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A population genetic study of the endangered plant species *Limonium dufourii* (*Plumbaginaceae*) based on amplified fragment length polymorphism (AFLP)

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Abstract

***Limonium dufourii* (*Plumbaginaceae*) is a triploid species with obligate apomictic reproduction and is endemic to the East Mediterranean coast of Spain, where it is present in only six populations, most of which have a very low number of individuals. Genetic variation and population structure in this species was studied using amplified fragment length polymorphisms (AFLPs) as markers, using the same individuals as in a previous study with random amplified polymorphic DNA (RAPD). Three different primers provided 252 bands of which 51 were polymorphic among the 152 individuals analysed. Those polymorphic bands were able to define 65 different phenotypes, of which all but two were present in only one population. The comparative analyses of data from AFLPs with those from RAPDs show a high degree of concordance. Additionally, and given the nature of these markers, we propose the estimation of nucleotide divergences from AFLP patterns. Relationships among the different AFLP patterns and the estimates of population genetic parameters obtained with this evolutionary distance are in good agreement with previous results. These analyses show that substantial genetic variability and differentiation exist within and among populations of *L. dufourii*. Their higher reproducibility and the possibility of obtaining estimates of nucleotide divergence make AFLPs a much better DNA fingerprinting technique.**

Keywords: AFLPs, conservation genetics, endangered species, genetic variation, *Limonium*, population structure

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Introduction

Genetic variability studies at the intra- and interspecific levels and population genetic analyses depend on several factors, among which the availability of markers with adequate variability is one of the most important and often difficult to achieve. The advent of polymerase chain reaction (PCR) techniques has largely removed this obstacle. Hypervariable microsatellite markers that use simple sequence repeat (SSR) polymorphisms (Litt & Luty 1989), minisatellite markers arising from repetitive sequences (Jeffreys *et al*. 1985), and PCR-based DNA fingerprinting or multiple arbitrary amplicon profiling (MAAP) techniques (Karp & Edwards 1997; Caetano-Anollés & Gresshoff 1994) are tools recently developed that promise to replace isozymes and restriction fragment length polymorphisms (RFLPs) in the study of genetic variation, because they provide effective methods for the simultaneous characterization of a large number of polymorphic markers. Particularly in plants, they have been used in many different applications, such as the genetic characterization of agronomic traits in plant breeding programmes, the identification of species, subspecies, hybrids, and clones, and the distinction between plant cultivars or individuals with important implications in phylogenetic analyses, conservation studies, parentage testing, and cultivar certification (Caetano-Anollés & Gresshoff 1994; Heun *et al*. 1997). Among the most commonly used PCR-based fingerprinting techniques are random amplified polymorphic DNAs (RAPDs; Williams *et al*. 1990), arbitrarily primed PCR (AP–PCR; Welsh &

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McClelland 1990), DNA amplification fingerprinting (DAF; Caetano-Anollés *et al*. 1991), and amplified fragment length polymorphisms (AFLPs; Vos *et al*. 1995). These techniques have some advantages over other methods for the analysis of DNA polymorphisms. They do not require prior sequence knowledge, cloned or characterized probes, or considerable experimental manipulation, and many loci of moderate polymorphism can be analysed simultaneously, constituting an alternative to the analysis of single, highly polymorphic loci, such as microsatellites. The dominant nature of these markers, their sensitivity to reaction conditions (which has made questionable the reliability of some of these techniques [e.g. Penner *et al*. 1993]), and the rigorous validation necessary when nucleotide divergence is to be estimated (Clark & Lanigan 1993) are among their drawbacks.

In this study, we used AFLP markers to characterize genetic diversity within and among all extant populations of the endangered plant species *Limonium dufourii* and to compare these results with those from a previous RAPD analysis (Palacios & González-Candelas 1997) based on the same individuals.

L. dufourii (Girard) O. Kuntze is an endangered species endemic to the east Mediterranean coast of the Iberian Peninsula. It is a perennial, rosulate, hemicryptophyte, densely hairy species, with racemose inflorescences, and it has an obligate apomictic reproductive system, corroborated by its triploid chromosome number ($2n = 27$; J. A. Rosselló, personal communication) and incompatible pollen–stigma combination (Baker 1966). Historically, this species was more widely distributed along the coast and salt marshes of Valencia and Castellón provinces in Spain (Crespo & Laguna 1993), but today only six natural populations remain (Fig. 1; Laguna *et al*. 1994), most of them with extremely few individuals, indicating problems for the species long-term survival (Laguna & Escribá 1996).

The results obtained in the RAPD study (Palacios & González-Candelas 1997) permitted the design of strategies for the preservation of the natural populations of *L. dufourii*, through the identification and quantification of their genetic variability. This has enabled us to prioritize for the preservation of some populations and to identify the most suitable source populations for future re-introduction. In addition, the discovery of the existence of two divergent groups of molecular phenotypes coexisting in the same populations has caused the possible single hybrid origin of this species to be questioned.

We believe that future conservation measures for this and other endangered species will benefit from the development of more informative and reliable markers such as AFLPs. However, before these relatively new markers are widely accepted by the scientific community, it is necessary to verify whether they provide the same kind of information and lead to the same conclusions as previously used markers. Furthermore, we have introduced estimates of evolutionary divergence among variants and compared the results obtained with those achieved with conventional approaches based only on the similarity of the different phenotypes.

Materials and methods

Population sampling and DNA extraction

The number of individuals sampled and census estimates for each *Limonium dufourii* population are shown in Fig. 1. DNA was extracted following a modified CTAB protocol described in Doyle (1991). Further details of both sample collection and DNA extraction are summarized in Palacios & Gonzáles-Candelas (1997). The same DNA extracts were employed as in the previous RAPD survey.

AFLP procedure

The AFLP technique (Vos *et al*. 1995) consists of two consecutive PCR reactions (preselective and selective amplifications, denoted PSA and SA, respectively), which amplify the DNA fragments generated by double-restriction digestion and subsequent ligation of specific oligonucleotide adapters. Only a selected group of fragments are amplified because selective nucleotides are added to the primers used (Table 1). AFLP reactions were performed using the Perkin-Elmer/Applied Biosystem (PE/ABI) AFLP™ plant mapping kit for small genomes following the manufacturer's instructions. They are based on the method of Vos *et al*. (1995) but use nonradioactive fluorescent dyes to label the primers. Modifications to the original protocol were as follows: a modified core mix was employed for the PSA and SA reactions which consisted of 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1× PCR Buffer II (PE), and 0.2 U *AmpliTaq* (Perkin-Elmer). The number of cycles in the last step of the SA reaction was increased for the JOE and TAMRA fluorescent dyes (Table 1). Eventually, all SA reactions from each individual were mixed for loading the three dye–primer combinations in one lane in the gel. The quantity of SA added to this reaction was dependent on the dye–primer (Table 1).

Primer selection and reproducibility experiments

Two preliminary studies were necessary to carry out the AFLP assays. One was performed to determine empirically which primer combinations from the kit were most appropriate (Janssen *et al*. 1996). Primers were selected (Table 1) taking into account the total number of fragments amplified without biasing in favour of the most polymorphic primers. This is essential for making com-

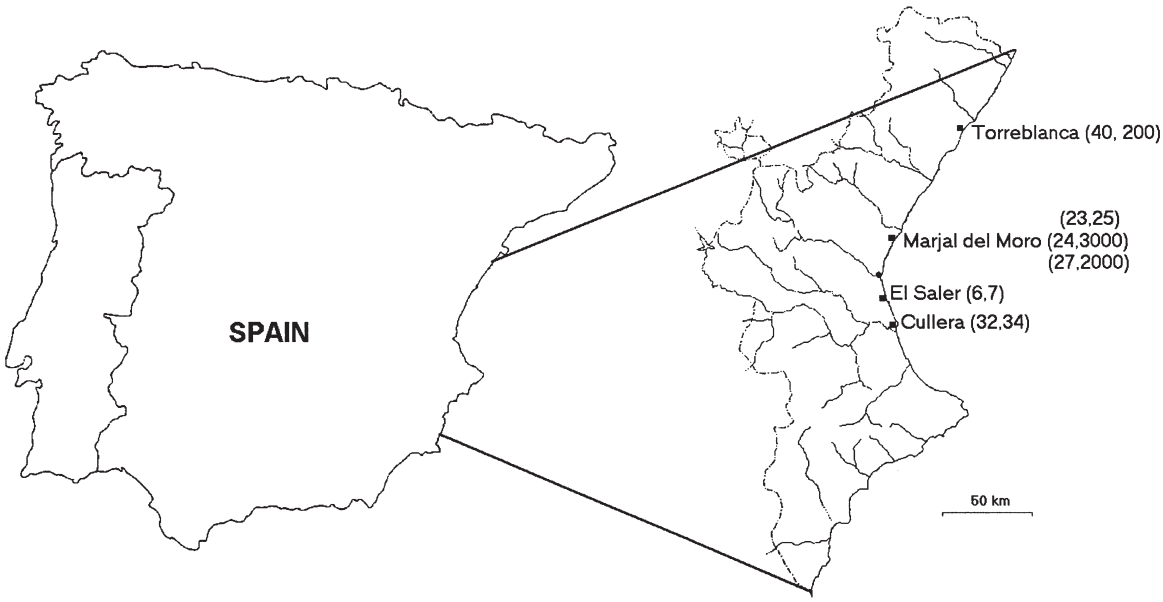

Fig. 1 Geographic location of *Limonium dufourii* populations. There are three populations, a few hundred metres apart, in Marjal del Moro. Sample and census sizes for each population are shown in parentheses.

parisons among different markers or with other species (Clark & Lanigan 1993).

Second, a multifactorial experiment was carried out to determine the reproducibility of the technique. It is possible that random differences among each of the three steps in the AFLP reaction (restriction–ligation, PSA, and SA) and/or the influence of changes in DNA concentration among individuals could produce artefactual differences in band patterns. Three different DNA quantities (50, 100, and 350 ng) were tested and each step of the AFLP reaction was repeated three times. A total of 14 reactions was performed for each DNA concentration.

Data collection and scoring procedure

AFLP reactions with each primer combination and gel multiplexing were performed as described for each *L. dufourii* individual. Next, 1.5 μL of the multiplexing reaction was mixed with 0.3 μL of GeneScan 500 ROX internal size standard (PE/ABI) and 2.0 μL of loading buffer (3 formamide: 1 loading buffer), heated at 95 °C for 5 min, placed on ice, and 3 μL was immediately loaded on 4% polyacrylamide gels. Electrophoresis was performed at constant voltage (3000 V) for 3 h at 51 °C using an automated DNA sequencer (Model 377, PE/ABI) equipped with GeneScan Analysis software (version 2.1, PE/ABI).

The presence/absence of each fragment within each individual, pooled over all primer combinations, was scored as a data matrix, assembled using GENOTYPER (version 1.1 PE/ABI). Further visual comparison of electrophoretograms using GENESCAN was performed to correct for any possible misinterpretations from the automated procedures. To assist the final scoring of data, some individuals from each population were always included in each gel.

Data analysis

AFLP phenotypes (patterns) from the original data matrix were extracted using the RAPDistance package

Table 1 Primers used, fluorescent dye label, number of cycles on the selective amplification reaction (SA), amount used on the multiplexing reaction (MR), and number of total and polymorphic bands obtained in the analysis of *L. dufourii* with AFLPs

AFLP primers	Colour dye	Cycles on SA	μL on MR	Total no. of bands	Polymorphic bands
*Mse*I-CTG EcoRI-AC	FAM	23	5	75	22
*Mse*I-CAA EcoRI-AG	JOE	25	10	84	16
*Mse*I-CTGEcoRI-AT	TAMRA	27	20	73	13

(Armstrong *et al*. 1996). The resulting matrix was used to ascertain the relationships among AFLP patterns following two approaches. First, pairwise comparisons among all AFLP patterns were assessed using Euclidean distance (Excoffier *et al*. 1992). In the second approach, we considered that AFLP data can be treated analogously to restriction-fragment presence/absence data (RFLP) because one single nucleotide substitution in the restriction site, or a mismatch in the selective nucleotides (that together form the actual restriction site), is sufficient to prevent the amplification of a particular fragment. This is equivalent to assuming, as in RFLP analyses, that the absence of a band is the result of a lack of amplification arising from one point mutation in either recognition site and not to a change in the size of the amplified product. Nei & Li's (1979) method for estimating nucleotide divergence (d; eqns 553 and 555 in (Nei 1987) between two phenotypes was employed. Nei & Li's approach gives a fairly accurate estimate of d when $d < 0.05$ (Nei 1987), because F (the expected proportion of polymorphic fragments) decreases when d increases because multiple hits are treated as a single substitution. Hence, this approximation will be valid only for closely related OTUs (operational taxonomic units), such as haplotypes within a species, and it will represent an estimate of the minimum distance among them. Other necessary assumptions for the analysis of DNA fingerprinting markers (Lynch & Milligan 1994; Stewart & Excoffier 1996) were applied for both approaches.

The pairwise distance matrices were analysed to determine phylogenetic relationships among AFLP patterns and the degree of differentiation among *L. dufourii* populations, following the same approach as for RAPD markers (Palacios & González-Candelas 1997). Unrooted trees were obtained from the previous distance matrices using the neighbour-joining procedure (Saitou & Nei 1987). An overall statistical assessment of these trees was performed using permutation test probability analysis (PTP; Faith & Cranston 1991), as more conventional approaches, such as bootstrapping, sometimes lack statistical power at the intraspecific level (Templeton *et al*. 1992). An alternative representation of the relationships among AFLP patterns was obtained with a minimum spanning network which represents all possible minimum length connections among them. We used the program MINSPNET (Excoffier & Smouse 1994) to perform such an analysis from the Euclidean distance matrix.

Pairwise population distances were calculated from the distance matrices using the expression $d'_{xy} = d_{xy} - (d_x + d_y/2)$, which corrects for intrapopulation diversity (Lynch & Crease 1990). A neighbour-joining tree was constructed using the resulting interpopulation distance matrices. The Euclidean matrix was also compared with linear geographical distances between populations by means of their correlation coefficient using the program DIPLOMO (Armstrong *et al*. 1996).

Two different analyses of molecular variance (AMOVA) were performed to study the partitioning of genetic variation within and among *L. dufourii* populations using the program WINAMOVA (available from L. Excoffier). First, we carried out a phenotypic AMOVA based on the Euclidean distances among phenotypes. This analysis considers each marker locus as a binary character, without further correction for dominance. Second, we performed a genotypic AMOVA as extended by Stewart & Excoffier (1996) for RAPD data and selfing species, taking into account estimates of the frequency of each allele both in the whole set of data as well as within each population. Because we were dealing with a triploid, apomictic species, additional assumptions and modifications to that method developed were necessary, as summarized in Palacios & González-Candelas (1997). Homogeneity of intrapopulation molecular variances (homoscedasticity) was tested using the HOMOVA procedure (Bartlett's test), also implemented in WINAMOVA. The AMOVA procedure provides an estimate Φ_{ST}, of population differentiation, which is equivalent to a F_{ST} statistic when the degree of relatedness among the genetic variants is considered. Additionally, and for comparative purposes, genetic diversity, average gene diversity per locus, and Wright's F_{ST} values (Wright 1951) without taking the relationships among markers into account, were obtained using program ARLEQUIN (available from L. Excoffier).

The nucleotide divergence matrix was also used for an analysis of molecular variance as described, but without considering any correction for dominance. Hence, we obtained an estimate of the degree of population structure Φ_{ST}, which can be compared with the nucleotide differentiation statistic among populations (N_{ST}) proposed by (Lynch & Crease 1990).

The distribution of pairwise differences, or mismatch distribution, between all pairs of molecular variants was computed in order to infer historical demographic patterns in these populations (Slatkin & Hudson 1991; Rogers & Harpending 1992; Majoram & Donnelly 1994; Lavery *et al*. 1996) and to compare them with those obtained with RAPD data. Additionally, a Monte-Carlo simulation was performed to obtain a null distribution for the raggedness parameter r (Harpending 1994), which characterizes the mismatch distribution, under the assumption of constant population size, thus allowing a test of this model in each population.

Finally, we investigated the correlation between RAPD and AFLP analyses of the same individuals, using the index of classification, I_C (Estoup *et al*. 1995). We considered the degree of monophyly of the individuals sharing the same RAPD pattern when compared to their corresponding AFLP pattern, i.e. we were interested in learn-

ing how well the classification obtained with one kind of molecular marker is maintained when a different marker is used on the same individuals. The index is defined as

$$I_C = \frac{d_T - d_G}{d_T - d_M},$$

where d_T, d_G, and d_M are the average distances between two OTUs (defined as the size of the smallest monophyletic clade encompassing both OTUs) taken from the total sample, from the group under study, and from a monophyletic group of the same size as the group being considered, respectively. This index takes values between 1 (perfect correspondence between two classifications) and – 0.5 (when a group includes only OTUs that are paraphyletic to all others).

Results

The only differences observed among reactions in the multifactorial reproducibility experiment were in the intensity of the bands, but they did not correlate with the quantity of DNA used. The immediate consequence was a discrepancy in the peaks detected by GENOTYPER between samples from the same individual. To minimize these errors, AFLP reactions below a certain intensity threshold were repeated. Subsequently, poor amplifications occurred systematically only with a few individuals; these were excluded from the analysis and they account for the different sample sizes of this and our previous RAPD study.

After pooling the data from the three AFLP primer combinations used for analysis (Table 1), 252 DNA fragments were scored, ranging from 75 to 500 bp, with an average of 219.4 fragments per individual and 84.7 bands per primer combination. In the six populations of *Limonium dufourii* there were 51 (20.2%) polymorphic markers that defined 65 AFLP phenotypes. Only two patterns (1 and 23) were present in more than one population; the rest were present in only one (see Appendix I). The large number of individuals with a unique pattern in the three Marjal del Moro populations is also noteworthy, in contrast with Cullera and Torreblanca. This is an indication of high diversity in the Marjal del Moro populations.

Two main clusters can be distinguished in the neighbour-joining dendrogram for the 65 AFLP patterns using the Euclidean distance (Fig. 2). Cluster I only includes individuals from Marjal del Moro populations (denoted group A for later discussion). The other, cluster II, is subdivided into two groups, one including individuals from Marjal del Moro and El Saler (denoted group B), the other exclusively with patterns from Torreblanca. Patterns from Cullera individuals, including pattern 1, which is also present in two other populations (see Appendix I), form a monophyletic group with the exception of phenotype 3. The PTP test gave a *Z*-value of 33.32 for the Euclidean

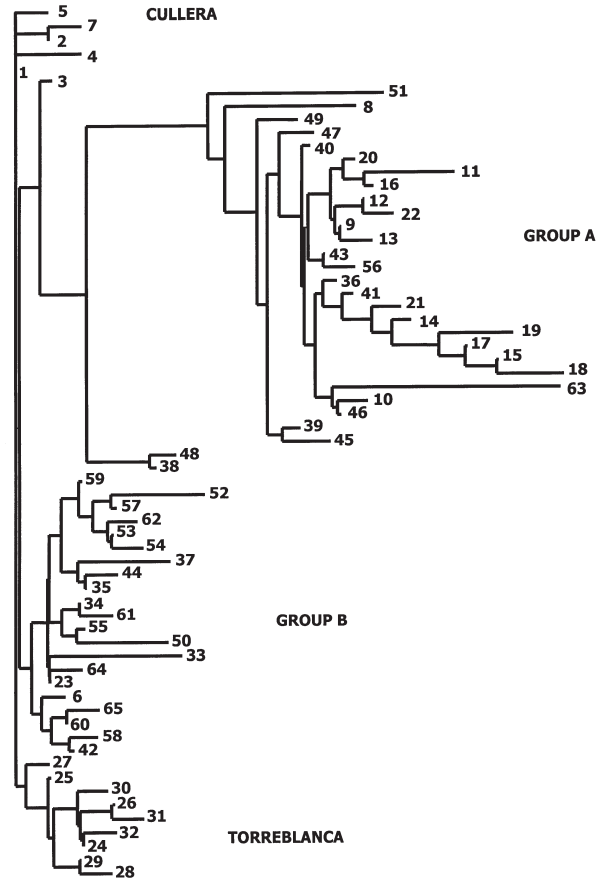

Fig. 2 Neighbour-joining tree derived from the Euclidean distance matrix among the AFLP patterns from *Limonium dufourii*.

tree, indicating an extremely low probability ($P < 0.001$) for this tree to have arisen by chance alone.

The nucleotide divergence estimated between pairs of AFLP patterns ranged from 0.0001 to 0.0024, with an average of 0.0009 substitutions/nucleotide. The unrooted neighbour-joining dendrogram (not shown) derived from the nucleotide divergence does not present major differences with the tree derived from the Euclidean distance (Fig. 2). The four main groups are consistent and encompass the same phenotypes. Only relationships within group A are slightly different.

The minimum spanning network derived from the Euclidean distance confirms the main relationships among phenotypes found in the cluster analysis (Fig. 3). Clusters I and II are clearly separate, with phenotypes 38 and 48 in an intermediate position. Patterns from cluster II form three separate groups that include, respectively, individuals from Torreblanca, from El Saler and Marjal del Moro, and those patterns, mainly from Cullera, that did not cluster monophyletically in the neighbour-joining trees.

The neighbour-joining tree obtained from the Euclidean interpopulation distance matrix is shown in Fig. 4, and it is

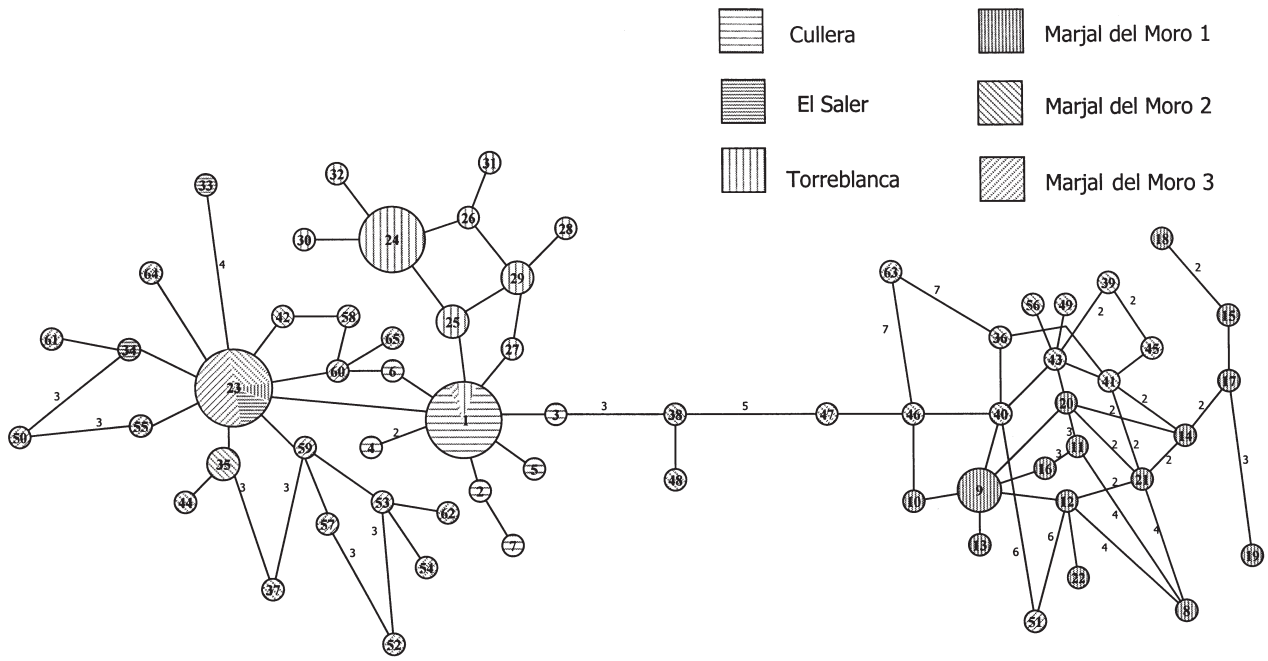

Fig. 3 Minimum spanning network obtained with MINSPNET showing the relationships among the 65 AFLP patterns found in *Limonium dufourii*. The area of each circle is an approximation to the frequency of that pattern in the species (see Appendix I). The numbers next to the segments connecting nodes represent the number of differences among them when they are larger than 1.

very similar to the one obtained from the nucleotide population divergence. Marjal del Moro populations form a monophyletic group, and Cullera and Torreblanca are also placed in the same cluster. The correlation between this distance matrix and the linear geographical distance between populations is not significantly different from zero ($r = -0.069$, d.f. = 13, $P > 0.5$). This is an indication that isolation by distance is not the process accounting for the distribution of genetic variation among populations.

Both phenotypic and genotypic AMOVA analyses reflect a significant population genetic structure (Table 2), with

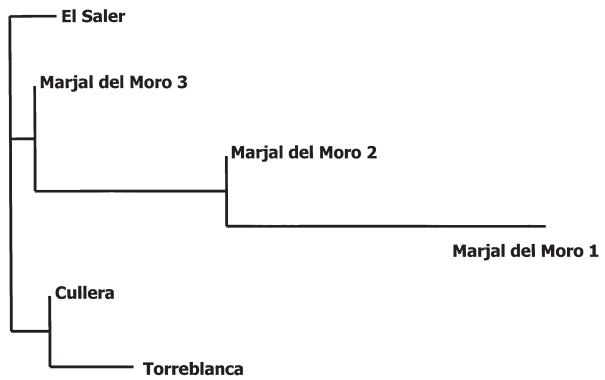

Fig. 4 Neighbour-joining tree of *Limonium dufourii* populations derived from AFLP analyses and using Euclidean interpopulation distance matrix.

Φ_{ST} values of 0.538 and 0.531, respectively, which means that *L. dufourii* populations are genetically differentiated, regardless of the actual selfing rate in the species (Palacios & González-Candelas 1997). All pairwise Φ_{ST} values between populations were also significant except for the Marjal del Moro-3 and El Saler comparison, possibly because they share the most frequent pattern (see Appendix I). When only the frequency of each pattern was considered, the resulting F_{ST} statistic for population differentiation had, as expected, a lower but still significant value (0.243, $P < 0.001$).

The intrapopulation variances derived from the AMOVA and genetic diversities are shown in Table 2. HOMOVA analyses showed significant differences in the amount of intrapopulation variation among populations as a whole, and in all but two pairwise comparisons (data not shown).

The AMOVA analysis performed with the nucleotide divergence matrix gave a Φ_{ST} value of 0.539 (Table 2). Pairwise genetic differentiation was significant for all except for the Marjal del Moro-3 and El Saler comparison, as in the previous analysis using the Euclidean distance. The alternative measure of population genetic structure, N_{ST}, had a value of 0.552, which is very similar to the one obtained with the AMOVA. As in previous analyses, Barlett's homoscedasticity index was highly significant ($P < 0.001$), indicating intrapopulation variance differences among populations. The levels of genetic variation were also generally consistent with previous analyses,

Population statistics	PE	GE	*d*	Genetic diversity
σ^2 Cullera	0.304	9.486	0.000032	0.440
σ^2 Marjal del Moro-1	2.507	39.058	0.000258	0.920
σ^2 Marjal del Moro-2	3.185	46.885	0.000331	0.932
σ^2 Marjal del Moro-3	2.220	51.207	0.000231	0.821
σ^2 Torreblanca	0.607	7.310	0.000061	0.667
σ^2 El Saler	1.095	25.198	0.000117	0.662
σ^2among	1.852***	31.995***	0.000194***	
σ^2within	1.593***	28.205***	0.000166***	
ϕ_{ST}	0.538***	0.531***	0.539***	
Bartlett's test	51.594***	47.735***	51.950***	
F_{ST}		0.243***		
N_{ST}		0.552***		

***$P < 0.001$.

Table 2 Results from the phenotypic and genotypic AMOVA analyses using the Euclidean distance (PE and GE, respectively), and for the same analysis using the nucleotide divergence matrix (*d*) are summarized in the three first columns. The first six rows are the within-population variance estimates for the six *Limonium dufourii* populations. These can be compared with the within-population genetic diversity estimates in the last column. Next rows are the ϕ_{ST} values from each AMOVA analyses, which can be compared with the F_{ST} and N_{ST} statistics in the bottom rows. Results from the HOMOVA analysis are shown in the 'Bartlett's test' row. Nonparametric tests of significance for variance components and homogeneity are based on 1000 random permutations

and no pairwise comparisons showed significant intrapopulation variance differences except for those between El Saler and Cullera, and El Saler and Marjal del Moro-3, which may be attributed to the small sample size from the El Saler population.

Although the different intrapopulation divergence analyses do not agree completely, they are highly correlated, and it seems clear that Marjal del Moro populations show the highest levels of variation; El Saler is next, despite its small population size, and the highest genetic uniformity is found in Torreblanca and Cullera populations.

The distributions of pairwise differences among AFLP patterns of *L. dufourii* populations are shown in Fig. 5. The expected distributions under a constant population size (Watterson 1975) and under the population expansion model (Rogers & Harpending 1992) are also represented. Most populations do not show departures in their pairwise distributions from the expectation under a constant population size model according to the values of Harpending's raggedness index. Only Marjal del Moro-3 departs marginally from the constant population size expectation using a Monte-Carlo approach for testing the null hypothesis. On the other hand, all Marjal del Moro populations show significant differences from the expectation under Rogers and Harpending's (1992) population expansion model.

L. dufourii individuals were classified into 44 different phenotype groups in the RAPD study (Palacios & González-Candelas 1997) which could be compared with the corresponding 65 AFLP patterns. However, only 16 of those 44 groups were composed by more than one individual, and these are the groups for which the comparison is meaningful, as a group with only one individual is necessarily monophyletic in any second classification. For those 16 groups, the average value of I_C was 0.685.

Discussion

AFLP analysis resulted in 20.2% polymorphic fragments (65 out of the 252 analysed), whereas the previous survey with RAPDs, using almost the same DNA samples, gave a proportion of 26.6% (44 out of 125) polymorphic bands. The average diversity per marker locus for AFLPs (0.0247 ± 0.0051) is significantly lower than for RAPDs (0.0763 ± 0.0128). This result contrasts with a study performed on another endangered *Limonium* species (C. Palacios, F. González-Candelas, unpublished), for which no variation was detected with RAPDs whereas some was present when the same individuals were assayed with AFLPs, and with a study in *Lens* (Sharma *et al*. 1996), where both the average fraction of polymorphic loci and the mean heterogeneity per locus were usually lower for RAPDs than for AFLPs in several populations of the genus. This implies that comparative results of variability levels between these techniques cannot be generalized, even for closely related species, at least until more comparative studies of this kind are available.

The intraspecific phylogeny obtained from the AFLP study confirmed the two main clusters defined in the neighbour-joining tree of the RAPD survey (Palacios & González-Candelas 1997). It is noteworthy that the particular individuals involved in those clusters are the same. Besides, those patterns that were not clearly grouped in the neighbour-joining tree (Fig. 2) form a compact group in the minimum spanning network (Fig. 4). This result was also observed in the network derived in the RAPD study, although involving other individuals. The concordance between the intraspecific relationships obtained with AFLP and RAPD markers is also reflected in the comparison by means of the index of classification, which

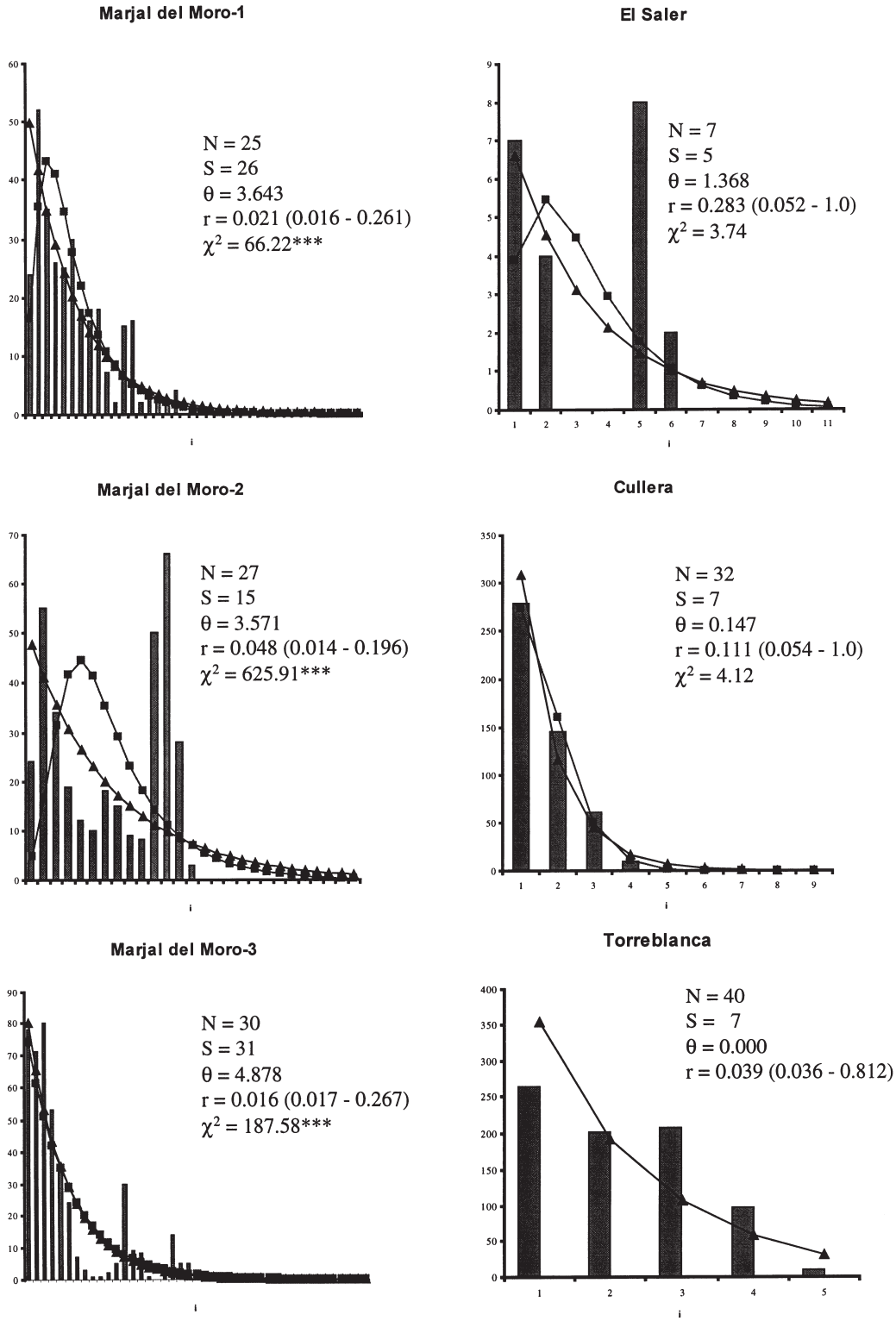

Fig. 5 Pairwise difference distribution for comparisons among individuals from *Limonium dufourii* populations. (a) Cullera; (b) Torreblanca; (c) Marjal del Moro-1; (d) Marjal del Moro-2; (e) Marjal del Moro-3. The continuous lines represent the expected distributions of pairwise differences under constant population size (triangles) and population expansion (squares) models. The parameters shown in each population are: N, the number of individuals sampled; S, the number of segregating sites; (the expected pairwise differences; r, Harpending's raggedness index with its 95% confidence interval obtained after 10000 simulations; and χ^2, chi-square values for the goodness of fit between the observed and expected value of mismatch counts under the population expansion model.

provides a quantitative estimate of the degree of coincidence between the clustering of the same individuals obtained from these two molecular techniques.

The degree of genetic structure observed from the AMOVA analyses when using RAPD or AFLP markers was very high (Φ_{ST} values of 0.516 and 0.531, respectively). This significant differentiation among populations is concordant with the expectation for an apomictic species, with small seed dispersal distance, and with soil requirements that lead to a patchy distribution. Also, the different approaches from AFLPs to determine the degree of population subdivision show very similar results. AMOVA analyses of AFLP patterns result in Φ_{ST} estimates of about 0.53, whereas the analysis of population differentiation based on nucleotide divergence estimates results in a N_{ST} value of 0.55. Only the simpler F_{ST}, the only statistic not taking into account the degree of similarity among the variants, presents a lower, but still significant, value (0.24). In this case, the introduction of an estimate of nucleotide divergence among AFLP patterns represents an advantage as it provides a means for direct comparison of population structure derived with other molecular markers.

Intrapopulation levels of variation obtained with AFLP and RAPD studies are quite similar, with the highest levels in Marjal del Moro populations and the lowest in Cullera and Torreblanca. In spite of its smaller size, the El Saler population showed intermediate levels of intrapopulation heterogeneity, but this is only a reflection of the evenness in the frequency of the different variants. Average gene diversity per marker locus in *L. dufourii* populations ranged from 0.0024 to 0.025. These values can be compared with those reported for other plant species. Hamrick & Godt (1990) report an average value of 0.06 from a review made on allozyme studies, when only species with endemic ranges similar to *L. dufourii* are considered. Travis *et al*. (1996) obtained values ranging from 0.02 to 0.13 in an AFLP study of several populations of the endangered plant *Astragalus cremnophylax*. These comparisons suggest that *L. dufourii* populations have a distinctly low genetic variation, even considering their endemic distribution. However, this conclusion is based on only a small number of studies based on DNA markers of endemic plant species.

Inferences about the historical population genetic and demographic processes that have affected *L. dufourii* populations can be drawn from the analysis of within-population diversity by calculating the pairwise mismatch distribution among patterns (Tajima 1983). No major differences have been observed from the comparison between the distributions from both AFLP and RAPD surveys. Although only the Marjal del Moro-3 population showed significant differences from the equilibrium model, a recent reduction in population size has been postulated for the Cullera population (Crespo & Laguna 1993), which agrees with the distribution obtained in the RAPD study. Some authors have pointed out that recent demographic events might not be discernible from this analysis of pairwise differences, and that their effects might be masked by older events (Lavery *et al*. 1996). This same reasoning applies for at least the El Saler population, for which a size reduction has been observed in the last few decades, and whose mismatch distribution does not show deviation from the equilibrium expectation. Therefore, it can be postulated that most *L. dufourii* populations were in equilibrium in the past. The current reduction of suitable habitats for the species has caused the observed decline in the extant populations, as well as the extinction of some others.

Summarizing, RAPD and AFLP analyses of the same *L. dufourii* individuals provide a very similar picture and lead to the same conclusions about the population genetic variation and structure in this species. Furthermore, the measures for the preservation of the species derived from both analyses are also essentially the same.

The high level of population subdivision detected in this species implies that transplantation of individuals or seeds from one population to another should probably be avoided, except in the case of the El Saler population, for which the extremely small population size and close phylogenetic proximity to Marjal del Moro populations make it advisable to re-introduce seeds from group B Marjal del Moro individuals. Not all *L. dufourii* populations currently hold the same amount of variability (Table 2, and table 4 in Palacios & González-Candelas 1997), and this could help in deciding which of them should be prioritized for preservation when resources are scarce. In this case, the Marjal del Moro populations do not simply hold the highest variability, but they also include variants from the two divergent groups, A and B. On the other hand, Cullera and Torreblanca populations are also 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. As is often the case, having access to more information does not ease the process of making hard decisions.

Should we use AFLPs (instead of RAPDs)?

In spite of the drawbacks and considerations imposed by the use of PCR-based DNA fingerprinting methods for population genetic analysis, the existence of other restrictions often encountered in the survey of natural populations still makes the use of these methods advisable. This is more so when the amount of biological material for analysis is small, closely related individuals are being compared, little (or no) sequence information

is known for the particular species, and general evaluations of variability at, for instance, the population level are sought. Among these techniques, AFLP markers, unlike other fingerprinting methods (Ellsworth *et al.* 1993; Caetano-Anollés *et al.* 1992; Micheli *et al.* 1994), have a high reproducibility (Vos *et al.* 1995; Janssen *et al.* 1996). This advantage of AFLP overcomes some limitations of other PCR-based DNA fingerprinting markers, especially with regard to its potential use in different laboratories, which can be of great importance when *ex situ* conservation management measures are to be established, and for the evaluation of phenotypes along several generations, as in re-introduction experiments. Besides, the flexibility of the AFLP technique (by using different enzymes and/or selective nucleotides) makes it superior to other methods because of the potentially large number of markers that can be detected simultaneously, especially after the evolution of AFLPs towards full automation.

Attempts to estimate evolutionary divergence relatedness from DNA fingerprinting data have been reported previously (Clark & Lanigan 1993). However, the similarity between AFLPs and RFLP markers makes it possible to apply confidently Nei & Li's approach, because the required assumptions are better satisfied in this case than with RAPDs. To our knowledge this is the first time that an evolutionary approach has been used for AFLP data analysis. Although our results did not show appreciable differences on the relationships derived from this evolutionary genetic distance and the phenetic distance, by using an evolutionary approach we can be more confident about the inferred phylogenetic relationships. Another advantage of this approach is that an estimate of nucleotide diversity provides a measure that makes comparisons among different species feasible. Molecular studies within plant species in which nucleotide divergence estimates have been used are strongly biased towards RFLPs (Böhle *et al.* 1994). Soltis *et al.* (1992) reported intraspecific nucleotide divergence values in a revision of several chloroplast DNA (cpDNA) studies ranging from 0.000 to 0.003. Milligan (1991) reported levels of intraspecific cpDNA divergence ranging from 0.0003 to 0.0015. Typical values for congeneric species range from 0.0004, for very closely related species, to 0.0214 for species in the same section of a genus, or even 0.0805 for species from different sections (Soltis *et al.* 1992; Milligan 1991; Wang & Szmidt 1993). A phylogenetic study (C. Palacios *et al.*, unpublished) also using RFLPs on cpDNA as markers with some *Limonium* species of the same section to which *L. dufourii* belongs shows nucleotide diversity values ranging from 0.0048 to 0.0347. Hence, nucleotide diversity in *L. dufourii* (0.0000–0.0024) corresponds to the expectation for within-species comparisons, thus indicating that individuals from groups A and B in the Marjal del Moro populations actually belong to the same species, a point of difficult definition when apomictic species are involved. Although we lack information about evolutionary rates at the nucleotide level for anonymous nuclear sites in plants, this low divergence may indicate either a paucity of the evolutionary rate at these positions or a relatively recent origin of this species.

Finally, although there are a number of features in AFLPs that make them better markers for population genetic studies than other DNA fingerprinting-based ones, there are still several questions (for instance, Hardy–Weinberg equilibrium, or levels of intra-individual heterozygosity) that cannot be addressed unless codominant markers are employed. The nature and opportunity of these questions for the specific problem to be tackled should be carefully considered before selecting any molecular markers.

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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 use 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. Dr Kresovich's research has focused on the application of genomics to resolve questions of agricultural resources conservation and use. He currently serves as Director of the Institute for Genomic Diversity at Cornell University.

Appendix I Frequency of each AFLP phenotype in the six *Limonium dufourii* populations studied

Phenotype	Cullera	Torreblanca	El Saler	Marjal del Moro-1	Marjal del Moro-2	Marjal del Moro-3
1	24	3	0	0	0	1
2	2	0	0	0	0	0
3	1	0	0	0	0	0
4	1	0	0	0	0	0
5	2	0	0	0	0	0
6	1	0	0	0	0	0
7	1	0	0	0	0	0
8	0	0	0	1	0	0
9	0	0	0	7	0	0
10	0	0	0	1	0	0
11	0	0	0	1	0	0
12	0	0	0	2	0	0
13	0	0	0	1	0	0
14	0	0	0	1	0	0
15	0	0	0	1	0	0
16	0	0	0	1	0	0
17	0	0	0	1	0	0
18	0	0	0	1	0	0
19	0	0	0	1	0	0
20	0	0	0	2	0	0
21	0	0	0	1	0	0
22	0	0	0	1	0	0
23	0	0	4	2	6	13
24	0	23	0	0	0	0
25	0	3	0	0	0	0
26	0	2	0	0	0	0
27	0	2	0	0	0	0
28	0	1	0	0	0	0
29	0	3	0	0	0	0
30	0	1	0	0	0	0
31	0	1	0	0	0	0
32	0	1	0	0	0	0
33	0	0	2	0	0	0
34	0	0	1	0	0	0
35	0	0	0	0	4	0
36	0	0	0	0	1	0
37	0	0	0	0	1	0
38	0	0	0	0	1	0

Appendix I *Continued*

Phenotype	Cullera	Torreblanca	El Saler	Marjal del Moro-1	Marjal del Moro-2	Marjal del Moro-3
39	0	0	0	0	2	0
40	0	0	0	0	1	0
41	0	0	0	0	1	0
42	0	0	0	0	2	0
43	0	0	0	0	2	0
44	0	0	0	0	1	0
45	0	0	0	0	1	0
46	0	0	0	0	1	0
47	0	0	0	0	1	0
48	0	0	0	0	1	0
49	0	0	0	0	1	0
50	0	0	0	0	0	1
51	0	0	0	0	0	1
52	0	0	0	0	0	1
53	0	0	0	0	0	1
54	0	0	0	0	0	1
55	0	0	0	0	0	1
56	0	0	0	0	0	1
57	0	0	0	0	0	1
58	0	0	0	0	0	1
59	0	0	0	0	0	1
60	0	0	0	0	0	1
61	0	0	0	0	0	1
62	0	0	0	0	0	1
63	0	0	0	0	0	1
64	0	0	0	0	0	1
65	0	0	0	0	0	1
Total	32	40	6	23	24	27