Molecular Ecology 1997, 6, 1107–1121

Analysis of population genetic structure and variability using RAPD markers in the endemic and endangered *Limonium dufourii* (Plumbaginaceae)

C. PALACIOS and F. GONZÁLEZ-CANDELAS
Departament de Genètica, Universitat de València, c/Dr Moliner, 50, E-46100 Burjassot, Valencia, Spain

Abstract

***Limonium dufourii* (Plumbaginaceae) is a triploid species, with apomictic reproduction, endemic to the east mediterranean coast of Spain, where it is present in only six populations with a few individuals in most of them. *L. dufourii* is included in the Red List of Endangered Species by the IUCN. Genetic variation and population structure in this species has been studied using RAPDs. Twelve different primers provided 124 reliable bands of which 33 were polymorphic among the 165 individuals analysed. Those polymorphic bands were able to define 44 different patterns, of which all but six were present in only one population. Several methods for statistical evaluation have been used for intra- and interpopulation analysis of genetic variability. Relationships among patterns have led to the identification of four main clusters. Two of them show a perfect correspondence to the population of origin of those individuals that present them (Cullera and Torreblanca), and the other two (Groups A and B) include patterns found in individuals coexisting in the same populations (Marjal del Moro populations) and in El Saler. Most of the variation found in this species is due to differences among populations as shown by the analysis of molecular variance. This agrees with the expectation for an apomictic species such as *L. dufourii*. The analysis of homogeneity of variance shows that substantial differences in the amount of genetic variability present in the six populations exist. These results have been used to understand the evolutionary and demographic history of *L. dufourii*, which is a requisite in order to establish efficient conservation measures for this species.**

Received 22 April 1997; revision accepted 5 July 1997

Introduction

The genus *Limonium* (nomen ambiguum *Statice*) of the family Plumbaginaceae is one of the plant genera in the Iberian Peninsula with a relatively large number of endemic, rare, and/or endangered species. The genus is distributed world-wide, comprising ≈ 400 species. It presents two centres of diversity: the Western Mediterranean region and the Asian steppes (Erben 1979).

The vulnerability of specialized ecosystems to sudden changes, such as those caused by extensive human impact on the Mediterranean coasts of the Iberian peninsula, has resulting in a high incidence of rarity throughout the highly endemic flora of the region, and extinction events are becoming more and more frequent (Gómez-Campo 1987; Laguna *et al.* 1994). The conservation of endangered species in the face of their perceived decline represents a major challenge in the halting of the current decrease of the world's biological diversity.

At the forefront of management decisions concerning critically endangered species is the necessity to understand the biology of the species and the factors acting against its natural survival. Population genetics provides critical guidance for interpreting the present status and future prognosis of threatened species as it allows us to study relevant parameters such as the genetic diversity within populations, the distribution of this genetic variability in species that occur in fragmented populations, and, with the use of appropriate markers, to infer historical processes that have moulded the current species structure. Population genetic analysis of endangered species has been identified as one of the main priorities for conservation biology (Holsinger & Gottlieb 1991; Ellstrand

Correspondence: Dr Fernando González-Candelas; Tel.: + 34-6386-4505; Fax: + 34-6398-3029; E-mail: fernando.gonzalez@uv.es

& Elam 1993) and the integration of these data with those from other disciplines has permitted the taking of better informed decisions for the appropriate management of endangered species (Schonewald-Cox *et al.* 1983; Avise 1994; Avise & Hamrick 1996; but see Schemske *et al.* 1994 for a different opinion).

One of the most threatened species of the genus *Limonium* native to Spain is *L. dufourii* (Girard) O. Kuntze, a perennial, rosulate, hemicryptophyte, densely hairy, and with racemose inflorescences, many of which, usually the lower ones, are sterile. It is an obligate apomictic species with self-incompatible pollen-stigma combination (Baker 1966) and a triploid chromosome number (2n = 27, J. A. Rosselló, personal communication).

Historically, the species was distributed along the Valencia and Castellón coasts where it occurs on salty soils (Crespo & Laguna 1993). *L. dufourii* was originally described from the El Saler population, where it was present in large numbers. In the past years, only a few individuals have persisted in this population. In general, most of the original populations have disappeared entirely and, based on numerous field surveys along the coast, only six populations of *L. dufourii* have been found to remain in the natural environment(Fig. 1). Three of these are at the brink of extinction given their extremely small size (Table 1). The decline of *L. dufourii* populations, which has occurred in the last 20 years, is mainly due to human activities such as tourism and environmental pollution, which have caused loss and alteration of the habitats where the species occurred (Laguna *et al.* 1994). The species has been declared under protection by the Valencia regional government in 1985, and catalogued as endangered, level E, by the IUCN.

In this study, which is part of a larger study started on this species, Random Amplified Polymorphic DNA (RAPD) analysis has been used to characterize population differentiation by quantifying the genetic variability within and between populations of *L. dufourii*, to infer the relationships among these populations, and to establish the relatedness among the different phenotypes found in the species.

In a previous study we were unable to detect restriction site variation in chloroplast DNA from different *L. dufourii* populations, using four frequently cutting restriction enzymes (Palmer 1986) and screening ≈ 70% of the genome with homologous probes. In fact, only one probe–enzyme combination was found that could discriminate *L. dufourii* from *L. angustebracteatum*, a species that belongs to a different subsection of the genus (Boissier 1848). Given the small quantity of variation found within and between species in this study, and in view of the advantages mentioned above we decided to use RAPDs for the genetic analysis of *L. dufourii* populations.

RAPD analysis has some advantages over other techniques for the genetic analysis of populations (Hadrys *et al.* 1992), and some of them make the technique potentially ideal in genetic studies of rare and endangered organisms (Gibbs *et al.* 1994; Rosseto *et al.* 1995; Nusser *et al.* 1996). Typical of PCR-based methods, only small quantities of template DNA are required, and the technique is simple, fast and relatively inexpensive. Moreover, the amplified fragments provide a large number of potentially polymorphic loci, which make RAPDs very useful for studies of species with low genetic variability. This is usually the case for endangered and/or nonsexual species (Williams *et al.* 1993), especially when other techniques fail to reveal differences among populations (Dawson *et al.* 1993).

One of the main drawbacks of RAPD analysis is the lack of reproducibility of banding patterns between reactions, which has been attributed to PCR artefacts (see for instance Riedy *et al.* 1992). Problems of this kind can be

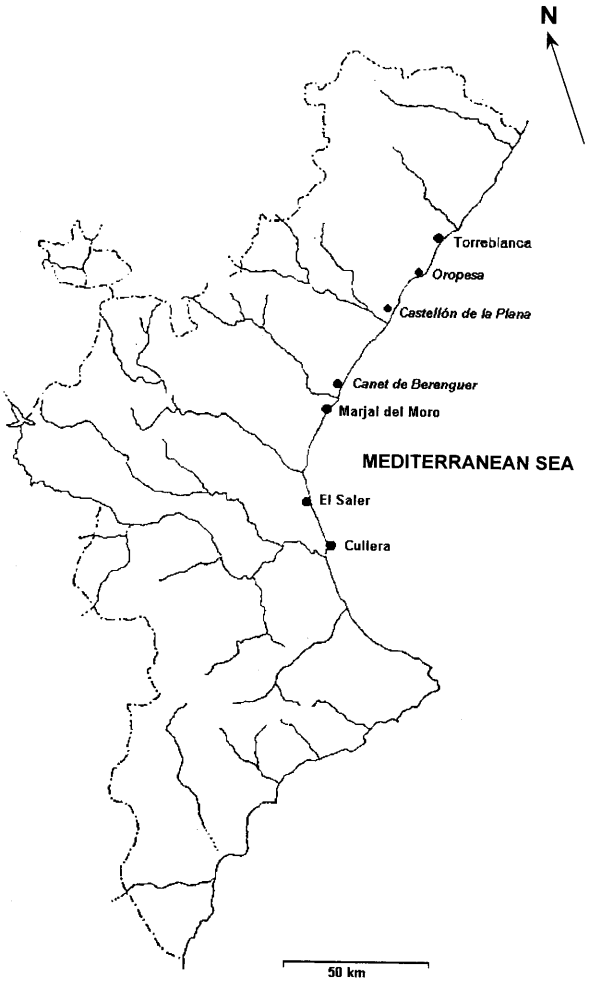

Fig. 1 Location of *Limonium dufourii* populations. Locations shown in italics represent historical sites in which the species is now extinct.

Table 1 Location, number of individuals analysed, and population sizes (approximate for populations larger than 100 individuals) of the six *Limonium dufourii* populations used in this study.

Population	Individuals analysed	Population size
Cullera	34	34
Torreblanca	40	200
El Saler	7	7
Marjal del Moro-1	25	25
Marjal del Moro-2	29	3000
Marjal del Moro-3	30	2000

reduced by careful laboratory practice, including reproducibility studies and keeping all possible variables between reactions unchanged (Hadrys *et al.* 1992). Another disadvantage of the method, that concerns the statistical data analysis, lies in the dominant nature of RAPD products. Recently, Stewart & Excoffier (1996) have proposed a method to circumvent this difficulty.

A second goal of this paper is to illustrate how RAPD markers can be used in a population genetics framework using the available statistical techniques developed for them or for other makers, even when some of the Hardy–Weinberg equilibrium assumptions do not hold, which is usually the case in nonsexual species.

Material and Methods

Population sampling, DNA isolation and quantification

The number of individuals sampled from each of the six remaining populations of *L. dufourii* is shown in Table 1. For the three populations in which the census was lower than 100 individuals, all individuals were sampled for analysis. From the other populations, a representative sample size was collected. Two or three small leaves ranging from 20 to 800 mg of tissue, depending on the availability of material, were taken from each individual. Samples were kept at – 80 °C until DNA extraction.

DNA was extracted using the CTAB protocol developed by Doyle (1991), with the only modification that an additional chloroform–isoamylalcohol (24 : 1) extraction was performed when samples were still turbid after the first extraction. DNA yields were estimated by direct comparison with standard DNA concentrations in 0.8% agarose gels stained with ethidium bromide (0.5 μg/mL). After quantification, DNAs were diluted to a final concentration of ≈ 1 ng/μL in distilled water.

DNA amplification and fragment visualization

Initial RAPD profiles were generated using 20 decamer primers (OPA1–OPA20) from Operon Technologies Primer Kit A. Amplification reactions were carried out in 20 μL total volume, containing 1 × *Taq* Buffer (Pharmacia), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 15 ng of primer, 1.0 unit of *Taq* DNA polymerase (Pharmacia), and 5 ng of template DNA. Negative controls with water instead of DNA were included in each run in order to verify the absence of contamination. DNA amplifications were carried out in a Perkin Elmer 2400 thermocycler. Cycling consisted of an initial denaturation step at 94 °C for 5 min followed by 45 cycles of 94 °C for 1 min, 39 °C for 2 min, and 72 °C for 2 min, and a final elongation step at 72 °C for 7 min.

Amplification products were separated on 1.4% agarose. Gels were run in 0.5 × TBE buffer for approximately 4 h at 7.5 V/cm and visualized under UV light. Monochrome negatives (Agfapan, APX100) were taken using a Polaroid camera. To help in scoring the bands, each 30-well gel consisted of alternating individuals from each of the six populations of the species.

Pilot study: primers used and reproducibility of banding patterns.

As noted previously, problems with band reproducibility, even using standardized conditions and reagents, seem to be the norm rather than the exception in RAPD studies. For unknown reasons, some primers give more reliable results than others depending on the species. A pilot study was therefore carried out to evaluate the suitability of the available primers with *L. dufourii*.

Initially, each primer was used on a subset of four randomly chosen individuals from different populations. Based on the clarity of the patterns obtained (i.e. bright staining, sharp, and not too many amplification products per lane), 14 primers were selected to test the reproducibility of their profiles. Each reaction was repeated three times using the same sample subset. Only those primers that gave reproducible banding patterns between reactions were chosen for the analysis of the whole sample set of the species (Table 2).

Furthermore, all the fragments included in the final analysis were tested for reproducibility. Whenever a new RAPD band that was not present in the pilot study appeared, two to four replicate PCR reactions were performed with that sample. The new band was included in the analyses only if it was present in all the reactions.

It is important to emphasize that primer selection was not biased in favour of those that revealed the most polymorphism. There is also no a priori reason to assume that there was a bias in scoring polymorphic vs. monomorphic bands [Clark & Lanigan (1993) discuss how these biases can influence measures of genotypic diversity].

By heeding all the precautions mentioned above, most of the assumptions usually made in the analysis of dominant markers (Lynch & Milligan 1994; Stewart & Excoffier 1996) could be validated, allowing us the use of RAPDs,

Table 2 Primers used, their sequences, base pair range scored, and number of polymorphic and monomorphic markers observed for each primer

Primer	Sequence	bp range	Bands	
			Monomorphic	Polymorphic
OPA-01	CAGGCCCTTC	600–2500	7	1
OPA-07	GAAACGGGTG	200–1500	10	5
OPA-08	GTGACGTAGG	250–2500	8	7
OPA-09	GGGTAACGCC	1000–2000	8	0
OPA-10	GTGATCGCAG	300–1250	6	5
OPA-12	TCGGCGATAG	500–2500	5	5
OPA-14	TCTGTGCTGG	600–1250	4	0
OPA-15	TTCCGAACCC	350–1250	12	0
OPA-16	AGCCAGCGAA	300–1300	4	0
OPA-18	AGGTGACCGT	350–1250	10	3
OPA-19	CAAACGTCGG	800–2500	6	4
OPA-20	GTTGCGATCC	800–2500	10	3

but at the cost of a decreased precision compared with codominant markers.

Data analysis

The presence or absence of homologous bands included in the final analysis was determined for all individuals and a matrix of the different RAPD phenotypes was assembled. Fifteen different metric distances for all pairwise combinations of RAPD patterns were computed using RAPDISTANCE (Armstrong *et al.* 1996). These distance matrices were compared with the Euclidean metric (Excoffier *et al.* 1992) using DIPLOMO (Weiller & Gibbs 1993) to test their correlation by means of a *t*-test (Sokal & Rohlf 1995). This distance was chosen for further analyses due to its adequacy for AMOVA (see below) and because it uses both presence and absence of matches, which may be considered as a safety margin when dealing with rare organisms (Rossetto *et al.* 1995).

Several approaches to assess relationships among RAPD phenotypes were used. First, the Euclidean distance matrix was used to construct a dendrogram using the neighbour-joining method (Saitou & Nei 1987) as implemented in NEIGHBOR from the PHYLIP package (Felsenstein 1993). Conventional approaches for the estimation of the reliability of inferred trees, such as bootstrapping or jackknife, were originally developed for interspecific studies. These techniques sometimes lack statistical power at the intraspecific level because they focus on differences among taxa rather than between individuals, which are expected to differ minimally (Templeton *et al.* 1992; Templeton & Georgiadis 1996). Techniques that make an overall assessment of the tree rather than generating confidence limits for individual branches are better in this situation. We have used two different methods for this goal. First, we have compared the distance matrix with the patristic distances in the resulting neighbour-joining tree using DIPLOMO by means of their correlation coefficient (Armstrong *et al.* 1996). Second, we have performed a Permutation Test Probability (PTP) analysis to test whether the resulting tree reflects an actual tree-like signal in the data or merely an artefact of the algorithm (Faith & Cranston 1991).

Even though parsimony cannot be applied directly to RAPD data except in haploid organisms (Clark & Lanigan 1993), we have also used the original RAPD phenotype data matrix to perform maximum parsimony analysis using PAUP (Swofford 1993).

The above mentioned techniques are the classic approaches to determine the relationships among different operational taxonomic units (OTUs), but a theoretical framework for integrating the information from gene genealogies and frequency distributions of the variants at the intraspecific level is arising (Excoffier & Smouse 1994; Templeton *et al.* 1995). Moreover, the finding of a large number of most parsimonious trees (see Results) suggested that an alternative way of representing the relatedness between the patterns obtained, not simply by means of strictly bifurcating trees, would be more adequate. We have therefore used estimation of a minimum spanning tree (MST, Dunn & Everitt 1982) as the third approach for inferring the intraspecific phylogeny of *L. dufourii* using NTSYS (Rohlf 1993). Alternative MSTs were obtained with MINSPNET, using the modified Prim procedure proposed by Excoffier & Smouse (1994). We have also used NTSYS to study the relationships among RAPD phenotypes using principal coordinates (PCO) analysis. The multidimensional scaling was completed with the superposition of the MST on the corresponding plot.

By using these last approaches we expected to be able to extract additional information from the plot obtained, such as the multidimensional relationships among the populations (Whitty *et al.* 1994; Travis *et al.* 1996), and to formulate a hypothesis on the intraspecific phylogeography of the species (Avise 1994), which cannot be inferred from a simple dendrogram representation.

The distance matrices between RAPD patterns for 15 different metrics available in RAPDISTANCE were used to calculate pairwise genetic distances between populations as:

$$d'_{xy} = d_{xy} - (d_x + d_y/2)$$

where d_{xy} represents the uncorrected distance between populations x and y, and d_x, d_y correspond to intrapopulation diversity measures. The resulting population distance matrices were compared to test their correlation with the Euclidean metric as in the previous section. The results of this correlation test allowed us to reduce the number of distance matrices to be employed in subsequent analyses, using only the Euclidean distance. This distance matrix was used to construct a neighbour-joining tree for *L. dufourii* populations. To test whether genetic and linear geographical distances between populations were correlated, the corresponding matrices were compared by means of the correlation test as described above.

For studying the genetic structure of *L. dufourii* populations we have used the extension of the analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) recently developed by Stewart & Excoffier (1996) to accommodate RAPD profile data by taking into account the dominant nature of these markers. The technique can be applied to diploid populations with assumed levels of self-fertilization. However, as *L. dufourii* is a triploid species, some further correction for dominance was necessary. In an apomictic species, departures from equilibrium result both from deviations in the founding populations and because of other evolutionary forces (selection, drift, mutation, etc.) which cannot be counteracted by random mating. Because Hardy–Weinberg cannot be checked with dominant markers, we either assumed random mating ($S = 0$) or complete self-fertilization ($S = 1$), as the two extremes for the range of possible values of the selfing rate, S. For the former case, the equations for the conditional expectations of the squared distance between individuals (7a)–(8b) in Stewart & Excoffier (1996) were transformed to be applied to a triploid species in Hardy–Weinberg equilibrium (Appendix 1). The two-step strategy proposed by the authors was applied to calculate the F-statistic analogue (Φ_{ST}) and its significance (Excoffier *et al.* 1992). For the case of $S = 1$, transformations were not necessary, because this condition is equivalent to a purely phenotypic comparison without correction for dominance (Huff *et al.* 1993), except for a proportion factor. Under conditions of complete selfing, any individual showing a band is assumed to be homozygous for that allele. When compared with another individual showing the same band, there would be no differences among them, and when compared with an individual lacking the band, there would be nine differences (3 × 3 chromosomes in each individual being compared). Finally, we used the nonparametric test for homogeneity of molecular variance (HOMOVA), based on Bartlett's statistic (Bartlett 1937), to test for within population genetic diversity. Both AMOVA and HOMOVA analyses were performed using WINAMOVA (available from L. Excoffier).

Results

RAPD profiles and their geographical distribution.

Fig. 2 shows examples of RAPD profiles generated with some primers. Negative controls, in which DNA was omitted, were always free of amplification products. Individuals that shared profiles with a polymorphic primer also shared their profiles with almost all the other polymorphic primers (e.g. compare patterns for primers 7 and 8 in Fig. 2). These results are characteristic for apomictic species, in which each individual is expected to be identical to its progenitor [i.e. they are 'ramets' from the same 'genet' according to Harper (1985)], unless a mutation event has occurred. Thus, if two individuals are descendant from the same progenitor, they are expected to present the same RAPD profile with all primers (barring mutation). This is corroborated by the fact that no band is exclusive to a single individual.

Primers used, their sequences, size range scored, and numbers of polymorphic and monomorphic markers observed for each primer are presented in Table 2. RAPD products in the middle molecular weight range usually

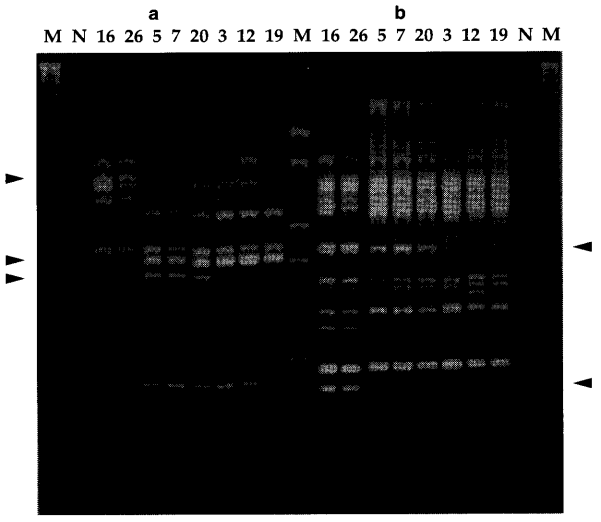

Fig. 2 Example of RAPD profiles in *Limonium dufourii* obtained with two different primers (a) OPA-8, (b) OPA-7. M and N lanes correspond to molecular size markers and to negative controls for each primer, respectively. Arrows indicate polymorphic bands. Note that individuals that share profiles with primer 8, also share their profiles with primer 7.

produced much more reliable results, as some authors have already pointed out (Penner *et al.* 1993; Stewart & Porter 1995). The 12 primers used in the final study of 165 individuals from *L. dufourii* gave a total of 124 markers, 91 of which (74%) were monomorphic. A total of 33 (26%) polymorphic markers allowed 44 different RAPD phenotypes to be distinguished(data not shown, available on request). Torreblanca and Cullera populations do not have any pattern in common with other populations, whereas El Saler and Marjal del Moro populations share some phenotypes. A total of six (13.6%) RAPD phenotypes are shared by more than one population, while the remaining 38 (86.4%) are confined to single populations. Hence, these results suggest a high degree of population differentiation, as expected for an apomictic species.

Pattern divergence and intraspecific phylogeny

Among the available measures to calculate distances among RAPD patterns we have used the Euclidean metric proposed by Excoffier *et al.* (1992), because the correlation among this and the remaining distance matrices was not significantly different from 1 and for the reasons mentioned in the Material and Methods sections. The resulting distance matrix for the 44 RAPD patterns is available upon request.

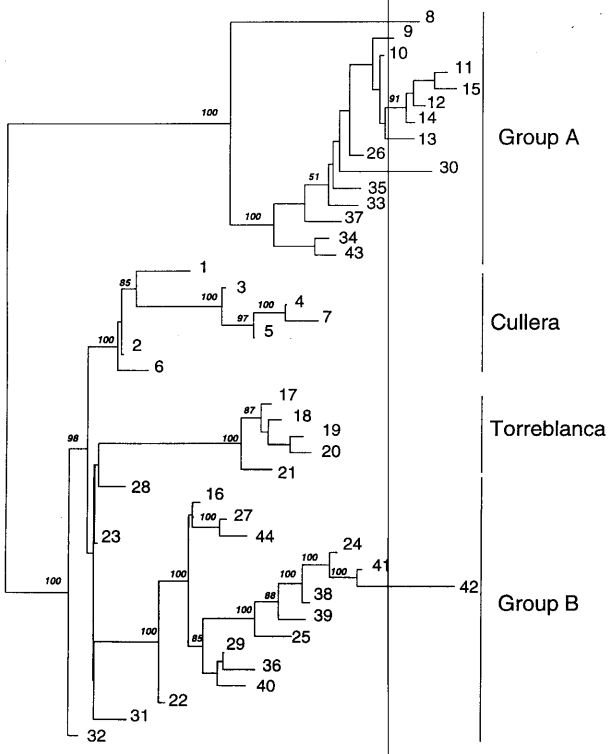

Fig. 3 Neighbour-joining tree showing the relationships among the 44 different RAPD patterns obtained in *Limonium dufourii*. The Euclidean distance matrix was used. Numbers at nodes represent the values of the majority-rule consensus tree obtained by parsimony analysis of the raw presence/absence matrix.

The unrooted dendrogram obtained using the neighbour-joining method (Fig. 3) shows the relationships among the 44 patterns based on RAPD analysis. The tree revealed clear differentiation of two major groups. One group includes patterns restricted to Marjal del Moro populations, denoted group A for further discussion. The remaining patterns are grouped in three subclusters, where phenotypes from Cullera and Torreblanca populations form two well-separated groups, and the remaining patterns form another group, except for phenotypes 23, 28, 31, 32 that are not clearly included in any of these subclusters. The last subcluster and the four separate patterns are denoted as group B. A majority-rule consensus tree derived from the 100 most parsimonious trees revealed similar relationships, including the two main clusters and the subclusters present in the neighbour-joining tree. Other minor groups with lower consensus values were also present in the neighbour-joining tree. The consensus values for groups coincident in both analyses are shown in the nodes of the tree depicted in Fig. 3.

The two methods used for testing the tree-like signal in the data gave consistent results. The comparison between the genetic and the patristic distance matrices gave a significant positive correlation value ($r = 0.976$, $P < 0.001$). The PTP test gave a Z-value of 44.61, implying that the probability of this tree to occur simply by chance is practically null.

Population frequency information and relationships among the patterns can be represented simultaneously on a minimum spanning network (Fig. 4). In general, the plot supports the clusters suggested by the phenetic method, but it shows that alternative relationships among patterns can be derived from this network, especially within groups. Consequently, a simple bifurcating tree is an inadequate representation of the evolutionary relationships at the intraspecific level.

The superposition of the MST on the bidimensional plot of the principal coordinates analysis derived from the Euclidean distance matrix between the 44 RAPD phenotypes (Fig. 5) confirms that the previous relationships are preserved in the multidimensional mapping. The first dimension of the PCO explains 51.5% of the total variation by separating group A of patterns found in the three Marjal del Moro populations from the rest. Note that in the minimum spanning network (Fig. 4) at least 11 mutational steps separate these groups. The second dimension explains an additional 14.2%, essentially spreading along its axis the remaining patterns and separating Torreblanca from the other populations. The third dimension (not shown) explains almost 10% of the total variation by enhancing the differences among Cullera patterns and the rest.

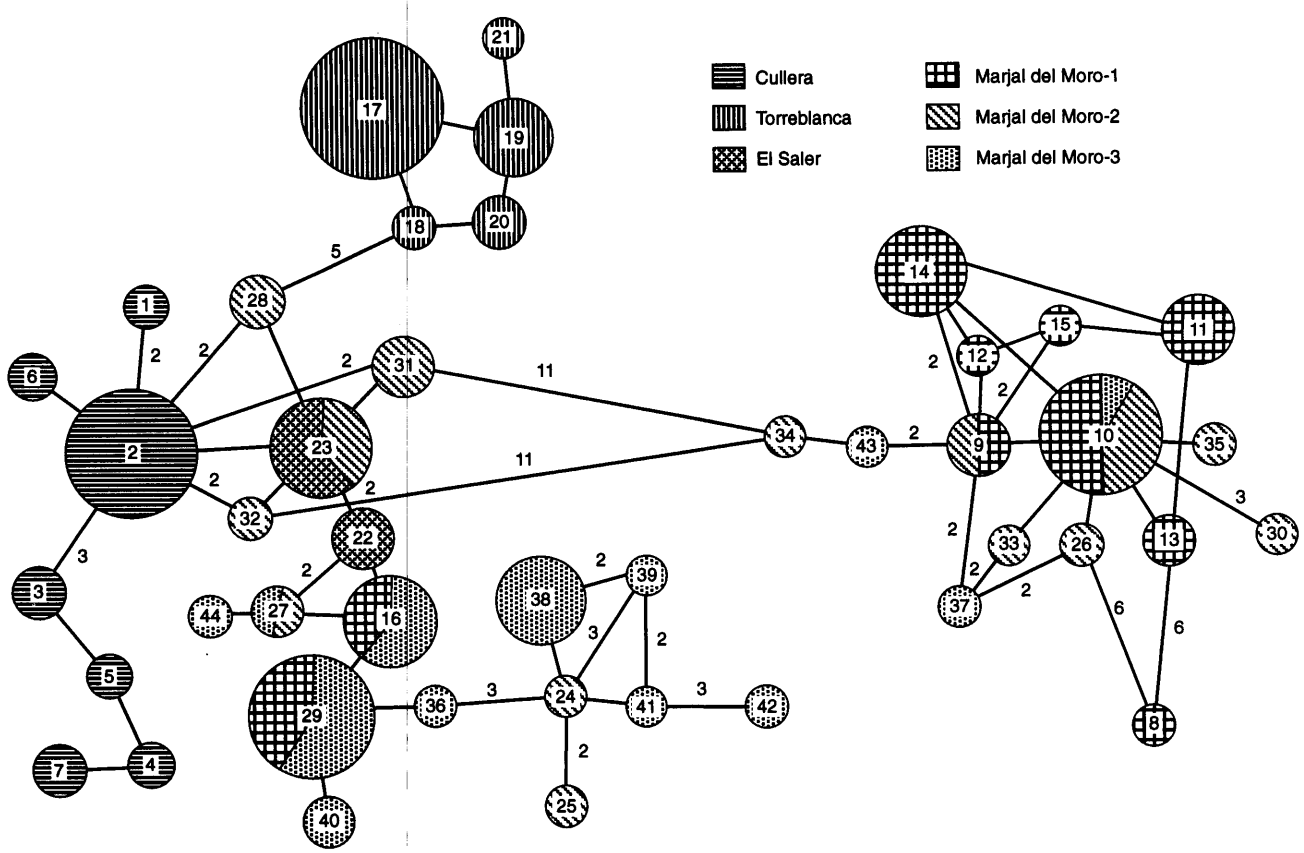

Fig. 4 Minimum spanning network obtained with MINSPNET showing the relationships among 44 RAPD patterns found in *Limonium dufourii*. The area of each circle is proportional to the frequency of individuals showing the corresponding pattern. Numbers above the segments connecting circles represent the number of differences among them when these are larger than 1.

It is remarkable that only one pattern is common to all three Marjal del Moro populations and only five patterns are found in two populations. This low incidence of pattern sharing among Marjal del Moro populations is a preliminary indication of genetic structuring among them.

Divergence at the population level

Interpopulation distances obtained using the Euclidean metric (Table 3) and the remaining metrics available in RAPDISTANCE showed a significant positive correlation, with the only exception of Russel and Rao metric (data not shown). This measure overestimates the distance between OTUs because it only considers mismatches in the denominator. It showed low correlation with the other distance measures, and we did not consider it adequate for RAPD data. The Euclidean distance previously used for comparing RAPD patterns was therefore used in comparisons at the population level.

The neighbour-joining tree (Fig. 6) constructed from the Euclidean distances among populations did not show concordance between the geographical location of the populations and the topology of the dendrogram. This result agrees with the correlation test performed, which did not reveal a significant correlation between genetic and the actual geographical distances between populations (Table 3, $r = 0.151$, $P > 0.05$).

Population genetic structure

Partitioning of RAPD variance within and among populations was performed using the AMOVA procedure with the modifications proposed for RAPD data by Stewart & Excoffier (1996). The three Marjal del Moro populations showed significant heterogeneity (data not shown) and were therefore treated as separate populations in subsequent analyses. The phenotypic distance matrix was used in the AMOVA analysis of the six *L. dufourii* populations according to Huff *et al.* (1993). This analysis is equivalent to the complete selfing case ($S = 1$). AMOVA analyses with distances among individuals corrected for the dominant nature of RAPDs (genotypic analyses) followed the two-step procedure from Stewart & Excoffier (1996) and were based on equations derived in Appendix 1 for the case of

Table 3 Interpopulation distances obtained using the euclidean metric (lower hemimatrix) and linear geographic distances (upper hemimatrix) among *Limonium dufourii* populations

	Cullera	Torreblanca	El Saler	Marjal del Moro-1	Marjal del Moro-2	Marjal del Moro-3
Cullera	—	63.072	12.530	28.000	28.003	28.001
Torreblanca	6.124	—	52.086	35.128	35.125	35.127
El Saler	1.495	5.364	—	18.026	18.029	18.027
Marjal del Moro-1	13.027	15.189	12.235	—	0.003	0.001
Marjal del Moro-2	4.136	7.207	3.085	3.515	—	0.002
Marjal del Moro-3	4.291	8.173	2.311	11.956	3.209	—

random mating ($S = 0$). The corrected distance matrix used in step 1 of the genotypic analysis is based on the frequency across all populations of each marker (available upon request). Because this analysis showed significant population structure, the second step was performed based on a new corrected distance matrix that takes into account the frequency of each marker in each population. A summary of the phenotypic and two genotypic AMOVA analyses is shown in Table 4.

AMOVA analyses show significant population differentiation in *L. dufourii*, with independence of the selfing rate (phenotypic and genotypic analyses). Φ_{ST} values range between 0.515 and 0.656 for the possible values of S. The proportion of variation attributable to population differences is very high, as a consequence of the reproductive system, the low seed dispersal rate, and the demographic history of these populations. In addition, all pairwise comparisons of population variance were significant (data not shown).

Bartlett's tests for homogeneity of variance indicate significant levels of variability among populations (Table 4). Intrapopulation variances for phenotypic and genotypic AMOVAS are also shown in Table 4. The lowest level of genetic variation was observed in the Torreblanca population, followed by the Cullera, El Saler and Marjal del Moro populations, respectively. Among the latter, Marjal del Moro-2 showed the highest diversity.

Discussion

Since their development early this decade (Welsh & McClelland 1990; Williams *et al.* 1990), RAPDs have been used in many different applications in evolutionary biology, ranging from genomic mapping (Al-Janabi *et al.* 1993; Rieseberg *et al.* 1993; Sobral & Honeycutt 1993), to the characterization of genetic resources in plants (Kresovich *et al.* 1992; Orozco-Castillo *et al.* 1994), or the identification of species, subspecies, hybrids, clones, or genotypes (Benito *et al.* 1993; Cenis *et al.* 1993; Transue *et al.* 1994), among others. However, their utilization in population genetic studies has been hampered by the severe restrictions posed by some of the assumptions underlying the methods proposed for their analysis (Clark & Lanigan 1993; Lynch & Milligan 1994). Nevertheless, some of these difficulties can be overcome by the use of new methods (Stewart & Excoffier 1996; cf. Martínez-Torres *et al.* 1997). In spite of all the remaining drawbacks and considerations, the existence of other restrictions often encountered

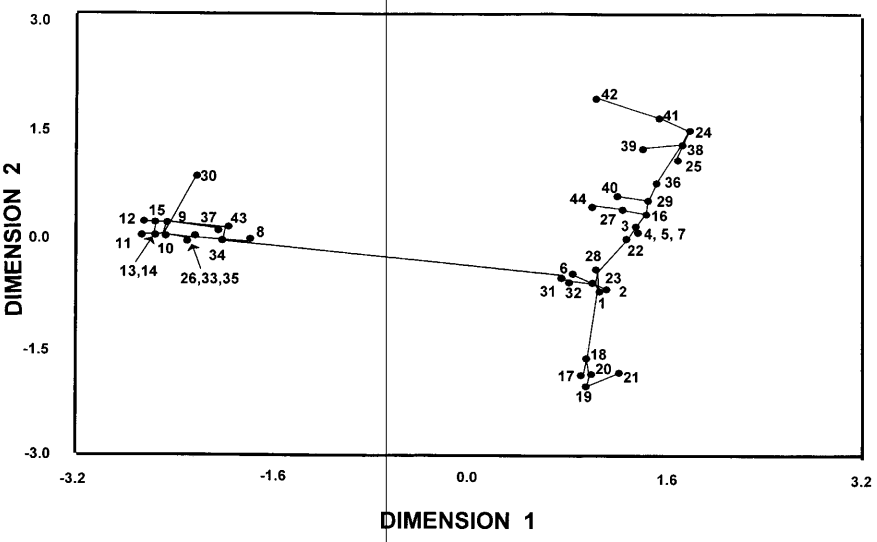

Fig. 5 Principal coordinates plot with the minimum-spanning tree superimposed for the two first dimensions in the principal coordinates analysis of the 44 RAPD patterns obtained in *Limonium dufourii*.

Table 4 Summary of the AMOVA and HOMOVA analyses. Population statistics are shown according to different assumptions on the data. DP, analysis based on the matrix of phenotypic distances between individuals. DG1 and DG2, analyses based on the matrices of genotypic distances among individuals when total or intrapopulation frequencies of each marker were considered (see text for further details). The first six rows refer to within population variance estimates for the six *L. dufourii* populations analysed. Φ_{ST} represents the F_{ST} statistic analogue from the AMOVA. Bartlett's test for the HOMOVA within populations is shown in the bottom row. Nonparametric tests of significance for variance components and homogeneity are based on 1000 random permutations

Population statistics	DP	DG1	DG2
$\sigma^2_{Cullera}$	0.777	9.267	3.770
$\sigma^2_{Torreblanca}$	0.311	8.275	1.253
$\sigma^2_{El\ Saler}$	0.571	11.590	8.796
$\sigma^2_{Marjal\ del\ Moro\text{-}1}$	2.120	26.092	52.470
$\sigma^2_{Marjal\ del\ Moro\text{-}2}$	4.684	33.255	92.396
$\sigma^2_{Marjal\ del\ Moro\text{-}3}$	2.668	27.245	50.706
σ^2_{among}	3.586***	16.690***	37.116***
σ^2_{within}	1.878***	19.213***	34.861***
Φ_{ST}	0.656***	0.465***	0.516***
Bartlett's test	73.074***	25.561***	159.886***

***$P < 0.001$.

in the survey of natural populations still makes the use of RAPDs advisable when there is only a small amount of biological material for analysis, closely related individuals are being compared, little (or none at all) sequence information is known of the particular species, and general evaluations of variability at, for instance, the population level is sought. All these conditions apply to the present study, in which RAPDs have been used to evaluate and characterize genetic variation within and among populations of an endangered plant species *Limonium dufourii*.

The application of a range of statistical techniques for RAPD data analysis allows a better understanding of the biological phenomenon under study. This is particularly clear in this case. A mere cluster analysis is an inadequate description of the relationships among the different patterns and populations, as it fails to resolve multifurcations. For instance, patterns 23, 28, 31 and 32 are not clearly included in any of the clusters in the neighbour-joining dendrogram (Fig. 3), but the minimum spanning network (Fig. 4) places all them together in a central place, where they act as connectors among patterns belonging to the four main groups: Cullera, Torreblanca and groups A and B. It is even possible to hypothesize which patterns have acted as ancestors. Patterns that are not fixed 9, 10, 16, 23, 27 and 29 usually act as the hub of star-like clusters, meaning that they are connected to several other group-specific phenotypes. This suggests that recurrent evolution from these phenotypes has taken place (Templeton *et al.* 1992; Excoffier & Smouse 1994). On the other hand, principal coordinates analysis allows a quantification of the diversity responsible for the appearance of the aforementioned groups.

The four main groups do not match with the observed population subdivision except for Cullera and Torreblanca, the two most extreme populations. This situation is unexpected for several reasons. First, the Cullera and El Saler populations are geographically much closer to each other than the El Saler population is to the three Marjal del Moro populations, but all the individuals from El Saler are included in group B patterns. This is unlikely to have been originated recently by gene flow given the low seed dispersal capacity of this species. Furthermore, the presence of a large urban area, Valencia, between El Saler and Marjal del Moro, would further contribute to the reduction of gene flow among these populations, at least in the last 2000 years. Second, phenotypes from Marjal del Moro populations are dispersed in two separate groups, A and B, hence resulting in large intrapopulation divergence. These patterns do not conform to any general structure. Furthermore, there is no correlation among geographical and genetic distances among populations (Table 3). Given

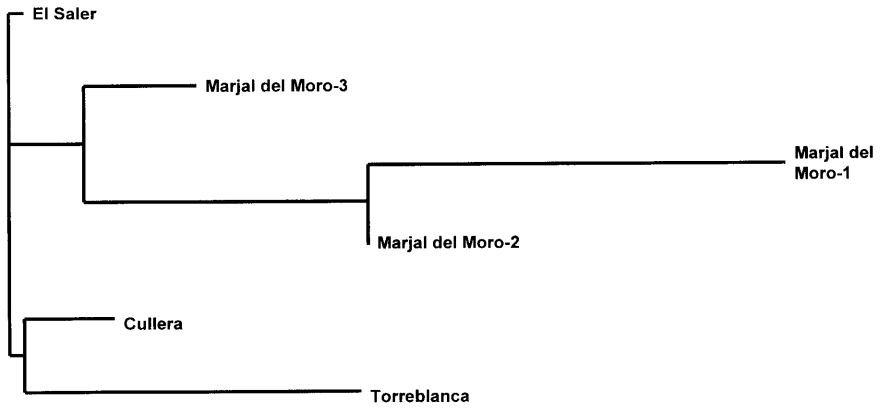

Fig. 6 Neighbour-joining trees for the six *Limonium dufourii* populations analysed in this study using Excoffier distance.

that there are no known forces currently acting on these populations that can explain these results, it seems plausible that historical processes in these populations have to be invoked.

The analysis of nucleon diversity within populations by means of the distribution of pairwise differences among molecular variants (Tajima 1983) has been used to infer historical demographic patterns (Slatkin & Hudson 1991; Rogers & Harpending 1992; Marjoram & Donnelly 1994). Although the most frequent application has been to nucleotide sequences, some studies have used this theory with other molecular markers, such as restriction sites (Lavery *et al.* 1996). Given the lack of recombination in the nuclear genome of apomictic species, which is the case for *L. dufourii*, and the large number of potential markers that can be studied by means of RAPDs, it is possible to consider the whole genome of that species as a single, nonrecombining gene with infinite alleles. We think that using this approach for the present study can shed some light on the historical processes that have occurred in *L. dufourii* populations.

Figure 7 shows the observed distribution of pairwise differences for individuals from *L. dufourii* populations (plots a–e), and for individuals from the three Marjal del Moro populations pooled together (plots f and g). El Saler population has not been considered due to its small

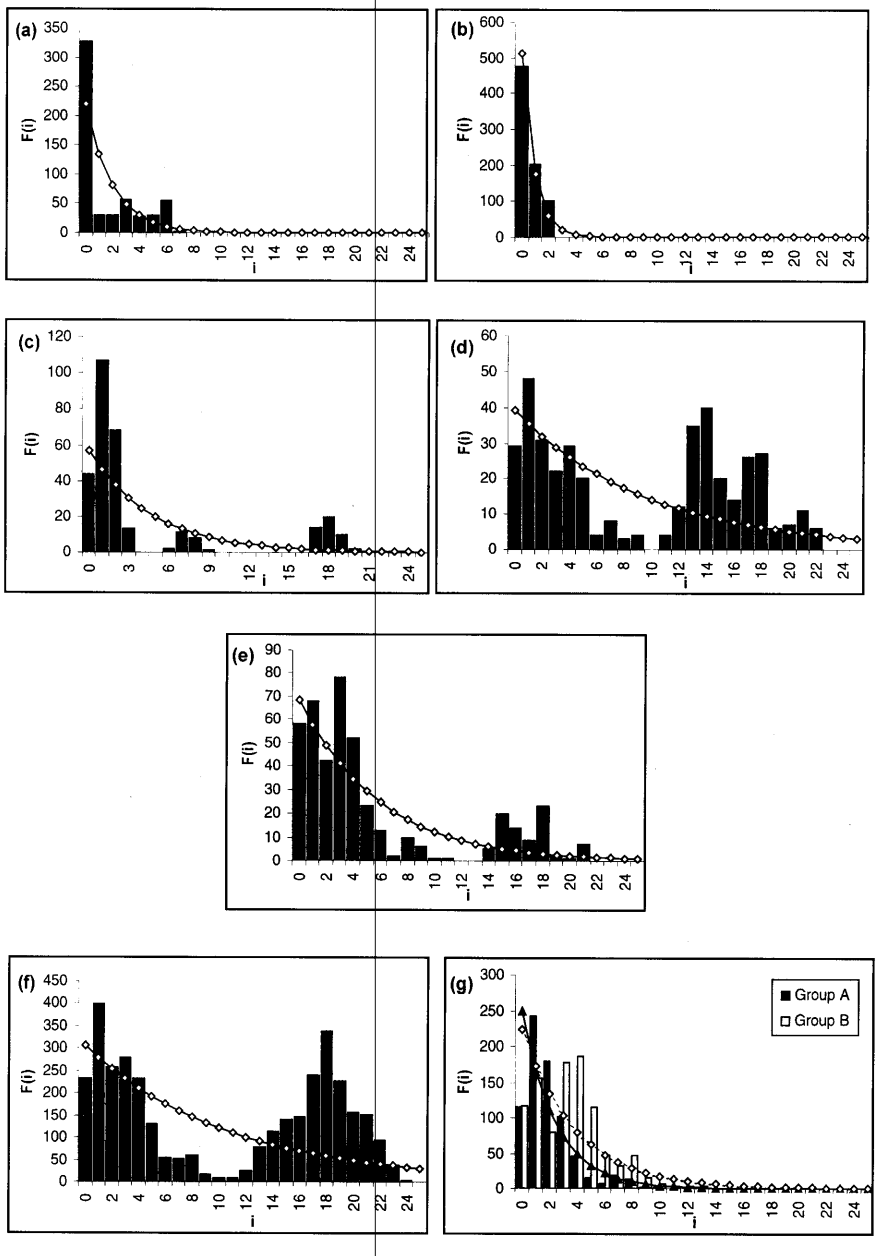

Fig. 7 Pairwise difference distribution for comparisons among individuals from *Limonium dufourii* populations. (a) Cullera, (b) Torreblanca, (c) Marjal del Moro-1, (d) Marjal del Moro-2, (e) Marjal del Moro-3, (f) individuals from the three Marjal del Moro populations pooled into one single population; (g) same as (f) when groups A and B are considered separately. The continuous line represents the expected distribution of pairwise differences under constant population size (Watterson 1975).

sample size. The expected distribution of pairwise differences under a constant population size (Watterson 1975) is also shown. Except for Torreblanca, all populations exhibit nonequilibrium distributions. However, there is also a clear difference between the Cullera and Marjal del Moro populations. The Cullera distribution shows a larger number of comparisons with zero differences than expected, while in the three Marjal del Moro populations there is a larger number of cases with large number of differences than expected. This is obviously due to the presence of patterns belonging to groups A and B in each Marjal del Moro population, thus generating a bimodal distribution in each of them as well as, and perhaps even more clearly, in the plot of the pooled populations (Fig. 7, plot f).

These signatures in the distribution of pairwise differences are indicative of different histories in Cullera and Marjal del Moro populations. The Cullera graph matches the distribution expected after a recent and sustained decline in population size (Rogers & Harpending 1992; fig. 10). This is a likely explanation in this case as a reduction in the size of this population due to expanding urban development is well documented (Crespo & Laguna 1993). The pattern observed in the Marjal del Moro populations requires a different kind of explanation. It is evident that the bimodality in these plots is due to the existence of two well-defined groups of patterns. Slatkin & Hudson (1991) argue that this situation is likely to arise in natural populations where the first split in a gene genealogy will give rise to two equally represented groups of variants. This is a plausible explanation given the hybrid nature of this species, but other alternatives should not be discarded. For instance, there is some evidence against this explanation in plot (g) from Fig. 7. This graph shows the observed and expected distributions of pairwise differences for all Marjal del Moro individuals when groups A and B are considered separately. If the above mentioned explanation was true, it would be expected that both plots overlap. The average number of pairwise differences between individuals with patterns from group A is 2.0 and for those from group B is 3.4, with variances 3.21 and 6.10, respectively. In consequence, it seems evident that group A is younger than group B, assuming that mutation rate and population size have been similar for both groups. It remains to be explained how these two groups arose in Marjal del Moro populations. Several processes, such as drift or recurrent hybridization of the progenitor taxa, could lead to the observed situation, but current evidence cannot provide support for any of the alternatives. Further analysis with other molecular markers that may shed some light into this problem is already under way.

We propose that the present genetic diversity pattern can be explained as follows. The three Marjal del Moro populations were united in the past and group B patterns arose first. The individuals dispersed, colonizing suitable habitats north- and southwards. The currently remaining borderline populations, Torreblanca and Cullera, present patterns related to those of group B. A second group of patterns appeared in the Marjal del Moro population, either derived from the existing one or from another hybridization event. In any case, this happened after the start of divergence of group B, which explains the younger age of group A and their absence from other populations. Subsequent reduction of suitable habitats caused decline in all populations, with the possible exception of Torreblanca. Reduction in population size led to the subdivision of the Marjal del Moro population into three subpopulations, and next genetic drift occurred resulting in the observed structuring of genetic diversity. This would explain the presence of individuals with patterns from both groups A and B in the three current populations. Reduction of the El Saler population has led to its almost complete extinction, while in Cullera, only a few individuals remain. The pairwise difference distribution shows this reduction of population size in Cullera, but not so in the three Marjal del Moro populations due to the persistence of patterns from two groups.

The conservation of Limonium dufourii

The original aim of this work was to provide some insight into the population biology from the genetic analysis of *Limonium dufourii* to possibly guide conservation measures. There has been much debate about the uses and virtues of population genetics in conservation (see for instance Avise & Hamrick 1996; and references therein). The discussion is especially pertinent in the case of nonsexually reproducing organisms. These organisms will eventually be composed of a series of more or less divergent clones, whose fate will be determined either by random forces or by the action of natural selection, but without any possibility of increasing their survival potential by means of establishing new genetic combinations through recombination and segregation. Hence, conservation of genes can only be achieved through conservation of whole genotypes, and this is usually unaffordable. The logic to this reasoning is that preservation of genes and their combinations will keep adaptability potential for future generations, hence decreasing the risk of extinction. However, this vision has been widely criticized (Lande 1988; Milligan *et al.* 1994; Lynch 1996) and we agree that it is possibly not the best way of using population genetics in conservation.

Several results from this study can be used in devising conservation measures for this species. Given the high level of population subdivision, transplantation of individuals or seeds from one population to another should probably be avoided, except in the case of El Saler population, for which the extremely small population size and close phylogenetic proximity to Marjal del Moro

populations would make it advisable to reintroduce seeds from group B Marjal del Moro individuals. However, this rule of thumb of avoiding artificial gene flow with high population structure is based on the assumption that genotypes are adapted to local environments. Ewens–Watterson tests of selective neutrality on the frequency distribution of RAPD patterns (data not shown) failed to reject the null hypothesis in five of the six populations studied, thus indicating that the current RAPD patterns frequency distribution does not seem to be due to adaptation to local conditions. It remains to be evaluated whether this is the case for other loci, which are or not selectively neutral as it is assumed for RAPDs, in order to sustain this rule. There is a risk in using neutral genetic markers for making inferences about adaptive processes, unless selection is still acting and/or there is a very close linkage between the selected locus and the neutral marker. In any case if asexual taxa do represent general-purpose genotypes (Bierzychudek 1989) then variation in neutral markers is not an indication of any adaptive process but these markers become very useful for the study of the evolutionary history of the corresponding populations and also for the monitoring of different conservation methods.

Not all *L. dufourii* populations currently hold the same amount of variability (Table 4). When this is an issue for choosing what natural populations to preserve, the analysis performed in this case is especially relevant. Populations can be ordered in terms of 'phylogenetic variability', as both frequencies and ancestrality relationships are used in evaluating within population diversity by means of the analysis of molecular variance. These results should not be taken as an absolute guidance for preserving some populations and disregarding others. Instead, they provide one more piece of information for taking a decision, but other considerations should be taken into account. For instance, the Cullera and Torreblanca populations are well differentiated from the rest and, although they do not rate as high in the variability scale as the Marjal del Moro populations, they should also be preserved due to their distinctiveness. So, Cullera is the only population whose individuals live on rocky soil, rather than in marshes as the rest. What the analysis is really useful for is in helping to decide, in this case, on which Marjal del Moro population should conservation measures be implemented first, or, alternatively, what sampling strategy should be used to setup an *ex situ* germplasm collection to complement other *in situ* preservation measures.

Acknowledgements

We wish to thank Drs E. Laguna and J.A. Roselló for their help on sample collection and valuable discussions on the biology of Limonium species. We wish to thank J. Felsentein for PHYLIP, L. Excoffier for WINAMOVA and MINSPNET, G.F. Weiller and A.J. Gibbs for DIPLOMO, and J. Armstrong *et al.* for RAPDISTANCE. Drs E. Barrio, R. van Ham, and A. Moya provided valuable comments on previous versions of the manuscript. Ministerio de Educación y Ciencia, DGICYT PB93–0350, and Conselleria de Medi Ambient, Generalitat Valenciana, have supported this work. CP has been recipient of a predoctoral fellowship from Conselleria de Cultura, Educació i Ciència, Generalitat Valenciana. Computer support was provided by Servei de Bioinformàtica, Universitat de València.

References

Al-Janabi SM, Honeycutt RJ, McClelland M, Sobral BWS (1993) A genetic linkage map of *Saccharum spontaneum* L. SES 208. *Genetics*, **134**, 1249–1260.

Armstrong J, Gibbs A, Peakall R, Weiller G (1996) RAPDistance Programs: Version 1.04 for the Analysis of Patterns of RAPD Fragments. Australian National University, Canberra.

Avise JC (1994) *Molecular Markers, Natural History and Evolution*. Chapman & Hall, Inc., NY.

Avise JC, Hamrick JL (eds) (1996) *Conservation Genetics: Case Histories from Nature*. Chapman & Hall, Inc., NY.

Baker HG (1966) The evolution, functioning and breakdown of heteromorphic incompatibility systems. I. The Plumbaginaceae. *Evolution*, **20**, 349–368.

Bartlett MS (1937) Some examples of statistical methods of research on agriculture and applied biology. *Journal of the Royal Statistical Society, Supplement* **4**, 137–170.

Benito C, Figueiras AM, Zaragoza C, Gallego FJ, de la Peña A (1993) Rapid identification of Triticeae genotypes from single seeds using the polymerase chain reaction. *Plant Molecular Biology*, **21**, 181–183.

Bierzychudek P (1989) Environmental sensitivity of sexual and apomictic *Antennaria:* do apomictics have general-purpose genotypes? *Evolution*, **43**, 1456–1466.

Boissier JP (1848) *The Candolle, A. P. Prodromus Systematics Naturalis Regni Vegetabilis, xii*. Paris.

Cenis JL, Perez P, Fereres A (1993) Identification of aphid (Homoptera: Aphididae) species and clones by random amplified polymorphic DNA. *Annals of the Entomological Society of America*, **86**, 545–550.

Clark AG, Lanigan CMS (1993) Prospects for estimating nucleotide divergence with RAPDs. *Molecular Biology and Evolution*, **10**, 1096–1111.

Crespo MB, Laguna E (1993) Nuevas localidades de *Limonium dufourii* (Girard) O. Kuntze (Plumbaginaceae). *Anales Jardín Botánico Madrid*, **51**, 154–155.

Dawson IK, Chalmers KJ, Waugh R, Powell W (1993) Detection and analysis of genetic variation in *Hordeum spontaneum* populations from Israel using RAPD markers. *Molecular Ecology*, **2**, 151–159.

Doyle JJ (1991) DNA protocols for plants. In: *Molecular Techniques in Taxonomy* (eds Hewitt GM, Johnston AWB, Young JPW), pp. 101–115. Springer-Verlag, Berlin.

Dunn G, Everitt BSE (1982) *An Introduction to Mathematical Taxonomy*. Cambridge University Press, Cambridge.

Ellstrand NC, Elam DR (1993) Population genetic consequences of small population size. Implications for plant conservation. *Annual Review of Ecology and Systematics*, **24**, 217–242.

Erben M (1979) Karyotype differentiation and its consequences in Mediterranean *Limonium*. *Webbin*, **34**, 409–417.

Excoffier L, Smouse PE (1994) Using allele frequencies and geographic subdivision to reconstruct gene trees within a species: molecular variance parsimony. *Genetics*, **136**, 343–359.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

Faith DP, Cranston PS (1991) Could a cladogram this short have arisen by chance alone? On permutation tests for cladistic structure. *Cladistics*, **7**, 1–28.

Felsenstein (1993) PHYLIP: Phylogenetic Inference Package, version 3.5. Department of Genetics, University of Washington, Seattle.

Gibbs HL, Prior KA, Weatherhead PJ (1994) Genetic analysis of populations of threatened snake species using RAPD markers. *Molecular Ecology*, **3**, 329–337.

Gómez-Campo C (1987) *Libro Rojo de especies vegetales amenazadas de España peninsular y Baleares*, pp. 370–371. Icona.

Hadrys H, Balick M, Schierwater B (1992) Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Molecular Ecology*, **1**, 55–63.

Harper JL (1985) Modules, branches, and the capture of resources. In: *Population Biology and Evolution of Clonal Organisms* (eds Jackson JBC, Buss LW, Cook RE), pp. 1–33. Yale University Press, New Haven, CT.

Holsinger KE, Gottlieb LD (1991) Conservation of rare and endangered plants: principles and prospects. In: *Genetics and Conservation of Rare Plants* (eds Falk DA, Holsinger KE), pp. 195–208. Oxford University Press, NY.

Huff KR, Peakall R, Smouse PE (1993) RAPD variation within and among natural populations of outcrossing buffalograss (*Buchloë dactyloides* (Nutt.) Engelm. *Theoretical and Applied Genetics*, **86**, 927–934.

Kresovich S, Williams JGK, McFerson JR, Routman EJ, Schaal BA (1992) Characterization of genetic identities and relationships of *Brassica oleraceae* L. via a random amplified polymorphic DNA assay. *Theoretical and Applied Genetics*, **85**, 190–196.

Laguna E, Aguilella A, Carretero JL, Crespo MB, Figuerola R, Gonzalo M (1994) *Libro de la Flora Vascular Rara, Endémica o Amenazada de la Comunidad Valenciana*. Generalitat Valenciana, Conselleria de Medi Ambient, Valencia.

Lande R (1988) Genetics and demography in biological conservation. *Science*, **241**, 1455–1460.

Lavery S, Moritz C, Fielder DR (1996) Genetic patterns suggest exponential growth in a declining species. *Molecular Biology and Evolution*, **13**, 1106–1113.

Lynch M (1996) A quantitative-genetic perspective on conservation issues. In: *Conservation Genetics: Case Histories from Nature* (eds Avise JC, Hamrick JL). Chapman & Hall, Inc., NY.

Lynch M, Milligan BG (1994) Analysis of population genetic structure with RAPD markers. *Molecular Ecology*, **3**, 91–99.

Marjoram P, Donnelly P (1994) Pairwise comparisons of mitochondrial DNA sequences in subdivided populations and implications for early human evolution. *Genetics*, **136**, 673–683.

Martínez-Torres D, Carrió R, Latorre A, Simon JC, Hermoso A, Moya A (1997) Assesing the nucleotide diversity of three aphid species by RAPD. *Journal of Evolutionary Biology*, in press.

Milligan BG, Leebens-Mack J, Strand AE (1994) Conservation genetics: beyond the maintenance of marker diversity. *Molecular Ecology*, **3**, 423–435.

Nusser JA, Goto RM, Ledig DB, Fleischer RC, Miller MM (1996) RAPD analysis reveals low genetic variability in the endangered light-footed clapper rail. *Molecular Ecology*, **5**, 463–472.

Orozco-Castillo C, Chalmers KJ, Waugh R, Powell W (1994) Detection of genetic diversity and selective gene introgression in coffee using RAPD markers. *Theoretical and Applied Genetics*, **87**, 934–940.

Palmer JD (1986) Isolation and structural analysis of chloroplast DNA. *Methods in Enzymology*, **118**, 167–186.

Penner GA, Bush A, Wise R, et al. (1993) Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. *PCR Methods and Applications*, **2**, 341–345.

Riedy MF, Hamilton WJ, Aquadro CF (1992) Excess of nonparental bands in offspring from known primate pedigrees assayed using RAPD PCR. *Nucleic Acids Research*, **20**, 918.

Rieseberg LH, Choi H, Chan R, Spore C (1993) Genomic map of a diploid hybrid species. *Heredity*, **70**, 285–293.

Rogers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution*, **9**, 552–569.

Rohlf FJ (1993) NTSYS: Numerical Taxonomy and Multivariate Analysis System, version 1.8. Exeter Software. Setauket, NY.

Rossetto M, Weaver PK, Dixon KW (1995) Use of RAPD analysis in devising conservation strategies for rare and endangered *Grevillea scapigera* (Proteaceae). *Molecular Ecology*, **4**, 321–329.

Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406–425.

Schemske DW, Husband BC, Ruckelshaus MH, Goodwillie C, Parker IM, Bishop JG (1994) Evaluating approaches to the conservation of rare and endangered plants. *Ecology*, **75**, 584–606.

Schonewald-Cox CM, Chambers SM, MacBryde B, Thomas L (eds) (1983) *Genetic and Conservation: A Reference for Managing Wild Animal and Plant Populations*. Benjamin-Cummings, Menlo Park, CA.

Slatkin M, Hudson RR (1991) Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics*, **129**, 555–562.

Sobral BWS, Honeycutt RJ (1993) High output genetic mapping of polyploids using PCR-generated markers. *Theoretical and Applied Genetics*, **86**, 105–112.

Sokal RR, Rohlf FJ (1995) *Biometry: the Principles and Practice of Statistics in Biological Research*. W. H. Freeman and Company, NY.

Stewart CN Jr, Excoffier L (1996) Assessing population genetic structure and variability with RAPD data: Application to *Vaccinium macrocarpon* (American Cranberry). *Journal of Evolutionary Biology*, **9**, 153–171.

Stewart CN Jr, Porter DM (1995) RAPD profiling in biological conservation: an application to estimating clonal variation in rare and endangered *Iliamma* in Virginia. *Biological Conservation*, **74**, 135–142.

Swofford DL (1993) PAUP: Phylogenetic Analysis Using Parsimony, version 3.1. Laboratory of Molecular Systematics, Smithsonian Institution.

Tajima F (1983) Evolutionary relationship of DNA sequences in finite populations. *Genetics*, **105**, 437–460.

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram Estimation. *Genetics*, **132**, 619–633.

Templeton AR, Georgiadis NJ (1996) A landscape approach to conservation genetics: Conserving evolutionary processes in the African bovidae. In: *Conservation Genetics: Case Histories from*

Nature (eds Avise JC, Hamrick JL). Chapman & Hall, Inc, NY.
Templeton AR, Routman E, Phillips C (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics*, **140**, 767–782.
Transue DK, Fairbanks DJ, Robinson LR, Andersen WR (1994) Species identification by RAPD analysis of grain amaranth genetic resources. *Crop Science*, **34**, 1385–1389.
Travis SE, Maschinski J, Keim P (1996) An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax*, a critically endangered plant, using AFLP markers. *Molecular Ecology*, **5**, 735–745.
Watterson GA (1975) On the number of segregating sites in genetical models without recombination. *Theoretical Population Biology*, **7**, 256–276.
Weiller GF, Gibbs AJ (1993) DIPLOMO: Distance Plot Monitor version 1.0. Australian National University, Canberra.
Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research*, **18**, 303–306.
Whitty PW, Powell W, Sprent JI (1994) Molecular separation of genera in *Cassiinae* (Leguminosae), and analysis of variation in the nodulating species of *Chamaecrista*. *Molecular Ecology*, **3**, 507–515.
Williams JGK, Hanafey MK, Rafalski JA, Tingey SV (1993) Genetic analysis using random amplified polymorphic DNA markers. *Methods in Enzymology*, **218**, 704–740.
Williams JGK, Kubelik AR, Livak KJ, Rafalsky JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **18**, 6531–6535.

The work described in this report is part of the research developed by C. Palacios to fulfil the requirements to obtain a PhD in Molecular and Evolutionary Genetics at the University of Valencia, under the supervision of Dr González-Candelas. The main interest of this group is the application of population genetics and evolutionary biology to the conservation of endangered plant species through the usage of different molecular markers. We currently work on several endemic species from the genus *Limonium*. Part of this work is performed in close collaboration with the Plant Biology Department and the Botanical Garden from the University of Valencia.

Appendix

We have followed Stewart & Excoffier (1996) approach for deriving the corrected distance estimates for RAPD markers when triploid individuals are being compared. We assume that only two alleles (presence = +; absence = –) are present in the s-th site with frequencies p_A and q_A in population A, respectively. For any individual at each site, there are four possible genotypes (+/+/+, +/+/–, +/ –/–, and –/–/–), and we further assume that the three genotypes that present at least one + allele are not distinguishable, and, in consequence, any individual can show only two phenotypes (+ or –) for that site. There are four different possible pairwise comparisons among individuals (+/+, +/–, –/+ and –/–), each involving nine (3 × 3) chromosomal comparisons. Assuming Hardy–Weinberg equilibrium for genotype frequencies in populations A and B, the conditional expectations for the interindividual distances are:

$$E(\Delta^2_{sjk} \mid j=[+], k=[+]) = \frac{1}{(p^3_A+3p^2_A+3p_A)(p^3_B+3p^2_B+3p_B)}$$

$$E(\Delta^2_{sjk} \mid j=[+], k=[-]) = \frac{9p_Aq^3_B(p^2_A+2p_Aq_A+q^2_A)}{q^3_B(p^3_A+3p^2_Aq_A+3p_Aq^2_A)} = \frac{9}{1+q_A}$$

$$E(\Delta^2_{sjk} \mid j=[-], k=[+]) = \frac{9p_Bq^3_A(p^2_B+2p_Bq_B+q^2_B)}{q^3_A(p^3_B+3p^2_Bq_B+3p_Bq^2_B)} = \frac{9}{1+q_B}$$

$$E(\Delta^2_{sjk} \mid j=[-], k=[-]) = 0$$

The corresponding values for intraindividual comparisons are

$$E(\Delta^2_{sjj} \mid j=[+]) = \frac{36q^2_A(p^2_A+q^2_A)}{p^4_A+p^2_Aq^2_A+q^4_A}$$

$$E(\Delta^2_{sjj} \mid j=[-]) = 0$$

By using these expectations, and summing up over m RAPD sites, we can obtain the expected number of RAPD differences among two individuals

$$E(\Delta^2_{jk}) = [+]) = \sum_{s=1}^{m} E(\Delta^2_{sjk})$$

The sum of squared deviations needed for the AMOVA can be obtained for all possible pairs of populations by means of

$$SSD(T) = \frac{1}{9N} \sum_{j=1}^{3N} \sum_{k=1}^{3N} E(\Delta^2_{jk}).$$