Low Levels of Allozyme Variability in the Threatened Species

Antirrhinum subbaeticum and A. pertegasii (Scrophulariaceae): Implications for Conservation of the Species

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INTRODUCTION

Theory predicts that levels of genetic variability depend on population size (Lewontin and Hubby, 1966), with small populations having lower levels of variability than larger ones. As pointed out by Gitzendanner and Soltis (2000), it is usually considered that rare species have low levels of genetic variability because of the generalized assumption that rare species have small population sizes. The available information does not always support that assumption, showing many examples of rare plants with both low (Arduino et al., 1996; Gemmill et al., 1998; Godt and Hamrick, 1998; Batista et al., 2001; Segarra-Moragues and Catalan, 2002) and high (Pedro-Monfort and Caujapé-Castells, 1994; El Mousadik and Petit, 1996; Young and Brown, 1996; Hoebee and Young, 2001) levels of variation.

Despite generalizations made by Hamrick and Godt (1989) regarding relationships between levels of genetic variation and its partition on the one hand, and the species range and biological traits on the other, other factors, such as habitat continuity, should be taken into account before making predictions about levels of genetic diversity and its partitioning. Ellstrand and Elam (1993) showed that in rare species with a small range, high levels of genetic diversity could be expected when population size is large and, similarly, Mateu-Andrés and Segarra-Moragues (2000) showed that the spatial distribution pattern of populations was an important factor in the partitioning of genetic diversity within and among populations of Antirrhinum valentinum F.Q. and A. charidemi Lange. Gitzendanner and Soltis (2000) shed light on several aspects regarding patterns of genetic variation in rare and widespread plants, leading to the conclusion that ‘while rare species do have statistically less genetic variation than their widespread congeners, there is a large range in values, and levels of diversity are highly correlated within a genus’. Specifically, the authors recommended comparing levels of variation among congeneric taxa with different ranges when trying to assess levels of variation in rare plants.

In this paper, data are reported on the levels of genetic variability of three related species of Antirrhinum: two threatened species, A. subbaeticum Güemes, Mateu and Sánchez and A. pertegasii Rothm., and, following the recommendation given by Gitzendanner and Soltis (2000), one related, but non-threatened species, A. pulverulentum Lázaro.

One of the main goals of conservation geneticists is to quantify levels of genetic diversity, as well as the distribution of genetic variability within and between populations,
since preservation of the evolutionary potential of endangered species is a primary aim in species conservation (Barrett and Kohn, 1991; Holsinger and Gottlieb, 1991). Knowledge of the genetic structure of the species will give us information about historical and contemporary patterns of gene flow among populations.

Both theory and experimental measurements show that processes that erode genetic variation, such as genetic drift, are more likely to affect small, geographically isolated populations (Young and Brown, 1996; Frankel et al., 1998; Oostermeijer, 2000) such as those of the three species considered here. Loss of genetic variation has traditionally been considered to decrease both the short- and long-term adaptability of populations in variable and changing environments.

Knowledge of genetic variability and its structure will provide a basis for the sustainable management and conservation of populations in threatened plants. Holsinger (1999) demonstrated how an understanding of the patterns of genetic variation within and between populations may help conservation biologists to identify evolutionarily distinct populations worthy of conservation, and Menges (2000) proposed the integration of genetics and demography to produce more realistic population viability analysis in plants and to use such analyses to guide conservation and management.

The aim of this paper is to study and compare levels of genetic variation of three related taxa with small ranges and similar ecology and biological traits, but with different population size and breeding system and to check the hypothesis that species with small total population size have lower levels of genetic variability than those with bigger ones. Additionally, these results will contribute to the development of conservation strategies for rare endemic species of *Antirrhinum*.

**Materials and Methods**

*Taxa studied*

All three species studied here are perennials, narrow-range endemics and specialized, with populations located on shaded limestone in the northeast, southeast and central parts of the Iberian Peninsula (Fig. 1).

Like all the species in the genus *Antirrhinum* (Sutton, 1988), *A. pertegasii*, *A. subbaeticum* and *A. pulverulentum* are perennial and diploid (Boscaiu et al., 1997, 2000). Both *A. pertegasii* and *A. pulverulentum* are self-incompatible, like the majority of species of *Antirrhinum* (Gruber, 1930, 1932), whereas *A. subbaeticum* has been recently reported as self-compatible (Jiménez et al., 2002).

Both *A. subbaeticum* and *A. pertegasii* are included in the Red List of Spanish vascular flora (VV.AA., 2000). *Antirrhinum subbaeticum* has been reported with only four populations and an overall total population lower than 500 individuals (Jiménez et al., 2002), is considered as endangered. *Antirrhinum pertegasii*, with no more than 10 populations and an overall total population estimated as less than 200 individuals (VV.AA., 2000), is considered as vulnerable. *Antirrhinum pulverulentum*, with many more populations, some of them of large size, is not considered as threatened.

**Plant material**

A total of 177 individuals were studied (Table 1). Individuals were grown under greenhouse conditions from seeds sampled from different plants in natural populations, with one individual per mother plant being studied. Sampled individuals represented between 23 and 63 % of the total population, except for PULV5 in which 10-5 % of the individuals were sampled (Table 1). Three out of four known populations were sampled for *A. subbaeticum*, four for *A. pertegasii* and five of *A. pulverulentum*, distributed throughout the species range (Table 1, Fig. 1). Data about populations are given in Table 1.

**Enzyme electrophoresis**

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 mM Na2S2O3, 1 mM MgCl2, 40 mg mL⁻¹ (w/v) PVP, and 4 mM mL⁻¹ mercaptoethanol. The material used for the extracts consisted of young leaves from plants grown in the greenhouse, and the extractions were absorbed onto 3 mm wicks of Whatman chromatography paper.

Ten enzymatic systems were assayed: aspartate aminotransferase (AAT, EC 2.6.1.1) aconitase (ACO, EC 4.2.1.3), diaphorase (DIA, EC 1.6.1.99), isocitrate dehydrogenase (IDH, EC 1.1.1.42), malate dehydrogenase (MDH, EC 1.1.1.37), menadione reductase (MRN, EC 1.6.99), 6-phosphogluconic dehydrogenase (6PGD, EC 1.1.1.44), phosphoglucone isomerase (PGI, EC 5.3.1.9), phosphoglucone mutase (PGM, EC 5.4.2.2), shikimic dehydrogenase (SKD, EC 1.1.1.25) and triosephosphate isomerase (TPI, EC 5.3.1.1). AAT and SKD could not be scored due to inconsistent banding patterns. The electrophoretic buffer system II from Wendel and Weeden (1989) was employed to resolve IDH, MDH, and SKD, their system VI was used for DIA, PGI, PGM, and TPI, and their system VII was used for AAT, ACO and MNR.

**Analysis of data**

Diversity parameters (A, mean number of alleles per locus; P05, P99, proportion of polymorphic loci at 95 % and 99 % criteria; H0, observed heterozygotes; He, expected heterozygotes), tests for significant deviations from Hardy–Weinberg expectation and Nei’s (1972) genetic distance measures within taxa between populations were calculated using Biosys-1 (Swofford and Selander, 1989). The total genetic variation (HT) was evaluated by a hierarchical gene diversity analysis following Chakraborty et al. (1982). The relative gene diversity (GST) was divided into two components: between samples within a region (GSR) and between regions (GTR). In *A. pertegasii* no regions can be differentiated, and so this hierarchical analysis was not performed for this species. A Mantel test (Mantel, 1967) was run by using NTSYS v.2.0 (Rohlf, 1998) to assess correlation between geographic and genetic distance.
FIG. 1. Map of the species’ ranges and the studied populations.
RESULTS

Eight out of the 10 enzyme systems studied could be interpreted (ACO, DIA, IDH, MDH, MNR, PGI, PGM and TPI), with a total of 14 loci. Pgi1 and Tpi1 had duplicated co-migrating loci, so they were not scored. The presence in diploid plants of an extra locus for Pgi1 and Tpi1 suggests a gene duplication (Gottlieb, 1982), which has been reported in species of other genera in Antirrhineae (Elisens and Crawford, 1988; Elisens, 1992; Elisens and Nelson, 1993). Mnr1 and Mnr2 were coincident with Dia2 and Dia1, while in A. pertegasii three out of 13 loci were polymorphic (Aco1, Mdh1 and Mdh2), all of them with two alleles, being Aco1-1 fixed in PERT1. In contrast, only two out of 14 loci (Dia2 and Dia3) were monomorphic in A. pulverulentum.

The loci that were resolved gave a total of 18 alleles in A. subbaeticum, 16 in A. pertegasii and 36 in A. pulverulentum. The mean number of alleles per locus through all three species ranged between 1 and 2-07 (Table 2). Among the three species, A. subbaeticum showed the lowest values, with all the studied loci being monomorphic in populations SBB1 and SBB3, and with a mean number of alleles per locus of 1-14 in SBB2. In A. pertegasii three out of 13 loci were polymorphic (Aco1, Mdh1 and Mdh2), all of them with two alleles, being Aco1-1 fixed in PERT1. In contrast, only two out of 14 loci (Dia2 and Dia3) were monomorphic in A. pulverulentum.

The proportion of polymorphic loci ranged between 0 and 36 in all populations with the only exception of Dia2 which showed three alleles in SBB2. In A. pertegasii three out of 13 loci were polymorphic (Aco1, Mdh1 and Mdh2), all of them with two alleles, being Aco1-1 fixed in PERT1. In contrast, only two out of 14 loci (Dia2 and Dia3) were monomorphic in A. pulverulentum.

Population size was calculated by a direct count of individuals.

* Only 12 individuals were reproductive in population PERT1.

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**Species** | **Population code** | **Province** | **Locality** | **Population size** | **% of population studied**
--- | --- | --- | --- | --- | ---
*A. subbaeticum* | SBB1 | Murcia | Benizar | 11 | 63-6
SBB2 | Albacete | Potiche | 78 | 34-6
SBB3 | Albacete | Bogarra | 23 | 43-4

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**Table 1. Parameters of genetic variability for the species studied**

| Species | Population | \( A_e \pm \sigma \) | \( P_{99} \) | \( P_{95} \) | \( H_o \pm \sigma \) | \( H_e \pm \sigma \) |
--- | --- | --- | --- | --- | --- | ---
*A. subbaeticum* | SBB1 | 1 | 0 | 0 | 0 | 0
SBB2 | 1-14 ± 0-14 | 7-14 | 7-14 | 0-024 ± 0-024 | 0-027 ± 0-027
SBB3 | 1 | 0 | 0 | 0 | 0

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*A. pertegasii* | PERT1* | 1-15 ± 0-10 | 15-38 | 15-38 | 0-103 ± 0-069 | 0-068 ± 0-046
PERT2 | 1-23 ± 0-12 | 23-08 | 23-08 | 0-019 ± 0-019 | 0-085 ± 0-046
PERT3 | 1-23 ± 0-12 | 23-08 | 15-38 | 0-045 ± 0-027 | 0-081 ± 0-050
PERT5 | 1-23 ± 0-12 | 23-08 | 15-38 | 0-032 ± 0-018 | 0-087 ± 0-054

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*A. pulverulentum* | PULV1 | 1-86 ± 0-23 | 64-29 | 64-29 | 0-298 ± 0-082 | 0-276 ± 0-073
PULV3 | 1-50 ± 0-14 | 50-00 | 50-00 | 0-314 ± 0-093 | 0-238 ± 0-067
PULV4 | 2-07 ± 0-32 | 64-29 | 64-29 | 0-164 ± 0-060 | 0-261 ± 0-067
PULV5 | 1-71 ± 0-24 | 50-00 | 42-86 | 0-102 ± 0-046 | 0-122 ± 0-046

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\( P_{99}, P_{95} = \text{proportion of polymorphic loci at 99 \% and 95 \% criteria, respectively; } A_e = \text{average number of alleles per locus; } H_o = \text{mean observed heterozygosity per locus; } H_e = \text{mean expected heterozygosity per locus.} \)
species as shown by ANOVA tests, even when populations of *A. subbaeticum* were excluded.

In *A. subbaeticum*, all loci were monomorphic in populations SUBB1 and SUBB3, and in SUBB2 the polymorphic loci were in Hardy–Weinberg equilibrium (χ² tests, with significance evaluated at *P* = 0.05 after sequential Bonferroni correction; Rice, 1989). Thirteen of the polymorphic loci in the other two species were in Hardy–Weinberg disequilibrium (Table 3), mostly due to an excess of heterozygotes in *A. pertegasii*, but invariably due to a deficit of heterozygotes in *A. pulverulentum*. Total diversity *H*ₜ in *A. subbaeticum* and *A. pertegasii* was very low, but was relatively high in *A. pulverulentum*. The genetic differentiation between populations was dramatically different among the three species, with most of the variation being accounted for in the between-populations component of *A. subbaeticum* (*G*₁ₛₚ = 0.87), being low in *A. pulverulentum* (*G*₁ₛₚ = 0.23) and negligible in *A. pertegasii* (*G*₁ₛₚ = 0.06) (Table 4). In *A. subbaeticum*, the between-regions component (*G*ᵣₜ = 0.71) accounted for most of the among-populations component of the total variation, while in *A. pulverulentum* the within-populations among regions (*G*ₛᵣ = 0.17) was higher.

Contrasting patterns were also found by correlation tests between genetic and geographic distances for all three species (Table 5). Mantel test showed a poor correlation between geographic and genetic distances in all three species, being positive in *A. subbaeticum* (*r* = 0.57) and negative both in *A. pertegasii* (*r* = −0.46) and *A. pulverulentum* (*r* = −0.33). These results agree with those obtained for differentiation between populations and gene flow estimates (Table 5), which were negligible (Nm = 0.04) for *A. subbaeticum*, high (Nm = 3.92) for *A. pertegasii* and moderately low (Nm = 0.83) for *A. pulverulentum*. This indicates that no gene flow exists between populations of *A. subbaeticum*, while those of *A. pertegasii* are genetically connected, and only low levels of gene flow exist between populations of *A. pulverulentum*.

A high correlation was found between within-population genetic variation (*Hₛ*) and population size in *A. subbaeticum* and *A. pertegasii* (*r* = 0.98 and 0.79, respectively) but not in *A. pulverulentum* (*r* = 0.18).

**DISCUSSION**

The three species show striking differences in levels of genetic diversity (Table 2) and its partitioning within and between populations (Table 4). The total genetic diversity (*H*ₜ) is 0.30 in *A. pulverulentum*, which can be considered as high but approaching values calculated for other species of the genus (Mateu-Andrés, 1999; Mateu-Andrés and Segarra-Moragues, 2000). In contrast, the values for *H*ₜ in *A. subbaeticum* (0.07) and *A. pertegasii* (0.08) are the lowest known in the genus. These low levels are more striking when compared with the high values found in other species of the genus, also among those considered as threatened (Mateu-Andrés, 1999; Mateu-Andrés and Segarra-Moragues, 2000). The partitioning of genetic diversity is also very different in the species studied here. The proportion of genetic differentiation between populations accounted for 85% of the total variation in *A. subbaeticum*, which is in agreement with results obtained with RAPD markers (Jiménez et al., 2002) and contrasting dramatically with the 6% value found in *A. pertegasii*, while the 23% estimated for *A. pulverulentum* indicates an intermediate level of differentiation. The high (71%) between-regions differentiation in *A. subbaeticum* contrasts with the low value found in *A. pulverulentum* (6%), indicating that populations of the two regions are genetically different in the former but not in the latter species.

The three species studied are perennials, a trait which, according to Loveless and Hamrick (1984) and Hamrick et al. (1979), is associated with a high migration rate and high levels of variation at the species level. *Antirrhinum subbaeticum* is self-compatible, with the converse effect of reducing genetic variability through inbreeding and drift. The contrasting coefficients of differentiation may result from the fragmentation of the species ranges: this being less of a factor for *A. pertegasii* whose populations are closely located, while those of *A. subbaeticum* are severely fragmented. The geographic proximity and continuity of the habitat in *A. pertegasii* allow genetic exchange between populations through pollen and/or seed dispersal, while the geographic distance between populations of *A. subbaeticum* makes this near impossible. Genetic similarities between populations are probably due to these being remnants of a wider range, when there were much higher numbers of populations and larger population sizes.

Population isolation, together with small population size, may lead to stochastic differentiation by genetic drift. The long-term evolutionary history of temperate plant species, including shifts in distribution, fragmentation and population isolation during and after the last glacial maximum, has a great effect on their population genetic structure (Mahy et al., 1999). In recent centuries, human activity has led to the fragmentation and isolation of the populations of many temperate plant species. In Mediterranean plants, human activity has had a dramatic influence on the fragmentation of habitats (Thompson, 1999). Both theory and empirical
Table 4. Partitioning of the total genetic variability

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<tr>
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<th>$H_S$</th>
<th>$H_R$</th>
<th>$H_{SR}$</th>
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***$P < 0.001$. $H_T$ = total genetic diversity; $H_S$ = mean genetic diversity within populations; $H_R$ = mean genetic diversity between regions; $H_{SR}$ = mean genetic diversity within populations among regions; $G_{ST}$ = ($H_T$ – $H_S$)/$H_T$, coefficient of genetic differentiation between populations; $G_{RT}$ = ($H_T$ – $H_R$)/$H_T$, coefficient of genetic differentiation between regions; $G_{SR}$ = ($H_R$ – $H_{SR}$)/$H_T$, coefficient of genetic differentiation between populations within regions.

Table 5. Estimates of gene flow and correlations among genetic and geographic distances

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Both $A. subbaeticum$ and $A. pertegasii$ could be threatened for natural reasons, including small population size, restricted habitat or climatic changes, similar to most of the endangered flora in Spain (Domínguez et al., 1996; Blanca et al., 1998). In the Mediterranean area, human activity has resulted in considerable fragmentation of habitats. Thompson (1999) concluded that, in some species, human influence has resulted in a fragmentation into habitat islands that is stronger than the separation of true islands. Jiménez et al. (2002) reported excessive herbarium collecting and grazing pressure as causes of the drastic reduction of individuals in three out the four known populations of $A. subbaeticum$. Other threats to populations close to rivers or small dams are wetland desiccation, recreational activities or construction of new infrastructures (roads, dams, etc.). The areas inhabited by $A. pertagasii$ are well preserved, and threats to that species are likely to be attributable to natural causes, such as small population size. However, other threats such as grazing by wild or domestic animals may also be important.

The consequences of small population size include increased inbreeding rates and genetic drift, with subsequent lack of variation within populations and increased differentiation between them (Ellstrand and Elam, 1993; Usher, 1997). These effects can be observed in $A. subbaeticum$, whose populations differ markedly in number of individuals. The smaller ones were fixed for all genetic diversity.
the loci studied, while more variability was found in the largest one. The fact that the biggest population shares alleles with one or two of the other populations supports the hypothesis of genetic drift, as reported by Jiménez et al. (2002). In *A. pertegasii*, lack of variation may also be seen as a consequence of the small size of the total population. Although populations may be genetically connected through pollen or seed flow due to their geographic proximity, the small number of individuals in total reduces the possibility for mutation to counterbalance the effects of genetic drift or bottlenecks.

Whatever the cause of the threat, differences in genetic diversity and its partitioning within and between populations may have important consequences for conservation. If populations of *A. subbaeticum* become extinct, recolonization is unlikely because of the large distances to the remaining ones. Conversely, estimated levels of gene flow indicate that recolonization may be possible in *A. pertegasii*.

For *A. subbaeticum*, all the known populations should be preserved, and reinforcement of small populations with individuals grown from seeds from the same populations and using special techniques favouring the establishment of seedlings are recommended.

Preservation of natural populations is the priority in plant conservation. It is thus implicit that any possible cause of threat, such as grazing, housing development or draining, should be avoided. Complementary strategies, such as *ex situ* preservation of seed, are also recommended. As levels of genetic variability assessed by some enzymatic loci may not reflect other types of variability (Eillstrand and Elam, 1993), seeds should be sampled from as many individuals as possible of *A. pertegasii* and in as many individuals as possible in all four known populations of *A. subbaeticum*. As individual plants of *Antirrhinum* can produce many fruits with tens of seeds in each, such a strategy will allow the preservation of the genetic diversity without losing the possibility for establishment of seedlings. Finally, high levels of genetic differentiation between populations of *A. subbaeticum* strongly suggest that any transplantation of seeds or seedlings among them should be avoided in this species.

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LITERATURE CITED


