

Genetic structure and population differentiation of the Mediterranean pioneer spiny broom *Calicotome villosa* across the Strait of Gibraltar

JUAN ARROYO*, ABELARDO APARICIO, RAFAEL G. ALBALADEJO,
JOAQUÍN MUÑOZ† and RITA BRAZA

Departamento de Biología Vegetal y Ecología, Universidad de Sevilla, Apartado 1095, E-41080 Sevilla, Spain

The region around the Strait of Gibraltar is considered to be one of the most relevant ‘hot spots’ of biodiversity in the Mediterranean Basin due to its historical, biogeographical, and ecological features. Prominent among these is its role as a land bridge for the migration and differentiation of species during the Pleistocene, as a consequence of the lowering of sea level and climate changes associated with the Ice Ages. In the present study, we report a multilevel hierarchical investigation of the genetic diversity of *Calicotome villosa*, a common pioneer legume shrub, at the regional scale. The results of genetic analysis of progeny arrays are consistent with a predominantly outcrossing mating system in all the populations analysed. Geographically, a pattern of population isolation by distance was found, but the Strait accounted for only approximately 2% of the among-population genetic differentiation. Consequently, extensive historical gene flow appears to be the rule for this species in this area. According to the natural history traits of *C. villosa* (pollination, dispersal, and colonization ability), we hypothesize that gene flow must be strongly influenced by seed dispersal because pollen flow is very limited. Based on the history of trade and land use, cattle and human movements across the Strait must have strongly favoured seed dispersal. We review and discuss these results and compare them with those of other reported studies of genetic and phylogenetic differentiation across the Strait of Gibraltar. It is stressed that colonization ability, which depends upon seed dispersal and life form, can be a more critical factor in gene flow than pollination. © 2008 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2008, **93**, 39–51.

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INTRODUCTION

In biogeography, some areas are of particular interest because their geographical settings and historical constraints are thought to strongly influence the geographical ranges of species. Among these areas, islands, refugia, and corridors are favourite targets because of the strong limitations on species movements, making it easier to interpret observed patterns (Hewitt, 2000; Holloway, 2003). The old theory

of land bridges attempted to explain global patterns of biogeographical similarities (for a botanical perspective, see van Steenis, 1962) until it was superseded by interpretations based on continental drift (Raven & Axelrod, 1974). Some particular areas, such as Beringia and Mesoamerica, are still considered as appropriate places to study the effects of land bridges as corridors for historical species movements. This is hypothesized to be the most plausible explanation of the current range of several animal and plant species (Cavers, Navarro & Lowe, 2003; Brubaker *et al.*, 2005; García-Moreno *et al.*, 2006). One of these areas is split by the Strait of Gibraltar, which separates the Eurasian and African plates at their westernmost

*Corresponding author. E-mail: arroyo@us.es

†Current address: Estación Biológica de Doñana, CSIC, Sevilla, Spain.

extremities. This region has had a convulsive geological history, involving recurrent closings and openings of the Strait until the late Tertiary, 5.5 Mya (Duggen *et al.*, 2003). Although there was no physical connection during the Pleistocene, the Ice Ages provoked a lowering of the sea level, resulting in the land masses being closer. It has been stressed that this complex history, in terms of its geological, climatic, and temporal heterogeneity, has determined most of the evolution of the plant lineages in the Mediterranean (Thompson, 2005). This geological instability, together with Pleistocene climate changes, may have increased the role of the Strait of Gibraltar as an active land bridge in the migration and differentiation of species (Quézel, 1978; Caujapé-Castells & Jansen, 2003).

A comprehensive biogeographical study of the regional flora is lacking, but studies focusing on the standing components of biodiversity and the ecology of woody communities across the Strait are available (Ojeda, Marañón & Arroyo, 2000; Ajbilou, Marañón & Arroyo, 2006). For example, short-term ecological processes associated with strong human disturbance regimes (especially from the last millennium to the present day; Mikesell, 1960; Reille, 1977; Moore *et al.*, 1998) explain the diversity of the lesser shrub and tree species observed in northern Morocco relative to that in southern Spain (Ojeda, Marañón & Arroyo, 1996a; Ajbilou *et al.*, 2006). On an evolutionary time scale, most phylogenetic and phylogeographical analyses undertaken of woody species (Arroyo *et al.*, 2004; Rodríguez-Sánchez *et al.*, in press) have shown some genetic differentiation across the Strait of Gibraltar, irrespective of taxonomic group, plant life form, or reproductive traits. However, none of these studies included widespread, pioneer species, whose large geographical ranges are due to their wide ecological niches (Morin & Chuine, 2006). Such a study would be useful to compare the effects of colonization ability on gene flow and on patterns of genetic variation at the regional scale.

Breeding systems and ecological requirements are reputedly associated with colonization and migration processes (Baker & Stebbins, 1965), and species with contrasted colonizing ability are particularly useful to test these associations. *Calicotome villosa* (Poir.) Link is a common pioneer shrub, the geographical range of which extends across both sides of the Strait of Gibraltar. This species usually grows in large, dense, and continuous populations in anthropogenic environments of intense land use (agriculture, herding). Although many ecological data are available for this species (Ojeda, 1995; Ajbilou, 2001), detailed information about its demography and reproductive biology remains very scarce and the breeding system of *C. villosa* in particular remains unexplored. Partial self-incompatibility or inbreeding depression has

been suggested in two other pioneer shrub legumes, *Cytisus* and *Retama* (Parker, 1997; Rodríguez-Riaño, Ortega-Olivencia & Devesa, 1999a), the closest relatives of *Calicotome* (Käss & Wink, 1997; Cubas, Pardo & Tahiri, 2002).

In the present study, we performed a multilevel hierarchical investigation of the genetic diversity, genetic identity, and mating system in populations of *C. villosa* at the regional scale of the Strait of Gibraltar. The aim was to compare the observed patterns with those previously reported for other species with different ecological requirements, contrasting biological traits (growth form, pollination and dispersal mechanisms) and historical and geographical ranges across the Strait of Gibraltar. Accordingly, we should be able to test the extent to which the Strait has represented a barrier to gene flow and promoted genetic differentiation. Our expectation was that, in a species with poor pollen dispersal, patterns of genetic differentiation should be related to seed dispersal and establishment, both of which are directly related to colonizing ability (DeWoody, Nason & Smith, 2004). Specifically, given their suitability in reflecting historical patterns of genetic differentiation and breeding systems (Hamrick & Godt, 1989), we used allozyme variation to determine: (1) the genetic diversity, genetic structure, and genetic identity of *C. villosa* at both the population and regional scales and (2) the breeding system of the species in selected populations using a mixed-mating model approach (Ritland, 2002) in relation to its migration ability. Finally, we discuss the results obtained in the light of the evidences gathered for other species in this same region of high biogeographical significance.

MATERIAL AND METHODS

STUDY SPECIES

Calicotome Link [Cytiseae (= Genisteae), Leguminosae] comprises four species of spiny brooms with Mediterranean distributions. The monophyly of this genus and its genetic differentiation from other genera have been demonstrated by Cubas *et al.* (2002). *Calicotome villosa* is a circum Mediterranean component of the lowland shrub vegetation in open woodlands (Greuter, Burdet & Long, 1984–89). Within the western Mediterranean area, the species is much more frequent in southern Spain and north-western Morocco, where it sometimes forms almost monospecific shrublands. In this range, *C. villosa* is associated with disturbance processes, mostly caused by cattle browsing, and to a lesser extent by fire and slashing. This species is very digestible by cattle (Anmar, López & González, 2005) and thus it occurs in open sites with low or no tree cover and extensive

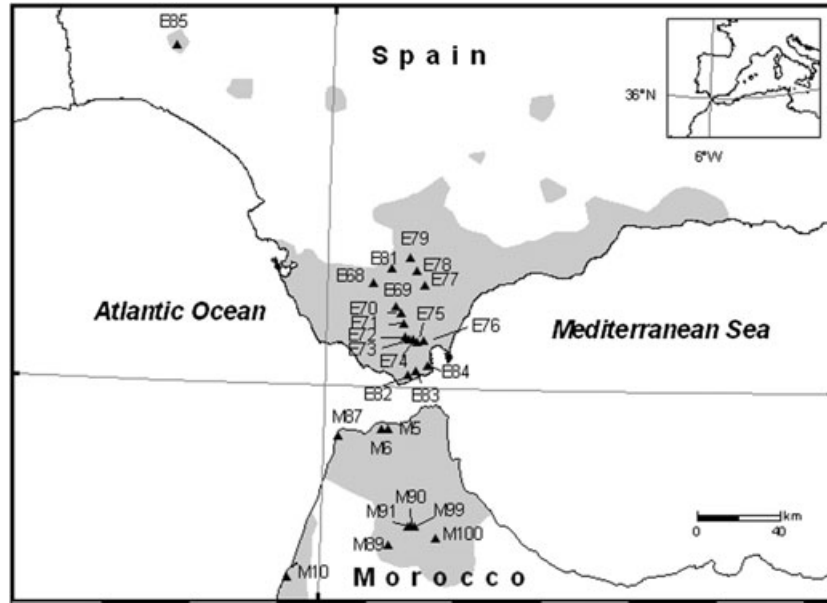


Figure 1. Distribution areas of *Calicotome villosa* on both sides of the Strait of Gibraltar (shaded areas) and the locations of the 26 populations studied.

cattle management (cows and goats). However, the species is quite resilient to serious damage because of strong spiny branches and profuse regeneration by seeds (R. Braza & J. Arroyo, unpubl. data). The species' tolerance is promoted by strong resprouting after damage. *Calicotome villosa* has a pollination mechanism usually triggered by bees of medium to large size (Arroyo, 1981; Rodríguez-Riaño, Ortega-Olivencia & Devesa, 1999b). The species is potentially dispersed by cattle when browsed during the fruiting season. Humans may also facilitate dispersal because the species is more frequent along roadsides and paths. Furthermore, the species survives the dry summer period because of its summer-deciduous habit (DeLillis & Fontanella, 1992).

STUDY AREA

The region of the Strait of Gibraltar is considered one of the most important 'hot spots' of biodiversity in the Mediterranean Basin (Médail & Quézel, 1997). On the one hand, geological instability and climatic changes (Duggen *et al.*, 2003; Pirazzoli, 2005) have actively shaped the floristic and community compositions, giving rise to a rich endemic woody flora restricted to poor sandstone-derived soils (e.g. *Satureja salzmannii*, *Teline tribracteolata*, *Drosophyllum lusitanicum*, *Thymelaea villosa*, *Genista tridens*; Arroyo, 1997; Médail & Quézel, 1997; Ojeda *et al.*, 2000) and a group of late Tertiary and Pleistocene relict species (e.g. *Rhododendron ponticum*, *Frangula alnus*; Hampe *et al.*, 2003;

Mejías, Arroyo & Marañón, 2007) typically restricted to deep, wet, and temperate gorges. On the other hand, open lowlands of fertile soils harbour most of the typical, widespread Mediterranean species (Ajbilou, 2001).

FIELD SAMPLING AND ALLOZYME ELECTROPHORESIS

Twenty-six populations of *C. villosa* were sampled across the Strait of Gibraltar, including nine in Morocco and 17 in Spain (Table 1, Fig. 1). The densities of the populations sampled in the study area approximately reflect the natural abundance of populations, with more samples taken in areas close to the Strait, and less in distant areas. This distribution is in part due to the fact that natural vegetation is steadily replaced by cultivated lands in low-altitude areas where the studied species grows.

The vegetative tissues of *C. villosa* (leaves and cotyledons) were discarded for electrophoresis because of their poor resolution and inconsistency in zymograms. Instead, seeds were collected and the embryos used for electrophoresis. In 1993 and 1994, seeds were picked from 30 mother plants in each population whenever possible and stored at 4 °C in dark until their proteins were extracted. Electrophoreses were run using one seed per mother plant, with 30 seeds per population (i.e. 30 mothers per population). In the small Moroccan populations M90 and M99, two to three seeds were used from each available mother plant (16 and 11, respectively) to complete 30 seeds per population.

Table 1. Allele frequencies for polymorphic loci of *Calicotome villosa* at the population and regional levels (M = Morocco, E = Spain), and for all populations combined

Locus	Allele	Populations													
		M5	M6	M10	M87	M89	M90	M91	M99	M100	E68	E69	E70	E71	E72
<i>Adh-1</i>	A	0.800	0.883	0.950	0.917	0.917	0.983	0.967	0.700	1.000	0.967	0.933	0.967	0.967	0.917
	B	0.200	0.117	0.050	0.083	0.083	0.017	0.033	0.300	–	0.033	0.067	0.033	0.033	0.083
<i>6-Pgd-1</i>	A	0.317	0.233	0.100	0.104	0.500	–	0.083	0.182	0.179	0.294	0.207	0.261	0.217	0.240
	B	0.683	0.767	0.900	0.896	0.500	1.000	0.917	0.818	0.821	0.706	0.793	0.739	0.783	0.760
<i>6-Pgd-2</i>	A	0.983	0.950	1.000	0.519	0.904	1.000	0.500	0.955	1.000	0.966	1.000	0.981	0.917	0.920
	B	0.017	0.050	–	0.481	0.096	–	0.500	0.046	–	0.034	–	0.019	0.083	0.080
<i>Pgm-1</i>	A	0.345	0.357	0.313	0.283	0.407	0.350	0.500	0.433	0.333	0.450	0.417	0.052	0.450	0.367
	B	0.655	0.643	0.688	0.717	0.593	0.650	0.500	0.567	0.667	0.550	0.583	0.948	0.550	0.633
<i>Pgm-2</i>	A	0.143	0.183	0.080	0.167	0.172	0.200	0.067	0.286	0.183	0.450	0.300	0.362	0.067	0.167
	B	0.786	0.683	0.920	0.783	0.793	0.783	0.850	0.714	0.783	0.533	0.700	0.569	0.467	0.717
	C	0.071	0.133	–	0.050	0.035	0.017	0.083	–	0.033	0.016	–	0.069	0.467	0.117

The seeds were scarified and soaked in distilled water overnight. The seed coat and the endosperm layer were then removed and discarded, and the embryos homogenized in four drops of DL-dithiothreitol (0.065 M) and Na₂HPO₄ (0.05 M) buffer at pH 7. Crude extracts were adsorbed onto Whatman 3MM paper wicks and stored at –80 °C until electrophoresis. Electrophoresis was performed following the general protocols of Wendel & Weeden (1989) on 12% starch and 2.5% sucrose gels. Sixteen enzyme systems were tested for activity and consistent banding patterns in three different electrode buffers following the staining recipes of Soltis *et al.* (1983). Only three gave sufficient consistent resolution in a histidine/citrate electrode buffer (pH = 6.5/6.5) (Cardy *et al.*, 1983): alcohol dehydrogenase (ADH; EC 1.1.1.1), phosphogluconate dehydrogenase (6-PGD; EC 1.1.1.43), and phosphoglucosyltransferase (PGM; EC 5.4.2.2). Zymograms were interpreted in terms of loci and alleles, which were labelled starting from the most anodally migrating band.

MATING-SYSTEM ESTIMATES

Sampling included the simultaneous collection of seed families for mating-system estimations. In particular, populations E68, M89, E81, and E84 (for locations, see Fig. 1) were selected for this purpose because they provided both adequate numbers of seeds and high or low H_o values. Ten pooled seeds from each of 15 mothers randomly selected in each population were screened for allozyme variation (i.e. 10 seeds ¥ 15 families ¥ 4 populations). All five polymorphic loci that were resolved (see Results) were used for this purpose.

DATA ANALYSIS

Genetic diversity statistics were hierarchically calculated at both the population and regional (Morocco and

Spain) levels using the Genetic Data Analysis program (GDA; Lewis & Zaykin, 2001), including the mean number of alleles per locus (A), percentage of polymorphic loci (P , at the 99% cut-off), mean number of alleles per polymorphic locus (A_p), and observed (H_o) and expected (H_e) heterozygosity under Hardy–Weinberg equilibrium. Weir & Cockerham's (1984) inbreeding coefficient (f) was computed for each population and its statistical significance was examined with a c^2 test (Li & Horvitz, 1953): $c^2 = f^2 N(k - 1)$, with d.f. = $k(k - 1)/2$, where N is the number of individual plants sampled and k is the number of alleles in the population.

Population differentiation (q) was also computed using GDA. The statistics f and q are similar to Wright's (1951) F -statistics and are unbiased with respect to small and uneven sample numbers and population sizes (Culley & Grubb, 2003). The inbreeding coefficient is a measure of the correlation of the genes of individuals within populations and is consequently analogous to F_{is} , whereas q is a measure of the amount of differentiation among populations relative to the total diversity and is therefore analogous to F_{st} . q was calculated across loci at the regional level in Morocco and Spain (q_{pM} and q_{pS} , respectively), among populations within regions (q_p), and between regions (q_r). To evaluate the relative influences of genetic drift and historical gene flow on the distribution of genetic variation, assuming a stepping-stone model of population structure (Hutchinson & Templeton, 1999), q was also calculated for each possible pair of populations using GDA. Their correlation with geographical distances was evaluated with the Mantel test in GENETIX, version 4.02 (Belkhir *et al.*, 2001). Slightly negative values of q were set to zero (Williams & Guries, 1994). A population pairwise geographical distance matrix was generated using ArcGis procedures (Environmental Research Institute, Redlands, CA, USA).

Populations

E73	E74	E75	E76	E77	E78	E79	E81	E82	E83	E84	E85	Morocco	Spain	All populations
1.000	0.917	0.817	0.950	0.967	0.950	0.967	0.967	0.967	0.983	1.000	1.000	0.902	0.955	0.937
–	0.083	0.183	0.050	0.033	0.050	0.033	0.033	0.033	0.017	–	–	0.098	0.045	0.064
0.259	0.317	0.433	0.339	0.214	0.103	0.150	0.217	0.375	0.183	0.133	0.367	0.191	0.254	0.233
0.741	0.683	0.567	0.661	0.786	0.897	0.850	0.783	0.625	0.817	0.867	0.633	0.809	0.746	0.767
0.948	0.933	0.850	0.946	0.982	0.862	0.917	1.000	1.000	0.967	0.967	0.967	0.870	0.947	0.922
0.052	0.067	0.150	0.054	0.018	0.138	0.083	–	–	0.033	0.033	0.033	0.130	0.053	0.078
0.450	0.350	0.109	0.160	0.400	0.417	0.217	0.067	0.259	0.217	0.207	0.383	0.370	0.297	0.322
0.550	0.650	0.891	0.840	0.600	0.583	0.783	0.933	0.741	0.783	0.793	0.617	0.630	0.703	0.678
0.150	0.417	0.200	0.111	0.233	0.167	0.233	0.067	0.077	0.150	0.133	0.017	0.165	0.195	0.185
0.750	0.567	0.733	0.870	0.433	0.567	0.550	0.433	0.846	0.783	0.817	0.333	0.786	0.625	0.680
0.100	0.017	0.067	0.019	0.333	0.267	0.217	0.500	0.077	0.067	0.050	0.650	0.048	0.180	0.135

Nei's (1972) genetic identity and distance were also calculated with GDA, and the linkage disequilibrium of individual loci and the effective number of alleles per locus (N_e) were estimated using POPGENE, version 1.32 (Yeh & Boyle, 1997). A Neighbour-joining (NJ) tree of populations based on Nei's (1972) genetic distances was constructed and branch support assessed by bootstrapping (1000 replicates) with PHYLIP, version 3.5 (Felsenstein, 1993).

For progeny, maximum likelihood multilocus and single-locus outcrossing rates (t_m , t_s), correlated paternity (r_p), and biparental inbreeding ($t_m - t_s$) were computed following Ritland's (1989) mixed-mating model and sibling-pair model with MLTR, version 3.0 (Ritland, 2002). Standard errors were calculated by bootstrapping over families (1000 replicates). Because most seeds could be assigned to a specific genotype, we applied the Newton–Raphson algorithm, which provides estimates of t_m from the theoretical bounds when dealing with multiple heterozygote fathered progeny arrays (Ritland, 2002).

For each population, the maternal multilocus genotypes inferred by MLTR were used to compute the 'observed' maternal inbreeding coefficient (f_{ob}) using GDA. 'Expected' maternal inbreeding (f_{ex}) was calculated with the equation $t_m = (1 - f_{ex}) / (1 + f_{ex})$ (Jain, 1979), given the t_m population outcrossing rate.

RESULTS

GENETIC DIVERSITY AND POPULATION GENETIC STRUCTURE

ADH, 6-PGD, and PGM, encoded by six loci (two loci each), showed consistent and interpretable bands on all zymograms. *Adh-2* was monomorphic across all populations and the rest of the loci were polymorphic in at least 20 populations. At the regional scale,

6-Pgd-1, *6-Pgd-2*, and *Adh-1* were fixed in at least one Moroccan population whereas *6-Pgd-2* and *Adh-1* were fixed in at least one Spanish population. Allele frequencies were not significantly different between Morocco and Spain, and no alleles were exclusive at either the regional or population level (Table 1). No linkage disequilibrium was detected between loci within any of the populations.

Measures of genetic variation did not differ significantly at the population or regional level. The percentage of polymorphic loci was in the range 50–83.3% but the number of alleles per locus was very low (range 1.66–2.00), and the number of alleles per polymorphic locus was also low (ranging 2.20–2.33). Most loci consisted of one frequent allele, so the effective number of alleles was in the range 1.208–1.561 (Table 2).

At the population level, most populations (81%) exhibited an excess of heterozygotes. This excess significantly differed from Hardy–Weinberg expectations in two populations in Morocco (M89 and M91) and in two in Spain (E68 and E71) (Table 2). Across loci, *Pgm-1* showed a significant excess of heterozygotes, whereas *Pgm-2* showed a significant deficiency. The same trend for these two loci was observed in both the Spanish and Moroccan subsets of populations. *6-Pgd-2* showed a significant excess of heterozygotes in Morocco (Table 3). Finally, the overall inbreeding coefficient (f) was not significantly different from zero at either the regional or species level, showing no departure from Hardy–Weinberg proportions.

Between-population genetic identities were high, ranging between 0.999 (M6/E72) and 0.866 (M91/E85) (overall mean \pm SD = 0.965 \pm 0.025). At the regional scale, populations in Morocco and in Spain showed exactly the same mean values. Accordingly, the NJ tree based on Nei's (1972) genetic distances showed

Table 2. Genetic diversity statistics for populations, regions and all populations combined of *Calicotome villosa*

Populations	<i>N</i>	<i>A</i>	<i>P</i>	<i>A_p</i>	<i>N_e</i>	<i>H_o</i>	<i>H_e</i>	<i>f</i>
M5	29.5	2.000	83.3	2.200	1.441	0.306	0.270	-0.134
M6	29.7	2.000	83.3	2.200	1.450	0.274	0.271	-0.010
M10	28.2	1.667	66.7	2.000	1.208	0.167	0.145	-0.157
M87	28.3	2.000	83.3	2.200	1.441	0.321	0.272	-0.185
M89	28.0	2.000	83.3	2.200	1.473	0.397	0.280	-0.428*
M90	27.7	1.667	50.0	2.333	1.233	0.117	0.141	0.177
M91	29.3	2.000	83.3	2.200	1.435	0.348	0.252	-0.394*
M99	27.0	1.833	83.3	2.000	1.483	0.332	0.289	-0.152
M100	29.3	1.667	50.0	2.333	1.293	0.193	0.185	-0.045
E68	29.7	2.000	83.3	2.200	1.480	0.365	0.263	-0.395*
E69	29.8	1.667	66.7	2.000	1.384	0.308	0.230	-0.344
E70	27.8	2.000	83.3	2.200	1.337	0.196	0.191	-0.027
E71	30.0	2.000	83.3	2.200	1.503	0.400	0.273	-0.476**
E72	28.3	2.000	83.3	2.200	1.433	0.273	0.267	-0.024
E73	29.7	1.833	66.7	2.250	1.399	0.281	0.234	-0.205
E74	30.0	2.000	83.3	2.200	1.490	0.289	0.283	-0.021
E75	28.8	2.000	83.3	2.200	1.449	0.303	0.281	-0.079
E76	28.0	2.000	83.3	2.200	1.283	0.194	0.194	0.002
E77	29.3	2.000	83.3	2.200	1.561	0.327	0.265	-0.241
E78	29.7	2.000	83.3	2.200	1.495	0.314	0.269	-0.172
E79	30.0	2.000	83.3	2.200	1.430	0.244	0.239	-0.025
E81	27.7	1.833	66.7	2.250	1.331	0.161	0.185	0.128
E82	28.2	1.833	66.7	2.250	1.325	0.249	0.202	-0.239
E83	30.0	2.000	83.3	2.200	1.268	0.178	0.186	0.043
E84	29.8	1.833	66.7	2.250	1.219	0.119	0.159	0.256
E85	30.0	1.833	66.7	2.250	1.451	0.300	0.249	-0.210
Morocco	28.6 ± 0.94	1.871 ± 0.16	74.1 ± 15	2.185 ± 0.12	1.384 ± 0.11	0.273 ± 0.09	0.234 ± 0.06	-0.170 ± 0.19
Spain	29.2 ± 0.88	1.931 ± 0.10	77.5 ± 8	2.203 ± 0.06	1.402 ± 0.10	0.265 ± 0.08	0.234 ± 0.04	-0.140 ± 0.19
All populations	29.0 ± 0.94	1.910 ± 0.13	76.3 ± 11	2.197 ± 0.08	1.396 ± 0.10	0.268 ± 0.08	0.234 ± 0.05	-0.150 ± 0.19

F-values significantly different from zero at **P* < 0.05, ***P* < 0.001.

Measures of genetic variation include sample size averaged over loci (*N*), mean number of alleles per locus (*A*), percentage of polymorphic loci (*P*, 99% cut-off), mean number of alleles per polymorphic locus (*A_p*), effective number of alleles per locus (*N_e*), observed heterozygosity (*H_o*), expected heterozygosity (*H_e*), and the fixation index (*f*) of Weir & Cockerham (1984).

Table 3. *F*-statistics for both regions (Morocco and Spain) and for all populations combined of *Calicotome villosa*, including the fixation index of Weir & Cockerham (1984) (*f*), the proportion of genetic diversity partitioned among populations within regions (q_{pM} and q_{pS} for Morocco and Spain, respectively), and the proportion of genetic diversity partitioned among populations (q_p) and regions (q_r) in the species (all populations)

Locus	Morocco		Spain		All populations		
	<i>f</i>	q_{pM}	<i>f</i>	q_{pS}	<i>f</i>	q_p	q_r
<i>Adh-1</i>	-0.2074	0.0923	-0.0758	0.0291	-0.1425	0.0768	0.0172
<i>6-Pgd-1</i>	-0.3946	0.1269	-0.3493	0.0340	-0.3617	0.0649	0.0047
<i>6-Pgd-2</i>	-0.6494	0.3521	-0.0790	0.0240	-0.3293	0.2148	0.0236
<i>Pgm-1</i>	-0.4126	0.0091	-0.4822	0.0844	-0.4554	0.0636	0.0063
<i>Pgm-2</i>	0.5628	0.0027	0.2762	0.1301	0.3559	0.1313	0.0350
Mean	-0.1667	0.0934	-0.1400	0.0811	-0.1487	0.1007	0.0175
95% CI _{lower}	-0.4819	0.0147	-0.4173	0.0302	-0.4129	0.0650	0.0064
95% CI _{upper}	0.3029	0.2279	0.2193	0.1170	0.2323	0.1502	0.0317

Upper and lower confidence intervals (CI) for mean values were estimated by bootstrapping (2000 replicates) across loci.

no pattern of geographical clustering of populations across the Strait of Gibraltar and very low bootstrap support for most branches (Fig. 2).

Within each region, the genetic differentiation among populations was not great. Overall, the population genetic differentiation was moderate ($q_p = 0.101$). Therefore, approximately 90% of the genetic variation occurred within populations. At the regional scale, the Strait of Gibraltar can only account for 1.8% of the total genetic variation of *C. villosa* ($q_r = 0.018$) (Table 3). The genetic differentiation in all the studied populations was significantly correlated with geographical distance ($r = 0.444$, $P = 0.004$), although the correlation coefficient decreased, but was still significant, when the geographically most distant population (E85) was excluded from the analysis ($r = 0.241$, $P = 0.027$). At the regional scale, the Moroccan populations did not show a significant correlation between pairwise q -values and geographical distance ($r = -0.230$, $P = 0.862$), whereas all Spanish populations showed a significant correlation ($r = 0.535$, $P = 0.010$), robust to the inclusion of the distant E85 population ($r = 0.224$, $P = 0.048$). Under the assumption of a stepping-stone population model, in which gene flow is most likely to occur between neighbouring populations, the scatter plot of q versus geographical distance is consistent with a lack of equilibrium between gene flow and genetic drift in *C. villosa*, with historical gene flow being much more influential than genetic drift (Fig. 3).

Multilocus outcrossing rates (t_m) were high for populations E68, E81, and M89 and moderate for population E84 (Table 4), indicating a general trend towards outcrossing in the studied populations of *C. villosa*. The levels of biparental inbreeding ($t_m - t_s$)

were consistently low and correlated paternity (r_p) was markedly different among populations, with 8–71% of seeds within a family being full sibs.

The observed maternal inbreeding coefficient (f_{ob}) was in the range -0.276 to 0.176, whereas the expected maternal inbreeding coefficient (f_{ex}) was in the range 0.00–0.234. When we compared f_{ob} with f_{ex} , a deficit of homozygotes became evident in the four populations studied, which was remarkably high in populations E81 and M89 (Fig. 4).

DISCUSSION

In the present study, we have shown that the Strait of Gibraltar has not acted as a biogeographical barrier for *C. villosa* on the historical time scale, as demonstrated by the lack of genetic structure across the studied populations on both sides of the Strait. Some ecological and biological traits of *C. villosa* may explain the observed results.

POPULATION GENETIC STRUCTURE, MATING SYSTEM, AND DISPERSAL

Most scored loci were polymorphic, with the number of alleles per polymorphic locus (A_p) and the effective number of alleles (N_e) per locus at the population level being similar to the average values for many other plant species with different life-history traits (Hamrick & Godt, 1989). The values for gene diversity (H_e) were high, consistent with averaged values for long-lived perennial plants with effective seed-dispersal mechanisms and outcrossing breeding systems (Hamrick & Godt, 1996). This is relevant because reproductive biology, together with other life-

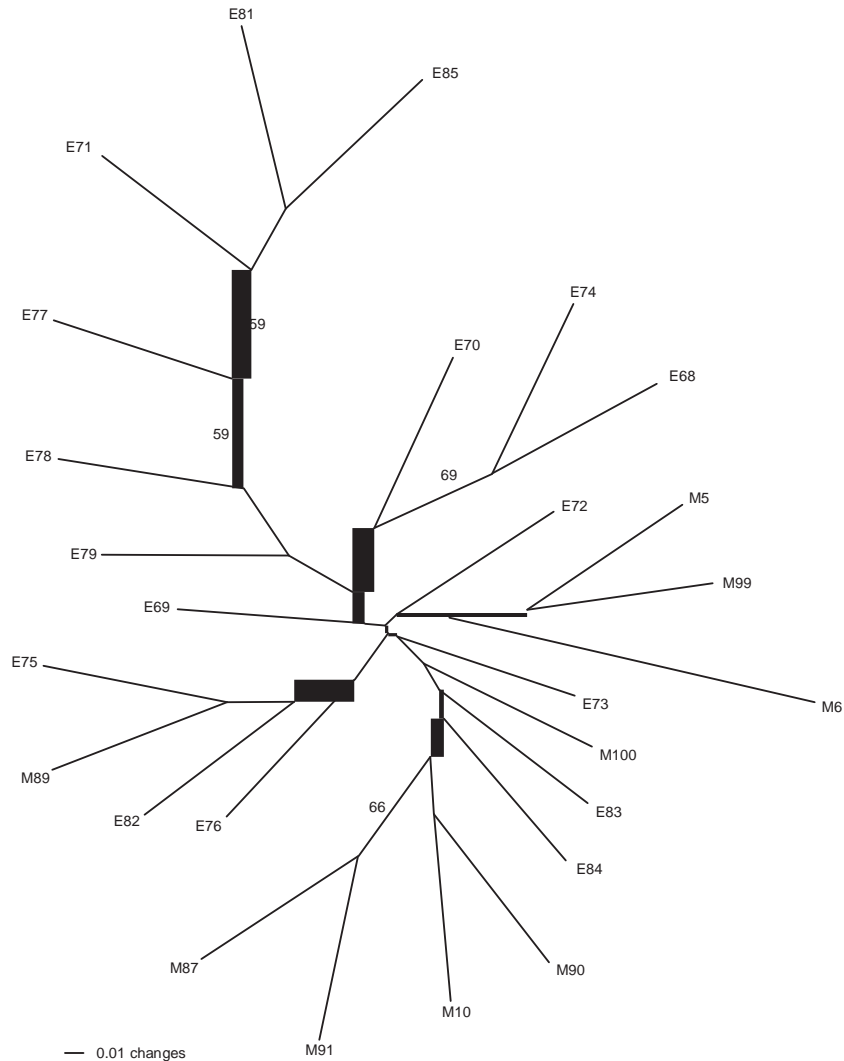


Figure 2. Unrooted Neighbour-joining tree for the 26 populations of *Calicotome villosa* studied across the Strait of Gibraltar based on Nei's (1972) genetic distances. Branch support was calculated by bootstrap analysis (1000 replicates). Only values above 50% are shown.

history traits of species, is a keystone in interpreting population genetic variation (Hamrick & Godt, 1989).

Self-incompatibility is relatively common in the Leguminosae, especially among woody species (Arroyo, 1981). Within the tribe Cytiseae, low fruit set after self-pollination has been interpreted in some species of *Cytisus* and *Retama* as partial self-incompatibility or late-acting inbreeding depression (Parker, 1997; Rodríguez-Riño *et al.*, 1999a). Detailed studies of the reproductive biology of *C. villosa* are lacking but the genetic data collected in the present study on seed progenies at the population level reveal high rates of outcrossing (t_m), supporting a major role for partial self-incompatibility in the breeding system of this species. In the present study, it was found that observed and expected heterozygosities did not exhibit

large discrepancies and, consequently, no significant departure from Hardy–Weinberg expectations was detected at the species level. Despite the overall negative value of the inbreeding coefficient (f) at the population level, only four populations (15.4%) showed a significant departure from random mating, with an excess of heterozygotes.

In *C. villosa*, we found high values of H_e and low values of q , typical of a species with potential long-distance dispersal events in seed flow and/or pollen flow (Hamrick & Godt, 1989, 1996). These events are dependent on the pollination and breeding systems, seed dispersal, habitat requirements, and the physical setting (barriers, habitat availability). First, honey bees have often been observed visiting many flowers in the mass flowering plants of *C. villosa*

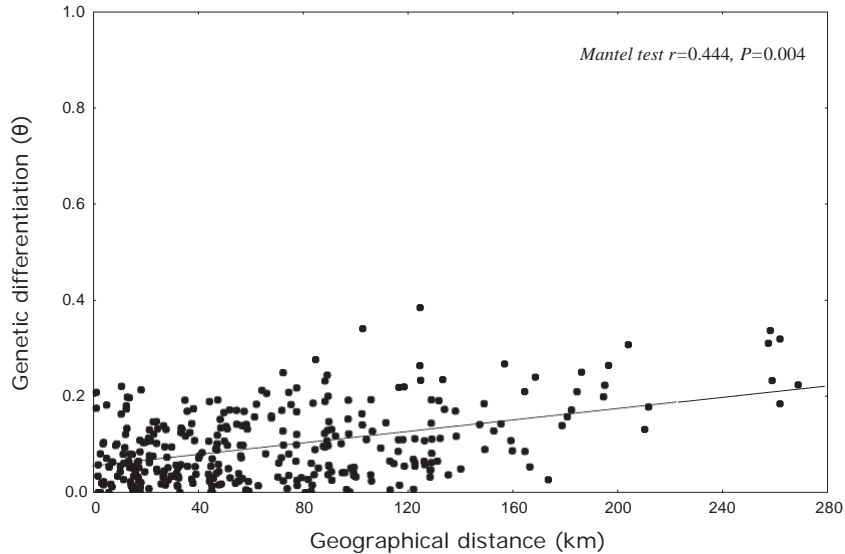


Figure 3. Mantel test, correlation coefficient and associated P -value, and scatterplot showing the relationship between pairwise genetic differentiation among populations of *Calicotome villosa* (q_p) in the region of the Strait of Gibraltar, and the geographical distances among them.

Table 4. Multilocus outcrossing rates (t_m) and levels of biparental inbreeding ($t_m - t_s$) in the four populations of *Calicotome villosa* selected for progeny analysis (for details, see Material and methods)

Population	t_m	$t_m - t_s$	r_p	f_{ob} (95% CI)
E68	1.200 (0.028)	0.052 (0.043)	0.153 (0.274)	-0.098 (-0.556–0.292)
E81	0.824 (0.129)	0.097 (0.054)	0.707 (0.333)	-0.236 (-0.471–0.104)
E84	0.620 (0.142)	-0.017 (0.034)	0.389 (0.362)	0.176 (-0.135–0.427)
M89	0.875 (0.116)	0.050 (0.061)	0.086 (0.244)	-0.267 (-0.450–0.180)

Standard errors based on bootstrap analysis (1000 replicates) are shown in parenthesis. CI, confidence interval.

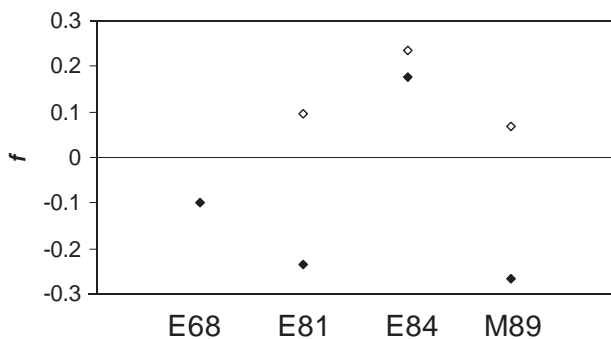


Figure 4. Observed (f_{ob} , filled diamonds) and expected (f_{ex} , open diamonds) maternal inbreeding coefficients in the four populations of *Calicotome villosa* selected for progeny analysis (for details, see Material and methods).

before moving to nearby plants, causing predominantly geitonogamy and cross pollination with relatives, whereas the much less frequent bee species of the genus *Anthophora* tend to move to more distant

plants (J. Arroyo, pers. observ.), as has also been observed in related plant species in the region with similar flower morphologies (Herrera, 1988). Thus, among-population gene flow by pollen movement should be low. Second, explosive pods, which have a limited dispersal distance, would also produce this pattern of mating. Many species have dispersal mechanisms that are not directly related to their inferred functional morphology (i.e. dispersal syndromes; Herrera, 2002). For example, capsules from some species of shrubby *Cistus* can be ingested and dispersed by deer that browse in similar communities (Malo & Suárez, 1998). The germinability of the woody legume *Cytisus scoparius* also increases after cattle gut passage (Manzano, Malo & Peco, 2005). The strong association of *C. villosa* with extensive herding and its palatability to cattle (Anmar *et al.*, 2005) could account for such seed dispersal over longer distances, as has been observed in other woody legumes (*Prosopis flexuosa*, Campos & Ojeda, 1997; *Acacia* spp., Argaw, Teketay & Olsson, 1999). More-

over, it has been demonstrated that Macaronesian broom species, also apparently limited in their potential seed dispersal, have repeatedly colonized oceanic islands (Percy & Cronk, 2002). Third, the association of this broom species with disturbed, often cultivated land and roadsides, may cause seed mixing with agricultural crops, and thus subject it to movement along trade routes. This may account for the lack of genetic differentiation across short distances and explain why most genetic diversity in *C. villosa* is within rather than among populations, even when the historical existence of the Strait of Gibraltar is taken into account. Only at the longest distances within the range does there appear to exist considerable genetic differentiation (as indicated by the Mantel test), probably because the process of range expansion is diffusive, which is consistent with a short-distance stepping-stone model.

ROLE OF HISTORICAL FACTORS

The area around the Strait of Gibraltar is one of the centres of diversity of the tribe Cytiseae (Gómez-González *et al.*, 2004). Many of the species of this tribe are endemic to the area, and many others, such as *C. villosa*, present their largest populations there because broom scrubland is one of the prominent woody communities in the region (Ojeda, Arroyo & Marañón, 1995). Although there has been no comprehensive phylogenetic or phylogeographical study of *Calicotome*, a wider study that included related genera (Cubas *et al.*, 2002) showed the monophyly and clear genetic differentiation of *Calicotome* with both nuclear and plastid markers, although the two samples of *C. villosa* differed in their nuclear sequences. It is interesting to note that, although many species of Cytiseae are narrowly endemic in the area, only a few are confined to one side of the Strait (e.g. *Teline tribracteloata* on the Iberian side, *Teline osmariensis*, on the Moroccan side). This may be due to the ancient origin of these taxa before the separation of the African and Iberian plates and an improbable lack of differentiation during the subsequent 5.5 Mya, or to their dispersal across the Strait once it had formed. A recent study of closely related *Ulex* species on both sides of the Strait of Gibraltar, using polymerase chain reaction-based plastid microsatellite markers (Cubas, Pardo & Tahiri, 2005), which indicate gene flow by seed, showed that the differences among species are very low compared to the differences among populations (on the long-distance dispersal of other broom species, see also Percy & Cronk, 2002). *Ulex* spp. probably have seed dispersal patterns similar to those of *Calicotome*, which suggests that the Strait is not a biogeographical barrier to these legume species. The 14-km width of the

Strait appears to be crossed by genes and, although the pattern could be consistent with pollen flow, this flow is most probably by seeds. A simultaneous study with appropriate molecular, nuclear, and organellar markers is required to resolve this question. Given the short distances over which pollen is dispersed by bee pollinators and over which seeds are dispersed by autochory, and in secondary dispersal by cattle, it is probable that the genetic exchange between the African and Iberian sides is recent and was aided by humans (with the movement of cattle and crops). There are very few studies of the patterns of genetic differentiation of wild species mediated by human influence in the studied region, except when they are deliberately cultivated (Tuomi & Lumaret, 1998). In this context, seed dispersal is a necessary, but not sufficient, condition to preclude genetic differentiation because seeds must germinate and seedlings become established. In this species, the seed germination rate is very high and seed viability long lasting, and seedling growth is very fast under greenhouse conditions (Braza, 2005). Although its behaviour under field conditions is not well known, we hypothesize that the colonization ability of the species is very high, as has been observed in other gorse and broom species in the area (Ojeda, Marañón & Arroyo, 1996b).

This pattern of differentiation across the Strait of Gibraltar contrasts with that observed in other species. Although the available information for plants straddling the Strait of Gibraltar is still scarce, there are interesting contrasting patterns. Traditionally, the Iberian Peninsula has been considered as one of the three main Mediterranean refugia (together with the Italian and Balkan Peninsulas) for plants and animals in harsh climatic periods since the late Tertiary (Hewitt, 2000). This refugium should probably be extended to include northern Morocco because its ecological and historical conditions are similar to those in southern Spain (Reille, 1977). The Strait has probably been a filter for some species through its expansion/retraction cycles during the Quaternary, insofar as the nearby African side has a tree and shrub flora poorer than that on the Spanish side (Marañón *et al.*, 1999; Ajbilou *et al.*, 2006). This filtering process must involve the biological traits and performance of species. Thus, some emblematic long-lived species of Mediterranean forests (*Quercus rotundifolia/ilex*, *Quercus suber*, *Pinus pinaster*) and some relict species (*Frangula alnus*) show a clear genetic discontinuity across the Strait, mostly attributable to limited seed dispersal (Tuomi & Lumaret, 1998; Burban *et al.*, 1999; Salvador *et al.*, 2000; Lumaret *et al.*, 2002, 2005; Burban & Petit, 2003; Hampe *et al.*, 2003). Although these species have fruits that are potentially dispersed by animals (Gómez, 2003; Hampe, 2004) or wind over longer

distances than are the seeds of *C. villosa*, they could not cross this effective barrier. Unlike our study species, those species are not associated with disturbances and their dispersal is not affected by human activities, except when they are deliberately planted. All of them are long-lived trees, late-successional species with reduced colonizing ability compared to that of short-lived, early successional species. However, herbaceous species show a variety of patterns, with no differentiation in the bulb geophyte *Androcymbium gramineum* (Caujapé-Castells & Jansen, 2003), and strong differentiation in the perennial herb *Saxifraga globulifera* (Vargas, Morton & Jury, 1999). Even within the same genus, *Bellis annua* (Compositae) shows genetic differentiation among European and African populations, whereas *Bellis microcephala* does not, although both species are annuals (Fiz, Valcárcel & Vargas, 2002). Because of the low number of species studied, we are far from determining the general properties of species filtered by the Strait. Nonetheless, it appears that the high seed dispersal and establishment potential of pioneer shrub species are key factors suppressing their genetic differentiation.

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