solis; precise coordinates of each locality are given in Hoballa et al. 2007, Table 2.1

et al. (2006) remarked that the two self-compatible accessions in their experiments produced less odor than self-incompatible lines. We did not find this trend analyzing a collection of *P. axillaris axillaris* accession samples ($n = 12$) that comprised both types of breeding systems (Fig. 2.6A).

Floral scents of plants measured in the field in Uruguay were not significantly different from those produced by seed offspring grown in the greenhouse (unpublished data). Growing plants in soils of varying nutrient content also did not show significant effects on scent production (ANOVA $P > 0.2$), while growth habit was clearly affected. Although this would suggest considerable buffering of scent production against environmental variation, there may also be considerable intraindividual variation, which could account for difficulties in demonstrating effects of environmental or genetic variation on scent production.

Fragrance intensity was quantified "by nose" in BIL of *P. hybrida* W138 x *P. integrifolia inflata* S6 (WI-BIL) and *P. hybrida* W138 x *P. axillaris parodii* S7 (WP-BIL) with *P. hybrida* W138 as recurrent parent (Stuurman et al. 2004). A QTL on chromosome VII was detected in WI- and WP-BILs (Stuurman et al. 2004). The gene *ODORANT1* (*ODO1*) (Verdonk, Haring, van Tunen, and Schuurink 2005) is a candidate for the regulation of scent production and has been mapped to chromosome VII (our unpublished results). However, in both BIL populations, the wild species allele at the QTL increased fragrance production, suggesting that the QTL represents a loss-of-function allele in the *P. hybrida* W138 line. More work will be required to evaluate *ODO1* as a candidate gene for controlling natural variations in scent production.

At present it is not established which genes required for *P. axillaris*–type scent are present in *P. integrifolia* or how many and which mutational changes would be required for gaining scent that would attract hawkmoths. Fortunately, the biochemical pathways leading to the floral volatiles emitted by Petunia flowers are under intensive study (see Chapter 3), so considerable progress in understanding natural variation in Petunia scent can be expected in the near future.

Not all volatiles produced by flowers are necessarily attractants for pollinators. Some compounds, such as isoeugenol, may rather have a function in protecting the flower from pathogens or herbivores (Hoballah et al. 2005). Moreover, volatiles that act as attractants to one pollinator may be repellents for others (Kessler and Baldwin 2007).

2.2.3 Nectar

Hawkmoth-visited flowers usually produce larger amounts of nectar that is more dilute and more sucrose rich than that of bee-pollinated flowers (Baker and Baker 1983). This difference has been observed between *P. axillaris* and *P. integrifolia* (Stuurman et al. 2004). Under greenhouse conditions, nectar concentration appeared largely independent of evaporation, and grafting experiments demonstrated that nectar production is dependent only on the scions and not on the grafting stock (Stuurman et al. 2004). However, the rate of nectar production is strongly affected

by environmental factors such as light and temperature. The total concentration of sugars (glucose, fructose, and sucrose) is about five times lower in *P. hybrida* W138 and about 10 times lower in *P. axillaris parodii* than in *P. integrifolia inflata*. The ratio of sucrose to hexose was almost 10 times higher in *P. axillaris parodii* compared to that in *P. integrifolia inflata*. In turn, the concentration of nectar is about five times higher in *P. integrifolia inflata* than in *P. axillaris parodii*.

QTL analysis of nectar volume in WI-BILs revealed two QTLs, on chromosomes II and VI; together the two QTLs account for about half the observed variation (Stuurman et al. 2004). Partial regression analysis showed that the QTL on chromosome II may be correlated with flower morphology. No QTLs were found in WP-BILs.

In a BC of *P. axillaris axillaris* N and *P. integrifolia inflata* S6, with the latter as recurrent parent, four QTLs for nectar volume, on chromosomes III, VI, V, and IV, have been detected (Galliot, Hoballah, Kuhlemeier, and Stuurman 2006b). Interestingly, all four nectar QTLs co-localized with QTLs affecting petal size. Similar correlations of nectar volume with morphology have been observed in *Nicotiana* species (Kaczorowski, Gardener, and Holtsford 2005). A sucrose–hexose ratio QTL was found in WI-BIL on chromosome IV explaining about 40% of the variation, with the *P. integrifolia inflata* allele decreasing the ratio, suggesting that it could control invertase activity (Stuurman et al. 2004).

A candidate gene for natural variation in nectar production could be *NEC1*, which affects nectar production (Ge et al. 2000, 2001). However, the RNAi knockout of *NEC1* in *P. hybrida* W115 (Petunia Mitchell) has large pleiotropic effects. Furthermore, *NEC1* and two homologs (*NEC2* and *NEC3*) have been mapped both in WI-BIL and in *P. axillaris axillaris* N x *P. integrifolia inflata* BC1 populations (Stuurman et al. 2004, unpublished data). *NEC1* is located on chromosome VII, *NEC2* on chromosome IV, and *NEC3* on chromosome VI (unpublished data), rejecting *NEC1* as a candidate, but suggesting that *NEC3* could correspond to a QTL.

Nectar may contain many components in addition to various sugars, such as amino acids, scent components, repellents, and antimicrobial substances. Some of these components may act as food or attractants; others might repel pollen robbers or inefficient pollinators. However, some substances may play a more complex role. Recently, it has been suggested that toxic repellents such as nicotine may also reduce specialist pollinator feeding time per visit and hence optimize the number of possible visits per volume of nectar produced (Kessler and Baldwin 2007).

2.2.4 Morphology

P. axillaris differs from *P. integrifolia* in numerous aspects of floral morphology (Fig. 2.7). The corolla of *P. axillaris* features a longer and narrower floral tube and a much larger floral limb, and appears less zygomorphic than that of *P. integrifolia*. In addition, the positioning of style and stamen differs between the species. The two ventral anthers in *P. integrifolia* exsert beyond the style so that the style is positioned between these and the three dorsal anthers, an arrangement often found in

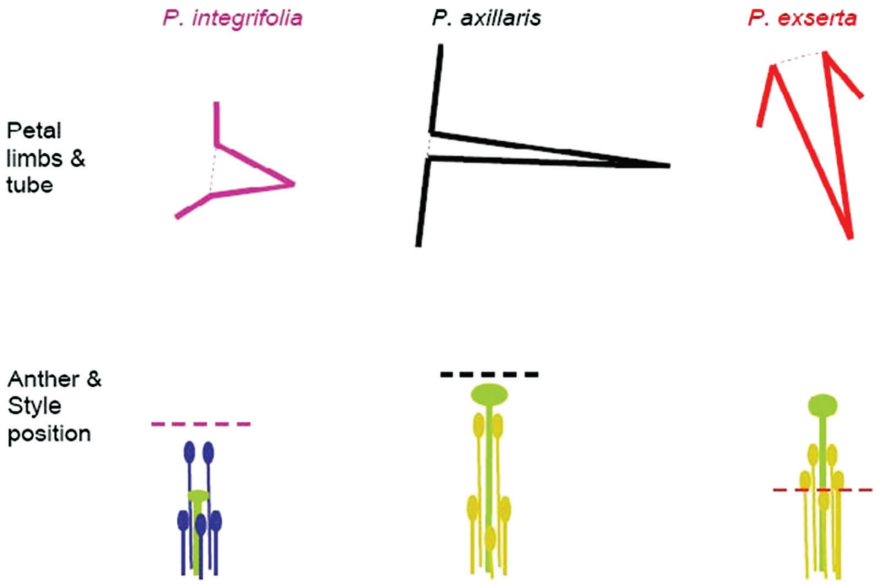

Fig. 2.7 Schematic overview of differences in corolla morphology and anther and style position in Petunia species representing different pollination syndromes

zygomorphic bee-pollinated flowers (Proctor, Yeao, and Lack 1996). In *P. axillaris* the stigma exserts beyond all anthers, putatively reducing the amount of self-pollen depositing on the style. The stigma of *P. axillaris* is also much broader than that of *P. integrifolia*, which in combination with the narrow tube could maximize the chance of non-self-pollen deposition by pollinators. In addition to floral morphology, the growth habit of the two species is different, with most *P. axillaris* accessions showing apical dominance and longer internodes while *P. integrifolia* plants are shorter, sometimes with a creeping habit. Although growth habit is certainly affected by other selective pressures such as shading and competition by other plants, hawkmoths may prefer taller plants with flowers unobstructed by vegetation, perhaps in order to avoid predators. Finally, *P. integrifolia* flowers also appear to close at night (Ando et al. 2001, MEH personal observation in Uruguay), which may be a thermo-protective strategy but may also exclude nocturnal pollinators. Empirical data confirming these notions of morphological functionality are lacking.

The genes underlying differences in the corolla phenotype in Petunia species have also been studied by QTL analysis (Stuurman et al. 2004; Galliot et al. 2006b). Three morphological domains can be defined (Fig. 2.8): D1, the length of the petal tube segment from the base of the corolla to the point where the stamina that are basally fused to the petals detach; D2, the distal tube domain between D1 and the boundary of the flower limb, defined as the maximal point of inflexion; and D3, the limb extending from D2. Among these measures, D1 shows the largest differences between *P. axillaris* and *P. integrifolia* (Stuurman et al. 2004). Stuurman et al. (2004) found five different QTLs for D1 in BIL populations in a *P. hybrida* W138 background derived from

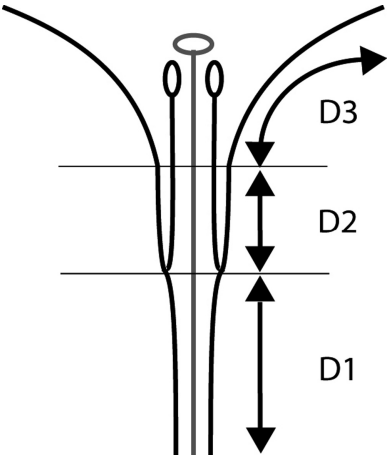

Fig. 2.8 Schematic diagram showing the three domains (**D1**, **D2,** and **D3**) in the corolla limb of Petunia flowers

crosses with *P. axillaris parodii* S7 or *P. integrifolia inflata* S6 (called WP or WI, respectively). In WI-BIL two QTLs, on chromosomes III and VII, were found. In WP-BIL three QTLs, one each on chromosomes II, IV, and VI, were detected, with the QTL on chromosome II explaining about 21% of the variation.

In a BC of *P. axillaris axillaris* N and *P. integrifolia inflata* S6 as recurrent parent, six QTLs for D1 on different chromosomes were found (Galliot et al. 2006b). The two largest QTLs (on chromosomes III and IV) contribute more than half the variation. In addition, four QTLs for D2 and four for D3 (L) were detected. Three QTLs for D2 and D3 (on chromosomes II, III, and IV) co-localize with each other and with QTLs for D1. This suggests that at least three QTLs contribute to the overall larger size of *P. axillaris axillaris* N petals compared to those of *P. integrifolia inflata*. In WI-BIL a single QTL with a large effect on the positioning of the two ventral stamens was detected on chromosome VI (Stuurman et al. 2004). This trait has been suggested to be subordinate to mechanisms controlling dorso-ventral asymmetry. To date none of the genes accounting for morphological differences in *P. axillaris* and *P. integrifolia* has been cloned.

Recently reported Petunia species such as *P. secreta* (Stehmann and Semir 2005) and *P. occidentalis* (Tsukamoto et al. 1998), with morphological phenotypes somewhat intermediate between those of *P. integrifolia* and *P. axillaris*, may represent either ancestral intermediates or hybrids of these taxa. In either case, knowledge about their pollination biology may shed some light on the pollination biology of intermediate morphs in the evolution from bee to hawkmoth pollination syndromes.

2.3 Evolution of Hummingbird from Hawkmoth Syndrome

As both hummingbirds and hawkmoths drink sucrose-rich, dilute nectar from flowers with long tubes, no large changes in these traits are required for shifting the pollination syndrome. Hummingbirds are mainly visually oriented and many

hummingbird-pollinated flowers have red pigmented corollas (Rodríguez-Gironés and Santamaría 2004). Most hummingbird-pollinated flowers have little or no scent (Proctor et al. 1996), but hummingbirds can also respond to scent components (Kessler and Baldwin 2007).

P. exserta is a recently discovered rare species endemic to southern Brazil, growing under sandstone cliff overhangs in only four locations (Stehmann 1987; see Chapter 1). *P. exserta* features typical hallmarks of hummingbird pollination: petal limbs have brilliant red pigmentation and are backward folded, style and anthers are exserted (Fig. 2.7), and the nectar is dilute, with a high sucrose–hexose ratio. We analyzed the scent of *P. exserta* and could not detect any odor compounds (unpublished data). There is anecdotal evidence suggesting that *P. exserta* is indeed pollinated by hummingbirds (Lorenz-Lemke et al. 2006). Considerable hybridization of *P. exserta* and *P. axillaris* has been observed, presumably the result of hummingbird visits to both species (Lorenz-Lemke et al. 2006). This notion is supported by our observation of hummingbirds visiting *P. axillaris axillaris* in the wild in Uruguay at sunset.

Nuclear markers suggest that *P. exserta* is closely related to *P. axillaris* and may even be nested among *P. axillaris* accessions (unpublished data). Hence, hummingbird pollination has most likely been derived from hawkmoth pollination very recently. This would be in contrast to the directional pollinator shifts suggested for the genus *Aquilegia*, in which pollination syndromes shifted from bee to hummingbird pollination and from hummingbird to hawkmoth pollination (Whittall and Hodges 2007). The putatively very recent shift to hummingbird pollination in *P. exserta* makes this species particularly appealing for testing the role of natural selection in the evolution of pollination syndrome traits. In particular, the apparent loss of scent and the accumulation of the red anthocyanin delphinidinin 3-rutinoside in *P. exserta* (Ando et al. 1999; Ando et al. 2000) are worthwhile traits for study.

Visitation by hummingbirds has been suggested for *P. reitzii* and *P. saxicola* (Ando et al. 1999). Compatible with hummingbird pollination, both species feature a different, more reddish pigment type (delphinidin 3-rutinoside-5-glucoside) than their closest known relatives. However, the morphological traits of these plants point more toward bee pollination. It would be interesting to establish the pollinator spectrum for these species and to test the significance of reddish petal pigmentation for attracting specific pollinators.

2.4 Conclusion and Outlook

Here we have tried to give an overview of our present understanding of the pollination syndromes in Petunia and to show its potential as a model system. The trait best understood at present is flower color variation. A handful of genes are known to account for much of the variation between *P. integrifolia* and *P. axillaris*. However, whether the divergence in these genes and traits was caused by natural selection or perhaps by drift is still not established. One shortcoming of this particular species

comparison is that both species are relatively divergent, which complicates the identification of the causal mutations and testing for natural selection.

The isolation of a growing number of genes involved in odor production (Schuurink et al. 2006; van Schie, Haring, and Schuurink 2006) will help to identify genes underlying species differences in this trait. In contrast to flower color and scent, we know of hardly any genes involved in establishing interspecific variation in nectar production and morphology. The prospect of isolating genes that underlie these traits would be greatly enhanced if more genomic tools for the Petunia system were available. Sequencing of genomic libraries, for example, bacterial artificial chromosomes (BACs) containing genes of interest, would be helpful and, furthermore, allow for the application of sophisticated approaches such as the testing of haplotype block structure to generate inferences about natural selection. Dissection of the genetic architecture of trait differences and isolation of major genes involved in establishing them will eventually allow us to reconstruct the major evolutionary steps in the transition from one pollination syndrome to another. One question concerning such a transition is whether particular traits such as scent or nectar have to change first and then other changes, for example, morphology or flower color, follow. Genetically reconstructing transitional phenotypes will also allow testing whether major shifts in attraction of specialized pollinators follow changes in just a few genes and whether intermediate phenotypes attract more generalized pollinators.

2.4.1 Trait Interactions

Often traits involved in different pollination syndromes co-vary, raising questions about the cause of co-variation. Do combinations of traits evolve strictly in parallel because they are selected for by pollinators, or are there functional or genetic interactions between traits? For instance, there is a correlation between tube length and nectar production. Is this the case because a longer tube simply allows the floral tube to hold more nectar, does an increase in tube length unavoidably lead to more nectar production, or is long tube length a secondary adaptation to exclude inefficient pollinators from a rich resource? Accumulation of flavonols appears inversely related to anthocyanin pigmentation. Does the competition of pigmentation pathways for the same substrate explain the correlation? Similarly, is there a functional link between scent production and reduced anthocyanin pigmentation? Do such functional correlates facilitate the evolution of fitting trait combinations?

2.4.2 Behavioral Choice Tests

Besides understanding the molecular evolution of pollination syndrome genes and genetically or transgenically manipulating pollination traits, it is crucial to assay the preferences of pollinators for floral phenotypes of interest in order to test hypotheses about the ecology and evolution of pollination syndromes. Behavioral tests can

be conducted either in the field or under controlled conditions with naïve pollinators. Both approaches have their merits and difficulties. Field experiments sample the broad diversity in pollinators and other interactors (e.g., nectar robbers and herbivores) occurring in nature and can give an understanding of how a newly evolved variant would fare under natural conditions. However, even field studies can be representative for only a given habitat and a given time frame. Testing in the wild is often very difficult (Hoballah et al. 2007), requiring favorable conditions for pollinator activity and sufficiently abundant pollinators. In contrast, testing in controlled conditions, such as a greenhouse or a flight tunnel with model pollinators, is limited to pollinators that can be reared in the laboratory. On the other hand, experiments can be replicated easily, plant material can be grown under highly standardized conditions, and transgenic plants can be used without encountering issues connected to the testing of genetically modified organisms in the field. Furthermore, testing innate pollinator preference arguably gives an indication of the genetically fixed and presumably adaptive preference in a given pollinator. Both the innate preference and the preferences modified by learning provide important information for assessing the adaptive value of floral traits.

2.4.3 Need for More Ecological and Phylogenetic Data

Obviously there is still much descriptive and experimental data needed for elucidating the ecology and evolution of the Petunia pollination syndromes. Pollinators were observed only on *P. axillaris* and *P. integrifolia* in Brazil (Ando et al. 2001) and in Uruguay (Hoballah et al. 2007). Furthermore, the importance of different pollinators for plant fitness was only partially studied in Uruguay with night and day exclusion experiments (Hoballah et al. 2007). Pollen movement between plants of the same species and between plants of different species can be studied with the use of a pollen dye. The quantity of pollen transferred by different pollinators can be studied in nature and in controlled experiments. Such experiments can reveal the degree of flower constancy of specific pollinators and the efficiency of pollinators in relation to flower types. Finally, improved taxonomic resolution of pollinators (bee species in particular) would be desirable.

2.4.4 Petunia as an Evolutionary Model System

There are a number of interesting questions that are related to, but go beyond, the issue of pollination syndromes. In *P. axillaris*, there is intraspecific variation in flower tube length. The subspecies *P. axillaris parodii* and *P. axillaris subandina* feature significantly longer floral tubes and narrower floral limbs than *P. axillaris axillaris*. It would be interesting to see whether long floral tubes are associated with a pollinator spectrum characterized by longer probosces, as would be predicted by co-evolutionary scenarios. Furthermore, does the inverse relationship between limb

size and tube length represent a genetic constraint on floral development, or is it constrained by mechanical properties of the flower, e.g., is a large limb on a long tube more easily damaged by wind?

Other aspects deserving more attention are the costs of particular traits and the tradeoffs between traits. Generating and maintaining floral tissue and nectar and scent production bear a metabolic cost (Pyke 1991), which needs to be compensated by an increase in fitness. Otherwise, variants reducing these costs should arise and spread throughout the population. Evolutionary theory would predict that where plants invest in costly traits such as nectar while pollinators cannot differentiate between cheating and non-cheating individuals, cheaters should evolve and reach a certain frequency in the population (Thakar, Kunte, Chauhan, Watve, and Watve 2003).

Besides its pollination syndromes and the importance of ethological isolation for speciation, the genus *Petunia* may also serve as an evolutionary model system to study the relevance of other prezygotic reproductive barriers, including ecophysiological and ethological barriers, and interactions of pollen with the stigma, the style and the embryo sac. As a few Petunia taxa cannot be crossed (e.g., *P. integrifolia inflata* S6 and *P. axillaris parodii* S7), but can be bridged via crosses to *P. hybrida* (Stuurman et al. 2004), introgression lines of each of these two wild species with *P. hybrida* as the recurrent parent may provide a useful means of identifying loci that are involved in establishing post-pollination barriers, and hence speciation genes *senso stricto* (Coyne and Orr 2004).

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