Genetic Diversity and the Reproductive System in Related Species of *Antirrhinum*

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**INTRODUCTION**

Plant species show a wide variety of breeding systems (Holsinger, 2000) ranging from obligate inbreeders to obligate outcrossers. In angiosperms, self-incompatibility is a widespread genetic mechanism to prevent self-fertilization due to the evolutionary advantages of out-crossing, and it is widely accepted that self-compatibility is repeatedly derived from self-incompatibility (Barrett and Harder, 1996). Recent data show that the gametophytic self-incompatibility system of Scrophulariaceae has a common phylogenetic origin to that of Solanaceae and Rosaceae (Uenoyma, 1995). Moreover, the independent loss of self-incompatibility has been shown in different groups of species of *Lycopersicon* (Kondo et al., 2002), supporting former theories on the derived origin of self-compatibility from self-incompatibility.

The reproductive system determines the way genes are transmitted between generations and, consequently, levels of genetic variability are closely related to this. It has long since been recognized that inbreeders show lower levels of genetic variability than outbreeders (Baker, 1959; Hamrick et al., 1979), and that such variability is mainly found among populations in the former while it remains within populations in the latter.

The genus *Antirrhinum* comprises species with different reproductive systems, as documented by Baur (1919, 1924, 1932), Gruber (1932) and Sherman (1939). Although most species are strictly self-sterile (Baur, 1932; East, 1940), *A. siculum* is completely self-compatible, *A. cirrhigerum* and *A. linkianum* are reported as partially self-compatible (Vieira and Charlesworth, 2002), and *A. latifolium*, *A. litigiosum*, *A. tortuosum* and wild populations of *A. majus* are self-incompatible. Despite all this, the breakdown of self-incompatibility is documented in *A. latifolium* and *A. majus*. (Baur, 1919, 1924, 1932) as well as in *A. valentinum* (Mateu-Andrés and Segarra-Moragues, 2003).

Understanding population genetic concepts such as diversity and linkage disequilibrium as well as within-species patterns of variability are important to all biologists (Charlesworth, 2003). Molecular markers are powerful tools to measure selfing rates within populations, and enable the species reproductive system to infer when extended to the species range, and to detect differences among populations. In this paper, data on levels of genetic variability and its partition within and among populations are presented, as well as linkage disequilibrium between pairs of loci, estimates of inbreeding coefficient and gene flow in seven related species showing different reproductive systems: *A. siculum*, *A. majus*, *A. latifolium*, *A. linkianum*, *A. litigiosum*, *A. cirrhigerum* and *A. tortuosum*, whose delimitation has been studied previously (Mateu and de Paco, 2005).

The aim was to check to what extent the levels of genetic variation of species are affected by differences in the reproductive system in these related taxa. The results will...
contribute to the knowledge of the reproductive system of these plant species and will help in the management of rare endemic taxa of the genus.

MATERIALS AND METHODS

Taxa studied

All seven species of *Antirrhinum* L. studied are perennials, and most of these species are unspecialised with populations growing on limestone crevices, roadsides, walls and roofs, while *A. cirrhigerum* grows on sands. *Antirrhinum siculum* Miller and *A. tortuosum* Bosc. are widespread taxa, distributed throughout a vast area in the Mediterranean basin, while *A. latifolium* Miller, *A. litigiosum* Pau, *A. cirrhigerum* Welw ex Rothm., *A. majus* L. and *A. linkianum* Boiss. et Reut. have small ranges.

Plant material

A total of 851 individuals from 49 natural populations were studied. Individuals were grown in a greenhouse from seeds sampled in different plants. Sampled individuals were between 10 % and 85.7 % of the total number of mature plants growing in each population. Sampled populations were distributed through the species range. Data on sampled populations are provided in Table 1, and the species distribution used is that of Mateu-Andrés and de Paco (2005).

Electrophoresis and analysis

Electrophoresis was carried out on horizontal 10 % starch gels. The extracting buffer consisted of 0.2 M Tris–HCl, pH 7.5, 2 mM EDTA, 0.12 M Na2S2O5, 1 mM Cl2Mg, 40 mg mL–1 (w/v) PVP, 4 μM μL–1 mercaptoethanol. The material employed for the extracts consisted of young leaves from plants grown in the greenhouse, which were absorbed onto 3-mm wicks of Whatman chromatography paper.

Nine enzyme systems were assayed: aconitase (ACO; EC 4.2.1.3), aspartate aminotransferase (AAT; EC 2.6.1.1), diaphorase (DIA; EC 1.6.99), isocitrate dehydrogenase (IDH; EC 1.1.1.42), malate dehydrogenase (MDH; EC 1.1.1.37), menadione reductase (MNR; EC 1.6.99), phosphoglucoisomerase (PGI; EC 5.3.1.9), phosphoglucomutase (PGM; EC 5.4.2.2) and triosephosphate isomerase (TPI; EC 5.3.1.1). All of them presented banding patterns. AAT and IDH could not be scored due to inconsistent banding patterns. The electrophoretic buffer system II of Wendel and Weeden (1989) was employed to test for significance (Rice, 1989). When more than two tests were involved, the sequential Bonferroni correction (Rice, 1989) was applied to the results of tests on the Hardy–Weinberg expectation in local populations and the total set of populations, respectively. The population genetic structure and Nm were calculated using FSTAT 2.9.3 (Goudet, 2001). Sequential Bonferroni correction (Rice, 1989) was applied to the results of tests on the Hardy–Weinberg expectations. Total genetic diversity Ht was computed for each species to make comparisons easier with others studied previously.

To compare whether parameters of genetic variability were significantly different among taxa, a one-way ANOVA test was applied to P95, P99 and A. The spatial pattern of genetic variation within taxa was illustrated by a cluster analysis, and the Mantel test was performed comparing the pairwise Nei’s genetic distances against the geographical distances between populations. Both were based on Nei’s genetic distance (Nei, 1972) and performed with the Ntsys 2.0 (Rohlf, 1998) program. An indirect estimate of gene flow was calculated based on the equation: Nm = (1 – GST)/4GST, where Nm is the number of migrants per generation (Wright, 1931).

A test for genotypic disequilibrium between pairs of loci was performed using FSTAT. Due to the fact that multiple tests were involved, the sequential Bonferroni correction was applied to test for significance (Rice, 1989).

RESULTS

Seven out of the nine enzymatic systems studied were interpreted, giving a total of 13 putative loci and 39 alleles. Only MN1 out of the 13 scored loci was monomorphic. PGI1 and TPI1 had duplicated conmigrant loci, also reported for other species of Antirrhininae (Elisens and Crawford, 1988; Elisens, 1992; Elisens and Nelson, 1993) and *Antirrhinum* (Mateu-Andrés, 1999; Mateu-Andrés and Segarra-Moragues, 2000), so they were not scored. The number of alleles was 10 in *A. siculum*, 18 in *A. majus*, 21 in *A. tortuosum*, 20 in *A. latifolium*, 17 in *A. cirrhigerum*, 20 in *A. linkianum* and 21 in *A. litigiosum*. Allele frequencies are shown in Mateu and de Paco (2005). No linkage
disequilibrium was found between pairs of loci after sequential Bonferroni correction.

Table 2 shows the diversity parameters, all of which are in the range previously reported for other species of Antirrhinum (Mateu-Andre’s, 1999; Mateu-Andre’s and Segarra, 2000, 2003a, b), and are similar to those obtained by Hamrick and Godt (1996) for Scrophulariaceae. The proportion of polymorphic loci ranged widely among taxa, varying between 0–77% and 0–84.6 for 95% and 99% criteria, respectively. The mean number of alleles per locus among taxa ranged between 1 and 2.1. $H_e$ ranged between 0 and 0.34, and $H_e$ ranged similarly between 0 and 0.31, among taxa. Among the populations showing variability in at least one locus, $F_{IS}$ values ranged between $-0.004$ and $0.004$ with different levels of signification. Antirrhinum siculum, showed the lowest levels of variability where all monomorphic loci in most populations were only AD2 and AS7 polymorphic, both at the $P_{95}$ and $P_{99}$ levels in the former case, and only at $P_{99}$ in the second; the mean number of alleles per locus was 1.15 and 1.08, respectively.
Correspondingly, $F_{IS} = 1$ was found in six out of eight populations and $F_{IS} = 0.904$ in one of the populations showing variability, while it was lower and non-significant in the other. The ANOVA tests performed to compare the genetic variability parameters revealed that $A$ ($P = 0.000$, $F = 12.936$), $P_{95}$ ($P = 0.000$, $F = 15.533$) and $P_{99}$ ($P = 0.000$, $F = 16.248$) were significantly different among taxa when all populations were considered in the analysis. The Tukey test performed to check differences between pairs of species revealed that all three parameters of diversity were significantly different when $A. siculum$ was compared with any other species ($P = 0.000$), and was lower ($P = 0.004$ for $A$ and $P = 0.001$ for $P_{95}$) when $A. siculum$ was compared with $A. cirrhigerum$. Antirrhinum litigiosum had a significantly higher variation than $A. latifolium$ in $P_{95}$ ($P = 0.010$) $P_{99}$ ($P = 0.017$), $A$ ($P = 0.014$) and $A. linkianum$ in $P_{95}$ and $P_{99}$ ($P = 0.011$ and $P = 0.047$).

Significant deviations from the Hardy–Weinberg expectations were detected for ten loci in different populations (Table 3). In most cases, deviations were due to a deficiency of heterozygotes, but deviations were due to an
excess of heterozygotes in MDH1 (population ALIT17), DIA1 (population AA2), MNR2 (population AA4) and MDH3 (populations AA6 and AA8).

$F_{IS}$ values were seen to be high (over 0.250) and positive in eight populations, positive with moderate or low values in another four populations, high and negative in only one population, and moderate or low and negative in nine. $F_{IS}$ values were highly significant ($P < 0.001$) in 17 populations. Throughout species, $F_{IS}$ was very high (0.904) in one of the two populations of *A. siculum*, showing genetic variability. Antirrhinum litigiosum and *A. majus* were in equilibrium or showed low values of $F_{IS}$, one (AC7) out of three populations of *A. cirrhigerum* showed high and positive $F_{IS}$. In *A. linkianum*, two populations showed positive $F_{IS}$, while this was negative in the other four. $F_{IS}$ values were high and positive in three (AL2, ALL2, ALL3) out of eight populations of *A. latifolium*, and they were moderate and positive in one (ALL6). In *A. tortuosum*, $F_{IS}$ estimates were positive in six out of eight populations of *A. tortuosum*, they were high in two of them (AT5 and AT6), while one population (AT10) showed high negative values, and moderate or low ones in another 5.

Total genetic diversity (i.e. diversity averaged across all the loci studied) $H_T$ ranged between 0.03 in *A. siculum* and 0.28 in *A. litigiosum* (Table 4). Mean values of $F_{IT}$ and $F_{IS}$ for the overall populations of each species were mostly positive and not significantly different from zero, despite departures from zero for some individual loci, indicating that both total and local populations were in Hardy–Weinberg equilibrium. All mean values of $F_{ST}$ per species were positive and not significantly different from zero. $F_{ST}$ values ranged between 0.061 and 0.270, indicating a low proportion of differentiation among populations in *A. tortuosum* (6.1%), *A. latifolium* (9.3%) and *A. majus*, a moderate proportion in *A. cirrhigerum* (13.2%), *A. litigiosum* (14.8%) and *A. linkianum* (17.6%), and a high proportion in *A. siculum* (27%).

Estimates of the number of migrants ($N_m$) gave high values for *A. majus* and relatively high values for *A. cirrhigerum*, *A. latifolium*, *A. linkianum*, *A. litigiosum* and *A. tortuosum* (Table 5), indicating high levels of gene flow among populations. However, low values were estimated for *A. siculum*. It is noted that the estimation of $N_m$ from $F_{ST}$ rests on numerous assumptions regarding meta-population dynamics which may not be met in all of the species studied, thus $N_m$ values must be interpreted with caution (Whitlock, 1992). Mantel tests detected a relationship among the geographic and genetic distance in *A. litigiosum* ($r = 0.76$, $P = 0.99$), although it was non-significant. It was weak and non-significant for *A. latifolium* ($r = 0.44$, $P = 0.99$), *A. majus* ($r = 0.44$, $P = 0.80$) and *A. tortuosum* ($r = 16$, $P = 0.83$), weak and negative ($r = -10$, $P = 0.38$) for *A. siculum*, and negligible for *A. cirrhigerum* ($r = 0.05$) and *A. linkianum* ($r = 0.004$, $P = 0.50$). Thus, no isolation by distance was detected in these six species. These results show that a low number of migrants is sufficient to genetically homogenize populations, according to Ellstrand and Elam (1993).

### DISCUSSION

In agreement with previously reported results for other species of *Antirrhinum*, the species studied show striking differences in parameters of genetic diversity, inbreeding coefficient (Table 2), total diversity levels ($H_T$) and its partition within and among populations (Table 4).

The total genetic diversity for *A. siculum* is 0.03, the lowest among the studied species of *Antirrhinum* (Mateu-Andrés, 1999; Mateu-Andrés and Segarra, 2000, 2003a,b; Mateu-Andrés, 2004) as well as among species of Antirrhineae (Elisens and Crawford, 1988; Elisens, 1992; Elisens and Nelson, 1993). Those for *A. litigiosum* (0.28) and *A. barrelieri* (0.25), can be considered as high, and are among those known for *Antirrhinum*, a genus for which $H_T = 0.50$ levels had been reported in *A. microphyllum* (Mateu-Andrés, 1999) and $H_T = 0.30$ in *A. pulverulentum* (Mateu-Andrés, 2004), two species with similar ranges to those of *A. majus* and *A. linkianum*.

Among the species studied, *A. siculum* is self-compatible, *A. cirrhigerum* and *A. linkianum* had been reported as showing variable levels of compatibility among populations (Vieira and Charlesworth, 2002; Zewtler et al., 2002), and *A. latifolium*, *A. litigiosum*, *A. tortuosum* and wild populations of *A. majus* are considered as self-incompatible, although a breakdown of the self-incompatibility system
<table>
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<tr>
<th>Loci</th>
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<th>F_ST</th>
<th>F_IS</th>
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**H_T**, total genetic diversity and **F** statistics; **F_1T** and **F_IS** measure of heterozygote excess (＜0) or deficit (＞0) relative to Hardy-Weinberg expectations for the total and local population, respectively; **F_ST**, coefficient of differentiation among-populations.

Significance levels: *P < 0.05; **P < 0.01; ***P < 0.001.
has been reported from the second year of cultivation onwards in the former species (Baur, 1919, 1924, 1932). The present results coincide with previous reports for A. siculum, although the low \( F_{IS} \) value indicates outcrossing among individuals in one population (AS7). In A. litigiosum, A. majus and A. tortuosum, \( F_{IS} \) values are either in Hardy–Weinberg equilibrium or are low and negative, in accordance with the obligate out-crossing in self-incompatible plants. Meanwhile, high and positive \( F_{IS} \) values are found in A. tortuosum (AT5 and AT6), as were high and negative values (AT10), indicating strong deviations from Hardy–Weinberg equilibrium both upwards and downwards. In A. latifolium, high and positive \( F_{IS} \) values in three (AL12, AL2, AL3) out of eight populations indicate different levels of inbreeding in some populations, and suggest that Baur’s (1919, 1924, 1932) results in cultivated plants could also be extended to wild plants. The inbreeding coefficient in A. linkianum shows that the species is out-crossing, although the selving rate is high in at least one of the populations studied, in agreement with that reported by Vieira and Charlesworth (2002). In A. cirrhigerum, \( F_{IS} \) values are low in two populations, indicating out-crossing among plants into populations, while high and positive values show a high level of inbreeding in a third population (AC7). Interestingly, populations AC6 and AC7 had been reported as being largely self-compatible, while AC5 is considered as largely self-incompatible (Vieira and Charlesworth, 2002; Zwettler et al., 2002). The present results agree with those of Vieira and Charlesworth (2002) and Zwettler et al. (2002) in both AC5 and AC7, while plants behave as out-crossers in AC6 despite being self-compatible.

Following Loveless and Hamrick (1984) and Hamrick et al. (1979), out-crossing reduces the effect of genetic drift and increases the migration rate, thus increasing the specific levels of variation and preventing divergence among populations. In self-pollinated plants on the other hand, genetic drift increases and species present high differences among sub-populations. In agreement with this, genetic differentiation among populations was high in A. siculum, the only self-compatible taxon, whereas it was low or moderate in the other species studied.

As in any other previously studied species of Antirrhinum, these taxa show patchily arranged populations. The low values of genetic diversity among populations in A. majus agree with the high estimates of gene flow, indicating little population divergence. The geographical proximity among populations, continuity of the habitat and the out-crossing breeding system, allow for a genetic exchange among populations through pollen and/or seed dispersal, as reported for A. charidemi (Mateu-Andrés and Segarra-Moragues, 2000).

Population isolation may lead to stochastic differentiation by genetic drift (Ellstrand and Elam, 1993), and to an increase of differences among populations. The low values of gene flow together with the low correlation among genetic and geographic distances in A. siculum, are consistent with the hypothesis that genetic drift has produced a stochastic differentiation of populations. A particular aspect worthy of consideration in that species is that, in the words of Sutton (1988), some authors ‘have interpreted the occurrence of A. siculum in places such as Perpignan and Jerusalem as introductions dating from the time of Crusades’. Although the only putatively introduced population studied (AS7) is genetically close to most of the others from a supposedly natural area of the species, the presence of allele MN23-2, present in that population in the species but common in several others in the group (Mateu-Andrés and de Paco, 2005), does not support the hypothesis of a relatively recent introduction. In any case, the present data cannot be conclusive in a species with such low levels of allozymic variability. It is considered that a study on the species phylogeography would shed light on this matter.

By way of conclusion, levels of genetic diversity and its partition were correlated with the reproductive system. Levels of genetic diversity were higher and differentiation among populations was lower in self-incompatible species than in self-compatible ones, in accordance with the assumptions of Hamrick and Godt (1989).

**Consequences for species conservation**

None of these species are included in red lists (A. linkianum is in the Spanish red list but not at the species range), and most of them show high levels of diversity, a common pattern in species of Antirrhinum, even in some with small and fragmented populations (Mateu-Andrés, 1999; Mateu-Andrés and Segarra-Moragues, 2000). Most species have a small range and small-sized populations, and many are located in places under human influence, which is the main cause of threat for plant species in the Mediterranean area (Domínguez-Lozano et al., 1996; Thompson, 1999). Such a scenario possibly leads to drastic reductions in size or even to the extinction of populations, owing to recreational activities, road construction, grazing etc. The small distance and high gene flow among populations makes the natural recovery of populations possible.

Both the extremely low levels of variability, indicating some genetic erosion of the species, and its reproductive system, make A. siculum a different case. Despite the wide range, the species is severely fragmented, most populations are of a small size and all of them are under human influence, which is likely to lead to drastic reductions or

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**Table 5. Estimates of gene flow (Nm) and correlation coefficients obtained in Mantel tests, within taxa**

<table>
<thead>
<tr>
<th>Species</th>
<th>Nm</th>
<th>Mantel test</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. cirrhigerum</td>
<td>2.2</td>
<td>( r = 0.05 )</td>
</tr>
<tr>
<td>A. latifolium</td>
<td>4.2</td>
<td>( r = 0.44; P = 0.99 )</td>
</tr>
<tr>
<td>A. linkianum</td>
<td>1.3</td>
<td>( r = 0.004; P = 0.50 )</td>
</tr>
<tr>
<td>A. litigiosum</td>
<td>1.8</td>
<td>( r = 0.76; P = 0.99 )</td>
</tr>
<tr>
<td>A. majus</td>
<td>15</td>
<td>( r = 0.44; P = 0.80 )</td>
</tr>
<tr>
<td>A. siculum</td>
<td>0.42</td>
<td>( r = -0.10; P = 0.38 )</td>
</tr>
<tr>
<td>A. tortuosum</td>
<td>1.7</td>
<td>( r = 0.16; P = 0.83 )</td>
</tr>
</tbody>
</table>

There is no estimate of \( P \) in A. cirrhigerum due to the low number of populations studied.
even local extinctions. For such reasons, preservation of natural populations is a priority for species conservation. Although low levels of gene flow among populations exist, recolonization of populations is uncertain owing to large distances. Self-compatibility and, probably the selving reproductive system, together with the high production of seeds, make the regeneration of populations from remaining seeds possible. The high genetic differentiation among populations indicates that the implementation of complementary strategies, such as *ex situ* preservation of seeds from different populations, is highly recommended.

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**LITERATURE CITED**


