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Polyploid origin, genetic diversity and population structure in the tetraploid sea lavender *Limonium narbonense* Miller (Plumbaginaceae) from eastern Spain

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Abstract *Limonium narbonense* Miller is a fertile tetraploid species with a sporophytic self-incompatibility system. This sea lavender is found in coastal salt marshes which have been under intense human pressure during the past decades resulting in significant habitat fragmentation. Eleven microsatellite loci specifically designed for this species were amplified in 135 individuals from five populations. These markers were used to investigate the polyploid nature, the levels of genetic diversity and population structure in this species. *L. narbonense* showed high levels of genetic diversity ($A = 7.82$, $P = 100\%$ $H_T = 0.446$), consistent with its likely autotetraploid origin revealed in this study and obligate outcrossing breeding system. Inbreeding (F_{IS}) values were low in the three southern populations (mean $F_{IS} = 0.062$), and higher in the northern populations (mean $F_{IS} = 0.184$). Bayesian analysis of population structure revealed that populations could be grouped into two genetic clusters, one including three southern populations and the other the two northernmost ones. Individuals from the two northernmost populations showed higher admixture of the two genetic clusters than individuals from the three southern ones. A thorough analysis of microsatellite electrophoretic patterns suggests an autotetraploid origin for *L. narbonense*. The genetic structure revealed in this study is attributed to a recent migration from the southern area. This result suggests a net gene flow from the south to the north, likely facilitated by migratory movements of birds visiting the temporary flooded ponds occupied by *L. narbonense*.

Keywords Autopolyploids · Genetic diversity · *Limonium* · Microsatellites · Pollen dimorphism · Plumbaginaceae · Self-incompatibility · SSR

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Introduction

Limonium Miller, sea lavenders, of the Plumbaginaceae is an example of a cosmopolitan genus with high diversification associated to saline habitats, including inland salt-steppe and gypsum soils and coastal cliffs, rocky and sandy seashores and salt marshes (Dolcher and Pignatti 1971; Erben 1993). It includes more than 150 species and two centres of diversification, the Asian steppes and the coastal habitats of the western Mediterranean (Dolcher and Pignatti, 1971; Erben 1979).

The reproductive biology of sea lavenders has been extensively studied (Baker 1948, 1953a, 1966; Bokhari 1971). *Limonium* species are known to have heteromorphic sporophytic self-incompatibility (SI) (Baker 1966). Generally, species showing stylar dimorphism (cob/papillate) have also pollen dimorphism (A, wide reticulum/B, narrow reticulum pollen types). In these species, individuals having papillose stigmata produce type B pollen and individuals having cob stigmata produce type A pollen (Bokhari 1971). For successful pollination, papillose stigmata require type A pollen, whereas cob stigmata require type B pollen, hence making them obligate outcrossers. In sexual species both morphotypes usually occur in similar ratios within populations, i.e. 50% (Erben 1979).

A reticulate diversification model involving changes in ploidy levels has been proposed for this genus, and is thought to be responsible for its high taxonomic complexity (Palacios et al. 2000; Lledó et al. 2005). Typically, *Limonium* taxa have two basic chromosome numbers $x_1 = 9$ ($2n = 2x_1 = 18$) and $x_2 = 8$ ($2n = 2x_2 = 16$). The latter results from a chromosomal translocation, producing a longer marker chromosome that allows tracking this genome in other *Limonium* taxa (Erben 1979; Castro and Rosselló 2007).

Triploid *Limonium* species are predominant in the genus and arise through hybridization (allopolyploids) of the two diploids types which supply the resulting hybrids with reduced and unreduced gametes ($2n = 3x_2 = 24$; $2x_2 + x_1 = 25$; $x_2 + 2x_1 = 26$ and $3x_1 = 27$). These taxa of hybrid origin usually form monomorphic populations with regards to the SI systems (A/cob or B/papillate) and have strong pollen sterility because of abnormal meiosis. In such species reproduction is via apomixis (Baker 1953a; Erben 1979) which reduces the possibility of generation of novel genetic variation after hybridization (Palop-Esteban et al. 2007).

Although in some of these taxa the absence of sexuality may pose a problem for gaining new genetic diversity (Palacios and González-Candelas 1997a, b 1999; Palop et al. 2000; Palop-Esteban et al. 2007), they may still benefit from higher seed-set through apomixis and higher molecular variability than their separate diploid counterparts due to their hybrid origin.

Higher-ploidy taxa also occur in *Limonium* including tetraploids, pentaploids and hexaploids. However, they are less frequent than triploids (Erben 1978, 1979). Tetraploids are the most abundant class among the higher-ploidy taxa (Erben 1978, 1993; Castro and Rosselló 2007).

Tetraploids in *Limonium* represent an interesting study case for the investigation of the processes leading to tetraploidy and their consequences in reproductive biology, genetic diversity and the final evolutionary outcome of the resulting taxa. Tetraploid *Limonium* include taxa with different chromosomal configurations, from simple duplications of the typical diploid counts ($2n = 4x_1 = 36$ and $2n = 4x_2 = 32$) to balanced tetraploids, i.e. consisting in the combination of the two diploid basic types ($2n = 2x_1 + 2x_2 = 34$) and unbalanced tetraploids, i.e. having three sets from one basic chromosome number and one set from another ($2n = 3x_1 + x_2 = 35$ and $2n = x_1 + 3x_2 = 33$), that correspond to allopolyploids (Erben 1979). Whereas the hybrid origin of unbalanced tetraploids is easily confirmed through karyotype analysis, in typical tetraploids and balanced tetraploids an autopolyploid or allopolyploid origin could be hypothesized. Hence, typical tetraploids and balanced tetraploids consist of dimorphic populations according to SI breeding types and have high male fertility. In consequence, sexual reproduction is not prevented whereas unbalanced tetraploids have high male sterility and reproduce exclusively via apomixis, as do as triploids (Erben 1979).

Limonium narbonense Miller, is a perennial rossulate chamephyte with broad leaves and racemose, showy inflorescences with purplish blue flowers actively visited by bees and butterflies (Palop-Esteban and Segarra-Moragues, personal observation). It is a tetraploid species with $2n = 4x_1 = 36$ chromosomes (Erben 1978, 1993; Brullo and Pavone 1981; Crespo-Villalba and Lledó-Barrena 1998). It is an obligate outcrosser due to the sporophytic self-incompatibility system described above (Erben 1979). For these reasons this species is presumed to be an autotetraploid in origin. Nonetheless, an allotetraploid origin cannot be ruled out solely by the presence of sexuality (note above that most *Limonium* allopolyploids are sterile), especially if hybridization occurred between two species with similar karyotypes.

Population genetic studies in polyploid species using codominant markers are hindered by the difficulty in scoring confidently polyploid genotypes in many individuals. Consequently, there is a predominance of genetic studies of diploid species in the literature, despite the wide incidence of polyploidy in angiosperms (Soltis and Soltis 1999, 2000; Otto and Whitton 2000). Studies using codominant markers, such as allozymes or microsatellites, often analyse genetic patterns as dominant markers (Pérez-Collazos and Catalán 2006), resulting in significant loss of information from these markers. From such analyses, testing hypotheses about the autopolyploid or allopolyploid origin of a taxon is challenging. Similarly, the levels of genetic diversity, which depend not only on the presence/absence of alleles but also on their frequencies can, to some extent, be imperfectly estimated. Nonetheless, several studies based on allozymes have inferred tetraploid genotypes based on the intensity of bands in the zymograms (López-Pujol et al. 2004, 2007) and novel analytical frameworks for microsatellite markers have been developed allowing the estimation of the number of allele copies and, consequently, individual genotypes in polyploid species (Esselink et al. 2004; Luo et al. 2006; Obbard et al. 2006; Liu et al. 2007). Some analytical methods have also implemented the modification of population genetic statistics to accommodate genomic attributes of polyploids and their different genetic behaviours (i.e. disomic inheritance in allopolyploids vs. polysomic inheritance and double reduction of autopolyploids) into different software packages (Ronfort et al. 1998; Pritchard et al. 2000; Thrall and Young 2000; Catalán et al. 2006; Luo et al. 2006; Markwith et al. 2006; Tomiuk et al. 2009; Clark and Jasieniuk 2011).

This study was specifically focused at (i) deciphering the origin of the tetraploidy in *L. narbonense* (i.e. auto vs.

allotetraploidy) through the investigation of microsatellite amplification patterns, (ii) describing levels of genetic diversity and population structure in the remnant fragmented populations in the eastern Iberian Peninsula, and (iii) comparing the genetic patterns of *L. narbonense* with other species of *Limonium* of the same geographical area to correlate differences in genetic patterns with variation in reproductive traits and habitat fragmentation.

For this purpose we used highly polymorphic nuclear microsatellite markers (SSR). Microsatellites have become the marker of choice for population genetics studies because individuals are likely to present more SSR alleles at any locus than allozymes due to the generally lower levels of polymorphism in these markers resulting from functional constraints.

We expected that, because of the presence of a SI breeding system, sexual reproduction and polyploidy, *L. narbonense* would show high levels of genetic diversity in spite of habitat fragmentation and population isolation. In general polyploid species are less sensitive to genetic erosion because of their higher capability to accumulate genetic diversity in their multiple chromosome sets, create alternative gene functions through loci duplication and lower sensitivity to the effects of deleterious alleles (Comai 2005). Nonetheless, given that *L. narbonense* is dependent on the presence of both reproductive morphotypes, population bottlenecks can have severe impacts on population dynamics and on the levels of genetic diversity, putting population persistence at risk.

Materials and methods

Plant material and microsatellite amplification

Limonium narbonense has a spotted distribution in the Mediterranean coast where it is linked to coastal salt marshes –Juncion maritimi, *Salicornietea*– (Boorman 1971; Pandža et al. 2007). It is ecologically specialized, occupying only a narrow coastal line in temporary flooded salt marshes behind the primary dune system. The habitat of *L. narbonenese* has been severely impacted in the eastern coast of the Iberian Peninsula because of human activities resulting in both habitat fragmentation and reduction of population sizes. The introduction of rice crops, building of coastal urbanizations and touristic resorts have contributed significantly to the fragmentation of coastal habitats. Some habitat transformations involved the desiccation of marshes to increase the agricultural or urban land area and to prevent insect (especially mosquitoes) proliferation in their surroundings.

One hundred and thirty-five individuals were sampled from five populations of *L. narbonense* (Fig. 1, Table 1).

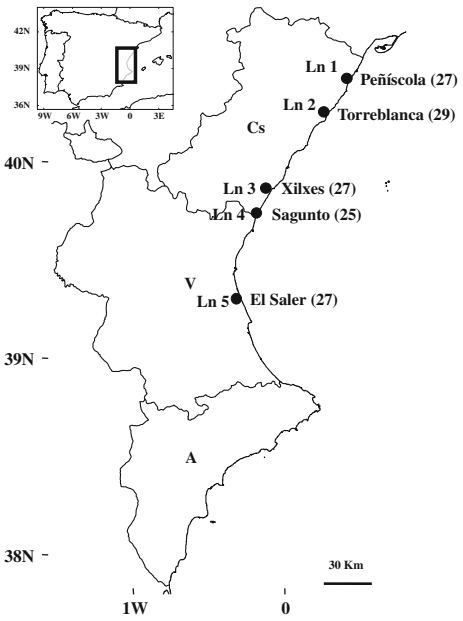

Fig. 1 Sampled populations of *Limonium narbonense*. A, V and Cs represent Alicante, Valencia and Castellón provinces, respectively

Fresh leaves were used as material for DNA extraction following the CTAB protocol of Doyle and Doyle (1990). DNA quality and quantity was estimated by electrophoresis in 0.5 × TBE 0.8% agarose gels and diluted to a final concentration of 10 ng/μl.

Eleven microsatellite loci specifically developed for this species (Palop et al. 2000) were used for the screening of genetic diversity. The microsatellite loci were combined for simultaneous (multiplex) amplification and analysis into 4 groups: Ln039, Ln045, Ln122 and Ln162, group I; Ln036, Ln044 and Ln149, group II; Ln041, Ln068 and Ln115, group III and Ln052, group IV. The PCR cocktail (25 μl) included 1 × buffer (Pharmacia), supplemented with 1–2 mM $MgCl_2$ depending on the multiplex grouping, 0.2 mM each dNTP, 0.5 U *Taq* DNA polymerase (Pharmacia), 2–6 pmol of each primer (depending on locus) and 20–30 ng genomic DNA as template. The PCR program consisted of one step of 4 min at 95°C for DNA melting, followed by 25 cycles each of 1 min at 95°C, 2 min at 55°C for annealing and 2 min at 72°C for extension. A final step of 72°C for 10 min was added to complete the extension of PCR products after which the reactions were kept at 4°C.

All PCRs were carried out in a PTC-100 thermal cycler (MJ Research). Allele sizing was carried out by automated fluorescent scanning detection in an ABI PRISM 377 DNA sequencer (Applied Biosystems) using ROX500 as internal lane size standard, and the software GENESCAN and GENOTYPER (Applied Biosystems). Allele sizes were converted into repeat units for further analyses taking into account the fragment size, the number of repeats and the

Table 1 Summary of genetic variability statistics for 11 microsatellite loci in five populations of *Limonium narbonense*

Population	Pop. size	N	A	A_i	P	H_O	$H_{E(ce)}$	$F_{(ce)}$	$H_{E(cd)}$	$F_{(cd)}$
Peñíscola	500	27	4.73 ± 3.41	1.85 ± 0.64	90.91	0.391 ± 0.253	0.478 ± 0.313	0.182 ± 0.149	0.446 ± 0.292	0.124 ± 0.139
Torreblanca	>1,000	29	5.64 ± 4.30	1.86 ± 0.53	90.91	0.412 ± 0.226	0.506 ± 0.267	0.186 ± 0.146	0.473 ± 0.249	0.128 ± 0.139
Xilxes	300	27	5.09 ± 3.05	1.94 ± 0.55	100	0.450 ± 0.219	0.480 ± 0.233	0.062 ± 0.063	0.448 ± 0.217	−0.005 ± 0.059
Marjal del Moro	200	25	4.82 ± 3.06	2.00 ± 0.63	100	0.459 ± 0.235	0.486 ± 0.215	0.055 ± 0.096	0.453 ± 0.201	−0.012 ± 0.097
Saler	200	27	5.55 ± 3.93	2.13 ± 0.58	100	0.519 ± 0.196	0.558 ± 0.222	0.070 ± 0.086	0.521 ± 0.207	0.004 ± 0.080
Total		135	7.82 ± 5.83	1.95 ± 0.50	100	0.446 ± 0.188	0.544 ± 0.245	0.180 ± 0.120	0.507 ± 0.228	0.122 ± 0.110

Pop. size, Estimated population size; N, sample size; A, mean number of alleles per locus; A_i, mean number of different alleles per individual and locus; P, proportion of polymorphic loci (at least two alleles per locus); H_O, observed heterozygosity; H_E, expected heterozygosity assuming random mating and random chromosomal segregation; $H_{E(ce)}$, and assuming random mating and non random chromatid segregation; $H_{E(cd)}$, i.e. a double reduction frequency of $\alpha = 1/7$ (Wrike and Weber 1986); F, Fixation indices under both random chromosomal segregation, $F_{(ce)}$, and non-random chromatid segregation, $F_{(cd)}$

microsatellite motif in the sequenced clone (Palop et al. 2000).

Genetic analyses of SSR data

Individual tetraploid genotypes were scored from microsatellite banding patterns in the electropherograms following the Microsatellite DNA Allele Counting-Peak Ratios (MAC-PR) method of Esselink et al. (2004). This method uses quantitative values for microsatellite allele amplification peak areas provided by the sizing software. For each locus, all alleles were analyzed in pairwise combinations to determine their dosages in the individual samples by calculating the ratios between peak areas for all allele-pairs that were amplified simultaneously. In tetraploids such as *L. narbonense*, conflictive phenotypes from which genotypes are most difficult to estimate are those of individuals having two bands (i.e. likely genotypes are ABBB, AABB and AAAB) or three bands (i.e. likely genotypes are AABC, ABBC and ABCC). We considered individuals with the maximum number of alleles (4) as a baseline with all pairwise comparisons of peak ratios equalling 1:1 in order to compensate for possible smaller amplification areas of larger-sized alleles (Esselink et al. 2004). In this manner genotypes could be reliably recorded by comparing observed pairwise peak ratios to the expected hypothetical configurations (see Esselink et al. 2004 for a full description of the procedure).

Encoding polyploid genotypes is potentially sensitive to the presence of null alleles. Unlike for diploids, there is currently no software available to test for the presence of null alleles in a population. In our case we have no empirical evidence for their presence in *L. narbonense* because (i) we have not detected any failure of amplification at any of the loci, which could be interpreted as a null homozygote, and (ii) triallelic individuals always showed one of their three simultaneously amplified alleles (i.e. AABC, ABBC and ABCC) with double the intensity than the others (Fig. 2). Should any of these individuals had had one null allele, the three amplification products would have shown the same intensity (i.e. ABC + 0). This is probably due to this set of microsatellites having been specifically designed for this species because, usually, the frequency of null alleles rapidly increases when SSR primers are transferred to other species (Li et al. 2003). Although we cannot totally discard the presence of null alleles by this procedure, it is likely that if present they should be in very low frequencies so that their effect on genetic parameters would be minimal.

From our detailed analysis of amplification patterns in *L. narbonense* we found no evidence favouring allotetraploidy over autotetraploidy (see results) and consequently, all further analyses were conducted assuming autopolyploidy.

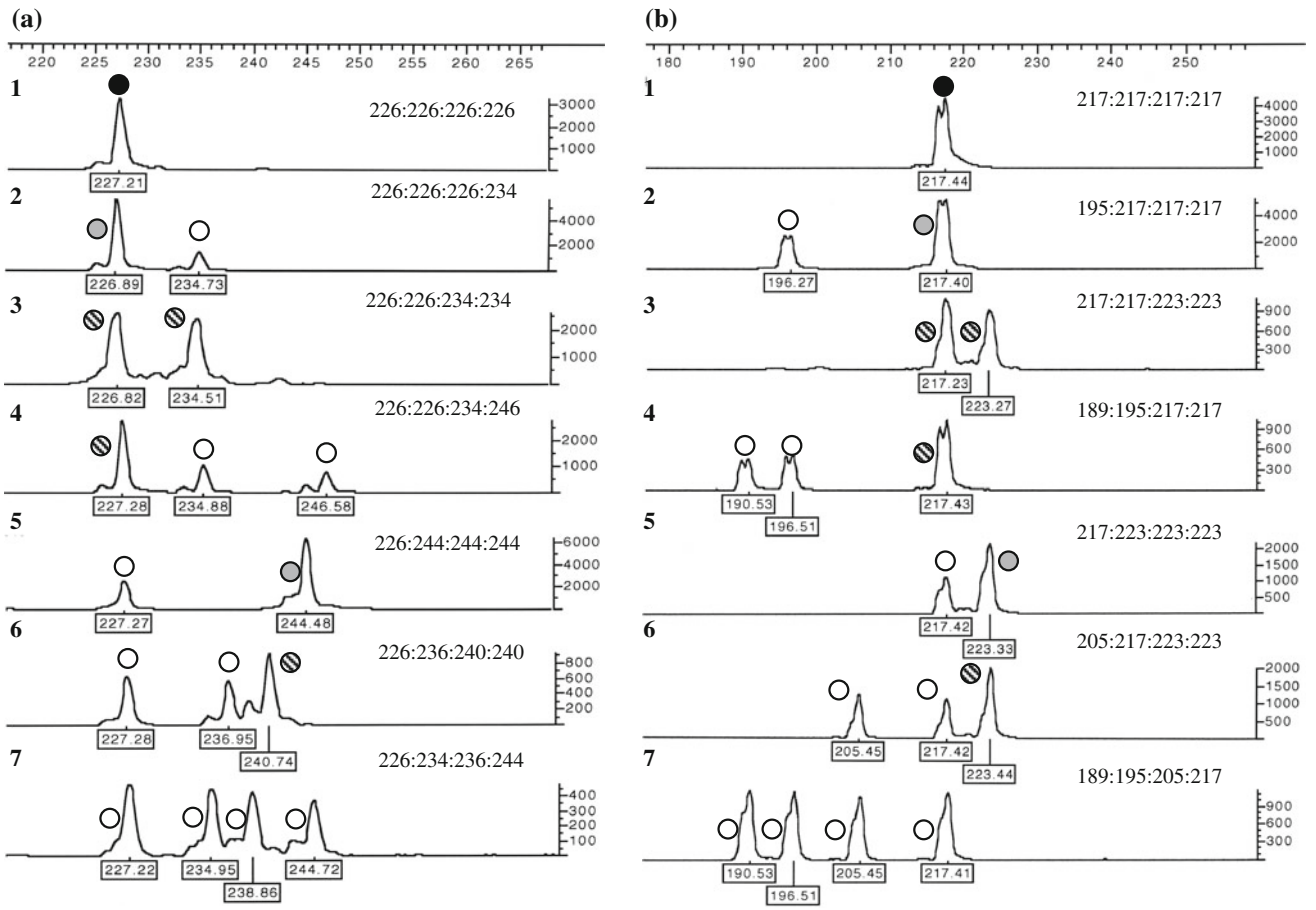

Fig. 2 Sample electropherograms of two microsatellite loci in *Limonium narbonense* showing different genotypic patterns. **a** Ln036; **b**. Ln044. Tetraploid genotypes (alleles designated in bp) were inferred from pairwise comparisons of amplification peak ratios of the different alleles present in an individual (Esselink et al., 2004). Alleles 226 and 217 were selected in loci Ln036 and Ln044, respectively to trace the change in the number of allele copies: *black circles* represent four copies; *grey* ones, three copies; patterned ones, two copies; and *white* ones, one copy

Allelic richness (A, the number of alleles at a locus) and allelic richness within individuals (A_I, the average number of alleles per individual at a locus), and observed heterozygosities (H_O) were calculated using AUTOTET (Thrall and Young 2000). Chromatid segregation is produced if sister chromatids segregate into the same gamete (i.e. double reduction), a phenomenon specific to autopolyploids and which is dependent on the amount of tetravalent formation and on the proximity of the locus to the centromere (Ronfort et al. 1998). As it is unknown if double reduction occurs in *L. narbonense*, expected heterozygosities were calculated considering both random mating and random chromosomal segregation ($H_{E(Ce)}$) and random mating and some level of chromatid segregation (i.e. the default value of maximum double reduction where $\alpha = 1/7$; Thrall and Young 2000), $H_{E(Cd)}$. Similarly, fixation indices were calculated assuming random chromosomal segregation ($F_{(Ce)}$) and non-random (i.e. $\alpha = 1/7$) chromatid segregation ($F_{(Cd)}$) using AUTOTET (Thrall and Young 2000). The proportion of polymorphic loci was calculated directly from the data by considering the fraction of loci with at least two alleles typed in a population over the total number of loci. Linkage disequilibrium (LD) among loci was assessed using LD4X (Julier 2009) and significance values were adjusted after applying Bonferroni's correction.

Population structure was analyzed by three different methods: first, the Bayesian approach implemented in STRUCTURE v. 2.1 (Pritchard et al. 2000) was used. This approach implements a clustering method to assign individuals and predefined populations to K inferred clusters each characterized by a set of allele frequencies at Hardy–Weinberg equilibrium, and to calculate the corresponding probabilities of membership to each group. Our analyses were based on an admixture ancestral model with correlated allele frequencies for a range of K values starting from one to seven. We used a burn-in and a run length of the Monte Carlo Markov Chain (MCMC) of 10^5 and 10^6

iterations, respectively. Twenty replicates of the analysis were conducted for each K value. We followed the guidelines of Evanno et al. (2005) to estimate of the optimal number of clusters. Second, global and pairwise ρ values, an analogue to F_{ST} specifically implemented for autotetraploid taxa, were computed using GENE4X (Ronfort et al. 1998, provided by the author). For autotetraploids ρ provides a more robust estimate for population differentiation than F_{ST} due to its lower sensitivity to selfing and the occurrence of double reduction (Ronfort et al. 1998). Significance of ρ values was tested with Fisher exact tests and Monte Carlo Markov Chain (MCMC) simulations using the default values of GENE4X. Third, a matrix of pairwise genetic distances (D_A, Nei et al. 1983) between individuals was computed with POPULATIONS v. 1.2 (Langella 2000). This distance matrix served as input to conduct Analyses of Molecular Variance (AMOVA, Excoffier et al. 1992) with ARLEQUIN v. 3.5 (Excoffier and Lischer 2010). AMOVA were conducted for *L. narbonense s.l.*, and at hierarchical levels according to the K optimal genetic clusters detected with STRUCTURE: between genetic clusters, among populations within genetic clusters, and within populations. In all instances, the significance of the variance components was obtained using 1000 permutations.

Isolation by distance was assessed by the correlation between the matrix of pairwise genetic distances (D_A Nei et al. 1983) obtained with POPULATIONS and the matrix of pairwise geographic distances between populations. Mantel tests based on one thousand permutations were performed with NTSYSpc v. 2.11a (Rohlf 2002).

Results

Eighty-six microsatellite alleles were scored from 11 amplified loci in 135 individuals of *L. narbonense* ("Appendix"). The number of amplified alleles per locus ranged from two alleles in the least polymorphic locus (Ln045) to 21 in the most polymorphic one (Ln036, see Appendix) with an average of 7.82 alleles per locus (Table 1).

Microsatellite amplification patterns were consistent with the tetraploid nature of *L. narbonense*, ranging from a minimum of one to a maximum of four alleles per amplified locus and individual (Fig. 2). No fixed heterozygous patterns were observed at any microsatellite locus in the 135 individuals analysed of *L. narbonense*. In these and also in the least polymorphic loci (amplifying up to 3 alleles in the 135 individuals) we identified both homozygous and heterozygous individuals. Furthermore, heterozygous individuals belonged to two different possible classes: balanced (i.e. AABB) and unbalanced heterozygotes (i.e. ABBB or AAAB). Similarly, the most polymorphic microsatellite loci were also consistent with this pattern and revealed all possible allelic combinations expected for an autopolyploid species with polysomic segregation (Fig. 2). We did not find fixed heterozigosity patterns as expected for allopolyploids.

Genetic diversity estimates were similar across the five studied populations. The number of alleles per locus and population ranged from 4.73 to 5.64 in the populations of Peñíscola and Torreblanca, respectively. The proportion of polymorphic loci was higher in the three southern populations. The observed heterozygosity ranged from 0.391 in the northernmost population of Peñíscola to 0.519 in the southernmost population of El Saler (Table 1).

Bayesian analyses of population structure of *L. narbonense* populations conducted with STRUCTURE (Fig. 3a–c) revealed a maximum ΔK value for two genetic clusters (Fig. 3b). Individuals from the northernmost populations of Peñíscola showed a high proportion of membership to cluster 1 whereas the individuals from the three southern populations (Xilxes, Sagunto and El Saler) showed a high proportion of membership to cluster 2 (Fig 3a). Individuals from the northern population of Torreblanca showed somewhat intermediate values of membership to clusters 1 and 2, although their proportion of membership was usually higher for cluster 1. The mean proportion of membership of populations to these two clusters was consistently high for cluster 1 (Peñíscola and Torreblanca) or cluster 2 (Xilxes, Sagunto and El Saler); nonetheless, the Torreblanca population also showed a moderate probability of membership to cluster 2, thus indicating that some of its individuals showed mixed origins, sharing some similarity to the southern genetic cluster, which could result from a net gene flow from South to North (Fig. 3c).

Genetic structure statistics revealed that most of the genetic variation was distributed within populations (79.25%) with a lower but significantly different from zero proportion of variation among populations ($\rho = 0.2075$, $p < 0.001$ calculated considering the statistic specifically implemented for autotetraploid species). The global F_{ST} value was 0.082. The proportion of variation explaining the differences between the two genetic clusters obtained in the STRUCTURE analysis was also low ($\rho = 0.096$), but significantly different from zero ($p < 0.001$).

Non-hierarchical AMOVA analyses provided similar results (Table 2) with most of the variance distributed within populations (81.18%) and a lower, but significantly different from zero ($p < 0.001$) proportion among populations (18.82%). Hierarchical AMOVA conducted with the predefined grouping of populations according to their genetic membership detected by STRUCTURE analyses also revealed a lower but significantly different from zero

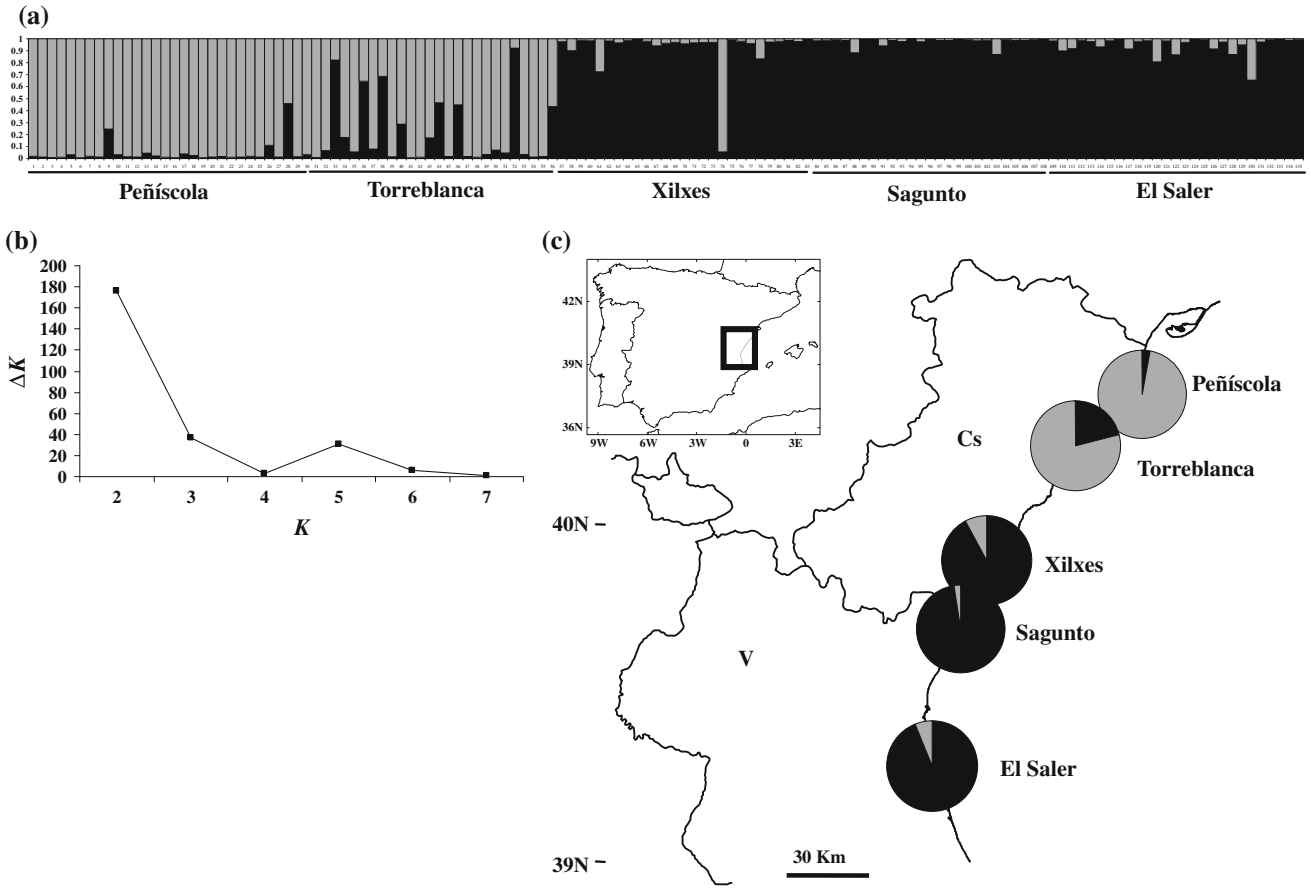

Fig. 3 Bayesian analysis of population genetic structure in *Limonium narbonense*. **a**. Proportion of membership for the 135 analyzed individuals from 5 populations to the two genetic clusters inferred with STRUCTURE. **b**. Magnitude of ΔK as a function of the range of K tested to infer the number of genetic clusters; the selected value of K is two. **c**. Mean proportion of membership of each studied population to the two genetic clusters inferred with STRUCTURE. *Grey shading*, cluster 1; *black shading*, cluster 2

Table 2 Analyses of molecular variance (AMOVA) of *Limonium narbonense* populations

Source of variation (groups)	Sum of squared deviations (SSD)	d.f.	Variance components	% of the total variance
1. *Limonium narbonense* s.l.				
Among populations	4.177	4	0.03336	18.82
Within populations	18.705	130	0.14389	81.18
2. *Limonium narbonense* genetic membership: Two clusters of STRUCTURE analysis; cluster 1 (Saler, Marjal del Moro, and Xilxes) *vs*. cluster 2 (Peñíscola and Torreblanca)				
Among clusters	1.242	1	0.00379	2.12
Among populations within clusters	2.935	3	0.03106	17.38
Within populations	18.705	130	0.14389	80.50

($p < 0.001$) differentiation between genetic clusters (2.12%), a higher proportion of variance among populations within genetic clusters (17.38%) and a larger amount of the genetic variance being distributed within populations (80.50%, Table 2).

Mantel tests revealed a moderate but significant correlation between genetic (D_A) and geographical distances ($r = 0.683$, $p < 0.01$) indicating that these populations show isolation by distance (Fig. 4).

Discussion

The polyploid nature of *Limonium narbonense*

Tracing back the origin of a polyploid species may involve diverse methods including chromosome fluorescent mapping of target DNA loci (FISH, Pires et al. 2004), genomic in situ DNA hybridization (GISH, Chase et al. 2003; Ran et al. 2001; Lim et al. 2007) or the study of progeny arrays to evaluate allele segregation with codominant markers such as allozymes or microsatellites (Olson 1997; Hardy et al. 2001; Scarcelli et al. 2005; Bousalem et al. 2006).

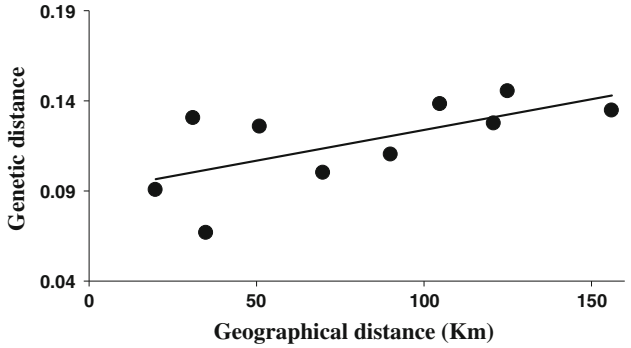

Fig. 4 Isolation by distance analyses. Correlations were performed between geographical distances in Km (x-axis) and D_A (Nei et al. 1983) genetic distance (y-axis). Significance of the correlation was assessed through 1,000 permutation Mantel tests. Correlation between matrices was $r = 0.683$, $p < 0.01$

Recently, electrophoretically detectable variation in individuals collected from wild populations has also been used for this purpose, based on the different patterns expected under the auto- and allopolyploidy scenarios (Catalán et al. 2006; López-Pujol et al. 2007) provided that molecular markers are variable enough to reveal as many alleles as expected for the ploidy level of the species in some loci and individuals (Kholina et al. 2004).

Species of sea lavenders with $2n = 36$ chromosomes, such as *L. narbonense*, have been hypothesized to be tetraploids of the basic chromosome number $x_1 = 9$. Polyploid *Limonium* taxa derived from species with a basic chromosome number $x_2 = 8$ can be identified by a chromosome number in factor of eight and the presence of longer marker chromosomes (Erben 1979), the number of these marker chromosomes equalling the number of $x_2 = 8$ subgenomes present in the polyploid. The karyotype of *L. narbonense* lacks these long chromosomes and is composed of four sets of 9 homologous chromosomes. A hybrid allopolyploid origin between species of different chromosome base numbers can be discarded based on chromosome number and morphology (Erben 1979; Castro and Rosselló 2007). However, this does not imply directly autopolyploidy nor rules out a possible hybrid (allopolyploid) origin for the species, since hybridization could have occurred between parents with similar ($x_1 = 9$) karyotypes.

Based on morphological and karyological grounds it seems unlikely that *L. narbonense* could have arisen from hybridization between any of the extant *Limonium* species present in the area (Erben 1993; Crespo-Villalba and Lledó-Barrena 1998). Besides, *L. narbonense* and the morphologically (Erben 1993) and phylogenetically (Palacios et al. 2000; Lledó et al. 2005) closest species, *L. vulgare*, do not share distribution areas, making hybridization between them highly unlikely in this area. These two species, formerly classified in subsection *Genuinae* (Boissier 1848), conform a monophyletic group separate from the vast majority of the species included in section *Limonium* (Palacios et al. 2000) and whose closest relatives are present in North America (Baker 1953b). Poor microsatellite transferability results also support the large phylogenetic distance from these two to other *Limonium* species (Palop et al. 2000). Therefore, if other unrelated *Limonium* species would have been involved in the origin of *L. narbonense*, microsatellite amplification patterns should have revealed a high proportion of null alleles, due to the amplification of only one subgenome (Palop-Esteban et al. 2007); fixed banding profiles, due to the absence of recombination between heterologous chromosome pairs; or absence of allele variability in one or both subgenomes (Palop-Esteban et al. 2007). None of these patterns was observed in the electropherograms of *L. narbonense* (Fig. 2).

In all the microsatellite loci analyzed, even in the least polymorphic ones, the observed individual amplification profiles were consistent with autopolyploidy, since different allelic configurations involving both balanced and unbalanced heterozygotes and absence of fixed heterozygotic profiles were observed. Nonetheless, tetra-allelic individuals were not scored for all loci but only in those that were more polymorphic and amplified at least 4 alleles per locus (7 out of 11 amplified loci, see Fig. 2 as an example). Furthermore, we were able to score individual genotypes and infer allelic dosages by combining the analysis of microsatellite amplification patterns and the amplification peak ratios (Esselink et al. 2004; Nybom et al. 2004) in individuals having two or three amplified alleles per locus (Fig. 2). Those cases represent the hardest challenge in terms of scoring genotypes reliably because they include at least two copies of one allele.

The patterns observed in *L. narbonense* contrasted with the expectations for allopolyploid taxa, where fixed heterozygotic profiles and balanced heterozygotes are typically observed (Soltis and Soltis 1993; Ramsey and Schemske 2002). Therefore, taking into account genetic (this study), karyological (Brullo and Pavone 1981; Erben 1993) and reproductive (Baker 1953a) data, the allopolyploid origin for this species can be definitely discarded.

Other factors lending support to an autotetraploid origin of this species are the presence of pollen-stigma dimorphism, high pollen fertility and sexual reproduction typical of diploid taxa. Conversely, *Limonium* species of hybrid origin usually show monomorphic, self-incompatible pollen-stigma combinations within populations, low pollen fertility and reproduce apomictically. These two very contrasting population dynamics of polyploid taxa of *Limonium*, sexuality associated with self-incompatibility vs. apomixis and associated clonality, are essential to explain the levels and distribution of genetic variation in sea lavenders and may ultimately determine the evolutionary

potential of the taxa. Therefore, identifying the origin of polyploidy in these taxa is central not only to describing correctly their genetic diversity but also to inferring their outcomes in terms of population dynamics and viability.

Genetic diversity of *L. narbonense*

Populations of *L. narbonense* showed high levels of allelic diversity (mean $A = 7.82$, Table 1), high proportion of polymorphic loci (90.91–100%) and heterozygosity ($H_O = 0.446$) and a higher proportion of genetic variance distributed within populations (Table 2), which can be attributed to the combined effect of tetraploidy and obligate outcrossing because of the sporophytic heteromorphic self-incompatibility system. General levels of diversity are consistent with those reported for other autotetraploid species analyzed with either allozymes (Brown and Young 2000; López-Pujol et al. 2004) or microsatellites (González-Pérez et al. 2004; Hochu et al. 2006; Kevin et al. 2004) but higher than for some narrow autotetraploid endemics (Buza et al. 2000; Kholina et al. 2004; López-Pujol et al. 2007). To our knowledge this is the first attempt made to describe genetic diversity in a tetraploid *Limonium* species with sexual reproduction using codominant markers. Therefore, comparisons of the levels of genetic diversity within the genus *Limonium* are premature. Many polyploid taxa of hybrid origin (i.e. allopolyploids) and autopolyploids, as confirmed in this study, exist in this genus, spanning from triploids to octoploids, as well as some diploid species. The number of taxa included in each category rapidly decreases with increasing ploidy levels, and the vast majority of *Limonium* species are triploids, which reproduce apomictically (Erben 1978, 1979; Castro and Rosselló 2007). Therefore, genetic diversity in *Limonium* may be conditioned not only by extrinsic factors, such as distribution ranges, but also by intrinsic factors such as ploidy levels and reproductive system types. Accordingly, *L. dufourii* (Girard) Kuntze, the only species studied to date using microsatellite markers, showed lower genotypic diversity and a stronger population differentiation than *L. narbonense* (72.06 vs. 18.82% of the variance distributed among populations; see Palop-Esteban et al. 2007 and Table 2), as consequence of triploidy, which involves male sterility, absence of recombination at meiosis, and exclusively apomictic reproduction.

Fixation indices (F_{IS}) do not provide support for non-random mating and asexual reproduction in *L. narbonense*. F_{IS} values were close to zero in the three southernmost populations despite their smaller population sizes (Table 1). Furthermore, even lower F_{IS} values, indicating some heterozygote excess, were obtained in these three populations (Table 1) when calculations were performed accounting for double reduction, a common phenomenon in autopolyploid species. This is not unexpected since the self-incompatibility system of *L. narbonense* precludes selfing and mating among individuals sharing the same reproductive morphotype. Nonetheless, the two northernmost populations of Peñíscola and Torreblanca, that also had larger population sizes, showed contrastingly higher F_{IS} values (Table 1). At this small geographical scale differences between populations driving such disparities in F_{IS} values between northern and southern populations were not expected. We cannot invoke different pollinator guilds, nor habitat differences to account for such differences; therefore, they could indicate local population substructure, probably mediated by net gene flow from southern populations towards northern ones (see below) and isolation by distance (Fig. 4).

Population structure and gene flow in *L. narbonense*

Our study has revealed a strong genetic structure among populations of *L. narbonense* in a narrow geographical area of the eastern Iberian Peninsula. The Bayesian analysis of genetic structure revealed that populations were aggregated into two clusters (Fig. 3a–c). These clusters included the two northernmost populations (Peñíscola and Torreblanca) in one cluster and the three remaining southern ones (Xilxes, Sagunto and El Saler) in another cluster (Fig. 3a).

Despite the mean proportion of membership of populations to the corresponding genetic cluster was high (>90%), we detected significantly more admixture from the southern cluster in the northern population of Torreblanca (21%, Fig. 3c), than to the similarly distant southern population of Xilxes in which only 8% mean membership corresponded to the northern cluster (Fig. 4c). While these results indicate that gene flow occurs among populations from both genetic clusters, and thus, genetic divergence between clusters was low (2.12%, Table 2), the net final outcome suggests an asymmetric predominant gene flow from southern towards northern populations of *L. narbonense*.

Population genetic structure in *L. narbonense* may be caused by different factors imposing barriers to gene flow among currently extant populations as shown by the isolation by distance revealed in this study (Fig. 4). The once continuous landscape of salt marshes along the coasts of Valencia and Castellón provinces has been subjected to increasing fragmentation for more than one century until present. Currently, the southernmost population of El Saler is separated from the remaining ones by the city of Valencia (an urban area of more than 10^6 people) that extends into the coast. Populations from Sagunto and Xilxes are similarly separated from the northernmost populations of Peñíscola and Torreblanca by the city of Castellón and also by numerous coastal urbanisations.

Therefore, despite the lack of significant natural geographical barriers in the past among all these populations, human activities that have dramatically fragmented the coastal landscape in recent times have likely contributed to the observed genetic pattern.

Gene flow may stem from pollen and seed dispersal; however both processes are not equally probable for this species, given the current patchy distribution and the geographical distances between areas of distribution (Fig. 1). Flowers of *L. narbonense* are mainly pollinated by bees and long-tongued insects, such as butterflies, that are not expected to cover the distances separating the populations of this species (mean pairwise distance, 67.19 km, Fig. 1). Therefore, gene flow due to pollen dispersal is expected to be less frequent than seed dispersal.

Seeds of *L. narbonense* are dispersed by gravity, as they lack specialized mechanisms for long-distance dispersal either by wind or animals. Hydrochorous dispersal can also be discarded for this species because it inhabits salt marshes separated from the sea, contrary to other sea-lavender with more coastal habitat preferences in sea shores or cliffs. However, hydrochorous dispersal through oceanic currents would result in similar unidirectional patterns as documented in *L. wrightii* (Hance) Kuntze from the Pacific Islands (Matsumura et al. 2009). Given that this species inhabits temporary flooded habitats we hypothesize that gene flow via seed dispersal could be facilitated by migratory animals such as birds. Migratory birds have been identified as important vectors for seed dispersal in coastal habitats by means of intentional dispersal, during seed or fruit ingestion, or through accidental transport of seeds present in mud attached to feet or feathers across large distances (Cain et al. 2000; Juan et al. 2004). The latter case is particularly likely in species that produce very small seeds, such as sea lavenders. While resident birds may contribute to seed dispersal at a local geographical scale around neighbouring populations, the involvement of migratory birds also could contribute to explain the observed population structure. Seeds of *L. narbonense* are set during winter months, when migratory birds are spending the cold periods in their southern ranges. During northward-bound spring migrations, birds stop in the flooded salt marshes to feed and may carry mud attached to their feet. This could result in an asymmetric and unidirectional transport of seeds which would explain the observed distribution of genetic diversity in these populations of *L. narbonense*. The intense urban activity in the coast of eastern Spain is likely to further increase the fragmentation of coastal habitats, including coastal wetlands inhabited by *L. narbonense*. Such habitat fragmentation would result in a direct reduction of population sizes of this sea-lavender and would also contribute to reduce the visits of aquatic birds. Both these factors could result in an increase of population differentiation as a result of genetic drift (Palop-Esteban et al. 2007) and also to increase the risk of local extinction through the reduction of gene flow and dispersal among populations.

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Appendix

See Table 3.

Table 3 Allele frequencies at 11 microsatellite loci in five populations of the tetraploid sea-lavender *Limonium narbonense*

Population		Peñíscola Ln1	Torreblanca Ln2	Xilxes Ln3	Marjal Moro Ln4	Saler Ln5
Locus	Allele					
Ln039	148	–	–	0.018	–	0.083
	151	0.935	0.802	0.806	0.560	0.685
	154	0.065	0.198	0.176	0.440	0.232
Ln045	337	0.907	0.733	0.630	0.590	0.731
	340	0.093	0.267	0.370	0.410	0.269
Ln122	256	–	–	0.009	–	–
	258	0.028	0.129	0.009	–	–
	260	0.352	0.569	0.787	0.690	0.185
	262	0.185	0.043	0.019	0.140	0.454
	264	–	0.026	–	–	–
	266	–	–	0.083	–	0.065

Table 3 continued

Population		Peñíscola Ln1	Torreblanca Ln2	Xilxes Ln3	Marjal Moro Ln4	Saler Ln5
	268	–	–	0.047	–	0.019
	270	–	–	0.009	0.020	0.157
	272	0.130	0.121	–	0.070	0.120
	274	0.231	0.069	0.037	0.080	–
	276	0.074	0.043	–	–	–
Ln162	77	0.093	0.035	0.028	0.038	–
	80	–	0.017	0.148	0.080	0.037
	83	0.296	0.146	0.019	0.010	0.194
	86	0.046	0.043	0.037	0.180	0.306
	89	0.306	0.440	0.694	0.270	0.278
	92	0.065	0.293	0.074	0.070	0.148
	95	0.037	0.009	–	–	0.028
	98	–	0.017	–	–	0.009
	101	0.009	–	–	–	–
	104	0.056	–	–	0.010	–
	110	0.092	–	–	–	–
Ln036	222	–	–	0.074	0.050	0.056
	224	0.037	0.009	0.019	–	–
	226	0.046	0.052	0.056	0.020	0.157
	228	0.306	0.319	0.195	0.670	0.194
	230	0.102	0.086	–	0.010	0.074
	232	0.102	0.026	–	–	0.074
	234	0.028	0.060	–	0.010	0.176
	236	0.102	0.198	0.009	–	0.019
	238	0.231	0.077	0.157	0.010	0.028
	240	0.037	0.017	0.259	0.040	0.074
	242	0.009	0.026	0.083	0.030	0.046
	244	–	0.026	0.130	0.140	–
	246	–	0.009	0.009	0.020	–
	248	–	0.009	–	–	–
	250	–	0.077	–	–	–
	252	–	0.009	0.009	–	0.009
	256	–	–	–	–	0.019
	268	–	–	–	–	0.009
	270	–	–	–	–	0.009
	274	–	–	–	–	0.037
	278	–	–	–	–	0.019
Ln044	189	0.121	0.173	0.111	0.030	–
	192	0.074	–	0.019	–	–
	195	0.120	0.181	0.111	0.120	0.176
	198	0.120	0.017	–	0.070	0.166
	202	0.093	0.147	0.046	0.040	–
	205	0.083	0.103	0.204	0.020	0.130
	208	0.037	0.103	0.130	0.010	0.130
	211	–	0.026	–	0.010	0.028
	217	0.083	0.198	0.176	0.580	0.120
	220	0.130	–	–	–	–
	223	0.139	0.026	0.194	0.090	0.204

Table 3 continued

Population		Peñíscola Ln1	Torreblanca Ln2	Xilxes Ln3	Marjal Moro Ln4	Saler Ln5
	226	–	0.017	–	–	–
	229	–	0.009	–	0.030	0.046
	235	–	–	0.009	–	–
Ln149	90	1.000	1.000	0.796	0.910	0.741
	96	–	–	0.195	0.090	0.185
	99	–	–	0.009	–	0.037
	101	–	–	–	–	0.037
Ln041	91	0.083	0.173	0.009	0.070	0.092
	94	0.250	0.224	0.204	0.070	0.065
	97	0.667	0.586	0.787	0.860	0.843
	103	–	0.017	–	–	–
Ln068	177	–	–	–	0.020	–
	179	0.667	0.698	0.565	0.340	0.769
	181	0.287	0.190	0.204	0.440	0.194
	187	–	–	–	0.050	–
	193	–	0.017	0.009	–	–
	199	0.046	0.095	0.222	0.150	0.037
Ln115	256	0.065	0.017	–	–	0.009
	259	0.870	0.905	0.963	0.920	0.806
	262	0.065	0.078	0.037	0.080	0.148
	265	–	–	–	–	0.037
Ln052	234	0.593	0.371	0.259	0.230	0.287
	240	0.046	0.078	0.074	0.210	0.130
	243	–	0.017	0.056	0.170	0.009
	249	–	0.060	–	–	–
	252	0.361	0.474	0.546	0.350	0.463
	255	–	–	0.065	0.040	0.111

Allele sizes are expressed in bp

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