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Genetic variability in *Apurimacia dolichocarpa* (Fabaceae), a narrow endemic species of Córdoba Hills, Argentina

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Apurimacia dolichocarpa (Fabaceae) is a narrow endemic species, restricted to Córdoba Hills, Argentina. At present, only two populations are known. In order to analyse the level and distribution of its genetic variability, allele frequencies in 16 loci coding for enzymes were estimated from starch gel electrophoresis patterns. The levels of genetic diversity ($P_{99\%} = 56.25$, $A = 1.81$, $H_e = 0.1125$) are higher than the mean for endemic species reported in the literature. The low level of genetic differentiation between the two populations ($\theta = 0.04$) may be the result of a recent fragmentation of an ancestral panmictic population. *Apurimacia dolichocarpa* does not appear as threatened from the genetic viewpoint, given that its populations preserve a moderate level of allozymic polymorphism. Artificial establishment of intermediate populations would facilitate pollen dispersal, a strategy that could favour the maintenance of the polymorphism levels.

Introduction

The genus *Apurimacia* (Fabaceae) comprises five species distributed mainly in the mountain regions of Peru and Bolivia, with one endemic species, *A. dolichocarpa*, restricted to Córdoba Hills, Argentina (Burkart 1952, Gómez Sosa 1999). Also other endemic species, such as *Aa achalensis*, *Adesmia cordobensis*, *Buddleja cordobensis* and *Croton argentinus* occur in this region (Funes & Cabido 1995, Vischi *et al*. 2004).

The total area of occupancy of *A. dolichocarpa* is less than 400 km^2 in the west of the *Sierras Grandes* (Great Hills) of Córdoba (Funes & Cabido 2008). The main orogenic event of these mountains began in the lower Paleozoic

and it was re-activated through tectonic movements in the Tertiary (Andean orogeny) and even in the Quaternary period. Most of the area is composed of metamorphic rocks, with granite batholits delimiting N–S oriented faults (Cabido *et al*. 1998). At present, only few aspects of the biology of *A. dolichocarpa* (inflorescence organization, floral vasculature, embryology and pollen morphology; Maldonado 1983) and its chromosome number (2n = 22; Cocucci 1961) are known. It is a woody subshrub up to 1-m tall; the flowers are violet, typical papilionaceous, and the fruit is a legume with bivalve dehiscence with 1, 2 or 3 seeds of 1 cm in diameter.

Apurimacia dolichocarpa occurs naturally on rock outcrops between 1300 and 1800 m, being thus exposed to high solar radiation and wind, water shortage and lack of soil; it is the dominant species in the saxicolous communities of the area (Funes & Cabido 2008). At present, only two populations are known. Rock outcrops represent a small extent within the region and provide a suitable habitat for local endemic and naturally rare plants (Cabido *et al*. 1990, Funes & Cabido 1995, De Lange & Norton 2004). Such species are of interest to plant biologists faced with the difficulty of preserving them from habitat degradation or fragmentation (Ge *et al*. 1997).

Levels of genetic diversity and gene flow among populations are of major concern in population genetics because they are considered to greatly influence the evolutionary potential of a species. Many factors are known to affect genetic diversity of plant species; one of them is their geographic range. Widespread species tend to maintain more genetic variability than those with a narrow distribution (Karron 1987, Hamrick & Godt 1989), although the geographic range appears to have little influence on the inter-population differentiation (Hamrick & Godt 1989). Besides, endemic species may become genetically depauperate owing to bottlenecking or strong directional selection for specialized niches (Williamson & Werth 1999). Assessment of the levels and distribution of genetic variation in endemic plant species may contribute to design appropriate management strategies for their preservation.

Biochemical markers such as allozymes have been used extensively in plant population genetic studies of geographically widespread (Hamrick & Godt 1989, Sanchez-Doreste *et al*. 2006) as well as narrow endemic species (Francisco-Ortega *et al*. 2000, Hamrick & Godt 2002, Caujapé-Castells 2009, Mora-Vicente *et al*. 2009). In endemics of the family Fabaceae, Oliva-Tejera *et al*. (2005, 2006) analysed the allozymic variation to clarify the relationships among taxa within the complex of Gran Canarian *Lotus* and to infer their patterns of diversification.

We employed this technique to estimate the levels of polymorphism in *Apurimacia dolichocarpa* and to analyse the degree of population structuring in this narrow endemic species.

Material and methods

Sampling

The study area is located on the western slope of Córdoba Hills, central Argentina (Fig. 1). Samples were taken from the two populations found in the area: Villa Benegas (31°36´S, 64°56´W) and Cumbres de Gaspar (31°19´S, 64°53´W), separated by 35 km. Within each location, individuals of the species were present between 1350 and 1800 m. The Cumbres de Gaspar population is substantially larger than the one in Villa Benegas.

Given the very difficult access to the populations, 9 individuals were sampled in Villa Benegas and 18 in Cumbres de Gaspar, separated by a minimum distance of 50 m to minimize the chance of collecting closely related or genetically identical individuals. The allozyme analyses were performed in cotyledons from five different seeds from each individual.

Allozyme analyses

Seven-day-old cotyledons (two-day-old for ADH) were homogenized in porcelain spot plates using Tris HCl buffer. Homogenates were introduced into gels immediately after homogenizing. Seed tissue extracts were analyzed by electrophoresis in 10% horizontal starch gels and 7% polyacrilamide gels. Electrophoresis was carried out according to Scandalios (1969) and Shaw and Prassad (1970).

Data were obtained for nine enzyme systems: esterase (EST), alcohol dehydrogenase (ADH), peroxidase (PRX), superoxide dismutase (SOD), asparatate aminotransferase (AAT), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), malic enzyme (ME) and shikimate dehydrogenase (SKD).

Staining procedures for PRX, ADH, AAT and EST were as described by Scandalios (1969) and those for IDH, MDH, EM, SOD and SKD, were performed according to Wendel and Weeden (1990).

Data analysis

Polymorphism estimations

Allozymic phenotypes were interpreted genetically according to standard principles (Wendel & Weeden 1990). The TFPGA computer program (Miller 1997) was used to estimate unbiased expected heterozygosity (H; Nei 1978), mean number of alleles per locus (A), percentage of polymorphic loci (P) and conformance to Hardy-Weinberg equilibrium. Expected heterozygosities were compared between populations using the Mann-Whitney test (Sokal & Rohlf 1995) implemented in InfoStat (Di Rienzo *et al*. 2007). A chi-square test was applied to detect significant difference in allele frequencies between populations for each locus.

The effect of sample size on the sampling distribution of the unbiased mean heterozygosity estimator (H) was studied by a re-sampling technique. For this purpose, the empirical association between the median and the amplitude of the 95% prediction interval of the sampling distribution of H was found for different sample sizes. The procedure was performed through a routine *ad hoc* implemented in the software Info-Gen (Balzarini *et al*. 2004).

Diversity within and between populations

Genetic structure was examined by means of F-statistics (Wright 1965) with the corrected method of Weir and Cockerham (1984), using the estimator θ for F_{ST} (the standarized variance of allele frequencies between populations) and f for F_{IS} (the inbreeding coefficient within populations). Significance of θ was obtained by permutation of alleles between samples, and that of f, by permutation of alleles within samples (1000 permutations). The Workman and Niswander (1970) procedure was used to test if F_{ST} values per locus differed significantly from 0. The FSTAT program (Goudet 2000) was used for these calculations. The effective migration rate was estimated as $N_m = (1/\theta - 1)/4$.

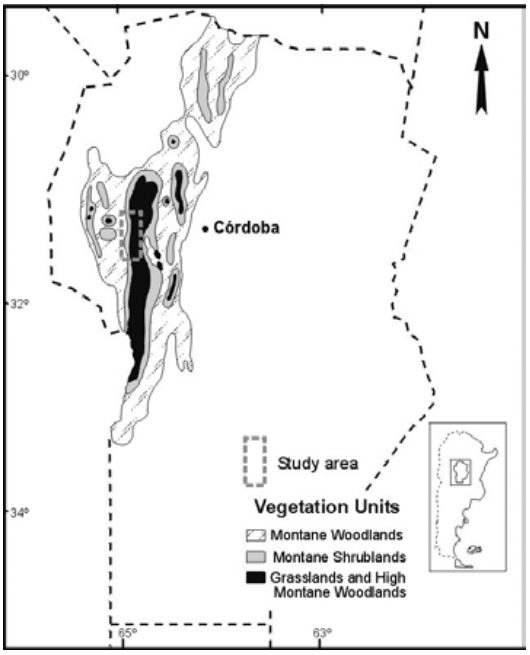

Fig. 1. Map showing the location of the study area in the Córdoba Hills, central Argentina.

An alternative method developed by Pritchard *et al*. (2000) and implemented in the software STRUCTURE allows to define clusters of invididuals on the basis of their genotypes at multiple loci using a Bayesian approach. The model assumes that within populations the loci are at Hardy-Weinberg equilibrium and linkage equilibrium, and attempts to define population groupings by maximizing both parameters. Ten independent runs of 2×10^5 MCMC cycles for burn-in and 8×10^5 for data collection were performed for values of K from 1 to 5, assuming correlated allele frequencies among populations, allowing for admixture (i.e. gene flow) and setting the allele frequency prior parameter λ to

one. Significance of analyses were tested over 50 000 permutations.

Results

Polymorphism estimations

The analysis of nine enzymes showed the product of 16 loci. Six of them were monomorphic in the two populations (*Aat1*, *Aat3*, *Prx2*, *Sod1*, *Sod2* and *Sod3*). In any of the polymorphic loci (*Aa2*, *Prx1*, *Prx3*, *Adh*, *Em*, *Idh*, *Mdh1*, *Mdh2*, *Skd* and *Est*) the observed genotype frequencies were not significantly different from those expected under the Hardy-Weinberg equilibrium (Table 1). Unique alleles were found within each population: allele 2 of *Adh* was present only in Villa Benegas, whereas allele 1 of *Prx* and allele 3 of *Skd* were detected only in Cumbres de Gaspar.

Table 1. Allele frequencies in ten polymorphic loci of two populations (Villa Benegas and Cumbres de Gaspar) of *Apurimacia dolichocarpa*. *n* = number of individuals (seeds) analyzed.

Locus	*n*	Villa Benegas	*n*	Cumbres de Gaspar
Adh	45		90	
1		0.900		1
2		0.100*		0
Est				
1	40	0.188	79	0.177
2		0.774		0.766
3		0.038		0.057
Aat2	41		90	
1		0.085		0.101
2		0.915		0.899
Idh	40		85	
1		0.163		0.041
2		0.837		0.959
Prx1	41		82	
1		0		0.032*
2		1		0.968
Prx3	40		89	
1		0.026		0.026
2		0.974		0.974
Em	43		85	
1		0.035		0.013
2		0.907		0.953
3		0.058		0.034
Mdh1	42		80	
1		0.049		0.016
2		0.951		0.984
Mdh2	40		80	
1		0.562		0.722
2		0.438		0.278
Skd	41		76	
1		0.829		0.848
2		0.171		0.106
3		0		0.046*

Locus abbreviations: *Adh* = alcohol dehydrogenase; *Est* = esterase; *Aat* = asparatate aminotransferase; *Idh* = isocitrate dehydrogenase; *Prx* = peroxidase; *Em* = malic enzyme; *Mdh* = malate dehydrogenase; *Skd* = shikimate dehydrogenase. The asterisks indicate unique alleles.

The mean number of alleles per locus was identical in the populations. *P* values were different under the criteria of 95% and 99% because different alleles in several loci were present in low frequency in both populations (Table 2). Although the expected heterozygosity in the Villa Benegas population was greater than in Cumbres de Gaspar, the difference was not significant ($p = 0.6227$).

The effect of sample size on the estimator *H* showed that the 95% prediction bands stabilize when sample size is > 10. Then, sample sizes of 9 (Villa Benegas) and 18 (Cumbres de Gaspar) would be adequate for the estimation of heterozygosity in the populations studied.

Diversity within and between populations

The average intra-population fixation index ($f = 0.043$) was not significant, confirming that there is no evidence of inbreeding in the species.

The estimated level of genetic differentiation between the populations ($\theta = 0.04$) was, on average, significantly different from 0, indicating population structuring (Table 3). The estimated gene flow (N_m) was of six effective migrants per generation (Table 3), considering an island model of population structure.

The analysis of the genetic differentiation level performed using STRUCTURE showed that the best hypothesis according to a maximun likelihood criterion is the one for $K = 1$, which means that the two populations are indistinguishable and that each population contributes the same proportion to the total diversity of the species. The probabilities for $K = 2$, 3 and 4 were 0.44–0.59, 0.30–0.38 and 0.24–0.26, respectively.

Table 2. Variability estimates in two populations of *Apurimacia dolichocarpa.*

	$P_{99\%}$	$P_{95\%}$	A	H_e	H_o
Villa Benegas	56.25	43.75	1.81	0.13	0.1207
Cumbres de Gaspar	56.25	25	1.81	0.095	0.0923
Average	56.25	34.37	1.81	0.1125	0.1065

P = percentage of polymorphic loci (95% and 99% criteria), A = mean number of alleles per locus, H_e = expected heterozygosity, H_o = observed heterozygosity.

Discussion

This is the first study on the genetic structure of *Apurimacia dolichocarpa*, an endemic species of the Córdoba Hills (Argentina). At the population level, the mean values of variability parameters found in *A. dolichocarpa* ($P_{99\%}$ = 56.25%, A = 1.81, H_e = 0.1125) were substantially higher than those cited by Hamrick and Godt (2002) for endemic species (P = 26.3%, A = 1.39, H_e = 0.063) and by Crawford *et al*. (2001) for species from the Juan Fernández archipelago (H = 0.042). Mean heterozygosity in *A. dolichocarpa* is similar to the average value of H_e = 0.156 reported in a review including 69 Canarian endemic species (Francisco-Ortega *et al*. 2000), but lower than the mean values reported by Oliva-Tejera *et al*. (2006) for the endemic *Lotus* species in Fabaceae (*L. kunkelii*, H_e = 0.306; *L. lancerottensis*, H_e = 0.254; *L. arinagensis*, H_e = 0.284). The authors explain these particularly high levels of polymorphism as a consequence of a considerable antiquity, habitat stability and recent habitat fragmentation. In Canarian woody endemics of the genus *Echium* (Boraginaceae) high levels of genetic variability were also reported, which are explained by combinations of ecological, geographical and reproductive factors (Mora-Vicente *et al*. 2009).

The level of polymorphism in *A. dolichocarpa* is noteworthy, given the extremely small geographic range of this species. Other authors have reported substantial levels of genetic variability in endemics occupying restricted areas, such as *Pterostylis aff. picta* (Orchidaceae; Sharma *et al*. 2003), *Cypripedium macranthos* (Orchidaceae; Izawa *et al*. 2007) and in a declining population of *Primula vulgaris* (Primulaceae) (Van Geert *et al*. 2008), reinforcing the idea that the degree of genetic diversity cannot be predicted only on the basis of the geographic range of a species.

Hamrick and Godt (1989) identified a number of species characteristics associated with high levels of genetic variation in plants, such as reproductive system, pollination, seed dispersal mode, geographical distribution, and local abundance. Among them, the breeding system has been addressed as a major determinant of patterns of genetic variation: outcrossing species have significantly higher levels of genetic variation within populations than selfing species (Hamrick & Godt 1989). In *A. dolichocarpa*, the genotype frequencies are in Hardy-Weinberg proportions in all the polymorphic loci and the values of F_{IS} are not significant, which indicate that outcrossing would be the prevalent mode of breeding. On the other hand, the persistence of multiple generations within populations observed in the

Table 3. Genetic structure estimates: F_{IS} (Wright's inbreeding coeficient); θ (Weir and Cockerham estimator of the standarized variance in allele frequencies between populations). N_m = effective number of migrants per generation, estimated by $(1/\theta - 1)/4$ (Wright 1951).

Locus	θ	F_{IS}
Adh	0.135*	0.143
Est1	0	0.078
Aat2	0	0
Idh	0.086*	0.099
Prx1	0.016	0
Prx3	0	0
Em	0.005	0
Mdh1	0.018	0
Mdh2	0.102*	0.017
Skd	0.001	0.154
Average	0.040*	0.043
N_m	6	

*$p < 0.01$.

field (M. A. Grossi pers. obs.) and, in spite of its restricted geographic range, the maintenance of locally important population sizes (Funes & Cabido 2008), may also contribute to the high levels of the detected genetic variation.

Although the value of θ was significant, it was very low ($\theta = 0.04$). When the analysis was performed using a Bayesian approach (which groups individuals according to their "posterior probability" of belonging to K populations that are in Hardy-Weinberg and linkage equilibria), all individuals were assigned to only one population, reinforcing the idea of very low genetic differentiation between the two geographical populations. The mechanism of pollination in *A. dolichocarpa* is not known; however, the morphology and color of the flowers suggest insects are the vectors (Fenster *et al*. 2004). In fact, small bees have been observed visiting the flowers (M. A. Grossi pers. obs.), but neither the specific status nor the flying capacity of those bees have been established, which would be essential to understand their role in pollen dispersal. However, inter-population pollen transfer by these insects appears very unlikely due to the geographical distance between Villa Benegas and Cumbres de Gaspar. The seeds are relatively heavy, they do not have structures to aid wind-dispersal and there is no evidence of dispersal by birds. The value of N_m estimated may not be the result of current active pollen and seed dispersal, but could indicate that the two sampled populations are the remnants of an ancestral panmictic population which suffered a recent fragmentation, having not had enough time to reach a higher degree of differentiation.

Segarra-Moragues *et al*. (2005) studied the effects of long-term reduced population size in the relictual species *Borderea chouardii* (Dioscoreaceae); the authors obtained more reliable results using RAPDs and SSR markers than allozymes. A study using other highly variable DNA markers (ISSR) is ongoing in our laboratory in order to assess the fine scale genetic structure of the extant populations of *A. dolichocarpa* on the basis of a higher number of polymorphic loci. The use of these markers would also allow to examine if young generations (recently germinated seedlings) maintain the same genetic diversity as their parents (sampled from leaves of adult plants) in order to distinguish between historical and current processes in the determination of the genetic population structure.

The high levels of heterozygosity within populations and the lack of evidence of inbreeding obtained using allozymic data suggest that *A. dolichocarpa* is not threatened from the genetic point of view, given that its populations are not genetically depauperated. In accordance with this idea are the facts that (1) the species is dominant in the outcrop communities where it occurs, and (2) the germination of the seeds is not constrained by temperature nor by light/dark conditions (Funes & Cabido 2008). However, the significant proportion of alleles in frequencies below 0.05 (as revealed by the difference between 99% and 95% P values) could indicate an ongoing loss of allele richness, which could restrain a rapid response to changing environmental conditions. Since *A. dolichocarpa* has a very restricted distribution, an increase in effective population size by extensive gene flow among several populations is virtually impossible. Artificial establishment of intermediate populations could facilitate pollen dispersal, a strategy that could favour the maintenance of the polymorphism levels. Taking into account that the access to natural populations is very difficult, manual pollen transfer would not be a feasible practice in this case.

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