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ORIGINAL ARTICLE

Genome sizes and phylogenetic relationships suggest recent divergence of closely related species of the *Limonium vulgare* complex (Plumbaginaceae)

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Abstract

Limonium vulgare and related species form a complex group, but until now cytological and genetic studies have been based on single species and specific geographical areas. We investigated genome size, karyological and genetic diversity in samples from Western Mediterranean and evaluated the phylogenetic relationships among the species of this complex. Genome size was assessed using flow cytometry on samples from natural populations of *L. vulgare*, *L. maritimum* and *L. narbonense*. Chromosome counts were conducted in plants obtained from seeds collected in the field. The internal transcribed spacer ITS1 of the nuclear rDNAs was used to assess ITS polymorphisms as well as the phylogenetic relationships within the *L. vulgare* complex. Our analyses showed that all species were tetraploid, with the chromosome number of *L. maritimum* being presented here for the first time. Significant differences were observed in genome size, with *L. narbonense* having lower genome sizes than the other two species, and possible aneuploids being detected. Ten new ITS sequences from *L. vulgare*, *L. narbonense* and *L. maritimum* were provided. Most species' populations showed unique ribotypes, and *L. narbonense* has the highest ribotype diversity. One of the *L. maritimum* populations presented a closer genetic relationship with *L. vulgare*, whereas the other two seemed to be more related with *L. narbonense*. Phylogenetic analyses confirmed that *L. vulgare* and *L. narbonense* form a monophyletic group, sister to the remaining *Limonium* species. Our results put into evidence that the studied species may represent a relatively early stage of divergence.

Keywords Chromosome number · Flow cytometry · ITS nrDNA · *Limonium vulgare* complex · Phylogenetic relationships · Polyploidy

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Introduction

Polyploidy, i.e., whole-genome duplications, hybridization and apomixis, have all played a major role in the evolution of flowering plants (Ramsey and Schemske 1998; Soltis and Soltis 2009). The fusion of unreduced gametes resulting in sexual polyploidization rather than somatic doubling has been assumed to be the main mechanism responsible for the origin and evolution of polyploid plants (Harlan and DeWet 1975; Bretagnolle and Thompson 1995). High-throughput ploidy analysis of natural populations by flow cytometry has enabled intensive studies at the population level, thus allowing the assessment of genome sizes and the estimation of cytotype frequencies within natural populations, as well as the detection of mixed-ploidy populations and rare ploidies (Husband et al. 2013). Cytogenetic diversity has been described for various plant groups, and 12–13% of the angiosperms and 17% of the ferns are estimated to be ploidy variable (Wood et al. 2009). However, for the vast majority of species, cytogenetic heterogeneity across their geographic range has not been investigated in detail (Husband et al. 2013; Marques et al. 2018). Additionally, cytogenetic diversity can also occur at the genome size without changes in ploidy levels. Differences among related homoploid species, either in absolute or in monoploid genome sizes (sensu Greilhuber et al. 2005), in combination with molecular and phenotypic approaches, have been shown to be a valuable tool in the biosystematics of several taxonomically difficult plant complexes (Kron et al. 2007; Loureiro et al. 2010). Genome size can vary among different (closely related) homoploid species, while it is constant within the same taxon (Greilhuber et al. 2005; Bennett and Leitch 2005).

The internal transcribed spacer (ITS) region of the 18S–5.8S–26S nuclear ribosomal cistron (nrDNA) is a standard molecular marker, which has been used for inferring the evolutionary relationships at the generic and interspecific levels in flowering plants (Álvarez and Wendel 2003; Calonje et al. 2009). In addition, the ITS region is a valuable tool for tracing hybridization or polyploidization events of diploid and polyploid plants (Sang et al. 1995; Tison et al. 2013) as well as for reconstructing the evolutionary history of taxonomical complexes in flowering plants with both sexual and apomixis (asexual seed production) reproductive modes (Kiefer and Koch 2012; Hodač et al. 2014). For example, the analysis of ITS polymorphisms in *Ranunculus auricomus* complex suggested that the natural polyploid apomict *R. variabilis* is an ancient hybrid that originated from crosses between the diploid sexual *R. notabilis* and the polyploid sexual *R. cassubicifolius* (Hodač et al. 2014).

Phylogenetic studies of Plumbaginaceae based on plastid and nuclear DNA sequences (ITS nrDNA) provided enough variation for inferring phylogenetic relationships among major clades even in closely related species (Lledó et al. 1998, 2005; Palacios et al. 2000; Akhani et al. 2013; Malekmohammadi et al. 2017). These studies confirm the classification of Plumbaginaceae into two subfamilies, Plumbaginoideae and Staticoideae (=Limonioideae, Malekmohammadi et al. 2017), the latter subfamily includes the *Limonium* genus (Baker 1966; Erben 1993; Lledó et al. 2005; Róis et al. 2016; Caperta et al. 2017). *Limonium* section *Limonium* (with six subsections) is the largest group in the genus, and it is clearly polyphyletic (Lledó et al. 2005). Attempts to classify *Limonium* at lower taxonomic levels present systematic and taxonomic misunderstandings associated with the difficulty in classifying species into discrete species, due to different modes of reproduction (sexual and/or uniparental reproduction), frequent hybridization and polyploidization (Ennos et al. 2005). Among the several taxonomical complexes described, the *Limonium vulgare* complex shows a remarkable diversity, with distinct but morphologically closely related polyploid species (*Limonium vulgare* Mill., *Limonium humile* Mill., *Limonium maritimum* Caperta, Cortinhas, Paes, Guara, Espírito-Santo and Erben and *Limonium narbonense* Mill. in the Iberian Peninsula; Erben 1993; Dawson and Ingrouille 1995; Cortinhas et al. 2015). A recent genetic study on *L. narbonense* is based on a specific geographical area (eastern Spain, Palop-Esteban et al. 2011), but the cytogenetic and genetic diversity information in a larger geographical scale of this and other species is still limited.

In this study, our goal was to investigate cytogenetic diversity, ITS nrDNA variability and phylogenetic relationships within species of *L. vulgare* complex. Populations of *L. vulgare*, *L. maritimum* and *L. narbonense* were sampled in southern France, eastern Spain, Portuguese coast and northern Morocco (i.e., Western Mediterranean). Flow cytometry, cytogenetic, genetic and phylogenetic analyses were combined to investigate: (1) cytotype diversity within and among populations and genome size variation among species; (2) ITS polymorphisms in natural populations; and (3) phylogenetic relationships among representatives of the studied *Limonium* species' populations.

Materials and methods

Study species

The *Limonium vulgare* complex is a group of halophytes with leaves with pinnate venation (Cortinhas et al. 2015). *Limonium vulgare s.s.* presents perennial, rosulate leaves, a chamaephyte habit with short spikes with densely arranged spikelets (Erben 1993). By contrast, *L. narbonense s.s.* forms taller plants with wider leaves and a longer scape

(Erben 1993), whereas *L. maritimum* is a small plant with few, nearly straight to slightly flexuous, stems and spikelets composed of a few flowers (Cortinhas et al. 2015). The species in this complex appear to be tetraploid with $2n = 4x = 36$ chromosomes (*L. vulgare* and *L. narbonense*) or hexaploid with $2n = 6x = 54$ (*L. humile*, *L. narbonense*) (Dawson and Ingrouille 1995; Georgakopoulou et al. 2006; Caperta et al. 2017). *Limonium vulgare s.l.* is distributed along the Atlantic coast of Europe (Dawson and Ingrouille 1995), whereas *L. narbonense s.l.* occurs mainly in the southwestern Mediterranean region (Erben 1993; Pandža et al. 2007; Cortinhas et al. 2015), and *L. maritimum* is found in the northwest coast of Portugal (Cortinhas et al. 2015). *Limonium vulgare* and *L. narbonense* are considered allogamous, inhabiting temporary flooded salt marshes (Erben 1993; Pandža et al. 2007; Costa et al. 2014), while *L. maritimum* appears to be a probable apomict (Cortinhas et al. 2015), which thrives on rocky tidal flats (A.D. Caperta and D. Espírito-Santo, unpublished data).

Plant material and field sampling

A total of 19 populations of *L. maritimum* ($n = 3$), *L. narbonense* ($n = 6$) and *L. vulgare* ($n = 10$) were sampled in the coasts of southern France, eastern Spain, Portugal and northern Morocco. All populations were tagged with Global Positioning System handheld (Garmin eTrex® 20). Species were identified using taxonomic keys available from Erben (1993), and herbarium specimens were deposited in the Herbarium Prof. João de Carvalho e Vasconcellos (LISI). Herbarium vouchers representative of all populations and species sampled are listed in Online Resource 1.

In the field, leaf material of five up to 21 individuals was sampled per population, totalizing 252 individuals. Two to three leaves per individual were stored in labeled hermetic plastic bags and maintained at 4 °C until flow cytometric (FCM) analysis. Seeds of *L. vulgare*, *L. narbonense* and *L. maritimum* mother plants were collected in selected natural populations to establish experimental populations under controlled conditions. They were germinated in a growth chamber (Phytotron Rumed) with a photoperiod of 18 h/6 h of light and dark, respectively, and a temperature of 25 °C until germination (Róis et al. 2012). Seedlings were transferred to jiffy pots and maintained in similar growth conditions. From these individuals, leaves and roots were collected for FCM analyses and chromosome counting, respectively. This procedure enabled to unambiguously assign genome sizes to each ploidy level detected.

Chromosome counts

Chromosome counts were performed in three to five individuals from each species to confirm the ploidy levels estimated based on the genome sizes obtained, following the procedure described in Caperta et al. (2017). Briefly, root tips were excised and cold-treated for 36 h at 0 °C. Then, root tips were fixed in a fresh absolute ethanol/glacial acetic acid (3:1) solution overnight and stored in 70% ethanol at − 20 °C. Afterward, root tips were digested in a pectolytic enzyme mixture [2% cellulase (Sigma), 2% cellulase "Onozuka R-10" (Serva), and 2% pectinase enzyme (Sigma) solution in 1xEB (40 ml 0.1 M citric acid-1-hydrate and 60 ml of 0.1 M sodium citrate dihydrate; pH 4.8)] for 2h30 at 37 °C. Chromosome preparations were made in 60% acetic acid and stained with 4′,6-diamino-2-phenylindole hydrochloride (DAPI) (1 mg ml^{-1}) in Vectashield (Vector Laboratories). Chromosomes were observed using a Zeiss Axioskop 2 fluorescence microscope and photographed with an AxioCam MRc5 digital camera (Zeiss).

Genome size and DNA ploidy-level estimations

Genome size and DNA ploidy levels were assessed using flow cytometry (FCM). Nuclei were isolated following the procedure of Galbraith et al. (1983) in which 0.5 cm^2 of fresh leaf tissue of *Limonium* was chopped with a razor blade simultaneously with 0.5 cm^2 of fresh leaf tissue of the internal reference standard, in a Petri dish containing 1 ml of WPB buffer (Loureiro et al. 2007a). *Pisum sativum* "Ctirad" (2C = 9.09 pg; Doležel et al. 1998) was used as internal standard. The nuclear suspension was filtered using a 50-µm nylon mesh and 50 µg ml^{-1} of propidium iodide (Fluka, Buchs, Switzerland) were added to stain the DNA. To avoid staining of double-stranded RNA, 50 µg ml^{-1} of RNAse (Fluka, Buchs, Switzerland) was also added. After a 5-min incubation period, samples were analyzed in a Partec CyFlow Space flow cytometer (532 nm green solid-state laser, operating at 30 mW; Partec GmbH., Görlitz, Germany). Results were acquired using Partec FloMax software v2.4d (Partec GmbH, Münster, Germany). The following graphics were obtained: histogram of fluorescence pulse integral in linear scale (FL); forward light scatter (FS) versus side light scatter (SS), both in logarithmic (log) scale; FL versus time; and FL vs. SS in log scale. For most samples, a polygonal region was defined in the latter graphic to include only intact nuclei, and this region was subsequently used to gate all the other graphics. At least 1300 particles per G_1 peak were analyzed per sample (Suda et al. 2007). Genome size estimates were only considered when the CV values of G1 peaks were below 5%. Samples with higher CV values were discarded, and a new sample was prepared. For most populations, it was possible to estimate genome size for every individual; however, given the high volume of samples collected from some populations and the fast degradation of leaf material, genome size was estimated in 5–9 individuals, while the remaining ones were analyzed using the pooled

sample strategy (i.e., 3–4 individuals analyzed simultaneously), enabling the estimation of ploidy level.

The value of genome size in mass units (2C in pg; sensu Greilhuber et al. 2005) was obtained using the following equation: *Limonium* sp. 2C nuclear DNA content (pg) = (*Limonium* sp. G_1 peak mean/reference standard G_1 peak mean) * genome size of the reference standard. Because the genome size value was obtained for several individuals that were also karyologically characterized (details above), DNA ploidy levels were inferred for all individuals analyzed.

Descriptive statistics were calculated for genome size data (mean, standard deviation of the mean, coefficient of variation, minimum and maximum values). Genome size outlier estimates were inspected and treated separately. Since data homoscedasticity was not achieved even after transformation, differences in genome size among species were assessed using a Kruskal–Wallis one-way ANOVA on ranks (Kruskal and Wallis 1952), followed by Dunn's test for all pairwise multiple comparisons. Statistical analyses were performed using SigmaPlot for Windows v. 12.5 (Systat Software).

DNA isolation, PCR amplification and sequencing

For genetic analysis, young leaves at the same developmental stage from a total of 106 individuals belonging to 14 populations previously analyzed by FCM were used. Leaves were cut, placed in paper envelopes and dried immediately at room temperature in sealed containers with abundant silica gel, prior to the DNA isolation procedure. Total genomic DNA was obtained and extracted according to Róis et al. (2016). The ribosomal ITS region (ITS1) was amplified and sequenced using universal primers (White et al. 1990). A volume of 10 µl PCRs was prepared using 50 ng of template DNA, 10 × NH_4 PCR buffer, 50 mM $MgCl_2$, 100 mM dNTPs, 10 mM of each primer and 5 U μl^{-1} Taq DNA polymerase (Bioline). The PCR amplifications began with an initial cycle of 2 min at 94 °C, 30 cycles of 1 min at 94 °C, 30 s at 55 °C and 45 s at 72 °C, and a final step of 9 min at 72 °C. The PCR product was verified on an agarose 1.5% Tris–acetate–EDTA (TAE) gel, and the products were purified using a Qiagen PCR purification kit and sequenced directly using the ABI PrismTM Big Dye Ready Reaction Terminator Cycle Sequencing Kit on an ABI 3700 genetic analyzer. Sequences were generated from at least two separate PCR amplifications per individual, and both strands of all fragments were sequenced.

Genetic analyses

Sequences were aligned using BioEdit 7.1.3.0 (Hall 1999; http://www.mbio.ncsu.edu/BioEdit/bioedit.html), examined by eye for nucleotide additivity (double peaks) that can suggest the presence of more than one repeat type, and concatenated using Concatenator V 1.1.0 (Pina-Martins and Paulo 2008). ITS ribotypes were defined using Network 4.6.1.1 (Bandelt et al. 1999; www.fluxus-engineering.com/), and a media-joining network was calculated under default parameters (weights¼10 and e¼0). Genetic diversity parameters, such as ribotype diversity (Rd), were calculated using DnaSP 4.0 software (Rozas et al. 2003). A Bayesian phylogenetic inference was implemented with MrBayes v.3.2.2 (Ronquist et al. 2012). Sequences available in GenBank (https://www.ncbi.nlm.nih.gov/) were used in comparison with the species of the *L. vulgare* complex (accession numbers are provided in Online Resource 2) belonging to 24 *Limonium* species, *Psylliostachys suworowii*, *Acantholimon acerosum* and *Plumbago europaea*, which was used as out-group.

Results

Chromosome numbers

Our results on chromosome counts made on metaphase spreads of *L. vulgare*, *L. narbonense* and *L. maritimum* revealed that all individuals had $2n = 4x = 36$ chromosomes (Fig. 1). In metaphase cells, four pairs of large submetacentric chromosomes, four pairs of medium submetacentric chromosomes, three pairs of medium metacentric chromosomes, and 14 pairs of small metacentric or telocentric chromosomes were detected.

Genome size and DNA ploidy-level estimates

Flow cytometric analyses enabled us to investigate the ploidy level of 252 individuals from a total of 19 populations of *L. vulgare* complex, including 10 populations of *L. vulgare*, six populations of *L. narbonense* and three populations of *L. maritimum* (Table 1; Fig. 2a). All three species were ploidy homogenous, with almost all individuals being tetraploid (Fig. 2b, c); a few individuals with outlier genome sizes were also detected for *L. vulgare* (Table 1). However, significant differences in genome size were detected among species ($H_2 = 121.9$, $P \leq 0.001$), with *L. narbonense* having significantly lower genome size estimates (mean ± SD, median: 5.43 ± 0.10 pg/2C, 5.41 pg/2C) than *L. vulgare* (5.75 ± 0.10 pg/2C, 5.69 pg/2C) and *L. maritimum* (5.80 ± 0.06 pg/2C, 5.78 pg/2C). Six individuals of *L. vulgare* with outlier genome sizes were detected in four populations (Table 1, Fig. 2a). These include two individuals with lower genome sizes (2C = 5.28 and 5.36 pg) than the average for the species, found in populations from Larache (LL, Morocco) and Aveiro (ARA, Portugal), respectively. The remaining four individuals presented higher genome

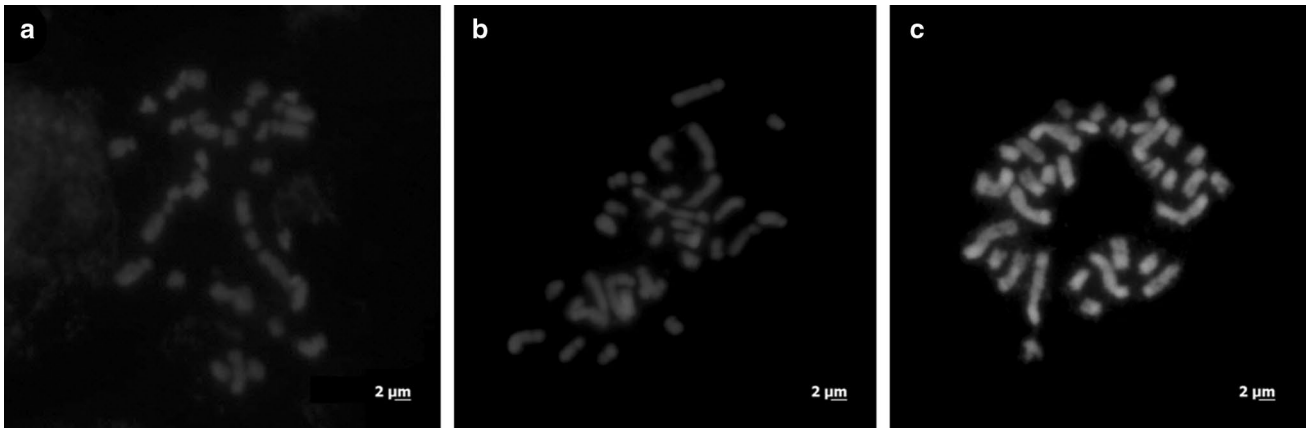

Fig. 1 Mitotic metaphase plates of DAPI-stained spreads from *Limonium vulgare* complex with $2n = 36$ chromosomes: **a** *Limonium vulgare*; **b** *L. narbonense*; **c** *L. maritimum*

sizes (ranging from 2C = 7.12 to 7.56 pg) than the average for the species, one individual from Aveiro (GE, Portugal), and the other three within the same population in southern Portugal, Tavira (TB, Table 1, Fig. 2a).

ITS polymorphisms and network relationships

The sequencing of the ITS region resulted in a final matrix of 106 sequences for a 195-bp fragment. New ITS sequences were provided belonging to eight *L. vulgare*, three *L. narbonense* and three *L. maritimum* accessions (for GenBank accessions, see Online Resource 3). A total of ten ribotypes (A–J) were identified in the 14 populations analyzed (Table 2). Most species showed a low number of ribotypes (maximum $n = 3$), representative of a low ribotype diversity, ranging from zero in populations SLCB, VCA, TB, GE, LMB, AES, PV1 and PV2, with unique ribotypes, to 0.83 in DES population. In all *L. vulgare* populations, a common ribotype (A) was detected, which was found in two *L. maritimum* (VCA) populations (Table 2).

The network analysis of ITS sequences indicated a clear genetic separation of *L. vulgare* and *L. narbonense* representatives (Fig. 3). Clustering was evidenced at species level, in which ribotype A was shared by *L. vulgare* and *L. maritimum*, although unique ribotypes were observed in both species (*L. vulgare*—B, C, D and G; *L. maritimum*—J). *Limonium narbonense* also presented exclusive ribotypes (E, F, G, H and I). Populations from each respective species appeared to be fairly homogeneous, as a result of very low differentiation within populations.

Phylogenetic analysis

In the few sequences that exhibited evidence of more than one ITS repeat type, as evident by the presence of double peaks, the signal of one repeat sequence was significantly stronger than the other and tended to be identical or nearly identical to other sampled sequence, which facilitates the resolution of the two repeats without cloning. We observed double peaks in almost all *L. vulgare* populations, and in one population of *L. narbonense*. No double peaks were detected in *L. maritimum* populations.

The Bayesian phylogenetic analysis was grounded on new ITS sequences from a matrix of 39 sequences for a 228-bp fragment, belonging to 24 species available from GenBank (for Alignments used to produce phylogeny, see Online Resource 4). The species of the *Limonium vulgare* complex were clustered in subfamily Staticoideae. There was a strong clustering of *Limonium* species (here termed, core *Limonium*, following Lledó et al. 2005 and Akhani et al. 2013), which form a strongly supported clade separated from the other genera of the subfamily, like *Psylliostachys* (AJ132446) and *Acantholimon* (LT714475) (PP = 100) (Fig. 4). The internal relationships of the core *Limonium* clade were resolved with two clades: (1) a well-supported *L. vulgare* clade (PP = 90) comprising species of "*Limonium*-type clade" (*Limonium vulgare* is the type of *Limonium*; Lledó et al. 2005; Akhani et al. 2013), *L. vulgare* and *L. narbonense*, with *L. latifolium* and *L. gmelinii* embedded in it; and another (PP = 91) clade that included species of *L.* section *Limonium* (*L. caspium*, *L. bellidifolium* and *L. iconum*), *L.* section *Siphonocalyx* (*L. sogdianum*) and *L.* section *Platyhymenium* (*L. sinense* and *L. nudum*); (2) a less-supported clade (PP = 77) which includes species of *L.* subsection *Limonium*, a subclade comprising species of *L.* section *Limonium* (*L. rigualii*, *L. furfuraceum*, *L. girardianum*, *L. dufourii*, *L. delicatulum*, *L. cossonianum* and *L. echioides*) and a subclade comprising species from section *Polyarthrion* (*L. caesium*).

Table 1 Genome size estimates, ploidy level and chromosome counts obtained in populations of the *Limonium vulgare*, *L. narbonense* and *L. maritimum*, sampled in France (FR), Spain (ES), Portugal (PT) and Morocco (MAR)

Species	Population/acronym	Genome size (2C, pg)						DNA ploidy level		Chromosome counts	
		Mean	SD	CV (%)	Min	Max	*N*	2*n*	*N*	Observed	Literature
L. vulgare											35, 36, 38[a,b]
	PT: Aveiro, Boco/ARA	5.79	0.06	1.03	5.71	5.91	11	4*x*	11	36	
		5.36					1	an.	1		
	PT: Aveiro, Gafanha da Encarnação/GE	5.67	0.05	0.86	5.61	5.74	8	4*x*	8	36	
		7.12					1	an.	1		
	PT: Aveiro, Ílhavo, Gafanha do Carmo	5.74	0.06	1.09	5.62	5.81	7	4*x*	7		
	PT: Aveiro, Ribeira da Aldeia	5.71	0.09	1.51	5.56	5.94	20	4*x*	20		
	PT: Setúbal, Alcochete, Sítio das Hortas/SHT	5.82	0.13	2.19	5.61	5.98	6	4*x*	16		
	PT: Setúbal, Mouriscas/SMM	5.76	0.05	0.83	5.69	5.86	9	4*x*	9		
	PT: Odemira, São Luís, Casa Branca/SLCB	5.71	0.06	1.06	5.59	5.79	6	4*x*	16		
	PT: Tavira, sapal do Barril/TB	5.80	0.14	2.43	5.63	6.06	9	4*x*	14		
		7.40	0.12	1.67	7.26	7.56	3	an.	3		
	MAR: Larache, Moulay Bousselham/LMB	5.79	0.08	1.35	5.71	6.00	17	4*x*	17		
	MAR: Larache, Loukkos/LL	5.67	0.04	0.79	5.61	5.72	8	4*x*	8		
		5.28					1	an.	1		
L. narbonense											36, 54, 72[a,b]
	FR: Vendres: Étang de Vendres/VE	5.48	0.05	0.96	5.41	5.56	5	4*x*	21		
	FR: Agde: Route de Béziers, Canal do Midi	5.54	0.06	1.14	5.44	5.64	5	4*x*	15		
	SP: Castellon, Torre de la Sal, Prat de Cabanes	5.58	0.04	0.70	5.53	5.61	5	4*x*	15	36	
	SP: Alicante, Denia, Devesas/DES	5.39	0.09	1.61	5.28	5.59	21	4*x*	21		
	SP: Valencia, El Saler, La Granja, Centro de Recuperación de Fauna 1/AES	5.39	0.09	1.58	5.25	5.58	18	4*x*	18		
	SP: Valencia, El Saler, La Granja, Centro de Recuperación de Fauna 2	5.47	0.09	1.68	5.33	5.56	5	4*x*	5		
L. maritimum											
	PT: Viana do Castelo, Areosa, Veiga/VCA	5.78	0.06	1.00	5.68	5.88	10	4*x*	10	36	
	PT: Viana do Castelo, Praia Porto de Vinha 1	5.78	0.06	0.96	5.68	5.87	8	4*x*	8		
	PT: Viana do Castelo, Praia Porto de Vinha 2	5.83	0.06	1.01	5.74	5.92	7	4*x*	7		

The following data are given for each taxa, population and ploidy level: mean, standard deviation of the mean (SD), coefficient of variation (CV, %) and minimum (Min) and maximum values (Max) of the holoploid genome size (2C, pg), followed by sample size for genome size estimates (N); DNA ploidy level (2*n*) and respective sample size (N) are also provided. DNA ploidy levels: 4*x*, tetraploid, an., aneuploid. Chromosome counts observed in this study (Observed) and reported elsewhere in the literature are also provided (Literature). Populations used in the genetic analyses are followed by the acronym to facilitate citation throughout the text

[a]Dawson and Ingrouille (1995)

[b]Cortinhas et al. (2015)

Discussion

Our study on genome size and genetic analyses on *L. vulgare* complex covers a broader geographic range (i.e., the Western Mediterranean) than previous studies limited to specific geographic areas. Flow cytometric and karyological analyses reveal that species from *L. vulgare* complex are homogenously tetraploid, including *L. maritimum* for which chromosome counts are provided here for the first time. However, *L. vulgare* and *L. maritimum* show larger genome sizes than *L. narbonense*. ITS data reveal that one *L. maritimum* population presents a closer genetic relationship with *L. vulgare*, while the other two have more genetic affinity with *L. narbonense*.

Considerations on the taxonomy and cytogenetics of the group

Taximetric studies on species of *L. vulgare* complex show a clear morphometric discrimination within it (Dawson

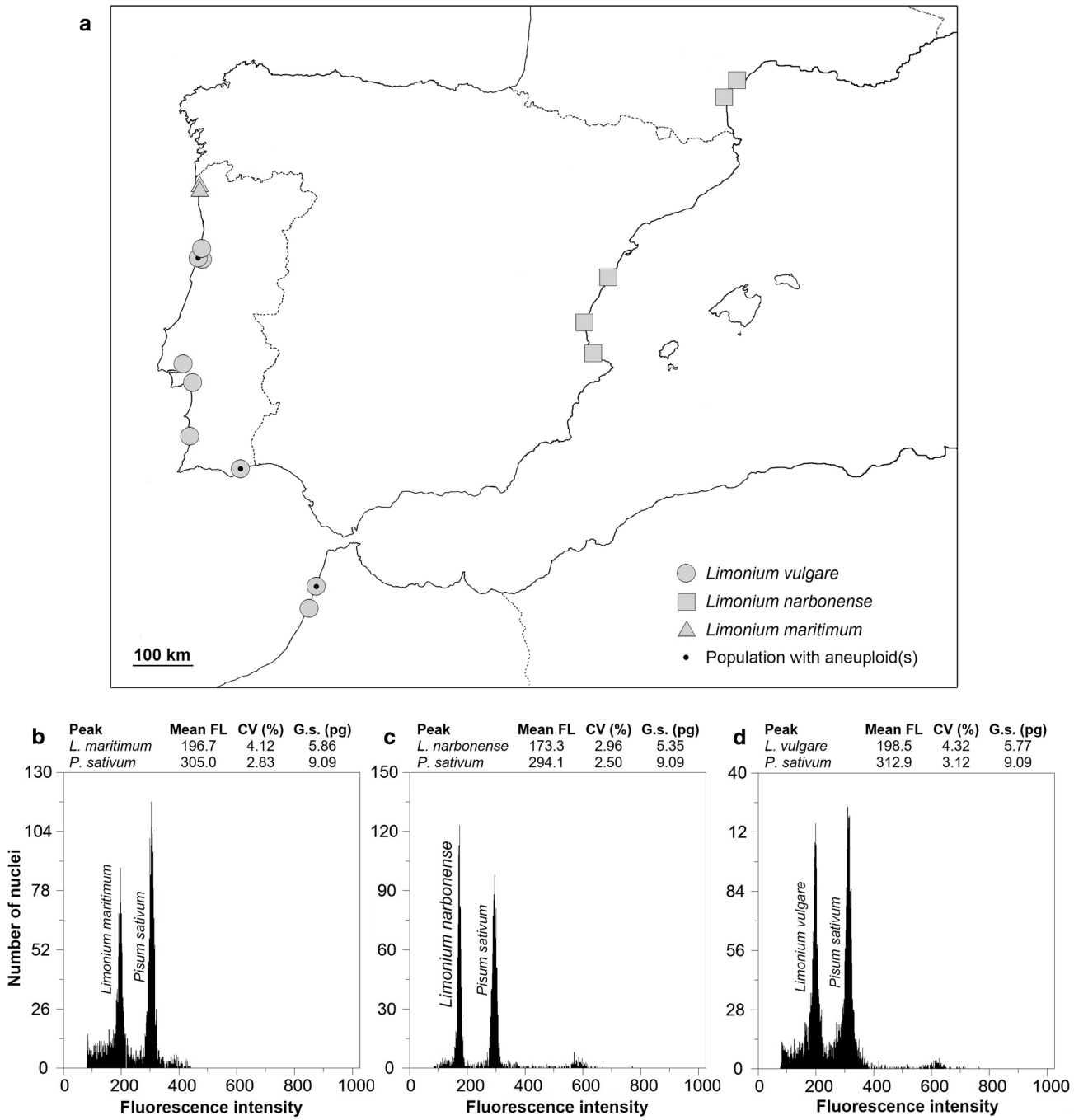

Fig. 2 Field surveys and flow cytometric analyses of *Limonium vulgare* complex. **a** Map of the locations of *L. vulgare* (circles), *L. narbonense* (squares) and *L. maritimum* (triangles) populations sampled in this study (all populations are tetraploid and populations with possible aneuploid individuals are denoted with a central black dot); **b–d** Flow cytometric histograms of propidium iodide stained nuclei of each *Limonium* species analyzed simultaneously with the internal standard *Pisum sativum* "Ctirad"

and Ingrouille 1995; Cortinhas et al. 2015). However, in the Iberian Peninsula morphotypes with some degree of intermediacy, representing *L. maritimum*, *L. narbonense* and *L. vulgare*, can be found in natural populations growing mixed with typical morphotypes (Cortinhas et al. 2015).

Combined cytological and cytometric analyses of *L. vulgare* complex revealed interesting patterns. First, *L. vulgare*, *L. narbonense* and *L. maritimum* show to be homogenously tetraploid through the surveyed geographic range. Previous chromosome counts in a few populations of these species

Table 2 ITS Ribotype diversity in *Limonium vulgare*, *L. narbonense* and *L. maritimum*

Species	Acronym	ITS Ribotype diversity			
		Ni	R	Rn	Rd
L. vulgare	PT: ARA	10	A; B	2	0.2
	PT: GE	8	A	1	0
	PT: SLCB	10	A	1	0
	PT: SHT	9	A; D	2	0.22
	PT: SMM	12	A; C	2	0.17
	PT: TB	8	A	1	0
	MAR: LMB	8	A	1	0
	MAR: LL	6	A	2	0.33
L. narbonense	FR: VE	6	E; F	2	0.28
	SP: DES	4	G; H; I	3	0.83
	SP: AES	5	G	1	0
L. maritimum	PT: VCA	10	A	1	0
	PT: PV1	3	J	1	0
	PT: PV2	5	J	1	0

N_i number individuals analyzed, *R* ribotypes, *Rn* number of ribotypes, *Rd* ribotype diversity

indicate that *L. vulgare* can be euploid and aneuploid tetraploid (Erben 1979; Cortinhas et al. 2015). This pattern is confirmed here for a wider distribution area and deeper within population screenings. *Limonium narbonense s.l.* is documented as tetraploid or hexaploid (Georgakopoulou et al. 2006); however, we only detect homogenously tetraploid populations in the geographical area surveyed. In this study, we provide the first evidence that *L. maritimum* also is a tetraploid species with $2n = 36$ chromosomes. Studies in other *Limonium* species reveal a constancy in ploidy composition across each species' geographic range, namely in *L. ovalifolium* ($2n = 16$), *L. algarvense* ($2n = 24, 25$) and *L. binervosum* ($2n = 35, 36$) (Caperta et al. 2017).

Similarly to other *Limonium* entities, some *L. vulgare* individuals showing either higher (GE and TB populations) or lower genome size values (ARA and LL populations) than the average are observed in four different populations in Portugal and Morocco. The magnitude of the differences among these individuals and the most common values obtained for the species does not seem to be large enough to reflect differences in ploidy levels. Thus, these values might be interpreted as possible aneuploidies with gain(s) or loss(es) of chromosome(s), although further chromosome counts are needed to confirm whether they represent aneuploids, or represent a wider genome size variation, or even different ploidy levels. However, the occurrence of aneuploid individuals is not surprising within the group since aneuploidy ($2n = 35, 38$) has already been documented in *L. vulgare* individuals sampled in Portugal (Cortinhas et al. 2015). High levels of karyological polymorphisms linked with polyploidy (spanning from triploids to octoploids) and aneuploidy have been previously reported in several *Limonium* species (Dolcher and Pignatti 1971; Erben 1993; Georgakopoulou et al. 2006; Castro and Rosselló 2007; Róis et al. 2012; Caperta et al. 2017). This karyological diversity is probably dependent on each species reproductive modes, which may reproduce sexually (selfing, outcrossing) and by apomixis (D'Amato 1940; Baker 1966; Róis et al. 2016).

Regardless of the fairly homogenous ploidy levels found across the studied species, their genome sizes present significant differences. Genome size has been suggested as a useful character for taxonomic decision making, not only in heteroploid complexes but also in particular homoploid plant groups (Loureiro et al. 2010). Genome size has been used to delimit species at various taxonomic levels (e.g., *Helleborus*,

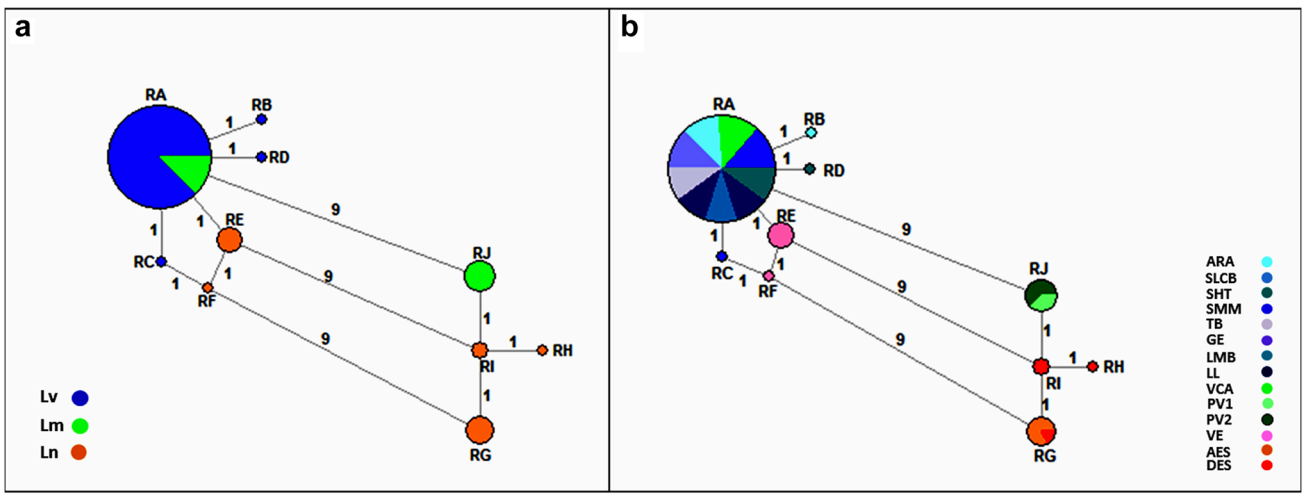

Fig. 3 Median-joining network of the ITS sequence regions for *Limonium vulgare* (Lv), *L. narbonense* (Ln) and *L. maritimum* (Lm). Representation of ribotypes by species (**a**) and populations (**b**). The circle size is proportional to ribotype frequencies

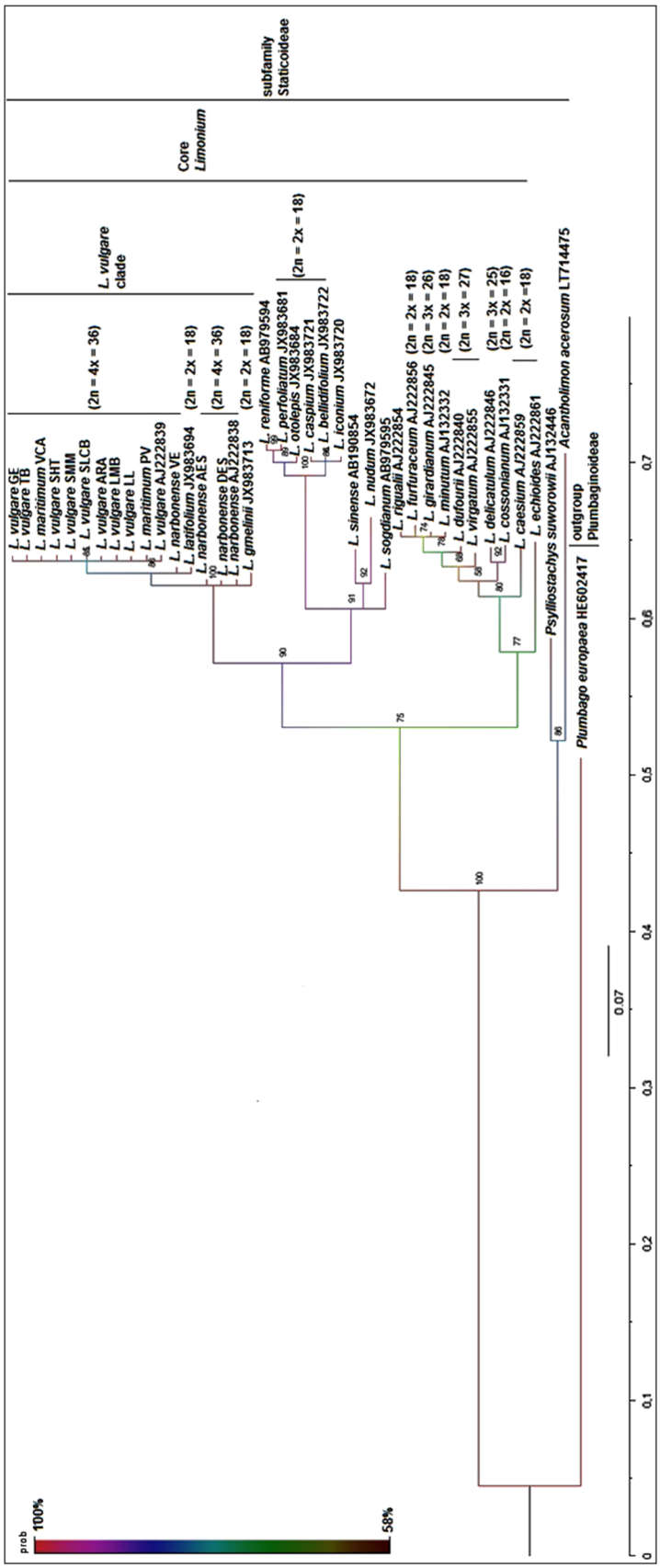

Fig. 4 Bayesian phylogenetic analysis of *Limonium* species. The accession numbers of the DNA sequences used from GenBank (https://www.ncbi.nlm.nih.gov/) are given in front of each taxon. *Plumbago europaea* (HE602417.1) was used as out-group. The plant chromosome numbers were obtained from the Chromosome Counts Database (Rice et al. 2015)

Zonneveld 2001; *Festuca*, Loureiro et al. 2007b), resolve complex low-level taxonomies (e.g., *Dryopteris carthusiana*, Ekrt et al. 2010), assess the frequency of interspecific hybridization (e.g., *Amaranthus*, Jeschke et al. 2003; *Elytrigia repens* and *E. intermedia*, Mahelka et al. 2005) or infer evolutionary relationships (e.g., *Musa* sp., Lysák et al. 1999; *Androsace brigantiaca*, Dixon et al. 2009) in homoploid plant groups. Our analyses reveal that *L. narbonense* has a consistently smaller and significantly the genome size than the other two species, *L. vulgare* and *L. maritimum*, which have similar genome sizes. More interestingly, the relationships among the species based on genome size agree with the genetic relationships observed using ITS markers (see below).

ITS polymorphisms

In this study, we report ITS data for ten new accessions (see Table 2) for *L. vulgare* complex. Although ours and other data support genetic differentiation between *L. vulgare* and *L. narbonense* (Palacios et al. 2000; Palop-Esteban et al. 2011; Cortinhas et al. 2015), surprisingly the levels of sequence divergence were low, with moderate to no diversity being found (Table 2). Mainly in *L. vulgare* populations, rDNA ITS analyses revealed the presence of double peaks, which is an indication of sequences characterized by heterozygosity between distinct genotypes and may indicate a hybrid origin. Heterozygosity is not found in *L. maritimum* and has a low frequency in *L. narbonense* studied populations. A study using eleven microsatellite loci specifically designed for *L. narbonense* reveals a possible autotetraploid origin for this species (Palop-Esteban et al. 2011). Although *L. vulgare* and *L. narbonense* can co-occur in mixed populations in the Portuguese coasts (Cortinhas et al. 2015), we are unable to detect interspecific genetic differences among individuals at the population level, nor species-specific genetic differences using the rDNA ITS marker. Similar patterns are observed in the closely related orchids *Platanthera bifolia* and *P. chlorantha* that have the same geographic distribution, habitat preferences and flowering times, in which ITS data failed to discriminate among species (Bateman et al. 2012). Moreover, our results did not support a direct relationship between ribotype diversity and geographic distribution, since from Portugal to Morocco, all *L. vulgare* populations shared the same ribotype, whereas *L. narbonense* populations from southern France and eastern Spain are more genetically differentiated than *L. vulgare* and *L. maritimum*. These findings suggest that at least in *L. vulgare*, substantial gene flow might occur and/or a recent divergence process within the group.

The network analyses show that all *L. vulgare* populations (Portugal and Morocco) and one *L. maritimum* population (northwest Portugal) share a super-ribotype that seems to be the common ancestral ribotype of the group. Unexpectedly, two of the *L. maritimum* populations have a closer genetic relationship with *L. narbonense*. In other *Limonium* complexes, genetic diversity assessed using chloroplast markers shows little to no genetic diversity in obligate apomicts (e.g., *L. multiflorum*), while moderate to high levels of genetic variation are observed in sexually reproducing *Limonium* species (e.g., *L. ovalifolium*) (Róis et al. 2016). Still, a comparative analysis of *Limonium* species using methylation-sensitive amplified polymorphism markers indicates that epigenetic rather than genetic variation is more likely to explain differences among species (Róis et al. 2013).

Phylogeny

Our results confirm previous molecular studies based on plastid and ITS markers in which *L. vulgare* and *L. narbonense* form a monophyletic group, sister to the remaining species of *Limonium* section *Limonium* (Lledó et al. 2005; Akhani et al. 2013; Malekmohammadi et al. 2017). The core *Limonium* clade is clearly separated from the other genera of Staticoideae (*Psylliostachys* and *Acantholimon*) and is polyphyletic, as previously detected using nrDNA and plastid markers (Lledó et al. 2005; Akhani et al. 2013; Malekmohammadi et al. 2017). We observe a mixture of ploidy levels (Fig. 4) in all formed clades. Whereas *L. reniforme* clade consists of Irano-Turanian area diploid species (e.g., *L. furfuraceum*, *L. caspium*, *L. reniforme*) (Rice et al. 2015), the western Mediterranean species includes a mixture of diploids and polyploids (e.g., triploid *L. girardianum*; Palacios et al. 2000; Lledó et al. 2005). The *L. vulgare* clade is represented by tetraploids from both Atlantic Europe and Mediterranean regions, i.e., *L. vulgare*, *L. maritimum* and *L. narbonense*, and the species embedded in it, i.e., diploid *L. latifolium* and *L. gmelinii* (Rice et al. 2015).

Nevertheless, it might be that the sole molecular marker used in this study is not sufficient to discriminate the possible complex and intricate genetic relationships among species in the *L. vulgare* complex. Indeed, combined in situ *hybridization* as well as molecular phylogenetic analyses with cpDNA and nrITS sequences revealed distinct genomically differentiated auto- and allotetraploids in *Prospero autumnale* complex (Jang et al. 2018), although without conspicuous morphological differentiation (Jang et al. 2013). Further studies in *L. vulgare* complex are necessary and should include more molecular markers to examine the origins and evolutionary trajectories of those closely related species.

Conclusions

Taken together, our results provide for the first time evidence of congruence between genome size and ITS relationships, which suggest that substantial gene flow might occur among

some of the species of the *L. vulgare* complex. Interestingly, taxonomic studies based on morphology show that at least in the Portuguese coasts *L. vulgare*, *L. narbonense* and *L. maritimum* can co-occur in some mixed populations (Cortinhas et al. 2015); however, we were unable to find genetic differences at species-specific and population levels. We conclude that the closely related species of *L. vulgare* complex probably evolved relatively recently.

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Author contributions ADC designed and coordinated the study. ADC, ASR, MG and LR collected plant material. ADC performed chromosome work. ASR and FS undertook the molecular genetic studies, and ASR processed the raw data and carried out the statistical analysis. SC and JL performed flow cytometric studies and statistical analyses. ADC, ASR, SC and JL drafted the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflicts of interests.

Information on Electronic Supplementary Material

Online Resource 1. Populations of the *Limonium vulgare*, *L. narbonense* and *L. maritimum*, sampled in France (FR), Spain (ES), Portugal (PT) and Morocco (MA). Representative specimens of some of the populations are deposited in the Herbarium of Universidade de Coimbra (COI) or in the Herbarium João de Carvalho e Vasconcellos (LISI), Instituto Superior de Agronomia, Lisbon.
Online Resource 2. DNA accession numbers of species used in phylogenetic analyses. Sequences available in GenBank (https://www.ncbi.nlm.nih.gov/) used in comparison with the *Limonium vulgare* complex comprising 24 *Limonium* species, *Psylliostachys suworowii*, *Acantholimon acerosum* and *Plumbago europaea*.
Online Resource 3. GenBank accession numbers for accessions included in this study. Sequences are available in GenBank (https://www.ncbi.nlm.nih.gov/).
Online Resource 4. Alignments used to produce phylogeny.

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