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ORIGINAL ARTICLE

Isozyme diversity in some Canarian woody endemisms of the genus *Echium* L. (Boraginaceae)

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Abstract Oceanic archipelagos are considered hot-spots of biodiversity because they harbor unique biota with a high level of endemicity. However, the endemic biodiversity of oceanic islands is very vulnerable to extinction. In recent decades, intensive exploitation of these territories and human-mediated introduction of alien species have posed unprecedented threats to the long-term survival of the endemic contingent. The very limited population genetic information available until now for the 28 Canarian endemic *Echium* taxa has hindered the development of conservation strategies for the ca. 25% of them that are under threat. In this paper, we analyze the levels and distribution of genetic isozyme diversity in 23 natural populations of three endangered endemics of restricted distribution (*E. acanthocarpum*, CR; *E. onosmifolium* ssp. *spectabile*, EN; and *E. callithyrsum*, VU), and two endemics of wide distribution and in principle free of threat (*E. decaisnei* and *E. onosmifolium*). Our results reveal high levels of genetic variability in all these taxa that have plausibly been reached despite a predominance of selfing. They also point out a high incidence of inbreeding in the reproductive dynamics of populations and suggest the potential value of hybridisation processes in shaping the genetic makeup of these species. Among-population differentiation, as estimated by *Gst*, and genetic distances within taxa are low overall, but they do support the current taxonomic separation between the two subspecies of *E. onosmifolium* and do not furnish evidence that the current status of the endangered species may be attributed to genetic factors. We use the genetic parameters to suggest some guidelines to help implement a conservation strategy of these taxa.

Keywords *Echium decaisnei* · *Echium callithyrsum* · *Echium onosmifolium* · *Echium onosmifolium* subsp. *spectabile* · Echium acanthocarpum · Tajinaste · Isozymes · Mating systems

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Introduction

Genetic diversity of species depends both on life history (Gitzendanner and Soltis 2000) and mating system (Hamrick and Godt 1989), which not only determines the frequency of the genotypes but also affects parameters such as gene flow and selection (Hamrick 1989). According to Hamrick and Godt (1989), selfing species often have lower genetic diversity and higher between-population genetic structure than outcrossing ones.

The apportionment of genetic variability among populations is usually estimated through the parameter *Gst* (Nei

1973), and is the result of interactions among several biological and stochastic factors that determine the evolution of the lineages (Slatkin 1985, 1987; Hamrick 1989). While selection and genetic drift usually increase differentiation among populations, gene flow tends to promote genetic cohesion (Slatkin 1985, 1987). Not unnaturally, the estimation of this parameter is of paramount importance for conservation biology; for example, the number of populations to conserve of a given taxon is directly related to the proportion of genetic diversity that resides among populations (Hamrick et al. 1991).

Although many Canarian plant endemics are outcrossers, the high *Gst* values reported for the taxa examined with allozymes in the latest published review (Francisco-Ortega et al. 2000) indicate diminished levels of gene flow between populations (average $Gst = 0.281$). As highlighted by Francisco-Ortega et al. (2000), this result entails that conserving the genetic variation of the Canarian flora would make it necessary to target a high number of natural populations. Nevertheless, some studies published subsequent to Francisco-Ortega et al. (2000) indicated lower *Gst* values (i.e., Oliva-Tejera et al. 2005, 2006; Sánchez-Doreste et al. 2006).

The genus *Echium* L. consists of about 60 taxa distributed in the Macaronesian and Mediterranean regions. More than half of them are endemic to Macaronesia, with the largest center of taxonomic diversity in the Canary Islands, where some 28 endemic taxa are recognized (approximately 25% of them are endangered or critically endangered) [Bramwell 1972, 1985; VV.AA 2001].

While the genus has been the subject of systematic studies based on morphological (i.e. Bramwell 1972) and molecular (Böhle et al. 1996) characters, there are still very few population genetic investigations. As far as we know, population genetic studies with isozymes have only been carried out in the widely distributed *E. plantagineum* L. (Brown and Burdon 1983; Burdon et al. 1988) and in the Canarian endemic *E. acanthocarpum* Svent. (Batista and Sosa 1998), and using RAPDs, in the Cape-Verdean endemics *E. stenosyphon* Webb and *E. vulcanorum* A. Chev. (Romeiras et al. 2007).

The lack of molecular data for the large majority of the Canarian endemic *Echium* prevents the necessary understanding of the influence of the biological characters on the levels and distribution of its genetic variability previous to the development of conservation strategies for the endangered taxa. Isozyme electrophoresis has been widely used to assess the genetic diversity of species (Hamrick and Godt 1989, 1996). In the Canarian Flora, it constitutes the largest population genetic database and it has been successfully applied in systematic (i.e., Pedrola-Monfort and Caujapé-Castells 1996 Oliva-Tejera et al. 2005), conservation (i.e., Batista and Sosa 1998; Francisco-Ortega et al. 2000), or taxonomic (i.e., Oliva-Tejera et al. 2006) studies. Therefore, we believe that this technique is a suitable first choice to address problems of microevolution and conservation in *Echium*.

In this work, we use isozyme electrophoresis to estimate the levels and distribution of population genetic variation in three Canarian *Echium* with different degrees of threat: *E. acanthocarpum* (*critically endangered*, Marrero Gómez et al. 2004), *E. onosmifolium* ssp. *spectabile* Kunk. (*endangered*, Mora-Vicente et al. 2004), and *E. callithyrsum* Webb ex Bolle. (*vulnerable*, Mora-Vicente 2004). Since a substantial part of the genetic variability of a species depends on its lineage ascription (Gitzendanner and Soltis 2000), we have also selected two widespread Canarian endemics which are not threatened at present (*E. onosmifolium* Webb & Berth. and *E. decaisnei* Webb & Berth.) to establish a meaningful comparative framework. Although there is consensus on the taxonomic identity of all these taxa, morphological differences among *E. onosmifolium* and *E. onosmifolium* ssp. *spectabile* are not always clear. In the case of the threatened taxa, key factors of threat are both the fragmentation processes of the species over its range due to population extinction, and the progressive diminution and fragmentation of their natural populations, as well as the small number of individuals surviving reproduction.

Our specific objectives are to use the isozyme data to (1) infer the influence of different reproductive parameters on the levels of genetic variation, (2) evaluate the relationship between the two subspecies of *E. onosmifolium*, and (3) give some conservation guidelines for the threatened species.

Materials and methods

Plant material

The five taxa selected are woody shrubs. *Echium decaisnei* belongs to the section *Decaisnea* and the others to the section *Virescentia*. All these taxa are exclusive insular endemics (*E. acanthocarpum* to La Gomera, and the rest to Gran Canaria) (Marrero Gómez et al. 2004; Mora-Vicente 2007). Reproductively, they can display floral heterochromy within and between populations, are non-strict protandrous (i.e., the androecium ripens before the gynoecium), and gynodioecy (i.e., coexistence of hermaphrodite and female individuals in the same population) usually has a high population incidence. They are partially self-compatible, but the viability of selfed seeds is lower than that of seeds produced by outcrossing (Mora-Vicente 2007). All these taxa develop large inflorescences, which promote geitonogamy (i.e., fertilization of a flower with pollen from

another flower of the same individual). The fruits are dispersed mainly through barochory (i.e., by gravity) although they can also be dispersed by exozoochory (i.e., adhered at the surface of animals) or anemochory (i.e., by the wind) (Bramwell 1972; Marrero Gómez et al. 2000).

Sampling

Several leaf buds of 741 individuals were collected from 23 natural populations (8 of *E. decaisnei*; 7 of *E. callithyrsum*, 5 of *E. onosmifolium*, 2 of *E. onosmifolium* ssp. *spectabile* and 1 of *E. acanthocarpum*) that thoroughly represent the distribution area of these five taxa (Table 1; Fig. 1). Each sampling was preceded by an exhaustive visual inspection in order to obtain an idea of the size and occupancy area of the populations. Homogeneous populations were sampled throughout transects between the extreme points of their occupation areas, and samples were collected at regular intervals. In heterogeneous populations, we carried out a previous sectorization and we proceeded in the same way for each zone defined. On average, we sampled 25–30 individuals per population, though we sampled higher numbers of individuals in some cases, according to the particular characteristics of certain taxa and/or populations. Each sample was stored in unambiguously labeled airtight plastic bags that were kept in a cool box until their storage at −80°C in the laboratories of the Department of Molecular Biodiversity at the Jardín Botánico Canario "Viera y Clavijo".

Electrophoretic analysis

The proteic extracts were obtained by grinding the leaf buds in a porcelain mortar with a pestle. We used liquid nitrogen to minimize the action of the secondary metabolites that in other cases (Batista and Sosa 1998) have interfered with the resolving power of the technique. We also used an extraction solution that prevents oxidation of proteins and guarantees their optimal conservation (after Shields et al. 1983). The resulting extracts were absorbed on Whatman no. 3 paper wicks that were stored at −80°C until they were subjected to 12% starch gel electrophoresis (Aldrich 23, 402–8).

Out of 25 tested enzymes, 11 were satisfactorily resolved in at least some of the taxa. Presumably as a result of the interference caused by the secondary metabolites, we were unable to achieve adequate resolution for all taxa in six of these. Consequently, studies of enzymatic diversity were only performed with the five enzymes that could be resolved in all taxa, using three gel/electrode systems: morpholine–citrate 6.5 (Clayton and Tretiak, 1972) resolved Acid phosphatase (ACP, EC 3.1.3.2); lithium borate 8.3 (Selander et al. 1971) resolved glutamate-oxaloacetate-transaminase (GOT, EC 2.6.1.1), malic enzyme (ME, EC 1.1.1.40) and phosphoglucose isomerase (PGI, EC 5.3.1.9); finally, histidine–citrate 5.7 (Stuber et al. 1977) resolved phosphoglucomutase (PGM, EC 5.4.2.2). All staining recipes were based on Wendel and Weeden (1989), though some of them underwent modifications in the amounts of substrate and final pH to enhance band resolution. The methodology used to prepare the gels and the gel/electrode buffer systems is described in Caujapé-Castells et al. (2008).

For each enzyme, gene loci were designated by numbers and alleles by letters (beginning with the most anodally migrating forms for each). Checking of allele mobilities among populations was carried out through side-by-side comparisons on the same gel. Gel interpretations were drawn and stored in the matrix provided by the Transformer-3 software (Caujapé-Castells and Baccarani-Rosas 2005).

Data analysis

The mean number of alleles per locus (A_l), percentage of polymorphic loci (P), observed and expected heterozygosities (H_e and H_o), and Nei's (1978) genetic identities (I) were calculated using BIOSYS-1, version 1.7 (Swofford and Selander 1989). The effective number of alleles (A_e), the estimates of gene flow between populations, and the Ewens–Watterson (E–W) test of neutrality were calculated using Popgene 1.32 (Yeh et al. 1997). Nei's (1973) population structure statistics and Wright's (1951) F statistics were calculated using GENESTAT-PC 3.31 (Lewis and Whitkus 1993) and BIOSYS-1 1.7, respectively. Nei's unbiased genetic identity (I, Nei 1978) was used to build a UPGMA cluster with NTSYS-PC 1.80 (Rohlf 1993) for all the taxa.

All the file formats needed to run the population genetic programs for all population groupings were obtained from the drawn interpretations of the isozyme patterns using the program Transformer-3 (Caujapé-Castells and Baccarani-Rosas 2005).

We made a correlation analysis to test the possible impact of sexual and chromatic floral heteromorphism on population genetic diversity. To that end, the diversity (Dv) of sexual forms and colors for each of the natural population sampled in the present work were calculated from the data in Mora-Vicente (2007) using the expression $\mathrm{Dv} = 1 - \sum f^2$ (Margalef 1986), where f is the frequency of the sexual and chromatic forms in each population (the color diversity was measured in the upper part of the corolla). Since this type of heterochromy is virtually nonexistent in the natural populations of *E. decaisnei* and *E. onosmifolium* ssp. *spectabile*, this calculation was only made for the natural populations of *E. callithyrsum* and *E. onosmifolium*. Since only two populations of

Table 1 Population sampling details and basic indicators of isozyme variation for the 23 where we resolved the five selected loci

Taxon/population	Code	ID	*N*	Basic indicators of isozyme diversity							Indicators of diversity of sexual and color floral forms	
				T	A_1	*P*	H_o	H_e	*F*	*t*	*Dvsx*	*Dvchr*
E. decaisnei												
Agaete	EDAG	1	–	9	1.6 (0.2)	57.1	0.168 (0.117)	0.213 (0.083)	0.365	0.465	0.20	–
Agüimes	EDAGÜ	2	–	10	1.7 (0.3)	57.1	0.096 (0.053)	0.150 (0.061)	0.479	0.352	0.16	–
Barranco de Mogán	EDM	3	–	15	2.6 (0.5)	57.1	0.153 (0.071)	0.240 (0.101)	0.229	0.627	–	–
Barranco de Tasartico	EDT	4	–	11	1.9 (0.3)	57.1	0.247 (0.135)	0.250 (0.096)	0.026	0.949	–	–
Caldera de Bandama	EDB	5	–	11	1.7 (0.4)	28.6	0.047 (0.025)	0.149 (0.094)	0.464	0.366	0.46	–
Hoya Pineda	EDHP	6	–	12	1.9 (0.4)	42.9	0.116 (0.065)	0.198 (0.103)	0.186	0.686	0.31	–
Montaña de Amagro	EDA	7	–	12	2.0 (0.4)	42.9	0.121 (0.075)	0.175 (0.092)	0.256	0.592	0.00	–
Pico Bandama	EDPB	8	–	13	2.0 (0.3)	71.4	0.105 (0.060)	0.267 (0.076)	0.667	0.200	0.39	–
Average *E. decaisnei*				11.6	1.9	51.8	0.132	0.205	0.334	0.530	0.25	–
E. callithyrsum												
Ariñez	ECUARI	9	250 (0.33%)	9	1.6 (0.2)	57.1	0.118 (0.058)	0.150 (0.057)	0.236	0.618	–	–
Barranco de Antona	ECUANT	10	9,950 (12.98%)	11	1.7 (0.4)	42.9	0.094 (0.050)	0.132 (0.070)	0.278	0.565	0.54	0.16
Barranco de Los Mocanes	ECMO	11	938 (1.22%)	14	2.0 (0.2)	57.1	0.174 (0.092)	0.226 (0.087)	0.09	0.835	0.2	0.49
El Hornillo-Fagagesto	ECH	12	8,163 (10.65%)	13	2.1 (0.3)	42.9	0.108 (0.062)	0.206 (0.105)	0.310	0.527	0.48	0.16
La Lechucilla	ECLE	13	200 (0.26%)	9	1.6 (0.3)	14.3	0.058 (0.035)	0.102 (0.077)	0.149	0.741	–	–
Lagunetas	ECLA	14	400 (0.52%)	9	1.7 (0.4)	42.9	0.133 (0.090)	0.173 (0.098)	0.292	0.548	0.00	0.37
Tenteniguada	ECT	15	55,200 (72.00%)	15	2.1 (0.3)	71.4	0.197 (0.100)	0.270 (0.097)	0.344	0.488	0.12	0.79
Average *E. callithyrsum*				11.4	1.8	46.9	0.126	0.234	0.243	0.617	0.27	0.4
E. onosmifolium												
Ayacata	EOA	16	–	9	1.6 (0.2)	57.1	0.065 (0.042)	0.217 (0.087)	0.517	0.318	0.44	0.24
Pico Bandama	EOAP	17	–	8	1.6 (0.3)	28.6	0.082 (0.070)	0.145 (0.086)	0.241	0.612	0.17	0.03
El Rincón	EORIN	18	–	15	2.4 (0.5)	57.1	0.126 (0.052)	0.278 (0.105)	0.490	0.342	0.53	0.03
Los Lajeales	EOL	19	–	8	1.4 (0.2)	42.9	0.381 (0.184)	0.301 (0.153)	0.833	0.091	–	–
Taidía	EOTA	20	–	15	2.4 (0.6)	71.4	0.171 (0.049)	0.275 (0.109)	0.146	0.745	0.47	0.07
Average *E. onosmifolium*				11	1.9	51.4	0.165	0.243	0.445	0.422	0.4	0.09
E. onosmifolium ssp. *spectabile*												
El Portezuelo	EOSP	21	976 (63.54%)	9	1.4 (0.3)	28.6	0.060 (0.060)	0.103 (0.076)	0.598	0.252	0.35	–
Guguy	EOSG	22	100 (6.51%)	10	1.7 (0.3)	57.1	0.115 (0.075)	0.308 (0.114)	0.522	0.314	0.51	–
Average *E. onosmifolium* ssp. *spectabile*	9.5	1.5	42.8	0.087	0.205	0.560	0.283	0.43	–			

Table 1 continued

Taxon/population	Code	ID	*N*	Basic indicators of isozyme diversity							Indicators of diversity of sexual and color floral forms	
				T	A_l	*P*	H_o	H_e	*F*	*t*	*Dvsx*	*Dvchr*
E. acanthocarpum												
Roque Agando	EAA	23	300 (12.74%)	10	1.9 (0.3)	57.1	0.180 (0.139)	0.242 (0.136)	0.214	0.647	–	–
Total average				10.0	1.8	50.0	0.138	0.226	0.359	0.5	–	–

ID numbers correspond to Fig. 1

For the threatened taxa, the total number of censused reproductive individuals (*N*) and the percentage that they represent within the taxon's census (between brackets) is indicated (after Marrero Gómez et al. 2004; Mora-Vicente 2004; Mora-Vicente et al. 2004]

Values in parentheses are standard deviations. We include the values of diversity indicators of sexual and color floral forms (*Dvsx* and *Dvchr*, respectively) for the populations where they could be calculated. The symbol "–" means that there were not sufficient data to make these calculations

T total number of alleles scored, A_l average number of alleles per locus *P* proportion of polymorphic loci (a locus is regarded as polymorphic when the frequency of the most common allele does not exceed 0.95), H_o and H_e observed and expected heterozygosity, *F* fixation index, *t* outcrossing rate [$t = (1 - F)/(1 + F)$]

E. onosmifolium ssp. spectabile could be included in the isozyme interpretations, we did not consider this taxon for this analysis. Both indicators have been correlated with the richness and genetic diversity of natural populations using Pearson's bivariate correlation (Sokal and Rohlf 1979).

Results

In all cases the isozyme patterns obtained could be interpreted in accordance with the hypothesis of mendelian codominance. The genetic interpretation of these isozymes allowed us to resolve seven putative loci, one of which was monomorphic in all populations (*Me-1*). Of the 30 alleles scored (Table not shown), five were exclusive to *E. decaisnei* (*Pgi-1f*, *Pgi-1 g*, *Acp-1a*, *Got-1a* and *Got-1c*), two shared between *E. decaisnei* and *E. onosmifolium* (*Pgi-1d* and *Pgm-1a*), and three among *E. decaisnei*, *E. callithyrsum* and *E. onosmifolium* (*Acp-1c*, *Acp-1d* and *Pbm-1b*). The remaining 20 alleles were shared by different combinations of the populations analyzed. We did not detect either diagnostic alleles (i.e., alleles that were monomorphic in one taxon and not present in the others), or alleles exclusively present in all populations of each taxon.

The basic indicators of isoenzyme variability (Table 1) showed generally moderate population diversity within populations of each taxon considered. The maximum average values of *T*, *Al* and *P* corresponded to *E. decaisnei* ($T = 11.625$; $A_l = 1.925$; $P = 51.775$), of H_o and H_e to *E. onosmifolium* ($H_o = 0.165$; $H_e = 0.243$), of *Fis* to

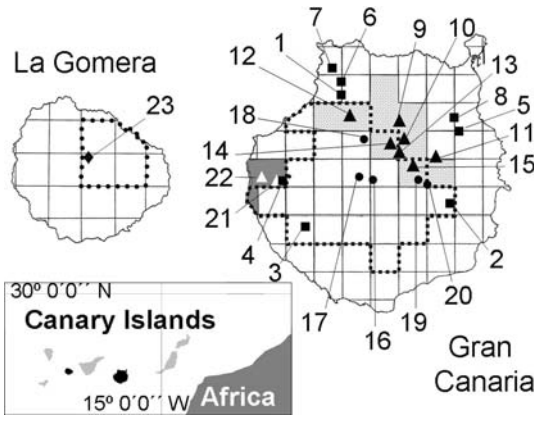

Fig. 1 Distribution area of taxa and location (UTM) of the populations studied in this paper. *La Gomera Island* distribution area (*dotted line*) of *E. acanthocarpum* and location (UTM) of the studied populations (*filled diamonds*). *Gran Canaria Island* distribution ranges of the taxa are symbolized by a *light gray area* (*E. callithyrsum*), a *dotted line* (*E. onosmifolium*), and a *dark gray area* (*E. onosmifolium* ssp. *spectabile*). *E. decaisnei*'s distribution range encompasses the entire island. Population labels are as follows: *E. decaisnei* = *filled squares*, *E. callithyrsum* = *filled triangles*, *E. onosmifolium* = *filled circles*, *E. onosmifolium* ssp. *spectabile* = *open triangles*. Population codes match Table 1

E. onosmifolium ssp. *spectabile* (*Fis* = 0.560) and of *t* to *E. callithyrsum* (*t* = 0.617) (Table 1). The minimum values of H_o and H_e were detected in *E. callithyrsum*, and the remaining ones in *E. onosmifolium* ssp. *spectabile*.

Most of the polymorphic loci did not fit Hardy–Weinberg proportions (data not shown) and all the loci resolved were neutral according to the Ewens–Watterson test (Table 2). The percentage of variation explained by the differentiation among populations (Table 2) was moderate, with a maximum *Gst* value of 0.225 in *E. decaisnei* and a minimum of 0.123 in *E. callithyrsum*.

Average between-population genetic distances of Nei (1978) were *D* = 0.078 for *E. decaisnei*, *D* = 0.027 in *E. callithyrsum*, *D* = 0.073 in *E. onosmifolium,* and *D* = 0.042 in the case of *E. onosmifolium* ssp. *spectabile*. The lowest genetic distance (*D* = 0.000; Nei 1978) was detected between populations of *E. callithyrsum* (ECLA–ECMO) and the highest (*D* = 0.380) between *E. callithyrsum* and *E. onosmifolium* ssp. *spectabile* (ECLE–EOSP).

The UPGMA cluster (Fig. 2) clearly separates *E. onosmifolium* ssp. *spectabile* from the other taxa, while the populations of *E. onosmifolium* ssp. *onosmifolium* are inter-

Table 2 Ewens–Watterson (E–W) neutrality test and population structure statistics following Nei (1973) and Wright (1951) for the polymorphic loci found in *E. decaisnei*, *E. callithyrsum*, *E. onosmifolium* ssp. *spectabile*

Taxon/locus	Multilocus structure statistics				Population structure statistics						
	Ewens–Watterson neutrality tests				Nei's (1973) unmodified				Wright's (1951) *F* statistics		
	Mean	SE	L95	U95	*Hs*	*Ht*	*Dst*	*Gst*	*Fis*	*Fit*	*Fst*
E. decaisnei											
PGI-1	0.465	0.024	0.261	0.823	0.447	0.502	0.055	0.109	0.615	0.657	0.109
ACP-1	0.805	0.028	0.501	0.973	0.019	0.020	0.001	0.041	0.300	0.328	0.041
GOT-1	0.654	0.032	0.357	0.947	0.078	0.085	0.007	0.081	0.571	0.606	0.081
PGM-1	0.550	0.028	0.304	0.896	0.470	0.707	0.237	0.335	0.244	0.497	0.335
PGM-2	0.656	0.031	0.370	0.947	0.132	0.141	0.009	0.062	0.747	0.763	0.062
PGM-3	0.660	0.029	0.370	0.947	0.193	0.273	0.081	0.294	−0.520	−0.073	0.294
Average	–	–	–	–	0.191	0.247	0.055	0.225	0.327	0.478	0.225
E. callithyrsum											
PGI-1	0.663	0.033	0.372	0.958	0.532	0.587	0.055	0.094	0.312	0.376	0.093
ACP-1	0.665	0.034	0.364	0.958	0.046	0.049	0.003	0.060	0.383	0.420	0.060
GOT-1					0.024	0.024	0.000	0.018	−0.031	−0.013	0.018
PGM-1	0.668	0.033	0.368	0.958	0.366	0.466	0.100	0.214	0.147	0.330	0.215
PGM-2	0.805	0.029	0.502	0.979	0.129	0.137	0.008	0.057	0.217	0.262	0.057
PGM-3	0.794	0.029	0.501	0.979	0.120	0.125	0.004	0.035	0.592	0.606	0.035
Average	–	–	–	–	0.174	0.198	0.024	0.123	0.276	0.365	0.123
E. onosmifolium											
PGI-1	0.378	0.011	0.240	0.663	0.427	0.558	0.131	0.235	0.779	0.831	0.235
ACP-1	0.748	0.023	0.503	0.926	0.059	0.063	0.004	0.063	0.579	0.606	0.062
PGM-1	0.463	0.017	0.287	0.787	0.427	0.558	0.131	0.235	0.258	0.432	0.235
PGM-2	0.750	0.024	0.503	0.926	0.327	0.408	0.082	0.120	−0.133	0.093	0.200
PGM-3	0.749	0.024	0.503	0.926	0.271	0.302	0.031	0.102	−0.281	−0.150	0.102
Average	–	–	–	–	0.216	0.270	0.054	0.200	0.236	0.389	0.200
E. onosmifolium ssp. *spectabile*											
PGI-1	–	–	–	–	0.545	0.572	0.027	0.046	0.295	0.327	0.047
PGM-1	–	–	–	–	0.222	0.278	0.055	0.200	1.000	1.000	0.200
PGM-2	–	–	–	–	0.265	0.273	0.008	0.029	0.144	0.170	0.030
PGM-3	–	–	–	–	0.250	0.375	0.125	0.333	1.000	1.000	0.333
Average	–	–	–	–	0.183	0.214	0.031	0.144	0.523	0.592	0.144

A locus can be regarded as neutral when its mean E–W value falls within the confidence interval

SE standard error, *L95* lower limit of the 95% confidence interval for the mean value of the E–W statistic, *U95* upper limit of the 95% confidence interval for the mean value of the E–W statistic

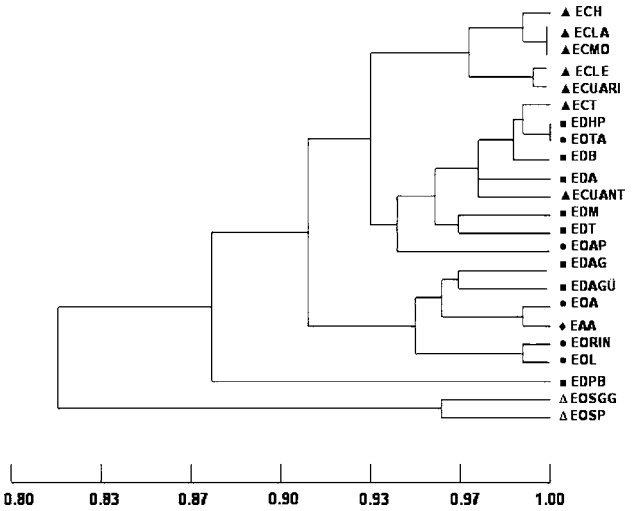

Fig. 2 UPGMA cluster built with Nei's (1978)genetic distance. Population codes match Table 1

mixed with those of *E. decaisnei*, *E. acanthocarpum*, and two populations of *E. callithyrsum* (ECT and ECUANT).

At a population level, chromatic floral diversity maintains a high significant correlation with the degree of polymorphism (*P*: $r = 0.948$; $P < 0.014$) and the observed heterozygosity (H_o: $r = 0.967$; $P < 0.007$) (Tables 1, 3). We also found a significant correlation between diversity of sexual forms and expected heterozygosity in *E. onosmifolium* ($r = 0.957$; $P < 0.043$). By contrast, we failed to find any significant correlation in *E. decaisnei*.

Table 3 Results of correlation analysis between diversity of sexual (*Divsx*) and chromatic (*Divchr*) forms and indicators of isozyme diversity, using Pearson's *r* correlation coefficient

Diversity indicators	Statistics	ED	EC		EO	
		Divsx	*Divsx*	*Divchr*	*Divsx*	*Divchr*
T	*r*	0.229	0.073	0.581	0.788	−0.395
	P	0.663	0.907	0.304	0.212	0.605
	N	6	5	5	4	4
A_l	*r*	−0.123	0.027	0.439	0.718	−0.490
	P	0.817	0.966	0.459	0.282	0.510
	N	6	5	5	4	4
P	*r*	−0.092	−0.434	0.948	0.891	0.271
	P	0.863	0.465	**0.014**	0.109	0.729
	N	6	5	5	4	4
H_o	*r*	−0.548	−0.694	0.967	0.501	−0.508
	P	0.260	0.194	**0.007**	0.499	0.492
	N	6	5	5	4	4
H_e	*r*	0.235	−0.453	0.827	0.957	−0.033
	P	0.653	0.444	0.084	**0.043**	0.967
	N	6	5	5	4	4
F	*r*	0.475	0.072	0.001	0.387	0.495
	P	0.341	0.909	0.998	0.613	0.505
	N	6	5	5	4	4
t	*r*	−0.447	−0.088	0.034	−0.346	−0.470
	P	0.374	0.888	0.957	0.654	0.530
	N	6	5	5	4	4

The significant correlations are indicated in bold ($P < 0.05$)

N number of populations sampled per taxon, *ED E. decaisnei*, *EC E. callithyrsum*, *EO E. onosmifolium*

Discussion

Genetic diversity and mating systems

The basic indicators of genetic variability (A_l, *P*, H_o and H_e) of *E. decaisnei*, *E. callithyrsum*, *E. onosmifolium*, *E. onosmifolium* ssp. *spectabile,* and *E. acanthocarpum* (Table 1) are higher than the average values offered by Hamrick and Godt (1989) for endemic plants ($A_l = 1.39$, $P = 0.26$, $H_e = 0.063$). Regarding the values of *Hs* (which is the parameter considered by Francisco-Ortega et al. 2000 in the latest published review of the isozyme diversity of Canarian plant endemics), its average values in the *Echium* species analyzed (Table 2) exceed the average value for the Canarian taxa calculated by these authors (*Hs* = 0.137), and are more than twofold of those found in Hawaiian Islands (*Hs* = 0.064—Dejoode and Wendel 1992—), or the Juan Fernández Archipelago (*Hs* = 0.042—Crawford et al. 2001—). Although the low number of loci interpreted (only seven) advises caution, it seems clear that these *Echium* taxa add to the already numerous evidences that indicate higher population genetic variability in Canarian endemic plants than in other oceanic archipelagos (i.e., Francisco-Ortega et al. 2000; Oliva-Tejera et al. 2005, 2006; Sánchez-Doreste et al. 2006).

In relation to other studies with the genus, levels of genetic diversity estimated by us for *E. acanthocarpum* (Table 1) exceed substantially those estimated in two populations of this taxon by Batista and Sosa (1998) ($A_l = 1.25$; $P = 25$; $H_e = 0.075$). Feasibly, the fact that this study was conducted with seedlings germinated from seeds collected on a very small number of adult individuals ($N = 2$) largely conditioned the detection of lower levels of genetic variation than with our sampling strategy that targeted adult individuals in a comprehensive population transect.

As regards mainland species of *Echium*, isozyme data on the widespread *E. plantagineum* (Brown and Burdon 1983) reveal considerably high levels of genetic variability ($A_l = 3.0$; $P = 56.52$; $H_o = 0.357$; $H_e = 0.380$) within Hamrick and Godt's (1989) group of dicots, which are generally much higher than those of the five taxa studied although some of the Canarian populations have very

similar values of A_l and higher values of P and H_o. Unlike the five taxa included in this paper (Table 1), the high genetic diversity in *E. plantagineum* is best explained by its very high levels of outcrossing ($t = 0.81 \pm 0.10$–1.15 ± 0.12, Burdon et al. 1988), that induce the generation of high genetic diversity (Loveless and Hamrick 1984; Hamrick and Godt 1989).

The genetic differences observed among the different taxa analyzed do not relate to factors of threat; rather, they seem to be influenced by different combinations of ecological, geographical, reproductive, and genetic factors. Geographic range is one of the features with greater impact on the patterns of genetic variability. It was generally believed that narrowly distributed species had less genetic variation than widely distributed congeners, although genetic differentiation among populations could be similar in both cases (Hamrick and Godt 1989 and 1996; Gitzendanner and Soltis 2000). Nevertheless, there are many examples of rare species whose indicators of genetic variability are higher than those of widely distributed congeners (Pedrola-Monfort and Caujapé-Castells 1996 and references therein). *Echium decaisnei* and *E. onosmifolium* are the analyzed taxa with a more widespread distribution. In the first case, this fact also agrees with a higher ecological heterogeneity. By contrast, *E. callithyrsum* and *E. onosmifolium* ssp. *spectabile* have a much more limited distribution range and their populations occupy more homogeneous habitats (Mora-Vicente 2007), especially in the second case. In agreement with Hamrick and Godt (1989), the distribution and ecological heterogeneity of these two groups, although faintly, is reflected in the indicators of genetic variability, which are usually somewhat higher in the widely distributed species (Table 1).

Another reason that could partly explain the lower genetic diversity of *E. callithyrsum* and *E. onosmifolium* ssp. *spectabile* is that the small size of most of their populations makes them more prone to the action of drift and inbreeding, resulting in reduced genetic variation levels (Barret and Kohn 1991; Ellstrand and Ellam 1993; Frankham 1998). Nevertheless, this relationship is not so clear in *E. onosmifolium* ssp. *spectabile*, where the highest levels of genetic diversity are found in the population EOSG, whose size is the second smallest one (Table 1). Therefore, ecological amplitude and population size are not sufficient to explain the genetic diversity differences observed.

Correlation analyses between the indicators of genetic diversity and populational diversity of sex forms and colors indicate that, in *E. callithyrsum* and *E. onosmifolium*, the incidence of floral heterochromy and sexual heteromorphism is significantly related to genetic diversity (Table 3), although there is no genetic parameter that consistently correlates with any of these two flower polymorphisms. In particular, the results of these tests in *E. callithyrsum* indicate that genetic diversity levels are significantly correlated with diversity of colors, whereas in *E. onosmifolium* population genetic diversity is correlated with the diversity of sexual forms. Therefore, it is possible that in these taxa sexual and chromatic floral heteromorphisms have an important impact on intra-population gene flow through promoting outcrossing (Stanton et al. 1989, Hamrick and Godt 1996).

Wright's fixation index (1951) for the polymorphic loci (F) is almost always positive and the corresponding values for the outcrossing rate (t) are generally rather lower (Table 1), which means that there is a predominance of selfing over outcrossing and, consequently, a deficiency of heterozygotes. Because there are three ways by which inbred crosses may occur in these taxa (mainly geitonogamy, biparental inbreeding and autogamy, Mora-Vicente 2007), and given the absence of evidence for selection (Table 2), this fact may be due to the existence of a strong population structure, with reproduction mainly taking place in familiar nuclei (through mating between relatives). However, given that we could not interpret all the individuals sampled, it is also possible that the estimated values of inbreeding were increased due to the Wahlund effect (Wahlund 1928).

Among-population genetic differentiation

The moderately low values of *Gst* (Table 2) and of intraspecific genetic distances strongly suggest a considerable genetic cohesion in these *Echium* taxa, probably fostered by the existence of moderate to high levels of between-population gene flow. This interpretation is also bolstered by the low number of population-exclusive alleles detected, which could indicate abundant genetic interchange. In particular, our *Gst* estimates are well below the average calculated by Francisco-Ortega et al. (2000) for the endemic taxa of the Canary Islands (*Gst* = 0.281). A possible explanation to this discrepancy is that many of the *Gst* values averaged by Francisco-Ortega et al. (2000) might be overestimated as a result of a biased intra-population sampling in terms of number and distribution of individuals (Caujapé-Castells 2007) while all populations of *Echium* in this paper were sampled thoroughly.

Although in these taxa seed dispersal is basically gravitational, at least a small percentage of the seeds may be dispersed at medium and/or long distances through anemochory or exozoochory (Bramwell 1972; Marrero Gómez et al. 2000). This small portion may be largely responsible for the moderate levels of gene flow detected, especially in the case of *E. callithyrsum* and *E. onosmifolium* ssp. *spectabile*, where the high fragmentation and the existence of important orographic barriers make inter-population pollen flow unlikely. In the case of *E. decaisnei* and

E. onosmifolium the geographical proximity of many of their populations makes genetic exchange via pollen-flow more probable. On the whole, our data suggest that in *E. decaisnei*, *E. callithyrsum*, *E. onosmifolium*, and *E. onosmifolium* ssp. *spectabile* the unifying action of inter-population gene flow overrides the differentiating effect of genetic drift induced by low population sizes.

Genetic differentiation as estimated by Nei (1978) genetic distance agrees with the expected values for the Canarian endemic plants (see summary in Sánchez-Doreste et al. 2006) and appears to support the separation of *E. onosmifolium* ssp. *spectabile* from the rest of the species (Fig. 2).

According to the ITS phylogeny of Böhle et al. (1996), the radiation of *Echium* in the Canary Islands is recent and can be explained by a single introduction from the mainland. However, the high levels of population genetic variation detected by us are at odds with this interpretation, unless there had been more than one introduction not detected by the low polymorphisms of ITS, or that hybridization phenomena had played a major role in shaping the genetic makeup of these taxa (Saunders and Gibson 2005). Given the high frequency of hybridisation detected in Canarian *Echium* (Bramwell 1972; Mora-Vicente 2007), perhaps the most plausible hypothesis at the present stage of knowledge is that this phenomenon has contributed to a higher degree to generate those levels of variation.

Knowledge of genetic diversity and the way it is structured in natural populations is essential in order to adopt suitable conservation strategies (Ellstrand and Ellam 1993). One general major problem for conservation of island endemics lies in their usually small population sizes. Among other things, this makes them especially prone to manifest the effects of genetic drift and inbreeding, namely loss of genetic richness and heterozygosity, as well as a lower inter-population genetic cohesion due to diminished reproductive interchange (Frankham 1998). In the case of *Echium acanthocarpum*, *E. onosmifolium* ssp. *spectabile*, and *E. callithyrsum*, human activity and the introduction of several invasive exotic species have produced intensive processes of fragmentation, reduction, and decline in their natural populations (Marrero Gómez et al. 2004; Mora-Vicente 2004; Mora-Vicente et al. 2004) that add up to those general conservation problems.

Although levels of isozyme variability estimated in many of the endangered taxa do not indicate genetic depauperation, *E. onosmifolium* ssp. *spectabile* does show the lowest values of the used parameters (Table 1), which rank clearly below those of its closest relative (*E. onosmifolium*), and surprisingly, are even lower than those found in the only analyzed population of *E. acanthocarpum* (presently considered *critically endangered*). However, all the populations of these three taxa are undergoing a process of strong regression (Marrero Gómez et al. 2004; Mora-Vicente 2004; Mora-Vicente et al. 2004), which apparently is not unambiguously reflected in genetic data.

Population genetics theory predicts that reductions in population size have as an immediate consequence the loss of alleles (Barret and Kohn 1991). However, in the case of the endangered endemics that we have investigated (*E. callithyrsum*, *E. onosmifolium* ssp. *spectabile* and *E. acanthocarpum*), the small size of most populations does not seem to affect their levels of genetic isozyme diversity. If we compare the levels of genetic diversity found in the single population of *E. acanthocarpum* studied (*critically endangered*) with those in the populations of *E. callithyrsum* (*vulnerable*) with a similar number of individuals ($N = 300$; see Table 1), we find that the latter populations have usually much lower levels of variation. On the basis of this comparison, it seems highly unlikely that genetic factors are among the main causes of current threat to this endemism.

The continuous decline in population sizes and recruitment rates, as well as the disappearance of some populations due to the expansion of allochtonous species, provide opportunities for the action of genetic drift and inbreeding, which can severely impair the response capacity of populations to potential environmental changes (Caujapé-Castells and Pedrola-Monfort 2004). These alterations in population dynamics of Canarian *Echium* are possibly very recent (Mora-Vicente 2007). Therefore, we need to be cautious in interpreting our genetic results, because perhaps the detection of the harmful effects of such processes on genetic diversity may require more time.

Consequently, in order to maintain the current levels of genetic diversity, it is advisable to mitigate the processes of fragmentation and continuous decline experienced by population of these taxa and, therefore, to halt the expansion of allochtonous species like *Arundo donax*, *Rubus* sp. and *Opuntia* sp., which is the most important cause for the population recession in *Echium* (Marrero Gómez et al. 2004; Mora-Vicente 2004; Mora-Vicente et al. 2004).

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