

Genetics of flower size and nectar volume in *Petunia* pollination syndromes

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Abstract The two related *Petunia* species, *P. axillaris* and *P. integrifolia*, are sympatric at various locations in South America but do not hybridise. Divergent pollinator preferences are believed to be in part responsible for their reproductive isolation. The volume of nectar produced and several components of flower morphology might contribute to pollinator-dependant reproductive isolation. In this study, we aimed to identify the genetic changes underlying the quantitative differences observed between these two *Petunia* species in flower size and nectar volume. We mapped quantitative trait loci (QTL) responsible for the different phenotypes of *P. axillaris* and *P. integrifolia* in an inter-specific backcross population. QTL of small to moderate effect control the differences in flower size and volume of nectar. In addition, we observed strong suppression of meiotic recombination in *Petunia*, even between closely related species, which precluded a fine resolution of QTL mapping. Thus, our data suggest that flower size and nectar volume are highly polygenic. They are likely to have

evolved gradually through pollinator-mediated adaptation or reinforcement, and are not likely to have been primary factors in early steps of pollinator isolation of *P. axillaris* and *P. integrifolia*.

Keywords Flower · Morphology · Nectar · *Petunia* · Pollination · Quantitative trait locus

Abbreviations

QTL	Quantitative trait locus
PVE	Percent variance explained
BC ₁	Backcross 1
BIL	Backcross inbred line
NIL	Near isogenic line

Introduction

Co-evolution of plants with their pollinators has contributed to angiosperms radiation and floral diversity (Grant 1994; Galen 1996). Distinct floral displays are adapted to animal pollinators which exert different selection pressure on floral traits such as flower shape, colour, odour, nectar and reproductive organs position that insure pollen transfer efficiency (Fenster et al. 2004). Such sets of traits, called pollination syndromes, can serve as guide marks for a particular type of pollinator (Proctor et al. 1996). These interactions between plants and animal pollinators are believed to be critical in assortative pollination and in the process of plant speciation. Pollination syndromes can be strikingly different, even among closely related plant species, raising the question how these complex adaptations evolve, and which genetic changes are responsible for transitions from one pollination system into another.

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Despite the importance of animal-mediated pollination in the reproductive biology of flowering plants, few studies have addressed the genetic bases of species differences in pollination syndromes (reviewed in Galliot et al. 2006). One thorough study was performed on monkeyflower, *Mimulus* (Bradshaw et al. 1998). In a mapping population involving *Mimulus cardinalis* (visited by hummingbirds) and *M. lewisii* (pollinated by bees), the authors found a major quantitative trait loci (QTL) for carotenoids content, and at least one QTL of large effect (> 25% PVE) for flower size traits and nectar volume. The authors suggest that these loci could have contributed to the rapid pollinator switch needed for sympatric speciation (Bradshaw et al. 1995, 1998). Although suggestive for a mode of inheritance that would allow rapid changes in pollination biology, most of these QTL have not been confirmed to be of major effect in near isogenic lines (NILs). The exception is the *YUP* pigmentation locus, which has large phenotypic and biological effects in allele swapping experiments (Bradshaw and Schemske 2003).

To allow a more general picture of the genetics of pollination syndromes, it is important that studies like those of *Mimulus* are carried out in a broader range of plant species, preferentially coupled to a set of molecular genetic tools that would allow in depth studies on the level of single genes. In *Petunia*, two species, *Petunia axillaris* and *P. integrifolia*, grow in sympatric populations on various locations in South America, although hybrids have not been found in the wild. Gametic isolation between these two species is incomplete and fertile hybrids are obtained by hand pollination (Ando et al. 2001). Thus, divergent pollinator preferences are believed to be responsible at least in part for their reproductive isolation (Ando et al. 2001). *P. axillaris* is pollinated by nocturnal hawk moths (*Manduca* sp., Ando et al. 2001). It displays large white flowers, with a long narrow corolla tube rich in nectar. It is strongly scented, following a circadian rhythm that peaks at dusk (Hoballah et al. 2005). *P. integrifolia* by contrast is mostly pollinated by bees (*Leioproctus* sp., Ando et al. 2001), it has small purple flowers, with a short and broad tube, little nectar and produces little scent (Hoballah et al. 2005). Interestingly, the genus *Petunia* contains a recently discovered species (*P. exserta*) that has all the hallmarks of a hummingbird pollination syndrome, although bird pollination has not been confirmed (Stehmann 1987).

The presence of at least three widely distinct pollination syndromes makes *Petunia* a favourable system for study. In addition, it is genetically well characterised by an extensive history of genetics and commercial breeding. Wide crosses are possible, many genotypes

can be easily transformed, and insertion mutagenesis is well developed (Gerats and Vandebussche 2005). Previously, a population of *P. axillaris parodii* and *P. integrifolia inflata* backcross recombinant inbred lines (BILs) in a *P. hybrida* W138 genetic background was used to map QTL for pollination syndrome-related traits. These analyses allowed the detection of several QTL controlling flower colour, scent, tube length, stamen length and nectar volume and composition (Stuurman et al. 2004). The flower colour and odour are under the control of a few relatively well-defined biochemical pathways of pigments and volatiles production, directing further investigation of species differences towards biochemical genetics and population genetics of several cloned genes (Quattrocchio et al. 1999; Stuurman et al. 2004; Hoballah et al. 2005; Verdonk et al. 2005). In contrast, flower size, morphology and nectar volume are less clearly defined. One possible shortcoming of our previous genetic study of these traits (Stuurman et al. 2004) is the use of *P. hybrida* which likely originates from a cross between *P. axillaris* and *P. integrifolia* and has undergone intensive breeding. Breeding and selection history may confound true species differences in QTL mapping. To gain better insight into these traits, additional QTL work is required on mapping populations directly obtained from inter-specific crosses.

In this study, we aimed to dissect in more detail QTL for morphology and nectar volume, using a cross between inbred derivatives of the wild species *P. integrifolia inflata* and *P. axillaris axillaris* to directly compare biologically relevant genotypes.

Materials and methods

Plant material and crosses

Different *Petunia* accessions obtained from botanical gardens were used: *Petunia integrifolia integrifolia* from the Botanical Garden of Dresden (Germany), *P. integrifolia violacea* from the Botanical Garden of Rostock (Germany), *P. integrifolia inflata* S6 and *P. axillaris parodii* S7 were from Vrije Universiteit (Amsterdam, The Netherlands). *Petunia axillaris axillaris* N, O, P and Q are accessions from the botanical gardens of Rostock, Dresden, Leipzig and Bern (Switzerland), respectively.

In previous work (Stuurman et al. 2004), QTL analyses used *P. axillaris parodii* S7 and *P. integrifolia inflata* S6 as wild accessions in crosses to *P. hybrida* W138. A direct QTL analysis in an inter-specific cross of *P. axillaris parodii* S7 to *P. integrifolia inflata* S6 was

impossible, as these accessions were fully cross-incompatible in reciprocal pollinations. Therefore, *P. axillaris axillaris* N and *P. integrifolia inflata* S6 were crossed, the first used as a seed parent. One of the F₁ hybrids obtained was backcrossed to *P. integrifolia inflata* S6 used as a pollen donor. We grew 188 of the BC₁ plants for QTL mapping.

To allow a comparison of QTL from *P. axillaris parodii* S7 and *P. axillaris axillaris* N, a second mapping population was made in order to make an integrated linkage map of AFLP markers derived from both accessions. In addition, the integrated map served to correlate linkage groups to the seven chromosomes of the haploid *Petunia* genome complement, as they were defined in Stuurman et al. (2004). In order to observe segregation of *P. axillaris parodii* S7 and *P. axillaris axillaris* N markers in a single AFLP-typed mapping population, a test cross scheme was used, in which a single F₁ plant (*P. axillaris axillaris* N × *P. axillaris parodii* S7) was outcrossed to the inbred line R27 of *P. hybrida*. Forty-four plants and twenty-six markers were used (nine of *P. axillaris axillaris* N and 17 of *P. axillaris parodii* S7) to produce a linkage map.

All plants were grown in a greenhouse with at least 16 h of light per day (natural light plus artificial light when necessary), the temperature ranged from 18°C by night to 35°C some days. Plants were kept in pots of 15 cm diameter with soil (peat-based substrate) and fertilised twice a week.

Phenotyping

For the QTL analysis, the flower tube segments D1 and D2 (Stuurman et al. 2004), and corolla limb diameter (L) were measured with calipers on six to seven flowers per plant. Nectar volume was measured as in Stuurman et al. (2004) on nine flowers per plant. Measurements were spread over 6 weeks and always done at the same floral stage: the second last flower at anthesis (about 3 h after watering the plants, for the nectar measurement). We measured also the morphology and nectar traits of different *P. axillaris* and *P. integrifolia* accessions (five plants per accession, two flowers each) to analyse if the plants used for our map were representative for each species.

Linkage maps and genotyping

DNA was extracted with the “DNAeasy 96 plant kit” from Qiagen. AFLP markers (amplified fragment length polymorphism) were generated according to Vos et al. (1995). Genomic DNA (300 ng) was digested with the enzymes *Eco*RI and *Mse*I, ligated with adap-

tors matching the *Eco*RI (E) and the *Mse*I site (M), and pre-amplified with primers matching the adaptors sequences plus a single base-pair extension (E–A and M–C). Nine combinations of selective primers with 3-bp extension were used for re-amplification (Stuurman et al. 2004). Specific primers that matched the E-adaptors were end-labelled with IRD-700 and analysed on a LiCor 4200 DNA sequencer. Markers were selected on the basis of their specific occurrence in *P. axillaris axillaris* N and according to apparent heterozygous segregation (all bands of same intensity). Apparent co segregating bands were eliminated. We built a linkage map by scoring 163 genetic markers on 188 backcross 1 (BC₁) plants.

The linkage analysis was performed with MapManagerQTX, version b20 (Manly et al. 2001), visually inspected by two-dimensional scaling in MAPL (Torgerson 1965) then the markers order was manually imported into MapManagerQTX and optimised with the “Ripple” function. For markers standing at the bottom or top of the chromosomes, to avoid false positive, we took stringent conditions (LOD > 5) as high evidence for their linkage to the adjacent markers. The map distances were computed from recombination value with the Kosambi mapping function. The markers were named from the primers combination and their size (EACCMCTC0149 was amplified with the primers E-ACC and M-CTC; its apparent size on a polyacrylamide gel was 149 bp).

Statistics and QTL analysis

Mann–Whitney test was performed in the statistics program SPSS (version 11.0), with a two-tailed significance value, on the mean of all pairs of accessions for the four traits to differentiate among phenotype classes and accessions. Correlations between the traits phenotypes were determined by Pearson’s correlation coefficient, *r*, at a significance level *P* < 0.01 with SPSS.

QTL were mapped using 188 plants of the BC₁ population and 163 AFLP markers. The QTLCartographer program, version 2.5 (Wang et al. 2005) was used to identify and locate QTL using a multi-trait composite interval mapping (CIM) method, preceded by an initial interval mapping scan to identify potential QTL for each trait. Cofactors were selected using forward/backward (*P* “in-out” = 0.01) regression in standard model 6 of QTLCartographer. The exclusion window size was reduced stepwise down from 10 cM, until the localisation and LOD scores (log₁₀ of the odds ratio) were optimised. Significant threshold levels for QTL detection were determined by 1000 permutations for each trait in QTLCartographer, using a simple interval

mapping model (Doerge and Churchill 1996). The LOD threshold was 2.92 at a confidence level $P < 0.01$ for all traits.

The most closely linked marker was selected for each QTL detected with QTLCartographer, and entered in a general linear model in SPSS. The mean of the floral traits measured for each backcross line was used. For the nectar volume data, the normality of the distribution was improved by transformation ($\log_{10}(\text{Nec} + 1)$). An analysis of covariance with continuous traits was performed by stepwise multiple linear regression, adding continuous traits as first covariates to estimate the effect of individual QTL in subsequent, complete multiple QTL models. The general linear regression model enabled an estimation of the relative contribution of the different QTL for each trait: the additive genotypic effect (a) and the percent of variance explained ($\text{PVE} = R^2$) were calculated under this model. Positive additive effects indicate that *P. axillaris axillaris* N locus increases the phenotype. Pairwise interactions were detected with a univariate factorial ANOVA in SPSS.

Results

Phenotypes

Besides flower colour and odour, the main phenotypic differences between *P. integrifolia* and *P. axillaris* reside in quantitative traits like flower size and volume of nectar. To determine how flower size components differ between *P. axillaris* and *P. integrifolia*, and to pinpoint general patterns that characterise the species, we measured the petal limb diameter L , and two sub-domains of the corolla tube, D1 and D2 as defined in Stuurman et al. (2004) in a series of accessions (Fig. 1). In addition, we measured the volume of nectar (Nec). In general, *P. integrifolia* clearly differs from *P. axillaris* accessions in D1, petal limb diameter (L) and volume of nectar (Nec) but not in D2 (Fig. 2). The tube segment D1 and the nectar volume are the most distinct traits between the two species (Fig. 2). The accessions used for QTL mapping (*P. integrifolia inflata* S6 and *P. axillaris axillaris* N) conform to the general pattern, and loci underlying differences between these accessions should be representative for the differences between the species in a broader sense.

The traits D1, L and Nec were intermediate in the F_1 hybrid (S6 \times N) phenotype (Fig. 3) suggesting a co-dominant effect of the alleles involved in these traits. In contrast, for D2 there was no significant difference between the parents (Fig. 2). Nevertheless, in the BC_1

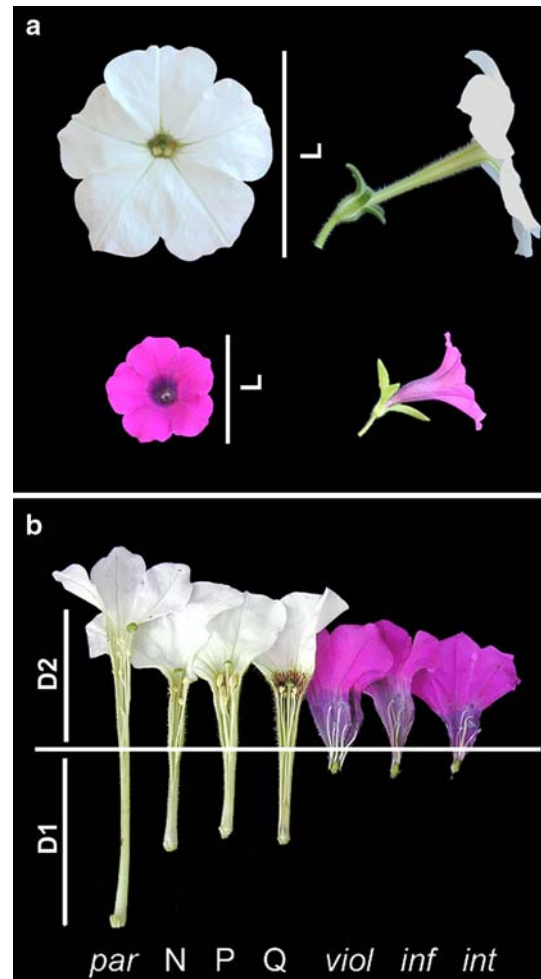


Fig. 1 The flowers of *P. axillaris axillaris* N (a, upper panel) and *P. integrifolia inflata* S6 (a, lower panel) in front and side view. L is the corolla limb diameter. **b** Tube length differences (D1, D2) in different accessions of *P. axillaris* (*P. axillaris parodii* S7, *P. axillaris axillaris* N, P, Q), and of *P. integrifolia* (*P. integrifolia violacea*, *P. integrifolia inflata* S6, *P. integrifolia integrifolia*)

population the length of D2 segregated with a striking transgression from the parents and F_1 phenotype, ranging from 15.6 to 27.1 mm (Fig. 3). In wide crosses, this is often explained by complementary gene action, due to new combinations of alleles or over-dominance (De Vicente and Tanksley 1993). This transgression allowed the mapping of QTL controlling D2. All the flower size traits showed a normal distribution, with a mean shifted towards *P. integrifolia inflata* S6 phenotype in the case of D1, L and Nec (Fig. 3) whereas the nectar volume follows a non-normal distribution in BC_1 plants, as indicated by a Kolmogorov–Smirnov test ($P < 0.001$; data not shown). Nectar volume distribution was skewed towards the lowest volumes (Fig. 3).

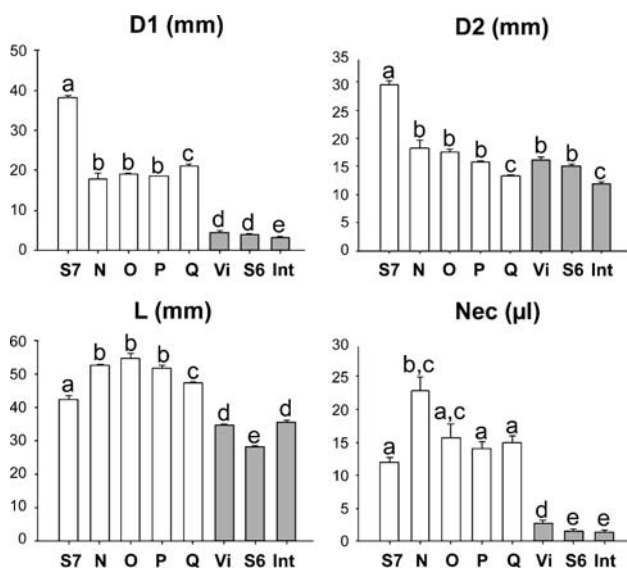


Fig. 2 Mean ± SD ($n = 10$) of flower tube lengths (D1, D2) in mm, limb diameter (L) in mm, and nectar volume (Nec) in μl in different *Petunia* accessions: *P. axillaris parodii* S7, *P. axillaris axillaris* N, O, P, Q, *P. integrifolia violacea* (Vi), *P. integrifolia inflata* S6, *P. integrifolia integrifolia* (Int). Different letters above the bars indicate significant differences among accessions (Mann–Whitney test)

Linkage mapping

To map the loci responsible for the species differences, we constructed a genetic map of the cross: (*P. axillaris axillaris* N × *P. integrifolia inflata* S6) × *P. integrifolia inflata* S6 based on *P. axillaris axillaris* N markers. The genetic map based on 188 plants of the BC₁ population

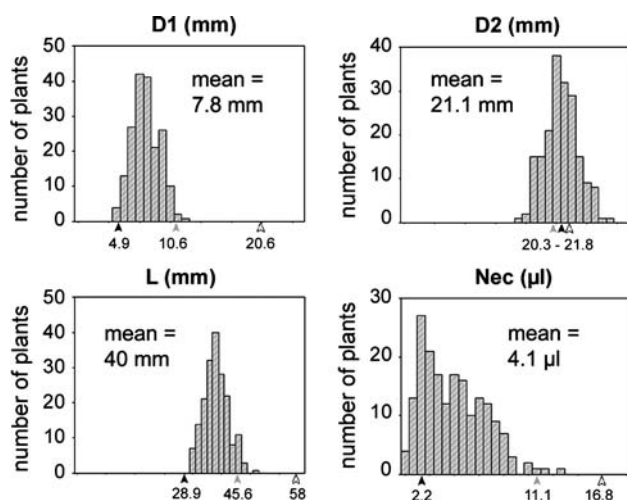


Fig. 3 Frequency distribution of the morphological traits (D1, D2, L) and of the nectar volume (Nec) measured in the mapping BC₁ population. The mean phenotypes ($n = 10$) of the two parental lines and of the hybrid are indicated on the x axis (black arrowhead = mean for *P. integrifolia inflata* S6, grey arrowhead = mean for the F₁ hybrid, and white arrowhead = mean for *P. axillaris axillaris* N). The mean of the trait measured in the mapping population is indicated (mean)

consists of 163 markers among which 159 are distributed over seven linkage groups, corresponding to the seven chromosomes of the genus *Petunia* (Fig. 4). Only four markers could not be attributed to the other linkage groups. The map characteristics are presented in Tables 1 and 2. The total map length is 404.6 cM. Resolution is strongly limited by intense marker clustering (Table 2a). Seventy-five per cent of the markers fall into dense clusters. The average and maximal distance between two adjacent markers are 6.42 cM (Table 1) and 37.1 cM, respectively. For instance, chromosome 6 shows a block of 21 markers and 84% of the markers lie within 7.43 cM of the chromosome. Chromosome 3 is the longest chromosome, with 148 cM.

Transmission ratio distortion occurs on the whole chromosome 2, and in 10 cM regions on chromosomes 4 and 5 (Fig. 4). *P. integrifolia* homozygote genotypes are over-represented in chromosome 2, with more than 62% of the genotypes instead of 50% expected in a BC₁ population, meaning a segregation distortion $k = 0.62$ at chi square = 10.83 ($P < 0.01$). *P. integrifolia inflata* S6 genotype is more frequent at marker EACCMCTC0099 (chr. 5; $k = 0.57$, chi square = 4.84, $P < 0.05$) whereas *P. axillaris axillaris* N genotype is predominant at the marker EACCMCAG0188 (chr. 4; $k = 0.67$, chi square = 21.79, $P < 0.01$). Segregation distortion is a common phenomenon in inter-specific crosses that has been attributed to structural polymorphism (chromosomes rearrangements) or loci that affect gamete transmission (Tanksley et al. 1992; Hall and Willis 2005).

In order to name the chromosomes, align the maps and compare our results to the previous QTL analysis achieved in a cross of *P. axillaris parodii* S7 and *P. hybrida* W138 (Stuurman et al. 2004), we performed a separate cross to build a linkage map combining markers of both *P. axillaris axillaris* N and *P. axillaris parodii* S7. Forty-four individuals from an F₁ (*P. axillaris parodii* S7 × *P. axillaris axillaris* N) outcrossed to *P. hybrida* R27 (isogenic to W138) were genotyped with AFLP markers (Fig. 4 and Table 2b). We did not use more than 26 markers, since clustering was so tight that increasing marker number would not increase map length appreciably (Fig. 4 and Table 2b). Interestingly, recombination was strongly suppressed although the two species *P. axillaris parodii* S7 and *P. axillaris axillaris* N are close relatives.

QTL mapping

To map the QTL underlying species differences in nectar volume and flower size, D1, D2, L and Nec were measured in all BC₁ plants. The four traits were pairwise positively correlated (Table 3). Floral tube

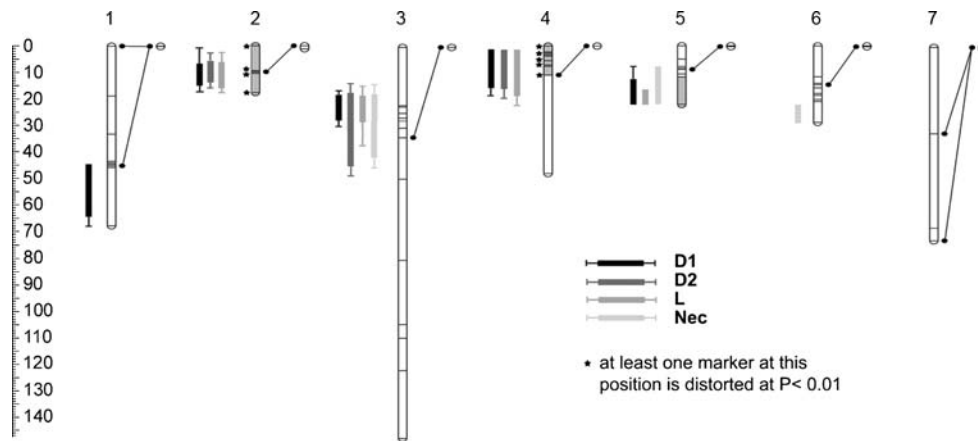


Fig. 4 Linkage map of *P. axillaris axillaris* N (left) based on the BC₁ population (*P. axillaris axillaris* N × *P. integrifolia inflata* S6) × *P. integrifolia inflata* S6 aligned with the map based on (*P. axillaris axillaris* N × *P. axillaris parodii* S7) × *P. hybrida* R27 (right). The distorted chromosome segments are indicated in grey

and one asterisk means that the segregation is distorted at a level of $P < 0.01$. Scale is in cM. QTL positions are shown and support intervals for significant LOD score peaks are indicated for each QTL. Boxes correspond to one LOD support intervals and thin line extensions to two LOD intervals

Table 1 Map of (*P. axillaris axillaris* N × *P. integrifolia inflata* S6) × *P. integrifolia inflata* S6 and marker's characteristics

Map length	404.6 cM
Number of markers	163 (4 unlinked)
Average number of markers per chromosome	22.7
Average distance between markers	6.42 cM
Recombination rate (rec/individual)	3.5
Maximum number of rec. per individual	9
Proportion of distorted markers (at $P < 0.05$)	31.4%
Frequency of the <i>P. ax. ax.</i> N genotype	53.3%
Frequency of the <i>P. int. inflata</i> S6 genotype	43.1%
Frequency of unknown genotype	3.6%

lengths D1 and D2, and corolla diameter *L* showed the highest association between D2 and D1, D1 and *L* or D2 and *L* with $r > 0.70$ ($P < 0.01$). In addition, all flower size related traits measured (D1, D2 and *L*) showed moderate to large association to the nectar volume (Nec). Therefore, we used all phenotypic data in a multi-trait interval mapping procedure. A total of four to six QTL ($P < 0.001$) were detected for each trait (Fig. 4 and Table 4). As expected from a highly correlated data structure, many QTL controlled more than one trait.

For D1 length, we found six QTL (*TBE6-11*). The strongest QTL, *TBE6*, is on chromosome 4, it accounts for 41.6% PVE and explains 9.5% of the difference between the parents' phenotypes. If this QTL's mode of action is co-dominant we can expect 19% (% delta *P*) of the phenotypic difference explained in a plant homozygous for *TBE6* ($2a = 3$ mm). It is followed by *TBE7* (25.2% PVE), on chromosome 3, which has an

apparent pleiotropic effect on the four traits measured. Four other QTL of smaller effect were detected: *TBE8* (PVE = 10.8%), *TBE9* (PVE = 6.6%), *TBE10* (PVE = 3.4%) and *TBE11* (PVE = 2.7%). *TBE9* controls D1 specifically and not any of the other traits (Fig. 4). All together, these six QTL explain 90.4% of the variance (PVE) within the BC₁ population that corresponds to 75.2% of the difference between the parents ($2a = 11.8$ mm) considering a co-dominant additive effect of the QTL. The high PVE value attributed to *TBE6* for the trait D1 (Table 4) does not compare with *TBE7* while their additive effect is similar. This may be artefactually due to pseudolinkage of the chromosomes 1 and 4 (inherited together more than random) where *TBE9* and *TBE6* are located, resulting in a confounding of their effects in a linear regression model.

Some QTL were detected that influence the length of the tube segment D2 although its phenotype did not differ significantly between the two parents (Fig. 4 and Table 4). QTL controlling D2 were mapped to the same markers as for D1 QTL apart from *TBE8* and *TBE9*. Together, they explain 77.7% PVE, which cannot be interpreted in terms of parental difference, the latter being nonexistent (Fig. 2). The observed effect of the QTL is likely due to a new combination of alleles which were in repulsion in the parents.

All QTL controlling diameter (*L*) appear to be responsible for other size traits as well (Fig. 4). The diameter is controlled by *TBE6*, *TBE7*, *TBE8* and *TBE10*. The cumulated effect of the diameter QTL is 61.9% PVE and accounts for about the same proportion of the parental difference: % delta $P = 68.7\%$, ($2a = 20$ mm) in a plant homozygote for all four QTL (Table 4).

Table 2 Distribution of the molecular markers on the linkage groups of the map (*Petunia axillaris axillaris* N × *P. integrifolia inflata* S6) × *P. integrifolia inflata* S6 (a) and (*P. axillaris axillaris* N × *P. axillaris parodii* S7) × *P. hybrida* R27 (b)

(a) (<i>Petunia axillaris axillaris</i> N × <i>P. integrifolia inflata</i> S6) × <i>P. integrifolia inflata</i> S6					
Chromosome	Length (cM)	Mean distance between bins (cM)	Number of markers (<i>P. ax. ax.</i> N)	Number of bins	Markers with LOD < 8.00
1	67.8	7.53	23	10	0
2	17.6	3.52	17	6	0
3	147.6	9.84	26	16	0
4	48	4.8	25	11	1
5	22.1	2.45	19	10	0
6	28.8	2.62	32	12	0
7	72.6	18.15	17	5	2
Unlinked	–	–	4	–	–
Total	404.6	–	163	70	3

(b) (<i>P. axillaris axillaris</i> N × <i>P. axillaris parodii</i> S7) × <i>P. hybrida</i> R27			
Chromosome	Length(cM)	Number of markers (<i>P. ax. ax.</i> N)	Number of markers (<i>P. ax. parodii</i> S7)
1	0.1	2	4
2	1.1	1	2
3	0	1	5
4	0	2	4
5	0.3	2	2
6	0.3	2	5
7	0.6	3	4
Total	2.4	13	26

Table 3 Pearson’s correlation coefficient (*r*) among traits (two tailed) *P* < 0.01

	D1	D2	<i>L</i>	Nec
D1	–	–	–	–
D2	0.75	–	–	–
<i>L</i>	0.71	0.75	–	–
Nec	0.58	0.35	0.43	–

Values in bold indicate large effect correlations, accounting for more than 25% of the variance

D1 and *D2* subdomains of the corolla tube, *L* petal limb diameter, *Nec* volume of nectar

The effect of the QTL *TBE6* and *TBE7* on flower size were confirmed in the progeny of the BC1 plants. For instance using graphical genotypes of all 188 BC1 plants, one individual (IL-28, Fig. 5) was identified to carry *TBE7* in addition to segments of *P. axillaris axillaris* N chromosomes 1 and 7, which did not carry any detectable QTL effect. The flower size of the BC1F1 plants segregated in a ratio of approximately 1:2:1 between small, intermediate and large flowers. This suggests co-dominance with a dosage effect of *TBE7*. Figure 5 shows the flower of one of these plants with large flowers.

More-than-additive interactions were found by two-way analysis of variance, performed between the significant markers linked to D1 QTL. The effect of the com-

bined QTL is higher than the predicted sum of their separate effects (data not shown). Yet a detailed description of QTL–QTL interactions cannot be inferred in such a primary segregating BC1 population and is limited also by a poor map resolution. Instead NILs would be best suited to separate QTL and combine them in repeated experimental designs for epistasis studies. For the volume of nectar, a QTL was detected at the same position as *TBE7* that we will call *TBE7/VOL4*. It is the strongest QTL (PVE = 39.1%) although it has an additive effect of 1.06 µl only as a heterozygote QTL (*2a* = 2.12 µl). Other nectar volume QTL (*VOL* loci) also co-located with loci for floral size components. *TBE11/VOL5*, *TBE8/VOL6* and *TBE6/VOL7* are small effect QTL with PVE ranging from 4.2 to 19.1%. The four nectar QTL together account for 70.9% of phenotypic variance, corresponding to 4.32 µl (*2a*) of nectar increase in a homozygote plant (*2 ΔP* = 29.6%).

For all QTL, *P. axillaris* QTL had a positive effect, increasing the phenotype, consistent with what is observed in the parents.

Discussion

To our knowledge, this is the first map based on a cross of pure *Petunia* species, *P. integrifolia* and *P. axillaris*,

Table 4 Quantitative trait locus (QTL)

QTL	Chromosome	Marker	Position (cM)	<i>a</i>	St. err.	PVE (%)	(Heterozygote) % ΔP (%)	(Homozygote) 2(% ΔP) (%)
D1								
<i>TBE6/VOL7</i>	4	EACCMCAG0188	3.26	1.5 mm	0.009	41.6	9.5	19
<i>TBE7/VOL4</i>	3	EACCMCTC0149	22.9	1.7 mm	0.008	25.2	10.8	21.6
<i>TBE8/VOL6</i>	5	EAGAMCCA0294	8.7	0.8 mm	0.008	10.8	5.1	10.2
<i>TBE9</i>	1	EACCMCCA0122	44.73	0.8 mm	0.009	6.6	5.1	10.2
<i>TBE10</i>	2	EAGAMCTC0227	10.28	0.6 mm	0.009	3.4	3.8	7.6
<i>TBE11/VOL5</i>	6	EAGAMCCA0430	21.27	0.5 mm	0.009	2.7	3.2	6.4
Total				+ 5.9 mm		90.4%	37.6%	75.2%
D2								
<i>TBE6/VOL7</i>	4	EACCMCAG0188	3.26	2.4 mm	0.020	36.9	–	–
<i>TBE7/VOL4</i>	3	EACCMCTC0149	22.9	2.2 mm	0.019	29.5	–	–
<i>TBE10</i>	2	EAGAMCTC0227	10.28	1.2 mm	0.019	6.9	–	–
<i>TBE11/VOL5</i>	6	EAGAMCCA0430	21.27	0.9 mm	0.019	4.3	–	–
Total				+ 6.7 mm		77.7%	–	–
L								
<i>TBE6/VOL7</i>	3	EACCMCTC0149	22.9	3.8 mm	0.037	30.6	13	26.1
<i>TBE6/VOL4</i>	4	EACCMCAG0188	3.26	2.5 mm	0.040	17.9	8.6	17.2
<i>TBE10</i>	2	EAGAMCTC0227	10.28	1.9 mm	0.038	6.9	6.5	13
<i>TBE8/VOL6</i>	5	EAGAMCCA0294	8.7	1.8 mm	0.038	6.5	6.2	12.4
Total				+ 10 mm		61.9%	34.4%	68.7%
Nec								
<i>TBE7/VOL4</i>	3	EACCMCTC0149	22.9	1.06 μ l	0.021	39.1	7.3	14.6
<i>TBE11/VOL5</i>	6	EAGAMCCA0430	21.27	0.55 μ l	0.021	19.1	3.8	7.6
<i>TBE8/VOL6</i>	5	EAGAMCCA0294	8.7	0.31 μ l	0.021	8.6	2.1	4.2
<i>TBE6/VOL7</i>	4	EACCMCAG0188	3.26	0.24 μ l	0.022	4.2	1.6	3.2
Total				+ 2.16 μ l		70.9%	14.8%	29.6%

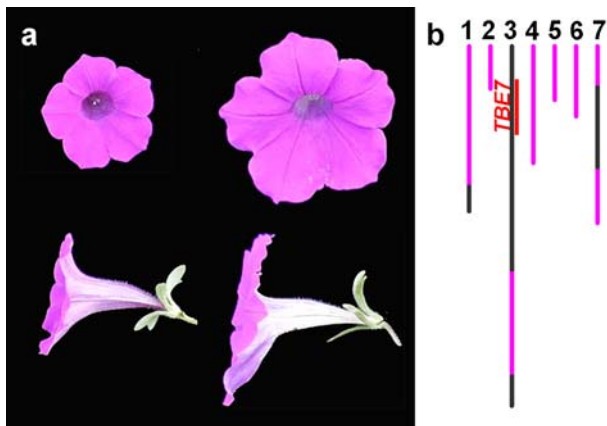


Fig. 5 Wild type *P. integrifolia inflata* S6 (**a**, left) and the flower of a homozygous introgression line (BC_1F_1) derived from IL-28 (**a**, right). **b** The genotype of the original line IL-28 is represented with purple chromosomes for *P. integrifolia inflata* S6 and black chromosome for *P. axillaris axillaris* N. The red bar indicates the position of the QTL *TBE7/VOL4*. In the BC_1F_1 line, D1 = 6.9 mm and L = 42.4 mm (measured on six flowers)

while previous maps have all included *P. hybrida*. The size of this map (404.6 cM) compares with the ones described before (Stuurman et al. 2004) and as found repeatedly for *Petunia* (Robbins et al. 1995; Strommer et al. 2000, 2002), genetic maps made from crosses

between divergent inbred lines do not resolve well. Because recombination rates can be high in crosses of isogenic inbred lines, it has been suggested that chromosomal differences such as inversions or translocations inhibit transmission of recombinant chromosomes, leading to short linkage maps (Robbins et al. 1995). Such chromosomal polymorphism may be frequent in *Petunia*. Interestingly, recombination rate was not increased in the intra-specific cross between *P. axillaris parodii* S7 and *P. axillaris axillaris* N, which produced the shortest linkage map in *Petunia* seen so far, affecting all chromosomes. Although it cannot be excluded that these species are highly divergent in genome structure, it may be that recombination is inhibited by specific factors that affect crossing over per se. The *Petunia Rm1* locus which regulates the rate of crossing-over through meiotic synapsis in pollen mother cells could be involved (Cornu et al. 1988; Abiracheddarmency et al. 1992). Its effects vary depending on the chromosome segment and is influenced by modifier genes (Robert et al. 1991). Although in the present study recombination occurred during female gametogenesis, a similar mechanism is conceivable.

In this study we estimated the number, the magnitude of the effect and the position of QTL controlling

the differences in flower size between *P. axillaris* and *P. integrifolia*. Differently from a previous QTL analysis on backcross inbred lines (BILs) of *P. hybrida* and *P. axillaris parodii* (WP-BILs) or *P. integrifolia inflata* (WI-BILs; Stuurman et al. 2004), we crossed directly the two wild species and analysed a BC₁ population. We have identified six QTL controlling these traits. The effect of these QTL on the length of the flower tube segment D1 explains 75.2% of the difference between the parental phenotypes. In the previous analysis, only three QTL that increased the tube length were detected, on the same chromosomes as *TBE6*, *TBE10* and *TBE11*. The poor map resolution, however, does not allow comparing QTL positions any further. In the prior analysis, the three D1 QTL explained 69.9% PVE (per cent variance explained) while the six present QTL account for 90.4% PVE. *TBE7*, *TBE8*, *TBE9* are three newly identified loci derived from *P. axillaris* that increase the length of the segment D1. It is noteworthy that the two QTL found previously in WI-BILs (involving *P. hybrida* and *P. integrifolia inflata*) on chromosomes 3 and 7 might also be involved in the difference between *P. integrifolia* and *P. axillaris*. *P. hybrida* displays an intermediate tube length between *P. integrifolia inflata* S6 and *P. axillaris parodii* S7 (Stuurman et al. 2004) and it may carry D1 QTL alleles derived from a *P. axillaris* ancestor. D2 and L which were not analysed before are regulated by at least four different loci that control D1 too. This is in accordance with the strong correlation between flower size traits. From our results, the genetic architecture of flower morphology appears polygenic; at least six loci control the flower size difference between these two species of *Petunia*. Most QTL have a small to moderate effect and could result from several genes in linkage. As almost all chromosomes carry a QTL controlling each trait it is likely that there is more than one locus on each of these chromosomes accounting for the effect.

In the present analysis, four nectar QTL account for 70.9% PVE. The individual, additive effects of *VOL4-7* are however low and explain only 29.6% of the difference between the parents (% ΔP). The discrepancy between variance explained (PVE) and parental difference (% ΔP) accounted for by the four QTL might best be explained by the skewed phenotypic distribution in the BC₁ population. Most individuals in the population were lower in nectar than expected on basis of average parental volumes, which may allow BC₁ variance to be explained to a good extent, but not the parental difference in microlitres. In addition, it may be that complex epistatic interactions, or undetected recessive QTL of *P. axillaris* contribute to nectar volume difference to

some extent. It is noteworthy that in a previous study (Stuurman et al. 2004), two nectar QTL of *P. integrifolia inflata* were found that explain 89% of parental difference in a *P. hybrida* background. The parental difference in this cross was smaller (6 μ l) and we have noted that the strongest of these (*VOL2*) is likely a consequence of defective anther dehiscence in certain recombinant genotypes, and may thus not reflect true allelic differences at a genuine nectar QTL but rather an artefact of the cross employed (unpublished observation). In general, therefore, nectar volume appears to be a complex polygenic trait, which, in conjunction with its sensitivity to environmental influences, remains a challenge for study (Mitchell 2004).

Most traits involved in *Mimulus* pollination syndromes were controlled by at least one QTL of large effect (> 25% PVE; Bradshaw et al. 1995, 1998). Bradshaw and Schemske (2003) suggested that a mutation at a major QTL could influence pollinators' attraction and rend sympatric speciation possible. In *Petunia* we have also found at least one QTL explaining more than 25% PVE for each trait; however, we would like to emphasise the fact that this does not translate into the same amount of explained parental difference. In the current work, only one QTL, *TBE7/VOL4*, can explain more than 25% of the flower limb diameter difference between *P. integrifolia inflata* and *P. axillaris axillaris*. We conclude that the flower size and nectar volume differences between *P. axillaris* and *P. integrifolia* are not controlled by mutations of large effect but rather by multiple mutations of small to moderate effect. Considering that allopatric speciation is predominant in plants (Coyne and Orr 2004), a possible scenario would be that two geographically isolated *Petunia* populations have gradually adapted to distinct pollinators under natural selection. Interestingly, Quattrocchio et al. (1998) have shown that the evolution of purple to white corolla colour has occurred several times independently in *Petunia*. With the current QTL data set, it seems unlikely that convergent morphological evolution has followed upon those colour changes. It seems more plausible that two distinct morphological types of *Petunia* existed as coloured ancestral species, which already had distinct animal pollinators. Switches to hawkmoth pollination probably occurred in the *P. axillaris* ancestral group, upon repeated loss of colour.

In the further study of the genetic bases of pollination syndromes in *Petunia*, the lack of recombination in primary segregating populations is a major limitation. As an alternative, one possibility would be to perform association mapping with candidate genes, using linkage disequilibrium in the large number of natural and cultivated accessions of *Petunia*. Future work should

also focus on evaluating the effect of QTL on pollinator behaviour. This can be assessed in the field with homozygous introgression lines. The strategy provides non-transgenic modified plants differing from the parents by only one introgressed chromosome segment.

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