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Historical and biological determinants of genetic diversity in the highly endemic triploid sea lavender *Limonium dufourii* (Plumbaginaceae)

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Abstract

Microsatellite markers were used to evaluate the genetic diversity and population genetic structure in the critically endangered *Limonium dufourii* (Plumbaginaceae), a highly endemic triploid species from the coasts of eastern Spain. Sixty-five alleles from 13 microsatellite regions were amplified in a sample of 122 individuals collected from the six extant populations. Microsatellite patterns were consistent with the triploid nature of *L. dufourii*. Alleles were unambiguously assigned to two different parental subgenomes in this hybrid species and the greater contribution of the diploid parental subgenome was confirmed. Eleven, 25 and 26 multilocus genotypes were recorded from the haploid, diploid and from the combined information of both subgenomes, respectively. Genetic diversity was mostly distributed among populations (72.06% of the total genetic variation). Genotypes from Marjal del Moro populations grouped into two highly structured clusters (88.41% of the total variance). The observed patterns of distribution of genetic diversity are interpreted to result from multiple hybridization events and isolation between populations. Threats to this species are mainly anthropogenic (urbanization and tourism pressure), although stochastic risks cannot be ignored. Therefore, in order to preserve extant genetic variation of *L. dufourii*, *in situ* strategies such as the preservation of its habitat are a high priority. Several recommendations in order to assist *ex situ* measures to guarantee the success of conservation strategies and maintain the relationships between individuals and populations are proposed.

Keywords: alloploidy, apomixis, genetic diversity, halophytes, microsatellites, plant conservation

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Introduction

Hybridization, polyploidy and apomixis have had significant impacts on the evolutionary history of angiosperms (Stebbins 1971; Soltis & Soltis 2000; Van Dijk & Vijverberg 2005). It has been demonstrated that many presumed diploids are in fact palaeopolyploids that have lost their polyploid genomic architecture through gene silencing and divergent evolution of initially homologous loci. Polyploidy is likely to occur after hybridization events, thus allowing the new hybrids to reproduce sexually because of normal chromosome pairing at meiosis. Enzyme multiplicity and biochemical diversity may provide newly arising hybrid taxa with increased evolutionary potential which might explain their persistence and increased colonization capabilities in areas where their parents have disappeared or were unable to colonize (Brochmann *et al*. 2004).

Apomixis is a reproductive mechanism in which plants produce agamospermous seed, and it is frequent in polyploid species that show abnormal meiosis because normal gamete production is severely reduced or non-existing. Most apomictic taxa are characterized by low pollen viability (Erben 1979) and their progeny are expected to be identical genetic replicates of the mother plants, that

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is, clonally produced ramets from a single genet. Agamospermous taxa usually consist of genetically uniform populations as a consequence of apomictic reproduction (Paun *et al*. 2006). Because of their low genetic diversity levels, apomicts often have short-term evolutionary potential and usually occupy derivative positions in phylogenetic trees (Richards 2003). Nonetheless, apomictic taxa may acquire genetic diversity through somaclonal mutation, backcrosses with parental taxa and occasional episodes of sexual reproduction (Van der Hulst *et al*. 2000). These processes can, to some extent, counteract genetic depletion within populations (Chapman *et al*. 2000). Additional processes leading to genetic differentiation among populations and/or to an increase of within-population genetic diversity are migration between different clonal populations or the input of individuals produced by recurrent hybridization events between the progenitors (Robertson *et al*. 2004). Despite the apparent long-term disadvantage of apomictic species, these taxa may still benefit from their ability to establish new populations from single individuals and from the dispersal and colonization capabilities derived from their hybrid origin (Van Dijk 2003; Hörandl 2006).

The genus *Limonium* Miller (sea lavenders) is the most species-rich genus of the Plumbaginaceae and includes more than 400 taxa (Erben 1993). *Limonium* species inhabit inland dry gypsum soils, coastal cliffs and salt marshes. The large diversification of this genus has been interpreted to be a consequence of the high hybridization potential among taxa, followed by habitat specialization coupled with the hybrids' ability to produce agamospermous seeds. This evolutionary model has been used to explain multiple series of complex aggregates of sexual diploid species and asexual polyploid hybrids which are perpetuated through gametophytic apomixis (Ozias-Akins 2006).

Limonium dufourii (Girard) Kuntze (Plumbaginaceae) is an endangered, highly endemic, triploid species ($2n = 3x = 27$) from the coasts of eastern Spain (Castellón and Valencia provinces). As for many triploids in the genus it has been proposed to have arisen through interspecific hybridization (i.e. allopolyploid origin), although the possibility of an autopolyploid origin has not been investigated (Erben 1979). It is a perennial, densely tomentose, rosulate, hemicryptophyte with racemose inflorescences of showy pink flowers. The taxonomic relationships of *L. dufourii* are still poorly understood. It was included in the subsection *Densiflorae* of the section *Limonium* by Boissier (1848) along with other diploid and triploid taxa. A phylogenetic analysis of chloroplast sequences suggested a close relationship with *Limonium densissimum* (Pignatti) Pignatti (Lledó *et al*. 2005), another triploid species morphologically well differentiated from *L. dufourii*, although this relationship lacked strong statistical support similarly to many other taxa included in the section *Limonium*. However, this and other phylogenetic studies of the genus (i.e. Palacios *et al*. 2000) were based on a limited sampling of taxa and therefore were unable to clarify species-to-species phylogenetic relationships within this complex genus which exhibits frequent hybridization.

Limonium dufourii has a monomorphic pollen-stigma combination (pollen type B/stigma papillate, Baker 1966; M. Palop-Esteban & J.G. Segarra-Moragues, personal observation). This combination is present in many other triploid taxa of *Limonium* and precludes self-fertilization. Neither the reciprocal morph (A/cob) nor the self-compatible combinations (A/papillate — B/cob) have been reported in this species. Further chances of intraspecific fertilization are also hampered by the high male sterility derived from its triploid nature. Therefore, *L. dufourii* apparently reproduces exclusively through gametophytic apomixis (Baker 1966).

Although *L. dufourii* was previously more widespread in this area, its distribution range has reduced because of habitat loss associated with urban development (Crespo & Laguna 1993). The extinction of three populations and the decimation of two others have been recently documented (Crespo & Laguna 1993; Laguna *et al*. 1994). Given its critical status, *L. dufourii* has been considered a priority species for conservation and it has been included as Critically Endangered in the Spanish catalogue of endangered plants (VVAA 2000; Crespo 2004). Several conservation initiatives have been promoted by national and regional governments including population censuses (Crespo & Laguna 1993), genetic studies (Palacios & González-Candelas 1997; Palacios *et al*. 1999) and *in vitro* cultures for *ex situ* conservation (Lledó *et al*. 1993; Martín & Pérez 1995).

Reduction in the number of individuals can have dramatic consequences on the viability of populations because the latter are more prone to genetic erosion which ultimately may reduce the adaptive capability and competitive strength of a species (Paschke *et al*. 2002; Pluess & Stöcklin 2004). Although apomictic taxa may withstand population bottlenecks and restore population sizes from just a few individuals, genetic diversity is recovered more slowly than in their sexually reproducing diploid relatives, and this could ultimately compromise their long-term survival (Richards 2003). The loss of entire populations can have more serious consequences given the strong genetic differentiation of apomictic populations (Gornall 1999).

In this study, we have used microsatellite (simple sequence repeats, SSR) markers to investigate the levels and distribution of genetic diversity within and among populations of *L. dufourii*. Because of their codominant nature, SSRs can provide additional clues about the genomic evolution of polyploid taxa of hybrid origin (Paun & Hörandl 2006). We have used two different analytical approaches to assign microsatellite alleles to

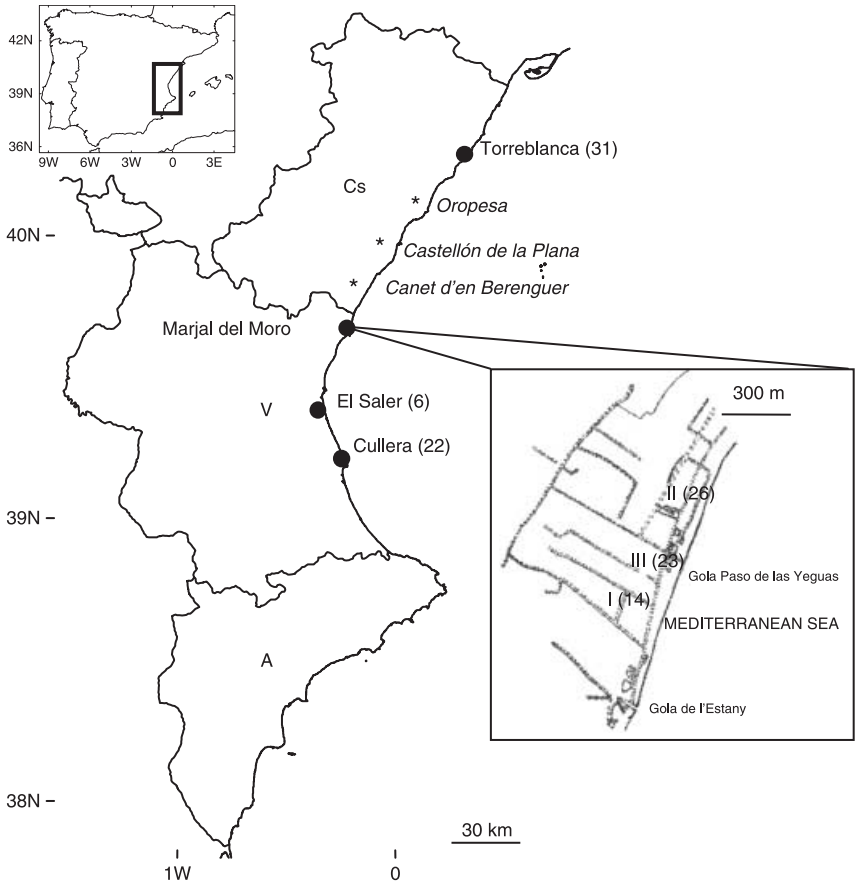

Fig. 1 Area of distribution of *Limonium dufourii*. Locations marked with an asterisk represent currently extinct populations. The zoomed area shows the three sampled subpopulations at Marjal del Moro. A, Alicante, Cs, Castellón and V, Valencia provinces. Numbers in brackets represent the number of analysed individuals from each population.

their corresponding parental genomes. This has allowed us to decipher for the first time in an asexual hybrid with unknown parents the relative genetic contribution of each putative parental taxon to the hybrid descendant. The observed genetic patterns have been interpreted in the light of single and multiple hybridization scenarios. Finally, some conservation strategies to minimize genetic erosion within populations are discussed.

Materials and methods

Plant material and microsatellite amplification

One hundred and twenty-two individuals were sampled from the six extant populations of *Limonium dufourii* (Fig. 1). Fresh leaves were used as material for DNA extraction (Doyle 1991) and corresponded to the same individuals previously analysed with random amplified polymorphic DNA (RAPD, Palacios & González-Candelas 1997) and amplified fragment length polymorphism (AFLP, Palacios *et al*. 1999) markers.

Thirteen microsatellite primer pairs of *L. dufourii* were used in this study (see Palop-Esteban & González-Candelas 2002 for amplification details). Allele scoring was carried out by automated fluorescent scanning detection in an ABI PRISM 310 DNA sequencer (Applied Biosystems) using ROX-500 as internal lane size standard, and the GENESCAN and GENOTYPER programs (Applied Biosystems). Allele sizes, excluding flanking regions, were converted into repeat units for further analyses.

Assignment of SSR alleles to parental subgenomes

Different allele amplification patterns were found in the analysed microsatellites, which could be attributed to an allotriploid origin for this species (see below). Under this hypothesis, in allotriploids such as *L. dufourii* ($3x = 27$) one parent is expected to contribute a haploid dosage ($x = 9$) whereas the other is expected to contribute a diploid one ($2x = 18$). In such a case, primer pairs amplifying a single variable allele across individuals are expected to represent the haploid-contributing parent (referred to as subgenome H), whereas those amplifying up to two variable alleles per individual should represent the diploid-contributing parent (subgenome D). Primer pairs amplifying three bands would correspond to the amplification of alleles from both genomes and in which the genome from the diploid parent is heterozygous. Genotype assignments

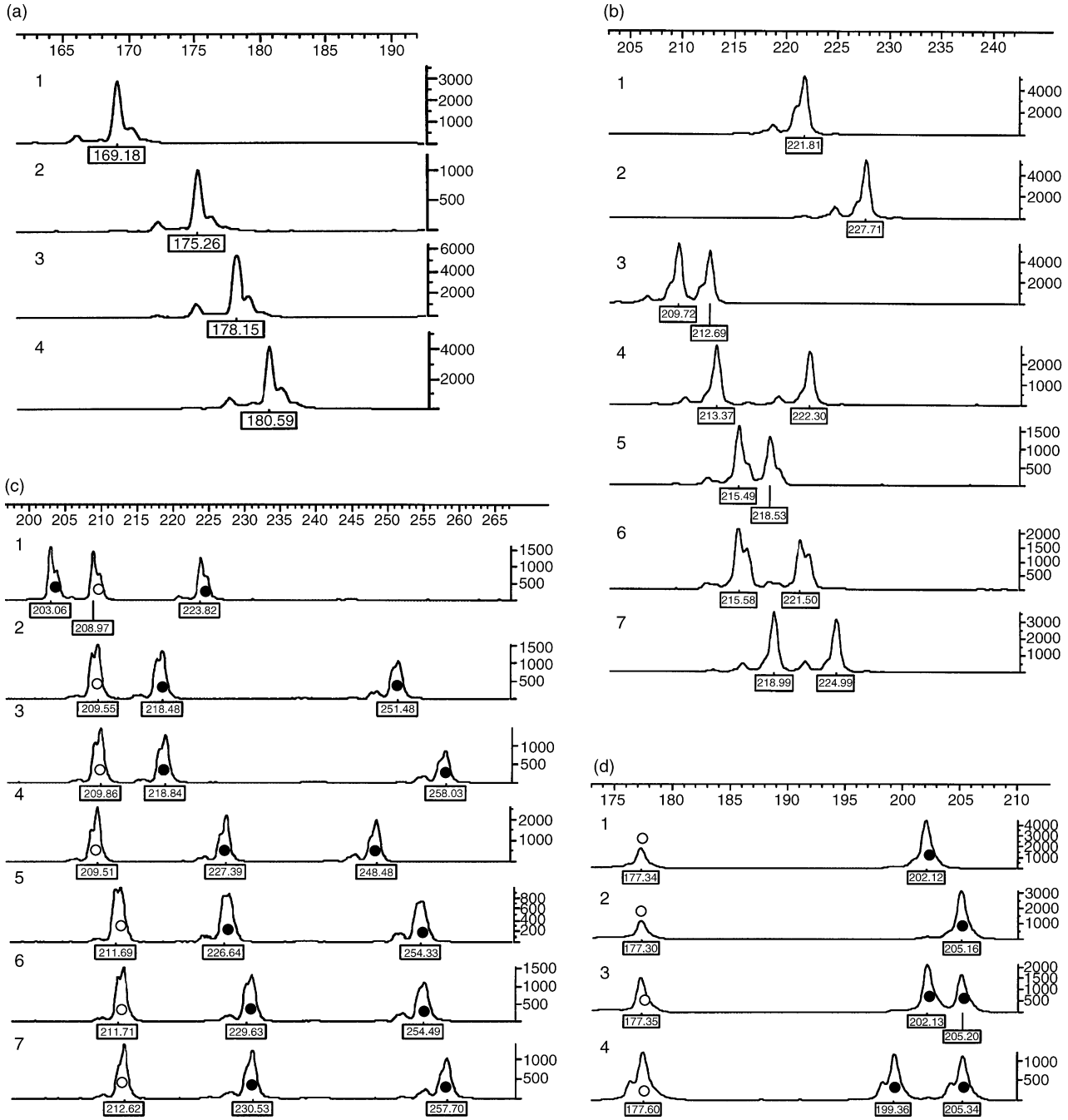

Fig. 2 Sample electropherograms obtained with GENOTYPER of amplification patterns from four SSR regions. (a) Ld478 amplifying a single genetic dosage (subgenome H), with individuals showing a single band. (b) Ld106 amplifying a single genetic dosage (subgenome D), with individuals showing up to two bands. (c) Ld418 and (d) Ld050 amplifying both genetic dosages (subgenomes H + D), individuals with up to three bands. In loci Ld418 and Ld050 open dots and black dots designate alleles from subgenomes H and D, respectively.

were checked for consistency using the microsatellite DNA allele counting-peak ratios method (MAC-PR, Esselink *et al*. 2004) using quantitative values for microsatellite allele amplification peak areas. In those cases in which up to two variable alleles per individual are amplified consistently, thus representing the diploid subgenome, equal peak ratios are expected for both alleles (Fig. 2b). For primer pairs that consistently amplify the two parental genomes, individuals with two or three bands can be found. Individuals with three bands should show equal peak ratios for all three bands, since each allele is present in a single dosage (Fig. 2c, d, samples 3 and 4), whereas in

those individuals with two bands, thus presumably homozygous for the diploid parental subgenome, a double peak ratio would be expected for alleles corresponding to the diploid subgenome, as these are present in two equal copies (Fig. 2d, samples 1 and 2). Differences in amplification patterns between primer pairs are most likely due to incomplete complementarity of the designed primers to the target regions in the heterologous chromosomes (Catalán *et al*. 2006). This is further supported by the relatively low transferability of microsatellite loci among species of *Limonium* (Palop *et al*. 2000), probably because of the rapid diversification of the genus (Lledó *et al*. 2005). If we were able to assign each allele to its corresponding genome then the relative contribution of each parent could be traced back from the hybrid species and unique or multiple parallel hybridization events could be identified.

A further attempt to assign microsatellite alleles to the corresponding subgenomes was conducted through cloning, sequencing and analysis of flanking regions. Amplification products of individuals with tri-allelic patterns were cloned into plasmids using the pGem-T Easy cloning kit (Promega). Positive colonies were screened by polymerase chain reaction (PCR) until all three individual alleles were captured and sequenced using the BigDye Terminator Kit version 3.1 (Applied Biosystems) on an Applied Biosystems 3700 DNA sequencer. The three different alleles were sequenced twice from different positive clones to ensure reproducibility, although only one sequence of each duplicate was used for subsequent analyses. Sixty-two microsatellite alleles from three different SSR loci were sequenced and a total of 23 different alleles were obtained. Allele sequences have been deposited in GenBank (accession nos EF434947–EF434969).

Flanking SSR sequences were aligned using CLUSTAL_X (Thompson *et al*. 1997) and alignments were further corrected by visual inspection. Aligned sequences were analysed using Jukes & Cantor (1969) distance and a neighbour-joining tree (Saitou & Nei 1987) was constructed with MEGA 3 (Kumar *et al*. 2004). Support for the nodes was assessed by 1000 bootstrap replicates (Felsenstein 1985).

Genetic analyses of SSR data

Because in triploid apomictic species individual loci cannot recombine, the most appropriate measure to quantify genetic variation is the diversity of multilocus genotypes (cf. Robertson *et al*. 2004). Given that both subgenomes are linked in a triploid species and that the genomic dosage contributed by the diploid parent becomes fixed because of lack of recombination, we would refer hereafter to all terms as multilocus genotypes and genotypic diversity. The number of individuals with each multilocus genotype was determined for each population and in the total sample and multilocus genotypic diversity was quantified as $H_g = 1 - \Sigma p_i^2$, where p_i is the frequency of multilocus genotype i, for the haploid genome (H_{gH}), the diploid genome (H_{gD}) and for the combined genomes (H_{gT}, total genotypic diversity).

In order to examine the partitioning of the total variance into variance components, we used ARLEQUIN version 2000 (Schneider *et al*. 2000) to perform analyses of molecular variance (AMOVA; Excoffier *et al*. 1992). For this purpose, we converted the multilocus genotype of each individual into a linear binary array and analysed as presence–absence data using F_{ST}-based distances. These analyses were conducted at different hierarchical levels: (i) within and among populations of *L. dufourii* with no geographical structuring, in order to quantify the proportion of genetic variation among populations; and (ii) among regions, among populations within regions and within populations to check whether the distribution of the genetic variance matched the geographical arrangement of populations. Separate AMOVA analyses were also conducted for the subset of populations from Marjal del Moro to assess levels of population differentiation within this location. The significance of variance components was obtained by non-parametric procedures using 1000 random permutations.

The relationships between individual genotypes and populations were visualized by means of cluster analyses using D_A genetic distance (Nei *et al*. 1983) using POPULATIONS (Langella 2000). The resulting distance matrices between individuals and populations were used to construct neighbour-joining (NJ) phylograms (Saitou & Nei 1987) with MEGA 3 (Kumar *et al*. 2004). The statistical robustness of the groupings was assessed by bootstrap analysis with 1000 replicates (Felsenstein 1985). Isolation by distance was assessed through the correlation of the pairwise genetic distances obtained with POPULATIONS and the geographical distances between populations. Significance values for the corresponding Mantel (1967) test were obtained with 1000 permutations using NTSYS-PC version 2.11a (Rohlf 2002). An alternative visualization of the relationships among SSR genotypes was obtained with a minimum-spanning network (MSN), which represents all possible minimum length connections among them, using ARLEQUIN with the Euclidean distance matrix between SSR genotypes. This analysis requires knowledge of the gametic phase. However, given that there is no recombination in *L. dufourii*, the individual multilocus genotypes for the diploid and combined subgenomes were converted into linear binary definitions and analysed as presence–absence data.

Results

Microsatellites were consistent with the triploid genome of *Limonium dufourii*. However, the amplification of alleles from the two subgenomes was not equally successful in

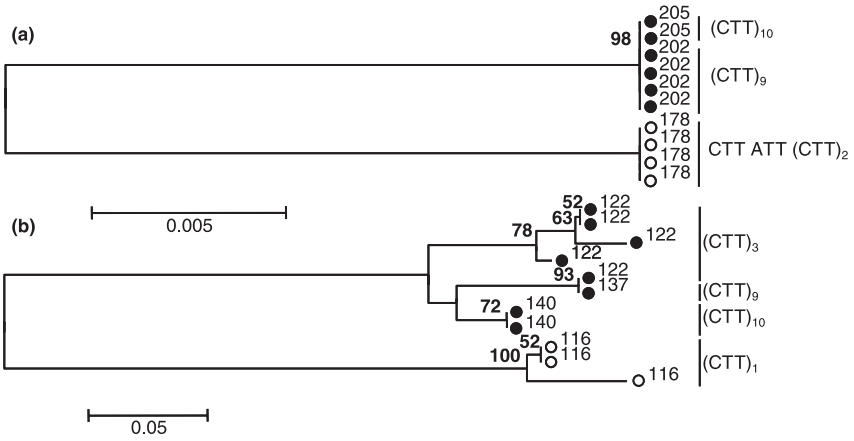

Fig. 3 Neighbour-joining trees constructed with Jukes & Cantor (1969) distance showing the relationship between cloned allele sequences of (a) region Ld050 and (b) region Ld103. The repeat motif in the microsatellite was excluded for the reconstruction. Numbers at the tips represent allele sizes. Numbers above nodes represent bootstrap values when higher than 50%. Black circles represent alleles from the diploid subgenome and open circles alleles from the haploid subgenome, respectively.

all the analysed microsatellite regions. In four of them (Ld032, Ld044, Ld445 and Ld448), a single variable allele was amplified in all individuals, suggesting that these regions corresponded to only the haploid parental genome, denoted subgenome H (Fig. 2a). In five regions (Ld059, Ld106, Ld412, Ld420 and Ld423), we found individuals with up to two bands; therefore these primer pairs were interpreted as amplifying alleles from only the diploid parental genome, denoted subgenome D (Fig. 2b). The remaining four regions analysed (Ld050, Ld081, Ld103 and Ld418) presented individuals with three bands, thus corresponding to alleles of both subgenomes (Fig. 2c, d). Allele sequences from tri-allelic individuals in microsatellite regions Ld050 and Ld103 illustrate the hybrid triploid nature of *L. dufourii* (Fig. 2c, d and 3). Furthermore, a phylogenetic analysis of the flanking sequences of these alleles allowed us to identify two allelic clusters corresponding to each parental subgenome (Fig. 3). Alleles from the four regions amplifying tri-allelic patterns were assigned to each subgenome and pooled when appropriate with data from subgenomes H and D, respectively. After sequencing some microsatellite alleles, we found that 20% of equal-sized alleles differed in base composition at the flanking regions. This size homoplasy is not uncommon in microsatellites (Scotti *et al*. 2002) and it implies deviation from a strict stepwise mutation model (SMM). Sequence divergence of alleles with identical size varied from 0 to 0.130 substitutions/site (s/s). Sequence variation between alleles considered in the same genomic complement (excluding differences in the number of microsatellite repeats) ranged from 0.0145 s/s (Ld050) to 0.1268 s/s (Ld103), whereas sequence divergence of alleles between genomic complements was much larger (from 0.087 s/s for Ld050–0.580 s/s for Ld103). A total of 65 SSR alleles were scored from the 13 microsatellite loci. Seventeen of these were assigned to subgenome H and the remaining 48 to subgenome D. Eleven multilocus genotypes were scored from subgenome H and 25 from subgenome D and only one additional multilocus genotype was revealed after their combination (Table 1).

Levels of multilocus SSR genotypic diversity for *L. dufourii* were high (H_{gT} = 0.93, Table 1). However, the amount of this diversity varied among populations, from a minimum in the small populations of Marjal del Moro I (H_{gT} = 0.13) and El Saler (H_{gT} = 0.44) to a maximum in the large population of Marjal del Moro II (H_{gT} = 0.85). Populations from the extremes of the distribution range of *L. dufourii*, Torreblanca and Cullera, presented intermediate values of genotypic diversity, H_{gT} = 0.65 and H_{gT} = 0.56, respectively. The highest amounts of genotypic variation (H_{gT} = 0.88) were harboured in the Marjal del Moro populations that altogether included 17 of the 26 SSR genotypes detected in the species. Genetic diversity within populations was significantly lower for subgenome H (H_{gH} = 0–0.68) than for subgenome D (H_{gD} = 0.13–0.85) and it did not increase when information from both subgenomes was combined, with the only exception of the Marjal del Moro III population (Table 1). Populations with high levels of genotypic diversity for subgenome D also showed high values for subgenome H. No genetic diversity was found in subgenome H for the populations of Cullera and Torreblanca.

The neighbour-joining tree showing the relationships among the 26 multilocus SSR patterns revealed a clear differentiation into two groups (Fig. 4). One group included a well-supported (75% BP) subcluster, encompassing genotypes from the southernmost population of Cullera and a sister group of genotypes from Marjal del Moro populations (denoted group A for further discussion). The other main cluster included genotypes from the northernmost population of Torreblanca and a sister cluster with the remaining genotypes from Marjal del Moro populations (denoted as group B). Both groups A and B from Marjal del Moro included individuals from the three populations from this location. The two genotypes from El Saler clustered separately; one was sister to the cluster conformed by

Table 1 Diversity at microsatellite loci in six *Limonium dufourii* populations. H_{gH}, H_{gD} and H_{gT} correspond to the multilocus genotypic diversities for the haploid, diploid and combined genomes, respectively. Sampling sizes (*N*): Torreblanca *N* = 31, Marjal del Moro I *N* = 14, Marjal del Moro II *N* = 26, Marjal del Moro III *N* = 23, El Saler *N* = 6, and Cullera *N* = 22

Genotype	Subgenome H												Subgenome D																									
Population	1	2	3	4	5	6	7	8	9	10	11	H_{gH}	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	H_{gD}
Torreblanca	31	—	—	—	—	—	—	—	—	—	—	0	12	13	4	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.65
Marjal Moro I	—	1	—	—	—	—	—	13	—	—	—	0.13	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	13	—	—	—	—	—	0.13
Marjal Moro II	—	6	12	6	1	1	—	—	—	—	—	0.68	—	—	—	—	1	7	4	3	4	2	1	1	2	1	—	—	—	—	—	—	—	—	—	—	—	0.85
Marjal Moro III	—	21	1	—	—	—	1	—	—	—	—	0.16	—	—	—	—	2	1	3	—	—	—	—	—	—	—	12	1	2	1	1	—	—	—	—	—	—	0.69
El Saler	—	—	—	—	—	—	—	—	4	2	—	0.44	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4	2	—	—	—	0.44
Cullera	—	—	—	—	—	—	—	—	—	—	22	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	8	12	2	0.56
Total	31	28	13	6	1	1	1	13	4	2	22	0.82	12	13	4	2	4	8	7	3	4	2	1	1	2	1	12	1	2	1	1	13	4	2	8	12	2	0.93

Genotype	Combined (subgenomes H + D)																												H_{gT}
Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26			
Torreblanca	12	13	4	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			0.65
Marjal Moro I	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	13	—	—	—	—	—			0.13
Marjal Moro II	—	—	—	—	1	7	4	3	4	2	1	1	2	1	—	—	—	—	—	—	—	—	—	—	—	—			0.85
Marjal Moro III	—	—	—	—	2	1	3	—	—	—	—	—	—	—	11	1	2	1	1	1	—	—	—	—	—	—			0.73
El Saler	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4	2	—	—	—			0.44
Cullera	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	8	12	2			0.56
Total	12	13	4	2	4	8	7	3	4	2	1	1	2	1	11	1	2	1	1	1	13	4	2	8	12	2			0.93

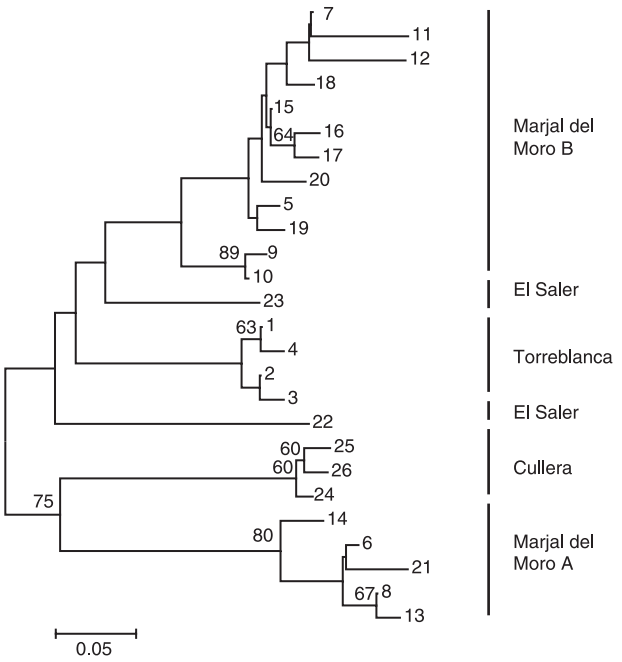

Fig. 4 Neighbour-joining tree showing the relationships among the 26 multilocus SSR patterns scored in *Limonium dufourii*. The Euclidean distance matrix was used. Numbers above nodes represent bootstrap proportions (over 1000 replicates) when higher than 50%.

genotypes from group B from Marjal del Moro whereas the other was sister to the (Torreblanca + Marjal del Moro B) group (Fig. 4). Mantel test revealed significant correlation between genetic and geographical distances among populations ($r = 0.60$, $P = 0.039$).

The MSN constructed from SSR patterns in *L. dufourii* also revealed a high level of genetic structuring. Molecular genotypes of subgenome H were more similar than those from subgenome D (Fig. 5a, b). For the former subgenome, the populations of Cullera and Torreblanca were characterized by a private genotype each, whereas those from Marjal del Moro were more diverse and clustered in an intermediate position (Fig. 5a). The two multilocus genotypes from El Saler clustered separately, one being related to genotypes found mainly in Marjal del Moro III individuals and the other to a cluster of genotypes from Marjal del Moro I and II (Fig. 5a). The SSR patterns revealed by subgenome D showed a clear geographical structure (Fig. 5b). Again, genotypes from Cullera and Torreblanca were clearly separated from the rest by a large number of differences (12 and 10, respectively). Molecular genotypes from Marjal del Moro populations grouped into two well-differentiated clusters. These two clusters corresponded to those identified in the NJ tree in which genotypes from Marjal del Moro A were closer to those from Cullera and genotypes from Marjal del Moro B were closer to those from Torreblanca. Individuals from El Saler presented two different molecular genotypes. One of these linked all major groups of multilocus genotypes whereas the other was related to the Torreblanca ones (Fig. 5b). The MSN constructed considering the combined information of the two subgenomes (Fig. 5c) was mostly coincident with that of the diploid subgenome (Fig. 5b), thus suggesting that there was little additional contribution of subgenome H to the genetic structure of *L. dufourii*. In this plot (Fig. 5c), the differentiation of genotypes from Torreblanca, Cullera and Marjal del Moro (A and B) was strengthened by an increased number of differences. One of the SSR genotypes from El Saler showed an intermediate position between these four main clusters of genotypes whereas the other linked closer to group B from Marjal del Moro (Fig. 5c).

Non-hierarchical analysis of molecular variance revealed that the largest proportion of genetic variation was found among populations (72.06%, Table 2). Hierarchical AMOVA with populations arranged in four groups predefined by geographical location attributed 25.44% of the total variation to differences between geographical ranges, whereas 48.14% of the variation was found among populations within the defined groups (Table 2). Most of the variance among populations was attributed to the genetic diversity of the three Marjal del Moro populations. In fact, when individuals from these populations were grouped according to their genetic membership to groups A and B, AMOVA attributed 88.41% of the variance to differences between these two groups whereas variance within groups was only 11.59% (Table 2).

Discussion

Genetic insights into the origin of Limonium dufourii

Triploid ($2n = 3x = 27$) species of *Limonium* such as *L. dufourii* have been proposed to arise either through interspecific fertilization between normal gametes of diploids ($2n = 18$) and tetraploids ($2n = 4x = 36$) or through fertilization between a normal haploid gamete and an unreduced diploid gamete, both gametes originating from two different diploid ($2n = 18$) species (Erben 1979). This second type of hybridization is more plausible in this genus given the rarity of tetraploid *Limonium* compared to diploid taxa (Erben 1979). In this study, we have been able to assign the different microsatellite alleles of each individual to the corresponding parental subgenome by combining the analysis of microsatellite amplification patterns (Fig. 2) and amplification peak ratios (Esselink *et al*. 2004) with the analysis of flanking regions of sequenced alleles (Fig. 3). To our knowledge, this is the first time that microsatellite alleles have been assigned to each parental species of a triploid hybrid without any information on the potential progenitors. In this particular case, it does not seem possible to identify these parental

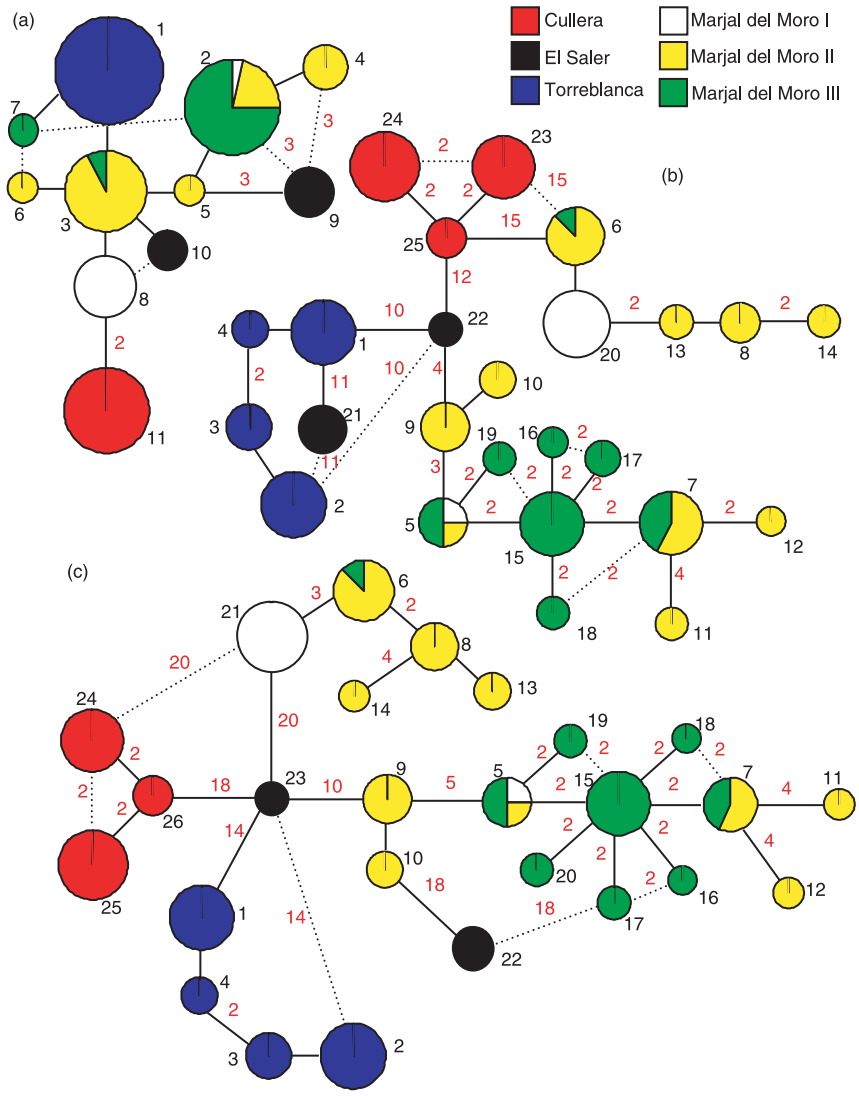

Fig. 5 Minimum-spanning networks (MSN) showing the relationships among the SSR genotypes found in *Limonium dufourii*. (a) 11 SSR genotypes of subgenome H (b) 25 SSR genotypes of subgenome D, and (c) 26 genotypes of combined information of subgenomes H + D. The size of the circles is proportional to the number of individuals with the corresponding genotype. Numbers above the segments connecting nodes represent the number of differences among them if larger than one. Dashed lines are additional links produced in the transformation of the minimum spanning tree into a MSN.

Table 2 Analyses of molecular variance (AMOVA) of *Limonium dufourii* populations. The analyses were conducted with three different hierarchy conformations: at no geographical grouping (panel 1), four groups defined by geographical location (panel 2), and considering only Marjal del Moro individuals grouped by molecular genotype (panel 3)

Source of variation (groups)	Sum of squared deviations (SSD)	d.f.	Variance components	Percentage of the total variance
1. *L. dufourii s.l.*				
Among populations	762.023	5	7.59623	72.06
Within populations	341.699	116	2.94568	27.94
2. Torreblanca vs. Marjal del Moro vs. El Saler vs. Cullera				
Among regions	537.355	3	2.83580	25.44
Among populations within regions	224.668	2	5.36719	48.14
Within populations	341.699	116	2.94568	26.42
3. Groups A and B from Marjal del Moro populations				
Between groups A and B	400.841	1	12.93527	88.41
Within groups	103.444	61	1.69581	11.59

species for the reasons mentioned above. However, this does not invalidate this procedure as a valuable strategy to search for unknown parental species of hybrids, provided two necessary conditions are met. First, primers designed for SSR amplification in the hybrid must amplify efficiently the homologous regions in the parental and related species. Second, the nucleotide sequences in these regions must hold enough phylogenetic signal to derive a reliable phylogenetic tree. If possible, sampling should include the considered parental species, in which case the identification will be very reliable, but, if this is not the case, the analysis will at least provide a reasonable working hypothesis on the phylogenetic position of those parental species.

We have tried to complete this process (M. Palop-Esteban, J.G. Segarra-Moragues and F. González-Candelas, unpublished results) but the primers designed for amplification of microsatellite loci in *L. dufourii* did not amplify in other *Limonium* species (Palop-Esteban & González-Candelas 2002). Furthermore, microsatellite primers designed for *Limonium narbonense*, a near relative of *L. dufourii*, which were able to amplify presumably homologous SSR regions in other *Limonium* species, failed to detect any difference in *L. dufourii* amplified regions and we could only hypothesize about one of its progenitor species (Palop *et al.* unpublished results). The closest identified relative of *L. dufourii* turned out to be *Limonium girardianum* (Guss.) Fourr., another triploid, apomictic species that might share a progenitor with *L. dufourii*, but other possible alternatives cannot be ruled out based on these partial results. In any case, this approach should not be based on inferences drawn from a single or a few loci, and caution must be exercised before drawing firm conclusions on the phylogenetic affiliation of unknown or unidentified species based on a relatively small sampling of their genomes.

Factors affecting genetic variation in L. dufourii

Apomictic taxa may present a wide range of within-population genetic diversity values, ranging from the complete absence of genetic variation as a consequence of founder events and asexual reproduction (Bayer & Minish 1993), to similar levels of variation such as those of sexual taxa. The latter have been explained by occasional events of sexual reproduction, that is, facultative sexuality (Carino & Daehler 1999; Paun *et al*. 2006), backcrossing with parental taxa, multiple hybridization events from sexual ancestors (Bayer 1990, 1991; Robertson *et al*. 2004) and accumulation of mutations within clones (Ellstrand & Roose 1987; Paun & Hörandl 2006). *Limonium dufourii* shows moderate to high levels of multilocus genotypic diversity within populations in the analysed microsatellite regions (Table 1), with most genotypes restricted to one or a few populations (Fig. 5), a common pattern in asexually reproducing plants (Ellstrand & Roose 1987).

It has been proposed that occasional events of sexuality and recombination could explain high levels of within-population genetic variation and differentiation between clones in apomictic species (Van der Hulst *et al*. 2000, 2003; Van Dijk & van Damme 2000; Paun *et al*. 2006). This scenario does not seem to apply to *L. dufourii* since it presents high male sterility, similarly to other triploid species of *Limonium*, because of abnormal meiosis (Erben 1979). Furthermore, populations of *L. dufourii* are characterized by a single monomorphic pollen-stigma combination which prevents fertilization between conspecific individuals (Baker 1966; Erben 1979). Even if *L. dufourii* were able to produce viable gametes, these would most likely participate in the production of new hybrids after fertilization of another *Limonium* species rather than new *L. dufourii* individuals.

Regarding apomicts as 'young' taxa, most genetic diversity in polyploid obligatory apomictic taxa is gained at the time of species formation through hybridization. Analysing the origins of genetic diversity in polyploids is a complex task that usually involves genomic comparisons of the polyploid taxon with its presumed progenitors (Robertson *et al*. 2004) or the production of artificial crossings between individuals and the study of multiple progeny arrays (Schranz *et al*. 2005; Nybom *et al*. 2006). Neither approach is feasible in *L. dufourii* because its parent species are unknown and it cannot reproduce sexually. On the coasts of the Valencian Community (Fig. 1), where *L. dufourii* lives, there are no karyologically compatible pairs of extant species that could hybridize to produce *L. dufourii* or they are so morphologically differentiated that they cannot be presumed to produce a species with the morphological traits of *L. dufourii* (Crespo-Villalba & Lledó-Barrena 1998). Additionally, crosses between *L. dufourii* individuals are prevented by both self-incompatibility and high male sterility, precluding the study of nonapomictic progeny in this species.

Hybridization and the structuring of genetic variation in L. dufourii

Individual genotypic configurations were consistent with the hybridization model proposed by Erben (1979) in which the *L. dufourii* genome would be composed of two sets of heterologous chromosomes: a haploid subgenome (subgenome H) and two sets of homologous chromosomes (subgenome D, Fig. 2). This proposal is supported by the separate clustering of alleles considered to arise from the haploid and diploid subgenomes based on the flanking sequences of microsatellites simultaneously amplified from both subgenomes (Fig. 3), thus reflecting the genotypic differentiation of both parental taxa and the allopolyploid origin of *L. dufourii*. The combination of both approaches in this study allowed us to score individual genotypes

confidently so that the relative contribution of each parental taxon to the genetic diversity of the resulting triploid *L. dufourii* could be evaluated. However, we note that multilocus genotypic diversity may have been underestimated because these statistics (Table 1) were calculated considering only differences in allele size and a number of multilocus genotypes could have passed undetected because of size homoplasy of microsatellite alleles, being only detectable through allele sequencing (Fig. 3).

The haploid subgenome showed lower diversity values than the diploid subgenome in all populations studied with the only exception being that of El Saler (Table 1). This is not unexpected given that the diploid subgenome contributed double the amount of information at the time of origin of the species, and that it has had twice the chance to accumulate mutations. Nonetheless this same pattern could also indicate that fewer individuals of the haploid genome-donor species were involved in the hybridization process. Torreblanca and Cullera populations present no variation at all in subgenome H (Table 1, Fig. 5a), although four and three multilocus genotypes could be distinguished in subgenome D in these populations (Fig. 4b), respectively. These genotypes were also very different from the rest for this subgenome, suggesting that several independent hybridization events are a more likely explanation for the observed patterns in this species than independent accumulation of mutations at many SSR loci.

A history of large population sizes and gene flow within the Marjal del Moro range could explain the high genotypic diversity found in this area. However, other factors apart from population dynamics must be invoked to explain the co-existence of two groups of divergent molecular genotypes (Marjal del Moro A and B) in the same area (Figs 4 and 5). These two groups are differentiated from each other by a large number of mutational steps (Fig. 5b, c) and these explain most of the genetic variance (88.41%, Table 2). It is unlikely that accelerated mutation rates in these populations could have produced such a pattern at 13 SSR loci simultaneously even though microsatellites are characterized as rapidly mutating markers (Schlötterer 2000). Similar results were previously obtained with RAPD (Palacios & González-Candelas 1997) and AFLP (Palacios *et al*. 1999) markers using the same individuals. A detailed study with a more exhaustive sampling of this area (Rodríguez *et al*. 2003) showed a correlation between the two groups of molecular genotypes and morphological differences. Although this could indicate hybridization events between different parental species, no morphological differentiation in any diagnostic character of *L. dufourii* was found and the assignment of these individuals to *L. dufourii* was undisputed. Therefore, the observed pattern is more likely the result of at least two hybridization events, probably at different evolutionary times, between the same parent species.

Other factors determining the genetic structure of L. dufourii

Limonium dufourii presents a strong population differentiation with a high proportion of the genetic variance distributed among populations (72.06%, Table 2) and significant isolation by distance. This pattern is not unexpected for apomictic taxa and can be explained by restricted gene flow between populations, founder events produced by a limited number of individuals, absence of recombination and spread of single asexual clones within populations (Paun *et al*. 2006). Reduced population sizes and bottlenecks may enhance this genetic pattern, especially in narrowly endemic taxa.

The reduced genotypic diversity found at the distant populations of Torreblanca and Cullera is consistent with a scenario of different founder events through a reduced number of individuals and further divergence of clonal lineages within each population with accumulation of mutations (Paun *et al*. 2006) since the different multilocus genotypes found within these populations are separated by only a few differences (Fig. 5b). Nevertheless, recent genetic impoverishment through population bottlenecks could also have contributed to the observed pattern. Genotypic diversity at El Saler population could be explained by a combination of hybridization producing one of the exclusive genotypes (genotypes 9, 21 and 22 for subgenomes H, D and combined, respectively, Fig. 5) and long-distance dispersal from Marjal del Moro (genotypes 10, 22 and 23, for subgenomes H, D and combined, respectively, Fig. 5). Apparently, recurrent gene flow via seed dispersal is likely to happen only among the three geographically close Marjal del Moro populations since both groups of multilocus genotypes (A and B) include individuals from these three populations (Fig. 5). Although alternative migration routes between populations of *L. dufourii* could be hypothesized on the basis of the genetic relationships between genotypes (Fig. 4), the most likely explanation is that different clonal lineages were formed through several hybridization events given the number of genetic differences separating the five groups of genotypes (Fig. 5b).

These patterns are consistent with the processes generating diversity in apomictic species (Gornall 1999). It seems that multiple hybridization events have had a major role in the generation of genetic diversity in *L. dufourii*. Additional factors such as mutation, migration, and drift have also contributed to shaping the observed pattern of structuring of genetic variation in this species. The observed morphological (Rodríguez *et al*. 2003) and genotypic divergence of individuals from the Marjal del Moro using other genetic markers with a genome-wide distribution (Palacios & González-Candelas 1997; Palacios *et al*. 1999; and this study) could indicate that some hybridization

events occurred at different evolutionary times and were related to adaptive processes. Although migration from other currently extinct populations could also help to explain the observed pattern of genetic differentiation within and among populations, this is more unlikely to occur in triploid *Limonium* taxa that usually occupy very restricted ranges or would result in a greater genetic similarity of individuals from different areas. Finally, it is likely that with changing environmental conditions, the two parental taxa of *L. dufourii* became extinct or were replaced by the more successful colonization ability of *L. dufourii*. Although in this self-incompatible triploid species, the generation of genetic diversity is certainly hampered, it may still benefit from high seed set gained through apomixis and high enzymatic polymorphism and biochemical versatility gained through hybridization. This would indicate that *L. dufourii* could have had higher fitness than its parental species in a stable environment as a consequence of the transmission of co-adapted gene-complexes through clonal reproduction as suggested for other polyploids and apomicts (Soltis & Soltis 2000; López-Pujol *et al*. 2004; Hörandl 2006; Kameyama & Ohara 2006).

Implications for conservation

Apomictic complexes may pose conservation problems because they may include numerous taxa in which morphological differentiation is not always correlated to genetic differentiation therefore causing taxonomic inflation (Pillon & Chase 2007). However, in *Limonium* polyploid apomicts of hybrid origin have received the same attention of conservationists as their sexual diploid endangered counterparts (VV.AA. 2000; Bañares-Baudet *et al*. 2004). Nonetheless, the genetic differentiation of most *Limonium* microspecies has not been studied in detail yet and some of these taxa, after more detailed genetic studies, could finally be shown to be mere phenotypically plastic forms of others. This is certainly not the case of *L. dufourii*, the taxonomic identity of which has been undisputed since its description (Girard 1842).

During the past decade, reduction in the number of populations and decline of population sizes has been documented for *L. dufourii* (Crespo & Laguna 1993). Population declines have resulted from habitat loss because of increasing urban development and the impact tourism. Even if some populations were included in natural parks and other protection categories (such as El Saler and Torreblanca), already demographically depleted populations might not recover from the incurred damages. From the genetic data at hand, it is obvious that none of the populations analysed could restore the genotypic diversity observed in any of the other *L. dufourii* populations. Therefore, habitat protection emerges as the top priority to ensure the *in situ* survival of the species and to prevent population extinction. Once *in situ* conservation is ensured, more refined conservation strategies could be implemented, for which the genetic data reported here will be very helpful.

One of the *ex situ* conservation strategies proposed for *L. dufourii* has been the collection and storage of seeds in germplasm banks for restoration of potential population losses or population reinforcements. Assuming that the collected material could be representative of the genetic diversity of the populations, there is no evidence that re-introductions could successfully strengthen the population genetic status based on the moderate diversity values observed in most populations, although further genetic screenings of populations could be performed in search for rarer genotypes (Table 1). If conservation strategies were aimed at maximizing the within-population genetic diversity, these could focus on two goals. A first goal could be equilibrating the frequencies of multilocus genotypes in the populations. In this sense, re-introduction of genotypes should not be performed at random but after prior genotyping of the re-introduced individuals. At this point, the genetic characterization of *L. dufourii* germplasm emerges as a top priority to evaluate whether the genetic diversity contained in these collections matches that in wild populations. Inadequate sampling schemes providing seeds for long-term storage would result in a biased genotypic representation and therefore would be of little use in the re-establishment of extinct populations. A second goal could be the translocation of individuals with different genotypes between populations. It is unlikely that translocated individuals could interbreed with the existing ones given the species' self-incompatibility. However, potential negative impacts on the populations could derive from a higher adaptive capability of foreign genotypes in the target population than that of the native ones because of the potential presence of co-adapted gene complexes. A better performance of native genotypes than the introduced ones would result in the waste of conservation resources and an overall reduction of population fitness (Gustafson *et al*. 2004), while a better performance of foreign genotypes could result in the displacement of native individuals. Therefore, this alternative scheme would need further research before being applied since it could change the evolutionary trends of the populations in unforeseen ways.

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References

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

Bañares-Baudet A, Blanca G, Güemes-Heras J, Moreno-Saiz JC, Ortiz S (2004) *Atlas y Libro Rojo de la Flora Vascular de España*. Ministerio de Medio Ambiente, Madrid, Spain.

Bayer RJ (1990) Patterns of clonal diversity in the *Antennaria rosea* (Asteraceae) polyploid agamic complex. *American Journal of Botany*, **77**, 1313–1319.

Bayer RJ (1991) Patterns of clonal diversity in geographically marginal populations of *Antennaria rosea* (Asteraceae: Inuleae) from subarctic Alaska and Yukon Territory. *Botanical Gazette*, **152**, 486–493.

Bayer RJ, Minish TM (1993) Isozyme variation, ecology and phytogeography of *Antennaria soliceps* (Asteraceae: Inuleae), an alpine apomict from the Spring mountains, Nevada. *Madroño*, **40**, 75–89.

Boissier E (1848) Plumbaginales. In: *Prodromus Systematis Naturalis Regni Vegetabilis* (ed. de Candolle AP), pp. 617–696. Treuttel et Wurz, Paris, France.

Brochmann C, Brysting AK, Alsos IG *et al*. (2004) Polyploidy in arctic plants. *Biological Journal of the Linnean Society*, **82**, 521–536.

Carino DA, Daehler CC (1999) Genetic variation in an apomictic grass, *Heteropogon contortus*, in the Hawaiian Islands. *Molecular Ecology*, **8**, 2127–2132.

Catalán P, Segarra-Moragues JG, Palop-Esteban M, Moreno C, González-Candelas F (2006) A Bayesian approach for discriminating among alternative inheritance hypotheses in plant polyploids: the allotetraploid origin of genus *Borderea* (Dioscoreaceae). *Genetics*, **172**, 1939–1953.

Chapman HM, Parh D, Oraguzie N (2000) Genetic structure and colonizing success of a clonal, weedy species, *Pilosella officinarum* (Asteraceae). *Heredity*, **84**, 401–409.

Crespo MB (2004) *Limonium dufourii* (Girard) Kuntze. In: *Atlas y Libro Rojo de la Flora Vascular de España* (eds Bañares-Baudet A, Blanca G, Güemes-Heras J, Moreno-Saiz JC, Ortiz S), pp. 352–353. Ministerio de Medio Ambiente, Madrid, Spain.

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

Crespo-Villalba MB, Lledó-Barrena MD (1998) *El Género Limonium en la Comunidad Valenciana*. Consellería de Medio Ambiente, Generalitat Valenciana, Valencia, Spain.

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

Ellstrand NC, Roose ML (1987) Patterns of genotypic diversity in clonal plant species. *American Journal of Botany*, **74**, 123–131.

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

Erben M (1993) *Limonium* Miller. In: *Flora Iberica III Plumbaginaceae (Partim)-Capparaceae* (eds Castroviejo S, Aedo C, Cirujano S *et al*.), pp. 2–143. Real Jardín Botánico. C.S.I.C., Madrid, Spain.

Esselink GD, Nybom H, Vosman B (2004) Assignment of allelic configuration in polyploids using the MAC-PR (microsatellite DNA allele counting-peak ratios) method. *Theoretical and Applied Genetics*, **109**, 402–408.

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.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.

Girard F (1842) Description de quelques espèces nouvelles de Statice appartenant à la flore de France. *Annales Des Sciences Naturelles, Botanique*, **Series 2**, 36.

Gornall RJ (1999) Population genetic structure in agamospermous plants. In: *Molecular Systematics and Plant Evolution* (eds Hollingsworth PM, Bateman RM, Gornall RJ), pp. 118–138. Taylor & Francis, London, UK.

Gustafson DJ, Gibson DJ, Nickrent DL (2004) Competitive relationships of *Andropogon gerardii* (Big Bluestem) from remnant and restored native populations and select cultivated varieties. *Functional Ecology*, **18**, 451–457.

Hörandl E (2006) The complex causality of geographical parthenogenesis. *New Phytologist*, **171**, 525–538.

Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: *Mammalian Protein Metabolism* (ed. Munro HN), pp. 21–132. Academic Press, New York.

Kameyama Y, Ohara M (2006) Genetic structure in aquatic bladderworts: clonal propagation and hybrid perpetuation. *Annals of Botany*, **98**, 1017–1024.

Kumar S, Tamura K, Jakobsen IB, Nei M (2004) MEGA 3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, **5**, 150–163.

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

Langella O (2000) POPULATIONS *(Logiciel de Genétique Des Populations)*. CNRS, Montepellier, France. Available from URL: http://bioinformatics.org/-tryphon/populations.

Lledó MD, Crespo MB, Amo-Marco JB (1993) Preliminary remarks on micropropagation of threatened *Limonium* species (Plumbaginaceae). *Botanical Gardens Micropropagation News*, **1**, 72–74.

Lledó MD, Crespo MB, Fay MF, Chase MW (2005) Molecular phylogenetics of *Limonium* and related genera (Plumbaginaceae): Biogeographical and systematic implications. *American Journal of Botany*, **92**, 1189–1198.

López-Pujol J, Bosch M, Simon J, Blanche C (2004) Allozyme diversity in the tetraploid endemic *Thymus loscosii* (Lamiaceae). *Annals of Botany*, **93**, 323–332.

Mantel NA (1967) The detection of disease clustering and generalized regression approach. *Cancer Research*, **27**, 209–220.

Martín C, Pérez C (1995) Micropropagation of five endemic species of *Limonium* from the Iberian Peninsula. *Journal of Horticultural Science*, **70**, 97–103.

Nei M, Tajima F, Tateno Y (1983) Accuracy of estimated phylogenetic trees from molecular data. *Journal of Molecular Evolution*, **19**, 153–170.

Nybom H, Esselink GD, Werlemark G, Leus L, Vosman B (2006) Unique genomic configuration revealed by microsatellite DNA in polyploidy dogroses, *Rosa* sect. *Caninae*. *Journal of Evolutionary Biology*, **19**, 635–648.

Ozias-Akins P (2006) Apomixis: developmental characteristics and genetics. *Critical Reviews in Plant Sciences*, **25**, 199–214.

Palacios C, González-Candelas F (1997) Analysis of population genetic structure and variability using RAPD markers in the endemic and endangered *Limonium dufourii* (Plumbaginaceae). *Molecular Ecology*, **6**, 1107–1121.

Palacios C, Kresovich S, González-Candelas F (1999) A population genetic study of the endangered plant species *Limonium dufourii* (Plumbaginaceae) based on amplified fragment length polymorphism (AFLP). *Molecular Ecology*, **8**, 645–657.

Palacios C, Rosselló JA, González-Candelas F (2000) Study of the evolutionary relationships among *Limonium* species (Plumbaginaceae) using nuclear and cytoplasmic molecular markers. *Molecular Phylogenetics and Evolution*, **14**, 232–249.

Palop M, Palacios C, González-Candelas F (2000) Development and across-species transferability of microsatellite markers in the genus *Limonium* (Plumbaginaceae). *Conservation Genetics*, **1**, 177–179.

Palop-Esteban M, González-Candelas F (2002) Development of microsatellite markers for the critically endangered *Limonium dufourii* (Girard) Kuntze (Plumbaginaceae). *Molecular Ecology Notes*, **2**, 521–523.

Paschke M, Abstract C, Schmid B (2002) Relationship between population size, allozyme variation, and plant performance in the narrow endemic *Cochlearia bavarica*. *Conservation Genetics*, **3**, 131–144.

Paun O, Hörandl E (2006) Evolution of hypervariable microsatellites in apomictic polyploid lineages of *Ranunculus carpaticola*: directional bias at dinucleotide loci. *Genetics*, **174**, 387–398.

Paun O, Greilhuber J, Temsch EM, Hörandl E (2006) Patterns, sources and ecological implications of clonal diversity in apomictic *Ranunculus carpaticola* (*Ranunculus auricomus* complex, Ranunculaceae). *Molecular Ecology*, **15**, 897–910.

Pillon Y, Chase MW (2007) Taxonomic exaggeration and its effects on orchid conservation. *Conservation Biology*, **21**, 263–265.

Pluess AR, Stöcklin J (2004) Genetic diversity and fitness in *Scabiosa columbaria* in the Swiss Jura in relation to population size. *Conservation Genetics*, **5**, 145–156.

Richards AJ (2003) Apomixis in flowering plants: an overview. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **358**, 1085–1093.

Robertson A, Newton AC, Ennos RA (2004) Multiple hybrid origins, genetic diversity and population genetic structure of two endemic *Sorbus taxa* on the Isle of Arran, Scotland. *Molecular Ecology*, **13**, 123–134.

Rodríguez S, Palop ML, Palacios C, González-Candelas F (2003) Molecular and morphological differentiation in *Limonium dufourii* (Plumbaginaceae), an endangered Mediterranean plant. *Conservation Genetics*, **4**, 383–391.

Rohlf FJ (2002) NTSYSPC, *Numerical Taxonomy and Multivariate Analysis System, Version 2.11a, User Guide*, p. 38. Exeter software, New York.

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

Schlötterer C (2000) Evolutionary dynamics of microsatellite DNA. *Chromosoma*, **109**, 365–371.

Schneider S, Roessli D, Excoffier L (2000) ARLEQUIN *V. 2.000: A Software for Population Genetics Data Analysis*. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Schranz ME, Dobes C, Koch MA, Mitchell-Olds T (2005) Sexual reproduction, hybridization, apomixis, and polyploidization in the genus *Boechera* (Brassicaceae). *American Journal of Botany*, **92**, 1797–1810.

Scotti I, Magni F, Paglia GP, Morgante M (2002) Trinucleotide microsatellites in Norway spruce (*Picea abies*): their features and the development of molecular markers. *Theoretical and Applied Genetics*, **106**, 40–50.

Soltis DE, Soltis PS (2000) The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences, USA*, **97**, 7051–7057.

Stebbins GL (1971) *Chromosomal Evolution in Higher Plants*. Arnold, London.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **24**, 4876–4882.

Van der Hulst RGM, Mes THM, den Nijs JCM, Bachmann K (2000) Amplified fragment length polymorphism (AFLP) markers reveal that population structure of triploid dandelions (*Taraxacum officinale*) exhibits both clonality and recombination. *Molecular Ecology*, **9**, 1–8.

Van der Hulst RGM, Mes THM, Falque M, Stam P, Den Nijs JCM, Bachmann K (2003) Genetic structure of a population sample of apomictic dandelions. *Heredity*, **90**, 326–335.

Van Dijk PJ (2003) Ecological and evolutionary opportunities of apomixis: insights from *Taraxacum* and *Chondrilla*. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **358**, 1113–1121.

Van Dijk PJ, van Damme J (2000) Apomixis technology and the paradox of sex. *Trends in Plant Science*, **5**, 81–84.

Van Dijk PJ, Vijverberg K (2005) The significance of apomixis in the evolution of angiosperms: a reappraisal. In: *Plant Species-Level Systematics: New Perspectives on Pattern and Process* (eds Bakker F, Chatroou L, Gravendeel B, Pelser PB), pp. 101–106. Gantner Verlag, Ruggell, Liechtenstein.

VVAA (2000) Lista roja de la flora vascular Española (valoración según categorías UICN). *Conservación Vegetal*, **6** (Suppl.), 11–38.

This work is part of a larger study aimed at understanding the mechanisms involved in the generation and maintenance of genetic variation in highly endemic and endangered species of *Limonium* and is part of the Ph.D. thesis of M. Palop-Esteban under the supervision of F. González-Candelas. J.G. Segarra-Moragues is interested in the application of molecular tools to understand the evolution of plant populations and to assist conservation activities in priority species. The research of F. González-Candelas is focused in understanding the evolutionary processes of population genetics and genomic evolution of a variety of organisms including plants, insects, bacteria and viruses.
