



# Biogeographical, ecological and ploidy variation in related asexual and sexual *Limonium* taxa (*Plumbaginaceae*)

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*Limonium* is a widespread genus of halophytes and taxa found on the Atlantic coast include sexual diploids of the *L. ovalifolium* complex, agamosperous tetraploids of the *L. binervosum* complex and the triploid *L. algarvense*. In this study, we investigated: (1) cytotype distribution and diversity within and among populations in an overlapping region of diploid and polyploid *Limonium* spp. in south-western Iberia and north-western Morocco; and (2) patterns of geographical parthenogenesis and ecological preferences across a latitudinal gradient on the Atlantic coast. We show here for the first time that *L. nydeggeri* and *L. algarvense* are found further south in Morocco than previously reported. Genome size and ploidy estimates showed that the distribution of these species is not random at the overlapping region studied: tetraploid apomicts tend to be found at higher latitudes than the sexual diploids and *L. algarvense* grows in sympatry at the southern boundaries of the diploids. Natural populations showed a constancy in ploidy in these complexes. However, we report for the first time the occurrence of mixed-ploidy populations of *L. ovalifolium* s.l., euploid triploids in *L. algarvense* and aneuploids in the *L. binervosum* complex. On the Atlantic coasts, *L. algarvense* followed by *L. ovalifolium* complexes occur significantly more frequently in thermomediterranean and dry ombrotypes habitats than the *L. binervosum* complex. Significant differences were also observed among taxa in the frequency of occurrences on the most common lithological groups. In conclusion, this work presents the first biogeographical insights for the group based in a coarse-scale analysis of data and it provides evidence of ecological differentiation between the studied *Limonium* complexes. © 2016 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2016

**ADDITIONAL KEYWORDS:** agamospecies – apomixis – biogeography – chromosome base numbers – cytotypes – ecological characteristics – geographical distribution – habitat – polyploidy.

## INTRODUCTION

Polyploidy, i.e. whole-genome duplications, is an important evolutionary process often associated with changes in the reproductive system of flowering plants (Grant, 1981; Otto, 2007). A major route for

polyploid emergence in several plant genera is through sexual polyploidization, a process in which polyploids are generated by the formation and fusion of unreduced gametes (Bretagnolle & Thompson, 1995; Ramsey & Schemske, 1998). Hybridization with polyploidy may lead to the establishment of reproductive barriers within a few generations, either via the formation of a diploid F<sub>1</sub> hybrid and

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subsequent chromosome duplication (allopolyploidy) or via a triploid hybrid bridge, in which a triploid cytotype serves as an intermediate step in the production of a new tetraploid entity (e.g. Ramsey & Schemske, 1998). The shift to apomixis (agamosperry; asexual reproduction via seed formation) is one possible mechanism to avoid hybrid sterility and stabilize these hybrid biotypes (e.g. Asker & Jerling, 1992).

In flowering plants, almost all apomicts are exclusively polyploid (Asker & Jerling, 1992; Carman, 1997), except in a few cases, such as diploid *Boechera* Á.Löve and D.Löve (Böcher, 1951) and *Paspalum* L. (Siena *et al.*, 2008). Apomicts are commonly allotetraploid (e.g. *Antennaria* Gaertn., Bayer, 1997; *Potentilla* L., Dobeš *et al.*, 2013; *Limonium* Mill., Palop-Esteban, Segarra-Moragues & González-Candelas, 2007) or autotetraploid (e.g. *Paspalum*, Hojsgaard *et al.*, 2008; *Ranunculus* L., Hörandl, 2011), whereas their sexual relatives are usually diploids. Reproductive differentiation and isolation between sexual and apomictic cytotypes is common in natural populations and their distribution is either allopatric (i.e. spatially separated from each other; van Dijk, 2007), or sympatric (i.e. co-occurring in the same population; Elzinga *et al.*, 1987; Talent & Dickinson, 2007; Cosendai & Hörandl, 2010; Dobeš *et al.*, 2013). Additionally, as most apomicts reproduce through facultative apomixis, i.e. the co-existence of apomictic and sexual seed production in the same plant, cytotype diversity can be higher than expected in these populations (e.g. Cosendai & Hörandl, 2010).

Different distributional patterns in related sexual and asexual organisms ('geographical parthenogenesis') have long been reported by several authors (Bierzychudek, 1985; Asker & Jerling, 1992; Kearney, 2005). Apomicts in *Taraxacum* F.H.Wigg. and *Chondrilla* L. (van Dijk, 2003), *Ranunculus* (Hörandl, 2006; Hörandl, Cosendai & Tensch, 2008) and *Limonium* (Róis *et al.*, 2016) have larger distributions at higher latitudes and elevations than their sexual relatives and tend to colonize previously glaciated areas. Geographical parthenogenesis has been attributed to several factors that may have contributed to replacement of sexuals by apomicts in sympatric areas. In particular, superior colonizing abilities of polyploid apomicts, i.e. the capacity to establish a new population from a single individual or seed (Richards, 2003), have been invoked to explain geographical parthenogenesis (Hörandl *et al.*, 2008). Apomixis also overcomes the need for mating partners after a long-distance dispersal event (Baker, 1967; Hörandl *et al.*, 2008; Pannel *et al.*, 2015). Autonomous apomicts, which do not need pollen at all, could provide an advantage over pseudogamous apomicts that still require fertile pollen for

endosperm formation (Hörandl *et al.*, 2008). For example, in Asteraceae, autonomous apomixis is predominant (Noyes, 2007) and a higher probability of population establishment in remote areas has been correlated with the patterns of geographical parthenogenesis frequently observed in the family (Bierzychudek, 1985; Hörandl *et al.*, 2008). Another theory hypothesizes a connection between geographical parthenogenesis and unidirectional hybridization between apomicts and sexuals which are expected to replace sexual reproduction by apomixis in sympatric areas and is thought to be a causal factor for the wide distribution of apomictic complexes (Mogie, 1992; Mogie, Britton & Stewart-Cox, 2007; Hörandl *et al.*, 2008). Other theories, such as the 'frozen niche-variation' model, suggest that apomictic taxa are adapted to slightly different niche optima and occupy larger and climatically more extreme areas than their closest sexual counterparts (Vrijenhoek & Parker, 2009). An in-depth study of niche differentiation between diploid and tetraploid cytotypes in the alpine *Ranunculus kuepferi* Greuter & Burdet showed that diploid and tetraploid populations differed in terms of niche optima and breadth, with a shift towards cooler conditions in tetraploids (Kirchheimer *et al.*, 2016). Rather than niche differentiation, it was assumed that a change towards facultative apomixis was decisive for tetraploid establishment, being an effective reproductive strategy to avoid minority cytotype exclusion.

*Limonium* (sea-lavenders, Plumbaginaceae) is a cosmopolitan species-rich genus of annuals, perennial herbs, shrubs and lianas, often adapted to extreme saline environments (Baker, 1966; Kubitzki, 1993). In this genus, several taxonomically complex groups (herein called 'complexes') have been identified (Erben, 1978, 1993). These are generally characterized by uniparental reproduction (e.g. self-fertilization, agamospermy, gynogenesis) and hybridization at some degree among its members, which with polyploidy make it difficult to classify the biodiversity into discrete and unambiguous species (Ennos, French & Hollingsworth, 2005; Cortinhas *et al.*, 2015; Róis *et al.*, 2016). A wide cytological diversity has been reported for this genus, ranging from euploid and aneuploid diploids to octoploids (Erben, 1978, 1993; Arrigoni & Diana, 1993; Georgakopoulou *et al.*, 2006; Castro & Rosselló, 2007; Róis *et al.*, 2012; Cortinhas *et al.*, 2015). In *Limonium* section *Limonium*, the majority of sexual species are diploid, such as those of *L. ovalifolium* Kuntze complex (Erben, 1999; Róis *et al.*, 2016), whereas most triploid and tetraploid species, like those in the *Limonium binervosum* (G.E.Sm.) C.E.Salmon complex, are apomicts (Erben, 1978; Ingrouille & Stace, 1985, 1986; Cowan, Ingrouille &

Lledó, 1998; Róis *et al.*, 2016). Triploid taxa appear to be highly concentrated in the western Mediterranean region, whereas tetraploid taxa and those with higher ploidy are found along the Atlantic coasts and in the eastern Mediterranean region (Erben, 1978, 1979, 1993; Ingrouille & Stace, 1985, 1986; Brullo & Erben, 1989). A recent study showed that diploid sexual and tetraploid apomictic *Limonium* taxa differed in their latitudinal distributional patterns (Róis *et al.*, 2016). Nonetheless, insights into biogeographical and ecological aspects of sexual and apomictic taxa in *Limonium* in the southern part of the distribution have been largely unexplored.

In this study, we investigated the distributional patterns of diploid sexual species of the *L. ovalifolium* complex, tetraploid agamosperous species of the *L. binervosum* complex and triploid *L. algarvense* Erben. First, we investigated cytotype distribution and diversity within and among populations in south-western Iberia and north-western Morocco, which is known to contain both diploid and polyploid *Limonium* spp. Individuals from natural populations were sampled and DNA ploidy was estimated using flow cytometry; chromosome counts were also made to confirm ploidy estimations. Second, because natural populations showed constancy in ploidy within these complexes, we analysed climatic and geological data available in online databases and the literature for the entire distribution range of the diploid and polyploid *Limonium* complexes studied here. Although a coarse-scale analysis, this constitutes the first biogeographical study for the group and provides the basis for subsequent fine-scale studies in selected populations.

## MATERIAL AND METHODS

### STUDY SPECIES

The perennial species studied belong to a group of parallel-veined *Limonium* spp., which are distributed in coastal habitats influenced by maritime winds and/or temporary soil submersion by seawater (Róis *et al.*, 2016). Among them are the diploid species of the *L. ovalifolium* complex ( $2n = 2x = 16$  chromosomes), which includes *L. ovalifolium* Kuntze, *L. nydeggeri* Erben and *L. lanceolatum* (Hoffmanns. & Link) Franco (Erben, 1978, 1993, 1999; Róis *et al.*, 2012, 2013), the tetraploid species of the *L. binervosum* complex ( $2n = 4x = 32, 35, 36$ ) comprising *L. binervosum*, *L. dodartii* Kuntze and *L. multiflorum* Erben (Erben, 1978, 1993; Ingrouille & Stace, 1986), and *L. algarvense* ( $2n = 3x = 25$ ) (Erben, 1978, 1993; Ingrouille, 1985). Molecular analyses using methylation-sensitive amplified polymorphism

markers and discriminant function analyses of morphometric data using specimens representative of the *L. ovalifolium* and *L. binervosum* complexes resulted in a clear separation within and between groups on the basis of the bract and calyx characteristics (Róis *et al.*, 2013). Moreover, the diploid species in the *L. ovalifolium* complex showed reasonable morphological differentiation and interspecific differences that were more evident when the analysis was focused on the epigenetic variation (Róis *et al.*, 2013).

Populations of the diploid species mostly reproduce by outcrossing, whereas tetraploids reproduce through apomixis (Róis *et al.*, 2016). There is no information on the reproductive system of triploids. *Limonium ovalifolium* s.s. extends from Atlantic France, through the south-western Iberian Peninsula to Morocco (Erben, 1978, 1993) and *L. nydeggeri* thrives on the west and south-west coasts of Portugal (Erben, 1999; Espírito-Santo *et al.*, 2012); in contrast, *L. lanceolatum* is distributed along the north-west to south-west coasts of the Iberian Peninsula and in northern Morocco [Franco, 1984; EUNIS (European Nature Information System), 2014; Fennane, Ibn Tattou & El Oualidi, 2014]. *Limonium binervosum* s.l. occurs in Britain and Ireland (Curtis & McGough, 1988; Stace, 2010), France (Lahondère & Biorét, 1995, 1996) and the Iberian Peninsula coasts (Pignatti, 1971; Erben, 1978), whereas *L. dodartii* grows on the French Atlantic coast, in north-western Spain, western Portugal (Erben, 1993; Lahondère & Biorét, 1995, 1996) and in Mediterranean France (Lahondère & Biorét, 1996) and *L. multiflorum* occurs in western Portugal (Erben, 1978, 1993; Espírito-Santo *et al.*, 2012; Caperta *et al.*, 2014). Finally, the triploid *L. algarvense* is distributed from south-western Portugal to southern Spain (Erben, 1993) and has been reported as having an uncertain presence in Morocco (Fennane *et al.*, 2014).

### PLANT MATERIAL AND FIELD SAMPLING

Thirty-nine populations of the *L. ovalifolium* and *L. binervosum* complexes and *L. algarvense* were sampled in the Iberian Peninsula (Portugal and Spain), Morocco and France. The species were identified using keys 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 taxa sampled are listed in Appendix 1. All populations were tagged with the Global Positioning System (Appendix 2).

In the field, leaf material from one to 32 individuals was sampled per population (290 individuals in total). Two to three leaves per individual were stored



in labelled hermetic plastic bags and maintained at 4 °C until flow cytometric analysis. Additionally, seeds of mother plants of *L. algarvense*, *L. binervosum* s.s., *L. dodartii* and *L. lanceolatum* were collected from the same natural populations to establish experimental populations under controlled conditions. These seeds were germinated in a growth chamber (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 flow cytometric analyses and chromosome counts, respectively. This procedure enabled us to assign genome sizes unambiguously to each ploidy detected.

#### GENOME SIZE AND DNA PLOIDY ESTIMATIONS

Genome size and DNA ploidy were assessed using flow cytometry. Nuclei were isolated following the procedure of Galbraith *et al.* (1983) in which 0.5 cm<sup>2</sup> of fresh leaf tissue of *Limonium* was chopped with a razor blade, simultaneously with 0.5 cm<sup>2</sup> of fresh leaf tissue of the internal reference standard, in a Petri dish containing 1 mL of WPB buffer (Loureiro *et al.*, 2007). As an internal standard, *Pisum sativum* 'Ctirad' (2C = 9.09 pg) or *Secale cereale* 'Dankovské' (2C = 16.19 pg) was used (Doležel *et al.*, 1998). *Pisum sativum* was used as primary reference standard for most of the samples, with the exception of 18 samples of *L. multiflorum* and two of *L. algarvense*, for which *S. cereale* was used instead. The nuclear suspension was filtered using a 50-µm nylon mesh and 50 µg mL<sup>-1</sup> propidium iodide (PI; Fluka) was added to stain the DNA. To avoid staining of double stranded RNA, 50 µg mL<sup>-1</sup> of RNase (Fluka) was also added. After a 5-min incubation period, samples were analysed in a Partec CyFlow Space flow cytometer (532 nm green solid-state laser, operating at 30 mW; Partec). Integral fluorescence and fluorescence height and width emitted from nuclei were collected through a 620-nm band-pass interference filter. After the initial analyses, the amplifier system was set to a constant voltage and gain. Each day, prior to analysis, the instrument stability and linearity were checked either with fluorescent beads or using PI-stained nuclei isolated from *P. sativum* 'Ctirad'. Results were acquired using Partec FloMax software v2.4d (Partec) in the form of four graphics: histogram of fluorescence pulse integral in linear scale (FL); forward light scatter (FS) vs. side light scatter (SS), both in logarithmic (log) scale; FL vs. time; and FL vs. SS in log scale. For most samples, a polygonal region was defined in the last-named graphic to include only intact nuclei and this region was

subsequently used to gate all the other graphics. At least 1300 particles per G<sub>1</sub> peak were analysed per sample (Suda *et al.*, 2007). Genome size estimates were only considered when the CV values of G<sub>1</sub> peaks were < 5%. Samples with higher CV values were discarded and a new sample was prepared. Up to 12 individuals per population were analysed for genome size and additional individuals were analysed using the pooled sample strategy, i.e. three or four individuals were analysed simultaneously; the latter analyses were used for ploidy estimation only.

The absolute 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<sub>1</sub> peak mean/reference standard G<sub>1</sub> peak mean) × genome size of the reference standard. Because genome sizes were obtained for several individuals that were also characterized karyologically (below), DNA ploidy could be inferred for all individuals analysed.

Descriptive statistics were calculated for genome size data (mean, standard deviation of the mean, coefficient of variation and minimum and maximum values). Differences in genome size between ploidies and between species within ploidies were assessed using a one-way ANOVA, followed by a Tukey test for multiple comparisons. For the comparison among ploidies, genome size was log<sub>10</sub>(x)-transformed to achieve normality and homogeneity of variances. A Mann–Whitney *U* test was used to compare genome size between tetraploids and aneuploids of *L. dodartii* and between triploids (including individuals with both 24 and 25 chromosomes for which a genome size continuum was observed) and aneuploids of *L. algarvense*. Statistical analyses were performed using SigmaPlot for Windows v. 12.5 (Systat Software).

#### CHROMOSOME COUNTS

Chromosome counts were made to confirm the ploidy estimated based on genome sizes obtained using flow cytometry. Chromosomes were counted for three plants of each species following the procedure described by Róis *et al.* (2012). Briefly, root tips were excised and treated with a 2 mM 8-hydroxyquinoline solution for 2 h at 4 °C in the dark and subsequently for 2 h at room temperature to induce c-metaphases. 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. Afterwards, 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 1× EB (40 mL 0.1 M citric acid-1-hydrate and 60 mL 0.1 M sodium citrate dihydrate; pH 4.8] for 3 h at 37 °C as described by Róis *et al.* (2012). Chromosome

spreads were prepared in 60% acetic acid and stained with 4',6-diamino-2-phenylindole hydrochloride (DAPI) (1 mg mL<sup>-1</sup>) in Vectashield (Vector Laboratories). Chromosome preparations were observed using a Zeiss Axioskop 2 fluorescence microscope and photographed with an AxioCam MRc5 digital camera (Zeiss).

#### OCCURRENCE DATA COLLECTION AND CHARACTERIZATION

In total, 1776 records were obtained for the *L. ovalifolium* and *L. binervosum* complexes from the Global Biodiversity Information Facility (<http://www.gbif.org/>). These encompassed the entire distribution range of the species from each complex, all over the Atlantic coasts in Europe, namely in Ireland, Britain, France and the Iberian Peninsula, and in Morocco (Baker, 1953; Pignatti, 1971; Erben, 1978, 1993, 1999; Franco, 1984; Ingrouille, 1985; Ingrouille & Stace, 1986; Curtis & McGough, 1988; Lahondère & Biorét, 1995, 1996; Stace, 2010; Espírito-Santo *et al.*, 2012; EUNIS, 2014; Fennane *et al.*, 2014). If no reliable location information could be found for some records, they were excluded from further analysis. Furthermore, erroneous GBIF (Global Biodiversity Information Facility) occurrence points (e.g. unlikely point locations such as those generated by coordinates outside the country border under which the species has been listed; coordinates located inland, and thus in non-coastal areas of the countries; and coordinates falling in the Atlantic Ocean or in the Mediterranean Sea) were removed through visual inspection in the desktop GIS environment. Additional occurrence data based on our own field observations (between 2009 and 2015) across the Iberian Peninsula and Moroccan coasts were added comprising a total of 219 records for both species complexes and *L. algarvense*. Since occurrence data from the GBIF for some of the species were missing (e.g. *L. algarvense*, *L. lanceolatum*, *L. nydeggeri*), the data analyses were made considering three species groups, i.e. *L. ovalifolium* complex, *L. binervosum* complex and *L. algarvense*. To map the extent of each species distribution, the added occurrence records were georeferenced using Desktop ArcGIS 10.0 (ESRI). After filtering and completion procedures, 618 occurrence records were considered (Supporting Information, Table S1; Fig. 1).

Occurrence data points were then overlapped with bioclimate maps with a grid size of 5000 m (<http://www.globalbioclimatics.org/>) to obtain bioclimate (Supporting Information, Table S2) and thermotype and ombrotype (Supporting Information, Table S3) of each location, according to the World Bioclimatic Classification System (Rivas-Martínez, Rivas-Sáenz & Penas, 2011). Additionally, the location points

were characterized by their surface lithology, which was based on information available at the OneGeology Europe Portal with a scale 1:1000 000 (<http://www.onegeology-europe.org/>). According to the information gathered on surface lithology, 18 different lithology groups were identified (Supporting Information, Table S4). Each *Limonium* taxon was characterized by the frequency of each thermotype, ombrotype and lithology group. Differences within and among taxa in the frequencies of climatic and lithological groups were statistically analysed using  $\chi^2$  tests. For the surface lithology, only the four most common lithological groups observed within each *Limonium* group were considered for statistical purposes. These statistical analyses were carried in R version 3.1.1 (R Development Core Team).

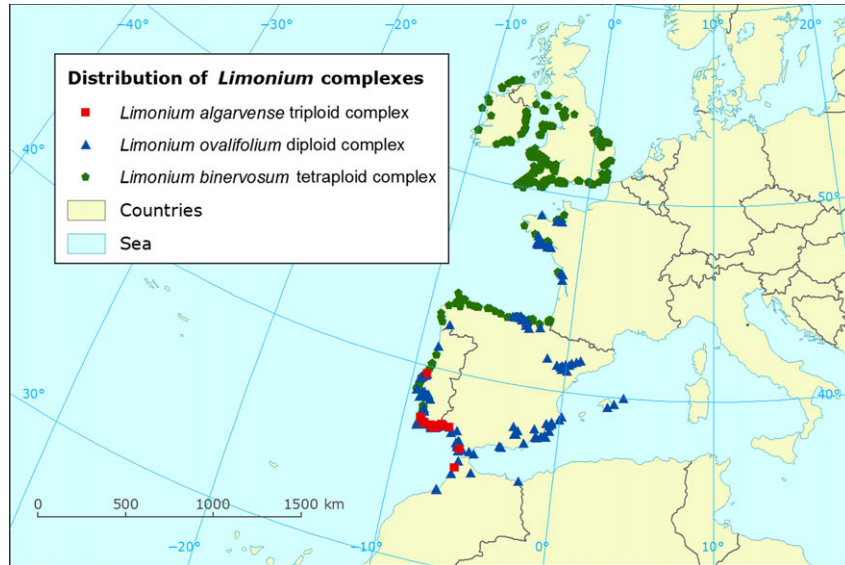
Available information for plant communities of the studied *Limonium* spp. is scarce and incomplete and this therefore led us to compile a summary (Supporting Information, Table S5) mainly discriminated by dominant plant species according to the available literature (Bendaanoun, 1991; Costa *et al.*, 1998, 2012, 2014; Hammada, 2007).

## RESULTS

#### CYTOTYPE COMPOSITION

Flow cytometric analyses enabled us to unambiguously assess the ploidy for 403 individuals (290 collected directly in the field and 113 from plantlets germinated from seeds) from 20 populations of the *L. ovalifolium* complex (*L. ovalifolium* s.s., *L. nydeggeri* and *L. lanceolatum*), ten populations of the *L. binervosum* complex (*L. binervosum* s.s., *L. dodartii* and *L. multiflorum*) and seven populations of *L. algarvense* (Table 1; Fig. 2). Generally, the quality of the flow cytometry histograms was good, with the CV values of the G<sub>1</sub> peaks of the sample and standard averaging 4.20 and 2.65%, respectively. Overall, three cytotypes were detected, namely diploids ( $2n = 2x = 3.55 \pm 0.07$  pg/2C), triploids ( $2n = 3x = 5.69 \pm 0.15$  pg/2C) and tetraploids ( $2n = 4x = 7.49 \pm 0.18$  pg/2C) (Fig. 2) and a few aneuploids (resulting from triploid and tetraploid individuals after the loss of some chromosomes). Not surprisingly, significant differences in genome size were observed between ploidies ( $F_{2,198} = 16\ 815.89$ ,  $P < 0.001$ ).

Despite a few exceptions, most species were ploidy homogeneous. The *L. ovalifolium* complex was uniformly diploid (Figs 2A–C, 3A), except in two mixed-ploidy populations of *L. lanceolatum* detected in the southern Iberian Peninsula (Figs 2D, 3A; Table 1), with individuals being either diploid (Fig. 2C) or triploid (Fig. 2D). Although only five individuals from



**Figure 1.** Distribution of diploid, triploid and tetraploid *Limonium* spp. in the Atlantic and Mediterranean coasts: 618 occurrence records based on data from the GBIF (399 records) and our own field observations (219 records). Diploid species of the *L. ovalifolium* complex include *L. ovalifolium* s.s., *L. nydeggeri* and *L. lanceolatum*; and tetraploid species of the *L. binervosum* complex include *L. binervosum* s.s., *L. dodartii* and *L. multiflorum*.

each of the two populations were analysed, the triploid cytotype was dominant in one of them (Fig. 3A; Table 1).

Analysis of *L. algarvense* confirmed that it was triploid (Figs 2E, 3B), although possible aneuploids were also detected in the Moroccan populations (Fig. 3B; Table 1), as they had significantly lower genome sizes (Mann–Whitney  $U = 0.00$ ,  $n_1 = 4$ ,  $n_2 = 63$ ,  $P < 0.001$ ; ranging from 5.22 to 5.46 pg/2C; Table 1). A high variation in genome size estimates was also observed in this taxon across the sampled area (Supporting Information, Fig. S1), suggesting that its chromosome number is more variable than previously envisaged or that some individuals have suffered DNA loss. The chromosome counts obtained from the Alvor population in Portugal (Fig. 4C, D; Supporting Information, Fig. S1) suggest that individuals with 24 or 25 chromosomes could occur across the sampled area.

*Limonium binervosum* s.s., *L. dodartii* and *L. multiflorum* were all tetraploid (Figs 2F–H, 3C, D), except for a few individuals of *L. dodartii* from two populations that appear to be composed of aneuploid plants with significantly lower genome sizes than the confirmed tetraploid individuals (possible aneuploids:  $6.87 \pm 0.10$  pg,  $4x$ :  $7.43 \pm 0.12$  pg; Mann–Whitney  $U = 0.00$ ,  $n_1 = 3$ ,  $n_2 = 14$ ,  $P < 0.001$ ; Fig. 3C; Table 1).

Differences in genome size between species of each ploidy were also explored, but no significant differences were observed in either the *L. ovalifolium*

complex ( $F_{2,85} = 2.80$ ,  $P = 0.066$ ) or the *L. binervosum* group ( $F_{2,41} = 0.35$ ,  $P = 0.709$ ).

#### CHROMOSOME NUMBERS

Our chromosome counts obtained from metaphase spreads of *L. lanceolatum* revealed, for the first time,  $2n = 2x = 16$  chromosomes for this species (Fig. 4A). Furthermore, we established differences in ploidy among *L. ovalifolium* s.s., *L. lanceolatum*, *L. algarvense*, *L. binervosum* s.s. and *L. dodartii* (Table 1; Fig. 4). For *L. lanceolatum* and *L. ovalifolium* s.s. (Fig. 4A, B), only diploid specimens with  $2n = 2x = 16$  chromosomes were found (Table 1). For *L. algarvense*, most specimens were triploids with  $2n = 3x = 25$  chromosomes (Fig. 4D), but individuals with  $2n = 3x = 24$  chromosomes (Fig. 4) were also observed. With regard to *L. binervosum* s.s., all individuals examined had  $2n = 4x = 35$  chromosomes (Fig. 4E), and *L. dodartii* individuals had  $2n = 4x = 36$  chromosomes (Fig. 4F), suggesting chromosome rearrangements.

#### BIOGEOGRAPHY AND ECOLOGY

Our findings showed that the apomictic *L. binervosum* complex tended to be distributed at higher latitudes (37–55°N) than the sexual *L. ovalifolium* complex (34–48°N), whereas *L. algarvense* occurs in an area that overlaps these two complexes and the southern boundaries of diploids (35–39°N). Differences in the frequency of the thermotypes

**Table 1.** Genome size estimates, ploidy and chromosome counts obtained in populations of the *Limonium ovalifolium* complex (*L. ovalifolium*, *L. nydeggeri* and *L. lanceolatum*), *L. algarvense* and the *L. binervosum* complex (*L. binervosum*, *L. dodartii* and *L. multiflorum*) sampled in France, Spain, Portugal and Morocco

| Species                                 | Population   | Genome size (2C, pg) |      |      |      |      | DNA ploidy level |           |          | Chromosome counts |                           |                   |
|---|--|----------------------|------|------|------|------|------------------|-----------|----------|-------------------|---------------------------|-------------------|
|   |  | Mean                 | SD   | CV   | Min. | Max. | <i>N</i>         | <i>2n</i> | <i>N</i> | Observed          | Literature                |                   |
| <i>L. ovalifolium</i>                   | Portugal, Cascais: Cabo Raso                           | 3.53                 | 0.05 | 1.45 | 3.44 | 3.59 | 5                |           | 20       |                   | 16 <sup>*,†</sup>         |                   |
|   | Portugal, Sines: Praia da Ilha do Pessegueiro          | 3.52                 | 0.06 | 1.62 | 3.44 | 3.60 | 5                | 2x        | 17       |                   |                           |                   |
|   | Portugal, Vila do Bispo: Cabo de Sagres                | 3.57                 | 0.06 | 1.66 | 3.47 | 3.62 | 4                | 2x        | 4        |                   | 16                        |                   |
|   | Portugal, Lagos: Luz                                   | 3.52                 | 0.04 | 1.10 | 3.49 | 3.57 | 3                | 2x        | 3        |                   | 16                        |                   |
|   | Portugal, Portimão: Ferragudo                          | 3.50                 | 0.04 | 1.26 | 3.46 | 3.55 | 2                | 2x        | 2        | 16                |                           |                   |
|   | Morocco, Rabat: Sidi Abed                              | 3.51                 | 0.06 | 1.71 | 3.39 | 3.57 | 6                | 2x        | 16       |                   |                           |                   |
| <i>L. nydeggeri</i>                     |  |                      |      |      |      |      |                  |           |          |                   | 15, 16, 17 <sup>*,‡</sup> |                   |
|   | Portugal, Peniche: Papôa                               | 3.56                 | 0.01 | 0.19 | 3.56 | 3.57 | 2                | 2x        | 2        |                   |                           |                   |
|   | Portugal, Peniche: Baleal                              | 3.57                 | 0.07 | 2.09 | 3.49 | 3.64 | 2                | 2x        | 2        |                   | 15, 16                    |                   |
|   | Portugal, Peniche: N. Sr <sup>o</sup> dos Remédios     | 3.57                 | 0.06 | 1.80 | 3.46 | 3.67 | 6                | 2x        | 6        |                   | 16                        |                   |
|   | Portugal, Cascais: Cabo Raso                           | 3.61                 | 0.05 | 1.33 | 3.49 | 3.65 | 8                | 2x        | 8        |                   | 16                        |                   |
|   | Portugal, Aljezur: Pontal da Carrapateira              | 3.54                 | 0.04 | 1.14 | 3.47 | 3.59 | 6                | 2x        | 18       |                   |                           |                   |
|   | Portugal, Vila do Bispo: Cabo São Vicente              | 3.55                 | 0.10 | 2.69 | 3.41 | 3.74 | 6                | 2x        | 15       |                   |                           |                   |
|   | Morocco, Rabat: Sidi Abed                              | 3.59                 | 0.04 | 1.06 | 3.54 | 3.65 | 5                | 2x        | 25       |                   |                           |                   |
| <i>L. lanceolatum</i>                   | Portugal, Setúbal: Pontal dos Musgos                   | 3.47                 | 0.04 | 1.29 | 3.39 | 3.53 | 10               | 2x        | 10       |                   |                           |                   |
|   | Portugal, Odemira: Vila Nova Milfontes                 | 3.57                 | 0.07 | 1.90 | 3.45 | 3.67 | 10               | 2x        | 22       | 16                |                           |                   |
|   | Portugal, Tavira: sapal do Barril                      | 3.61                 | 0.08 | 2.10 | 3.53 | 3.78 | 10               | 2x        | 24       | 16                |                           |                   |
|   | Spain, Ayamonte: marshes of Isla Cristina              | 3.54                 | 0.03 | 0.98 | 3.48 | 3.57 | 4                | 2x        | 4        |                   |                           |                   |
|   | Spain, Cádiz: Toruños                                  |                      | 5.38 | –    | –    | –    | –                | 1         | 3x       | 1                 |                           |                   |
|   |  |                      | 3.53 | –    | –    | –    | –                | 1         | 2x       | 1                 |                           |                   |
|   | Morocco, Assilah: Oued Tahadart                        |                      | 5.61 | 0.03 | 0.55 | 5.56 | 5.65             | 4         | 3x       | 4                 |                           |                   |
|   |  |                      | 3.58 | 0.06 | 1.71 | 3.47 | 3.67             | 10        | 2x       | 32                |                           |                   |
|   | Morocco, Larache: Moulay Bouselham                     |                      | 3.58 | 0.04 | 1.15 | 3.53 | 3.66             | 5         | 2x       | 25                |                           |                   |
|   |  |                      |      |      |      |      |                  |           |          |                   |                           | 25 <sup>*,†</sup> |
| <i>L. algarvense</i>                    | Portugal, Aljezur: Praia da Amoreira                   | 5.66                 | 0.09 | 1.61 | 5.53 | 5.79 | 6                | 3x        | 6        |                   |                           |                   |
|   | Portugal, Portimão: Alvor                              | 5.80                 | 0.09 | 1.56 | 5.62 | 5.90 | 8                | 3x        | 8        | 24, 25            |                           |                   |
|   | Portugal, V. R.St <sup>o</sup> António: Castro Marim   | 5.67                 | 0.04 | 0.75 | 5.61 | 5.73 | 4                | 3x        | 4        |                   |                           |                   |
|   | Portugal, V. R.St <sup>o</sup> António: Ponta da Areia | 5.68                 | 0.04 | 0.62 | 5.65 | 5.73 | 4                | 3x        | 4        |                   |                           |                   |
|   | Spain, Huelva: Spit El Rompido                         | 5.86                 | 0.04 | 0.64 | 5.82 | 5.95 | 8                | 3x        | 8        |                   |                           |                   |
|   | Spain, Cádiz: El Tómbolo de Trafalgar                  | 5.89                 | 0.10 | 1.67 | 5.73 | 5.99 | 6                | 3x        | 6        |                   |                           |                   |
|   | Morocco, Larache: Loukkos 1                            |                      | 5.66 | 0.09 | 1.61 | 5.53 | 5.78             | 12        | 3x       | 12                |                           |                   |
|   |  |                      | 5.41 | 0.03 | 0.50 | 5.39 | 5.44             | 2         | an.      | 2                 |                           |                   |
|   | Morocco, Larache: Loukkos 2                            |                      | 5.63 | 0.09 | 1.64 | 5.54 | 5.77             | 8         | 3x       | 14                |                           |                   |
|   |  |                      | 5.46 | –    | –    | –    | –                | 1         | an.      | 1                 |                           |                   |
| Morocco, Larache: Loukkos 3             |  | 5.61                 | 0.06 | 1.09 | 5.55 | 5.72 | 5                | 3x        | 5        |                   |                           |                   |
|   |  | 5.22                 | –    | –    | –    | –    | 1                | an.       | 1        |                   |                           |                   |
| <i>L. binervosum</i>                    |  |                      |      |      |      |      |                  |           |          |                   | 27, 35 <sup>*,†,§</sup>   |                   |
|   | France, Brittany: Saint-Benoit-des-Ondes               | 7.25                 | 0.03 | 0.45 | 7.21 | 7.29 | 5                | 4x        | 9        |                   |                           |                   |
|   | France, Brittany: Pointe du Grouin                     | 7.26                 | 0.07 | 1.00 | 7.17 | 7.34 | 5                | 4x        | 10       |                   |                           |                   |
|   | Portugal, Aveiro: São Jacinto                          | 7.44                 | 0.12 | 1.67 | 7.22 | 7.64 | 6                | 4x        | 6        |                   |                           |                   |
| Portugal, S. Pedro de Moel: Praia Velha | 7.50   | 0.15                 | 1.95 | 7.27 | 7.65 | 7    | 4x               | 7         | 35       |                   |                           |                   |



**Table 1.** *Continued*

| Species               | Population                            | Genome size (2C, pg) |      |      |      |      | DNA ploidy level |            | Chromosome counts |                             |
|-----------------------|---------------------------------------|----------------------|------|------|------|------|------------------|------------|-------------------|-----------------------------|
|                       |                                       | Mean                 | SD   | CV   | Min. | Max. | <i>N</i>         | 2 <i>n</i> | <i>N</i>          | Observed                    |
| <i>L. dodartii</i>    | Portugal, Sines: Praia da Oliveirinha | 7.51                 | 0.03 | 0.00 | 7.48 | 7.54 | 2                | 4x         | 2                 | 35, 36 <sup>*,†</sup>       |
|                       |                                       | 6.92                 | 0.13 | 0.02 | 6.75 | 7.08 | 3                | an.        | 3                 |                             |
|                       | Portugal, Sines: Porto Covo           | 7.46                 | 0.06 | 0.84 | 7.40 | 7.56 | 5                | 4x         | 5                 |                             |
|                       | Portugal, Odemira: Cabo Sardão        | 7.44                 | 0.04 | 0.54 | 7.39 | 7.50 | 6                | 4x         | 6                 |                             |
|                       |                                       | 6.96                 | –    | –    | –    | –    | 1                | an.        | 1                 |                             |
| <i>L. multiflorum</i> | Portugal, Lourinhã: Vale de Frades    | 7.56                 | 0.04 | 0.49 | 7.53 | 7.61 | 3                | 4x         | 3                 | 32, 35, 36 <sup>*,†,‡</sup> |
|                       | Portugal, Mafra: Foz do Lizandro      | 7.52                 | 0.07 | 0.89 | 7.42 | 7.64 | 6                | 4x         | 6                 |                             |
|                       | Portugal, Cascais: Cabo Raso          | 7.70                 | 0.17 | 2.25 | 7.32 | 7.87 | 12               | 4x         | 12                |                             |

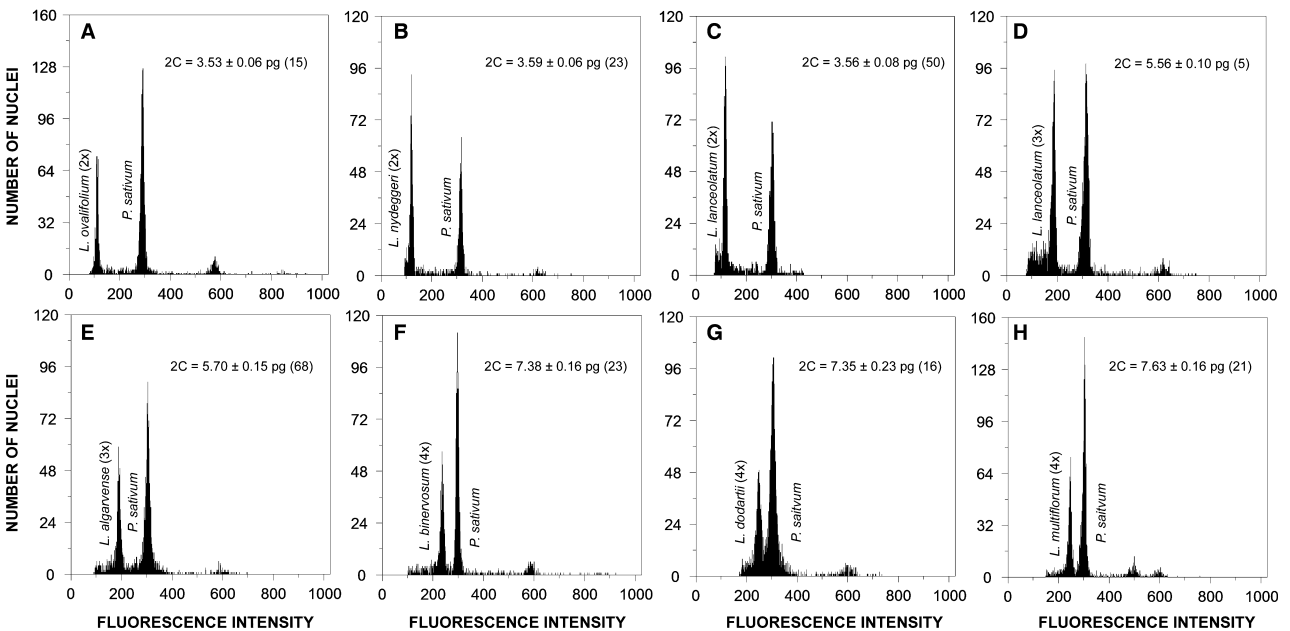
The following data are given for each taxon, population and ploidy: mean, standard deviation of the mean (SD), coefficient of variation (CV, %), and minimum (Min.) and maximum values (Max.) of the holoploid genome size (2C, pg), followed by sample size for genome size estimates (*N*); DNA ploidy (2*n*) and respective sample size (*N*) is also provided. DNA ploidy levels: 2x, diploid; 3x, triploid; 4x, tetraploid. Chromosome counts observed in this study (Observed) and reported in the bibliography are also provided (Literature).

\*Erben (1978).

†Erben (1993).

‡Róis *et al.* (2012).

§Ingrouille & Stace (1986).

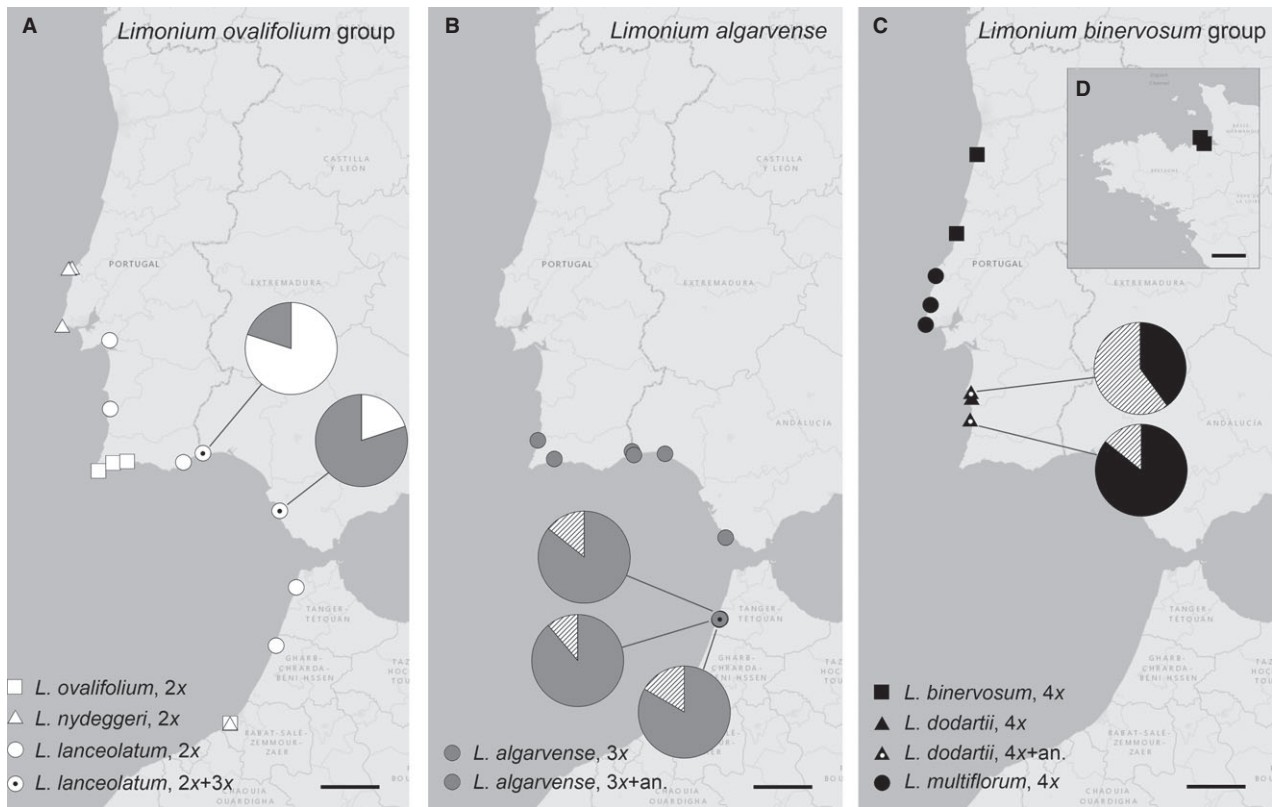


**Figure 2.** Flow cytometric histograms of propidium iodide-stained nuclei of diploid, triploid and tetraploid ploidy of the *Limonium ovalifolium* complex (A–D; *L. ovalifolium* – A, *L. nydeggeri* – B and *L. lanceolatum* – 2x, C; 3x, D), *L. algarvense* (E) and the *L. binervosum* complex (*L. binervosum* – F, *L. dodartii* – G and *L. multiflorum* – H) analysed simultaneously with the internal standard *Pisum sativum*. The genome size (pg/2C) for each species is indicated.

were observed among the three *Limonium* groups (Ther-momediterranean:  $\chi^2 = 29.18$ ,  $P < 0.001$ ; Mesomediterranean:  $\chi^2 = 7.40$ ,  $P = 0.025$ ; Mesotemperate:

$\chi^2 = 123.30$ ,  $P < 0.001$ ), with the Supratemperate type only being detected in *L. binervosum* (Fig. 5A; Supporting Information, Tables S2, S3). *Limonium*





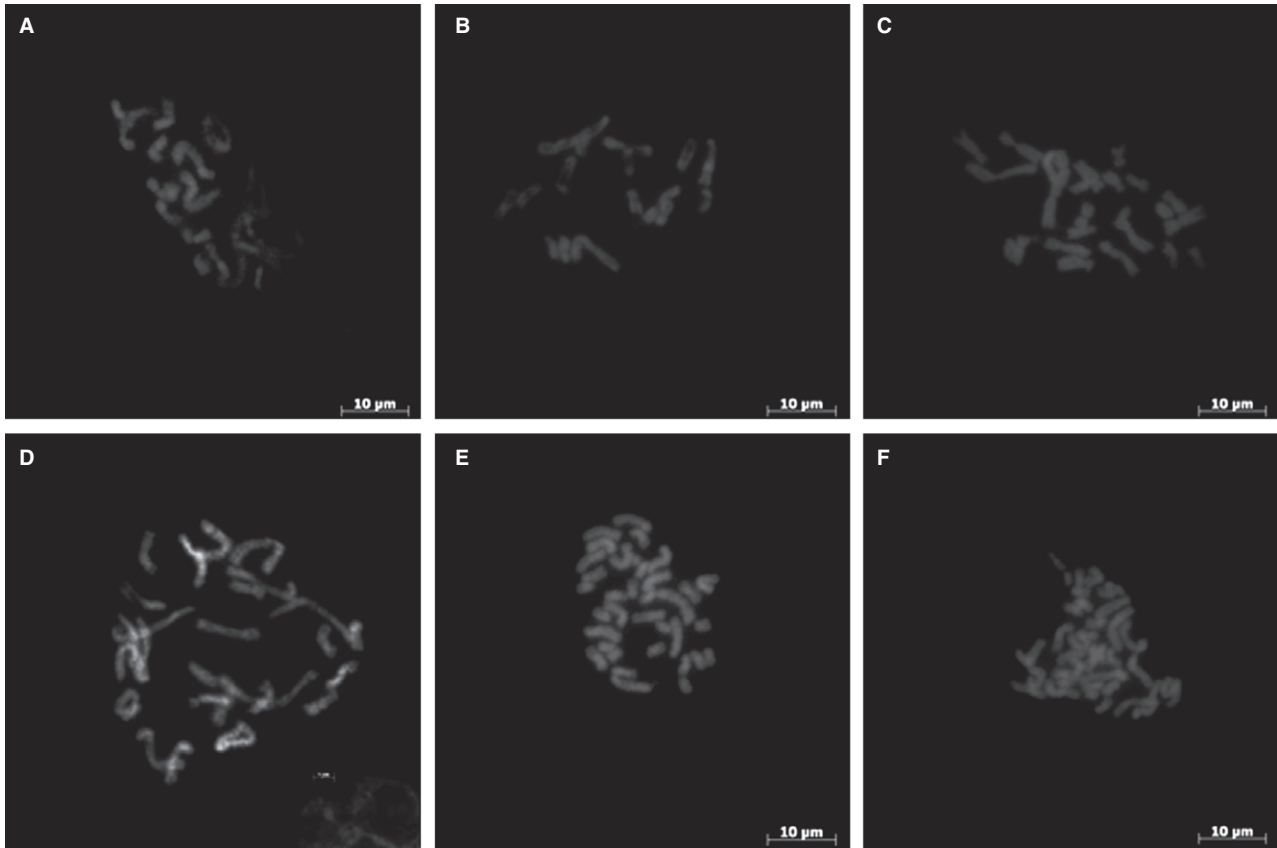
**Figure 3.** Geographical locations of diploid and polyploid *Limonium* populations studied in the overlapping region of south-western Iberia and north-western Morocco. A, *Limonium ovalifolium* complex: *L. ovalifolium* s.s., white squares; *L. nydeggeri*, white triangles; *L. lanceolatum*, white circles. B, *Limonium algarvense*, grey circles. C, *Limonium binervosum* complex: *L. binervosum* s.s., black squares; *L. dodartii*, black triangles; *L. multiflorum*, black circles. Cytotypes are represented by different colours: white, 2x, diploid; grey, 3x, triploid; black, 4x, tetraploid; stripes, an., aneuploid. Mixed-ploidy populations, i.e. populations with more than one ploidy detected, are denoted with a dot within the symbol and the proportion of cytotypes are provided in an inset pie diagram.

*algarvense* had the highest occurrence in the Thermomediterranean type followed by the *L. ovalifolium* complex, whereas the *L. binervosum* complex had significantly higher occurrences in the Mesotemperate type ( $P < 0.05$ ). The same patterns were evident when analysing frequencies of thermotypes within each *Limonium* group (*L. algarvense*:  $\chi^2 = 96.15$ ,  $P < 0.001$ ; *L. ovalifolium*:  $\chi^2 = 190.53$ ,  $P < 0.001$ ; *L. binervosum*:  $\chi^2 = 402.30$ ,  $P < 0.001$ ).

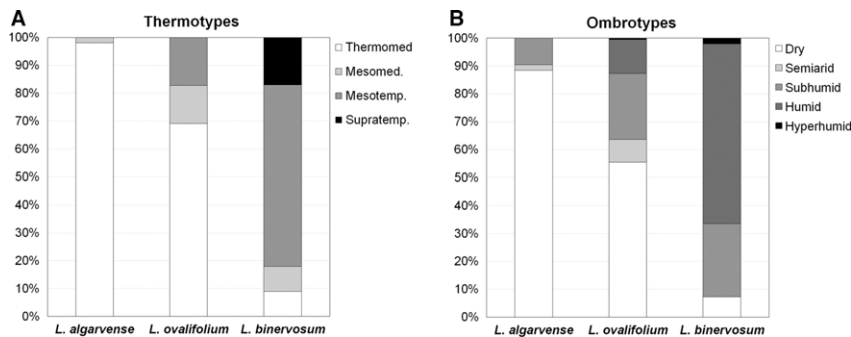
There were also differences between *Limonium* taxa in the frequency of occurrence records in some ombrotypes, in particular for Dry ( $\chi^2 = 232.25$ ,  $P < 0.001$ ) Subhumid ( $\chi^2 = 6.93$ ,  $P = 0.031$ ) and Humid types ( $\chi^2 = 148.48$ ,  $P < 0.001$ ). No differences were found for the remaining types (Semi-arid:  $\chi^2 = 2.53$ ,  $P = 0.111$ ; Hyperhumid:  $\chi^2 = 2.37$ ,  $P = 0.123$ ) (Fig. 5B; Supporting Information, Tables S2, S3). Under the dry ombrotype, *L. algarvense* followed by the *L. ovalifolium* complex had the highest frequencies of records ( $P < 0.05$ ). The

frequency of records in the semi-humid ombrotype was significantly higher in *L. ovalifolium* and *L. binervosum* complexes than in *L. algarvense* and the frequency of *L. ovalifolium* records in the humid ombrotype ( $P < 0.05$ ). The same patterns were evident when analysing frequencies of ombrotypes within each *Limonium* group (*L. algarvense*:  $\chi^2 = 107.37$ ,  $P < 0.001$ ; *L. ovalifolium*:  $\chi^2 = 254.60$ ,  $P < 0.001$ ; *L. binervosum*:  $\chi^2 = 443.24$ ,  $P < 0.001$ ).

Significant differences were observed in the frequency of the lithological groups within *Limonium* taxa (*L. algarvense*:  $\chi^2 = 67.72$ ,  $P < 0.001$ ; *L. ovalifolium*:  $\chi^2 = 19.31$ ,  $P < 0.001$ ; *L. binervosum*:  $\chi^2 = 9.44$ ,  $P = 0.024$ ), with *L. algarvense* occurring more frequently on alluvium, *L. ovalifolium* complex on limestone and *L. binervosum* complex on alluvium, limestone and till surfaces in comparison with other classes observed in each group (Fig. 6; Supporting Information, Table S4). Significant differences were



**Figure 4.** Mitotic metaphase plates of DAPI-stained metaphase spreads from *Limonium* spp. A, *Limonium lanceolatum* ( $2n = 2x = 16$  chromosomes); B, *L. ovalifolium* ( $2n = 2x = 16$ ); C, *L. algarvense* ( $2n = 3x = 24$ ); D, *L. algarvense* ( $2n = 3x = 25$ ); E, *L. binervosum* ( $2n = 4x = 35$ ); F, *L. dodartii* ( $2n = 4x = 36$ ).

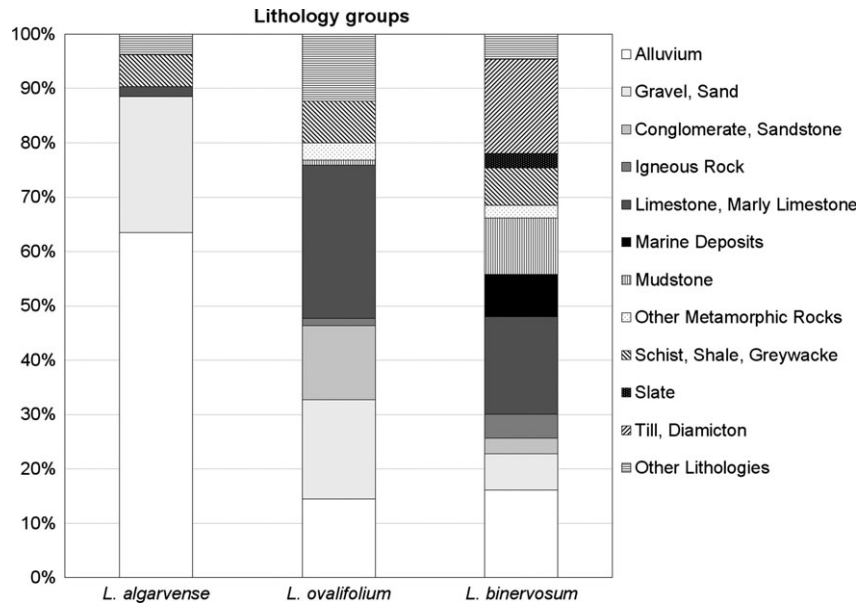


**Figure 5.** Frequency (%) of occurrence records in thermotypes (A) and ombrotypes (B) in *Limonium algarvense* and the *Limonium ovalifolium* and *Limonium binervosum* complexes. Data are based in GBIF occurrence points from GBIF and our own occurrence data. Thermomed., Thermomediterranean; Mesomed., Mesomediterranean; Mesotemp., Mesotemperate; Supratemp., Supratemperate.

also observed in the frequency of the most common surface lithology groups among *Limonium* taxa (alluvium:  $\chi^2 = 69.67$ ,  $P < 0.001$ ; limestone:  $\chi^2 = 20.57$ ,  $P < 0.001$ ; gravel:  $\chi^2 = 25.08$ ,  $P < 0.001$ ; conglomerate:  $\chi^2 = 23.64$ ,  $P < 0.001$ ; mudstone:  $\chi^2 = 19.36$ ,

$P < 0.001$ ), with till being observed only in *L. binervosum*.

Available data in the literature about the floristic composition of each *Limonium* community did not allow a discriminant statistical analysis (Supporting



**Figure 6.** Frequency (%) of occurrence records in surface lithology groups in *Limonium algarvense* and the *L. ovalifolium* and *L. binervosum* complexes. Data are based in GBIF occurrence points from GBIF and our own occurrence data.

Information, Table S5). Both *L. ovalifolium* and *L. binervosum* complexes have the same dominant species, i.e. *Frankenia laevis* L. or *Halimione portulacoides* (L.) Aellen (Costa *et al.*, 1998, 2012, 2014). However, in Morocco, other species can be dominant, including *Mesembryanthemum crystallinum* L., which associates with *L. nydeggeri* (our own unpublished observations), or *Sarcocornia perennis* (Mill.) A.J.Scott and *Juncus rigidus* Desf., which associate with *L. lanceolatum* (Bendaanoun, 1991). *Limonium binervosum* and *L. dodartii* communities are generally composed of only a few other species and in the Portuguese coast they are rare in salt marshes and on sea cliffs.

## DISCUSSION

In this study, we report for the first time the presence of *L. nydeggeri* on coastal cliffs and of *L. algarvense* in saltmarshes in Morocco. We provide novel data on the distribution of cytotypes and the diversity of diploid, triploid and tetraploid *Limonium* populations in a contact zone in the southern parts of their distribution range, i.e. south-western Iberia and adjacent north-western Morocco. Furthermore, using occurrence data available from the GBIF and our own data across a latitudinal gradient in the Atlantic coasts, we show ecological differentiation between the *L. ovalifolium* and *L. binervosum* complexes and *L. algarvense*.

## TAXONOMY AND CYTOGENETICS OF THE GROUP: WHAT IS KNOWN SO FAR?

*Limonium* is remarkably variable as a result of frequent hybridization and polyploidization events and different reproductive strategies (Baker, 1966; Erben, 1978; Costa *et al.*, 1998; Lledó *et al.*, 2005; Róis *et al.*, 2016). In the group of species addressed in this study, recent research has shown that the *L. binervosum* complex presents fairly close taxonomic relationships among its members (Erben, 1978; Ingrouille, 1985; Ingrouille & Stace, 1985; Róis *et al.*, 2013) and is clearly phenotypically distinct from the species belonging to the *L. ovalifolium* complex (Róis *et al.*, 2013). These observations are also supported by the published cytogenetic data. The latter complex has been described as being mainly diploid (Erben, 1978, 1999; Róis *et al.*, 2012; results herein), although aneuploid individuals have been detected in natural populations (Róis *et al.*, 2012); the species of the first complex are mostly tetraploid or aneuploid (Erben, 1978; Ingrouille & Stace, 1985; Róis *et al.*, 2012; results herein), although aneuploid triploid individuals ( $2n = 3x = 27$  chromosomes) have also been reported (Ingrouille & Stace, 1986). In contrast, *L. algarvense* is considered to be a homogeneously triploid taxon ( $2n = 3x = 25$ ) that hypothetically arose from crosses between *L. ovalifolium* and *L. binervosum* (Baker, 1953; Erben, 1978; Ingrouille, 1985).

Overall, we detected cytotype homogeneity in most populations and species, with these being either

diploid (*L. ovalifolium* complex), triploid (*L. algarvense*) or tetraploid (*L. binervosum* complex), which is consistent with previous cytogenetic reports noted above (Erben, 1978, 1993; Ingrouille, 1985; Ingrouille & Stace, 1986; Róis *et al.*, 2012). Remarkably, diploid and triploid species are more widespread and occupy the southernmost latitudes compared with the tetraploids, with *L. algarvense* occupying the southernmost limits of the distribution of the tetraploids and extending beyond the distribution range of the diploids.

Mixed-ploidy populations composed of diploid and triploid individuals were recorded for the first time in *L. lanceolatum*. Although the detection of triploid individuals is surprising, it is not completely unexpected. In fact, flow cytometric seed screening in *L. ovalifolium* s.s. and *L. nydeggeri* populations demonstrated the production of 2C or 3C seeds in variable proportions (Róis *et al.*, 2012). However, none of the karyotyped seedlings, which were able to grow and produce further seeds, was triploid (Róis *et al.*, 2012), suggesting that triploid seeds might have a fitness disadvantage. Indeed, this could be the reason for their apparent absence from the survey of natural populations studied here (which included 68 individuals from nine localities of *L. ovalifolium* s.s. and *L. nydeggeri*, and showed that all individuals were diploid). The triploid individuals may have arisen as a result of the fusion of one reduced ( $x$ ) with one unreduced ( $2x$ ) *L. lanceolatum* gamete. It is noted that the related *L. ovalifolium* s.s. and *L. nydeggeri* have been reported to produce reduced and unreduced gametes which can form viable pollen grains of various sizes (Róis *et al.*, 2012). Production of unreduced gametes is quite frequent in nature and is one of the main mechanisms of polyploid formation (Bretagnolle & Thompson, 1995; Ramsey & Schemske, 1998; Levin, 2002). Alternatively, triploid individuals could have originated through hybridization between *L. lanceolatum* with other *Limonium* spp., although this remains to be tested. Because mixed-ploidy populations were not expected, our sampling within populations was not extensive enough to unravel the proportion of different cytotypes in natural populations; nonetheless, in one of the populations the triploids seemed to be dominant.

A high variation in genome size estimates was observed in *L. algarvense*, suggesting that the occurrence of different chromosome numbers for this species might be more frequent than previously reported and hypothesized. In addition to the generally assumed triploid plants with  $2n = 25$  chromosomes, we describe here, for the first time, triploid plants with  $2n = 24$  chromosomes. Future studies should focus on increasing the number of chromosome counts made from different individuals to

evaluate how widespread these contrasting chromosome numbers are for this taxon. The few examples of speciation via aneuploidy observed so far in plants showed the prevalence of aneuploidy in interspecific hybrids and polyploids, which may contribute to the establishment of new karyotypes (De Storme & Mason, 2014).

Several aneuploid individuals have also been reported previously in *L. dodartii* populations, although none was found in the current work. Chromosome losses or gains have already been detected in other *Limonium* spp. for the same species and within the same population (Dolcher & Pignatti, 1967, 1971; Diana, 1995; Castro & Rosselló, 2007; Róis *et al.*, 2012). This suggests that chromosome rearrangements resulting in dysploidy or non-disjunction during cell division (De Storme & Mason, 2014). Additionally, a connection between aneuploidy and apomixis has been confirmed in polyploid complexes such as *Boechera* (Kantama *et al.*, 2007), *Potentilla* (Asker, 1971), *Manihot esculenta* Crantz (Nassar *et al.*, 2009) and *Limonium* (D'Amato, 1940, 1949; Róis *et al.*, 2016). Polyploidization might enable novel ecological adaptations and lead to immediate reproductive isolation from the parental plant(s) (Levin, 1975, 2002; Husband, Baldwin & Suda, 2013). In this context, apomixis is considered to be one of the mechanisms by which newly formed cytotypes can persist and coexist with other cytotypes in the same population (van Dijk, 2007; Dobeš *et al.*, 2013). Additionally, a mate-free reproductive strategy such as apomixis might increase the fitness of a newly generated cytotype and enable its fixation and spread beyond the parental populations (Hojsgaard & Hörandl, 2015).

#### GEOGRAPHICAL PARTHENOGENESIS

A recent phylogeographical and reproductive study in the *L. ovalifolium* and *L. binervosum* complexes has provided evidence for a pattern of 'geographical parthenogenesis' (Róis *et al.*, 2016). Here, we show that these taxa present cytotype differentiation along the coasts of the Iberian Peninsula and adjacent North Africa, with tetraploid apomicts tending to be distributed at higher latitudes than the diploid sexuals, and with the triploid *L. algarvense* occupying areas of sympatry at the southern limits of diploids. The *L. binervosum* complex was shown to be distributed in north, west and south-west Iberia but was never found at the southern limits of diploids and triploids. It is possible that the observed geographical pattern was driven by different ecological preferences, palaeoclimatic patterns of aridity, ocean currents and/or dispersal patterns by biotic and abiotic vectors.



Other biogeographical studies of apomictic taxa and complexes (e.g. *Arnica alpina* Salisb., *Paspalum simplex* Morong, *Taraxacum officinale* F.H.Wigg. aggregate or *Ranunculus auricomus* L. aggregate) have also shown similar patterns of 'geographical parthenogenesis', with polyploid apomicts having larger distributions at higher elevations/latitudes than their diploid sexual relatives or having colonized areas differentially (Bierzchudek, 1985; Kearney, 2005; Hörandl *et al.*, 2008). For example, *Ranunculus kuepferi* sexual diploids are confined to the south-western parts of the Alps, whereas tetraploid apomicts dominate in previously glaciated areas and geographically isolated areas (Cosendai & Hörandl, 2010).

#### ECOLOGICAL PATTERNS

Our analysis of climatic data across the entire range showed that *L. algarvense* followed by the *L. ovalifolium* complex occurred significantly more frequently in the thermomediterranean type and the dry ombrotypes than the *L. binervosum* complex in the Atlantic coasts, which instead was distributed across a much more variable range of thermotypes and ombrotypes than the first two taxa. Furthermore, significant differences between taxa were observed in the frequency of occurrences on the most common lithological groups, with *L. algarvense* occurring more frequently on alluvium, the *L. ovalifolium* complex on limestone and the *L. binervosum* complex on alluvium, limestone and till sites. Although it is recognized that the analysis was conducted at a coarse scale, these results suggest a wider ecological plasticity and greater use of the habitat spectrum in apomicts than in sexual species. Still, this hypothesis should be tested through more detailed, finer-scaled vegetation and geological analyses, which should also incorporate soil texture, pH, electrical conductivity and other types of data, to clarify more precisely the ecological preferences and plant communities within and between each complex. In *Antennaria parlinii* Fernald, a dioecious species that exhibits wide variation in sexuality at the population level, habitat differentiation was described for sexual and asexual populations, with the asexuals frequently found in disturbed roadside ditches and fallow fields, in contrast to the sexual populations that preferred wooded habitats (O'Connell & Eckert, 1999). At the microspecies level, differences at the level of the plant community in which species occurred were pronounced in *Rubus fruticosus* L. (Weber, 1995) and species of *Alchemilla* L. (Fröhner, 1990). Similarly, habitat differentiation between sexual and apomictic populations was also reported for the alpine species *Ranunculus*

*auricomus*, in which apomicts showed a fairly even partitioning among habitat types in contrast to their sexual relatives (Hörandl & Paun, 2007). A fine-scale study in diploid and tetraploid *R. kuepferi* populations demonstrated different niche optima and breadths between cytotypes, with tetraploids occupying new niche spaces and leaving some of the diploid niche space unoccupied (Kirchheimer *et al.*, 2016).

#### CONCLUSIONS

The cytotype distribution and differentiation of *Limonium* diploids, triploids and tetraploids appeared not to be random along the studied latitudinal gradient. Coarse-scale data analysis of climatic factors and surface lithology data points to a broader ecological range of tetraploids than diploids and triploids. Future studies should be directed towards exploring the role of fine-scale factors, such as local edaphoclimatic conditions and relevés to clarify better habitat preferences of these highly specialized species of saline environments. This work, which offers the first biogeographical approach of this *Limonium* group, supports a geographical parthenogenesis phenomenon and suggests an ecological differentiation between the studied taxa.

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#### CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Genome size variation in natural *L. algarvense* populations.

**Table S1.** Synthesis of *Limonium* population occurrence records considered in this study. The three distinct species groups (the *Limonium ovalifolium* and *L. binervosum* complexes and *L. algarvense*) are discriminated, as well as the distinct countries studied and the record sources.

**Table S2.** Synthesis of bioclimates considered in this study (adapted from Rivas-Martínez *et al.*, 2011).

**Table S3.** Synthesis of thermotypes and ombic types considered in this study (adapted from Rivas-Martínez *et al.*, 2011).

**Table S4.** Synthesis of surface lithology groups considered in this study (adapted from <http://www.onegeology-europe.org/>).

**Table S5.** Synthesis of plant communities with presence of the studied *Limonium* taxa (from the *Limonium ovalifolium* and *L. binervosum* complexes and *L. algarvense*) according to its dominant species (Bendaanoun, 1991; Costa *et al.*, 1998, 2012, 2014; Hammada, 2007).

**Appendix 1. List of herbarium specimen vouchers representative of *Limonium* populations sampled for genome size and ploidy studies; sampling location and date are also provided**

| Species              | Voucher          | Site location/Country                             | Collection date | Collectors                                     |
|----------------------|------------------|---|-----------------|--|
| <i>L. algarvense</i> | LISI – 876/2012  | Aljezur: Praia da Amoreira/Portugal               | 03/07/2012      | A. Caperta, A. P. Paes, S. Murra, S. Róis      |
|                      | LISI – 264/2010  | Portimão: Alvor/Portugal                          | 25/08/2009      | D. Espírito-Santo, R. Caraça                   |
|                      | LISI – 678/2014  | Vila Real de St° António: Castro Marim/Portugal   | 15/09/2014      | A. Caperta, S. Conceição, A. P. Paes           |
|                      | LISI – 680/2014  | Vila Real de St° António: Ponta da Areia/Portugal | 15/09/2014      | A. Caperta, S. Conceição, A. P. Paes           |
|                      | LISI – 659/2014  | Huelva: Spit El Rompido/Spain                     | 16/09/2014      | A. Caperta., S. Conceição, A. Paes, R. Freitas |
|                      | LISI – 681/2014  | Cádiz: El Tómbolo de Trafalgar/Spain              | 17/09/2014      | A. Caperta., S. Conceição, A. Paes, R. Freitas |
|                      | LISI – 721/2015  | Larache: Loukkos/Morocco                          | 06/06/2015      | Caperta, Rhazi, Paes, El Madihi, Róis          |
| <i>L. binervosum</i> | LISI – 769/2015  | Brittany: Pointe du Grouin/France                 | 03/07/2015      | P. Arsénio, P. Rodríguez-González              |
|                      | LISI – 770/2015  | Brittany: St. Benoit des Ondes/France             | 03/07/2015      | P. Arsénio, P. Rodríguez-González              |
|                      | LISI – 1173/2013 | Aveiro: São Jacinto/Portugal                      | 26/07/2013      | S. Murra, A. Caperta, A. P. Paes, P. Arsénio   |
|                      | LISI – 695/2014  | Marinha Grande: São Pedro Moel/Portugal           | 13/06/2013      | A. Caperta, A. Paes, S. Murra                  |
| <i>L. dodartii</i>   | LISI – 573/2010  | Sines: Oliveirinha/Portugal                       | 07/07/2010      | A. Caperta, S. Róis, A. P. Paes                |
|                      | LISI – 574/2010  | Sines: Porto Covo/Portugal                        | 07/07/2010      | A. Caperta, S. Róis, A. P. Paes                |
|                      | LISI – 591/2010  | Odemira: Cabo Sardão/Portugal                     | 23/05/2009      | A. Caperta, A. P. Paes                         |

Appendix 1. *Continued*

| Species               | Voucher          | Site location/Country                                    | Collection date | Collectors                                    |
|-----------------------|------------------|--|-----------------|---|
| <i>L. lanceolatum</i> | LISI – 558/2010  | Odemira: Vila Nova Milfontes/Portugal                    | 22/05/2009      | A. Caperta, A. P. Paes                        |
|                       | LISI – 890/2012  | Tavira: Sapal do Barril                                  | 31/07/2012      | A. Caperta, D. Espirito Santo, J. C. Costa    |
|                       | LISI – 716/2015  | Assilah: Oued Tahadart/Morocco                           | 05/06/2015      | Caperta, Rhazi, Paes, Madihi, Róis            |
|                       | LISI – 722/2015  | Larache: Moulay Bouselham/Morocco                        | 06/06/2015      | Caperta, Rhazi, Paes, El Madihi, Róis         |
| <i>L. multiflorum</i> | LISI – 350/2010  | Lourinhã: Vale dos Frades/Portugal                       | 28/06/2009      | A. Caperta                                    |
|                       | LISI – 1004/2013 | Cascais: Cabo Raso/Portugal                              | 12/06/2012      | A. Caperta, A. P. Paes                        |
| <i>L. nydeggeri</i>   | LISI – 891/2012  | Peniche: Ilha do Baleal/Portugal                         | 27/07/2012      | A. Caperta, A. P. Paes                        |
|                       | LISI – 169/2011  | Peniche: N <sup>a</sup> S <sup>a</sup> Remédios/Portugal | 13/07/2011      | A. Caperta, A. P. Paes, S. Róis, A. Cortinhas |
|                       | LISI – 689/2014  | Cascais: Cabo Raso/Portugal                              | 12/06/2012      | A. Caperta, A. P. Paes                        |
|                       | LISI – 1033/2012 | Aljezur: Pontal da Carrapateira/Portugal                 | 04/06/2012      | A. Caperta, A. P. Paes, S. Róis, S. Murra     |
|                       | LISI – 254/2010  | Vila do Bispo: Cabo S. Vicente/Portugal                  | 15/05/2010      | A. Caperta, S. Róis                           |
|                       | LISI – 704/2015  | Rabat: Sidi Abed/Morocco                                 | 10/06/2015      | Caperta, Rhazi, Paes, El Madihi, Róis         |
| <i>L. ovalifolium</i> | LISI – 581/2010  | Cascais: Cabo Raso/Portugal                              | 23/06/2010      | A. Caperta, S. Róis, A. P. Paes               |
|                       | LISI – 1009/2013 | Sines: Praia da Ilha do Pessegueiro/Portugal             | 02/07/1982      | J. G. Pedro, J. F. Costa                      |
|                       | LISI – 341/2010  | Vila do Bispo: Ponta de Sagres/Portugal                  | 23/08/2009      | A. Caperta, A. R. Antunes                     |
|                       | LISI – 343/2010  | Lagos: Praia da Luz/Portugal                             | 16/10/2009      | A. Caperta, A. R. Antunes                     |
|                       | LISI – 536/2010  | Ferragudo: Ponta do Altar/Portugal                       | 06/07/2010      | A. Caperta, S. Róis, A. P. Paes               |
|                       | LISI – 702/2015  | Rabat: Sidi Abed/Morocco                                 | 10/06/2015      | Caperta, Rhazi, Paes, Madihi, Róis            |

**Appendix 2. Localities of the studied populations of the *Limonium ovalifolium* complex (*L. ovalifolium* s.s., *L. nydeggeri* and *L. lanceolatum*), *L. algarvense* and of the *L. binervosum* complex (*L. binervosum*, *L. dodartii* and *L. multiflorum*) sampled in France, Spain, Portugal and Morocco**

| Species               | Population                                    | Geographical coordinates |              |
|-----------------------|---|--------------------------|--------------|
|                       |   | Latitude                 | Longitude    |
| <i>L. ovalifolium</i> | Portugal, Cascais: Cabo Raso                  | 38°42'6.96"N             | 9°28'34.44"W |
|                       | Portugal, Sines: Praia da Ilha do Pessegueiro | 37°49'42.71"N            | 8°47'27.94"W |
|                       | Portugal, Vila do Bispo: Cabo de Sagres       | 36°59'39.27"N            | 8°56'55.52"W |
|                       | Portugal, Lagos: Luz                          | 37°5'14.79"N             | 8°43'44.74"W |
|                       | Portugal, Portimão: Ferragudo                 | 37°6'23.77"N             | 8°31'10.39"W |
|                       | Morocco, Rabat: Sidi Abed                     | 33°55'02.52"N            | 6°58'41.44"W |

## Appendix 2. Continued

| Species               | Population   | Geographical coordinates |              |
|-----------------------|--|--------------------------|--------------|
|                       |  | Latitude                 | Longitude    |
| <i>L. nydeggeri</i>   | Portugal, Peniche: Papôa                               | 39°22'26.87"N            | 9°22'38.74"W |
|                       | Portugal, Peniche: Baleal                              | 39°22'44.11"N            | 9°20'27.54"W |
|                       | Portugal, Peniche: N. Sr <sup>a</sup> dos Remédios     | 39°22'11.66"N            | 9°23'44.63"W |
|                       | Portugal, Cascais: Cabo Raso                           | 38°42'34.33"N            | 9°29'9.77"W  |
|                       | Portugal, Aljezur: Pontal da Carrapateira              | 37°11'42.14"N            | 8°54'39.97"W |
|                       | Portugal, Vila do Bispo: Cabo São Vicente              | 37°1'21.40"N             | 8°59'47.63"W |
| <i>L. lanceolatum</i> | Morocco, Rabat: Sidi Abed                              | 33°55'02.52"N            | 6°58'41.44"W |
|                       | Portugal, Setúbal: Pontal dos Musgos                   | 38°32'1.53"N             | 8°46'48.02"W |
|                       | Portugal, Odemira: Vila Nova Milfontes                 | 37°43'39.92"N            | 8°46'15.35"W |
|                       | Portugal, Tavira: Sapal do Barril                      | 37°5'26.18"N             | 7°40'21.77"W |
|                       | Spain, Ayamonte: Marshes of Isla Cristina              | 37°12'5.73"N             | 7°22'53.73"W |
|                       | Spain, Cádiz: Toruños                                  | 36°30'42.74"N            | 6°14'1.37"W  |
| <i>L. algarvense</i>  | Morocco, Assilah: Oued Tahadart                        | 35°35'1.80"N             | 5°59'16.14"W |
|                       | Morocco, Larache: Moulay Bousseham                     | 34°52'24.90"N            | 6°17'13.62"W |
|                       | Portugal, Aljezur: Praia da Amoreira                   | 37°20'57.51"N            | 8°50'42.26"W |
|                       | Portugal, Portimão: Alvor                              | 37°7'36.58"N             | 8°36'6.91"W  |
|                       | Portugal, V. R.St <sup>o</sup> António: Castro Marim   | 37°13'10.57"N            | 7°26'14.75"W |
|                       | Portugal, V. R.St <sup>o</sup> António: Ponta da Areia | 37°10'38.04"N            | 7°24'34.07"W |
| <i>L. binervosum</i>  | Spain, Huelva: Split El Rompido                        | 37°11'25.23"N            | 6°56'35.57"W |
|                       | Spain, Cadiz: Cabo Trafalgar                           | 36°11'0.05"N             | 6°2'6.07"W   |
|                       | Morocco, Larache: Loukkos 1                            | 35°11'52.44"N            | 6°6'49.56"W  |
|                       | Morocco, Larache: Loukkos 2                            | 35°11'18.12"N            | 6°7'50.40"W  |
|                       | Morocco, Larache: Loukkos 3                            | 35°11'39.54"N            | 6°7'44.28"W  |
|                       | France, Brittany: Saint-Benoit-des-Ondes               | 48°37'21.20"N            | 1°51'13.8"W  |
| <i>L. dodartii</i>    | France, Brittany: Pointe du Grouin                     | 48°42'34.38"N            | 1°50'35.0"W  |
|                       | Portugal, Aveiro: São Jacinto                          | 40°40'26.51"N            | 8°43'17.63"W |
|                       | Portugal, S. Pedro de Moel: Praia Velha                | 39°46'19.76"N            | 9°1'36.50"W  |
| <i>L. multiflorum</i> | Portugal, Sines: Praia da Oliveirinha                  | 37°55'25.08"N            | 8°48'21.53"W |
|                       | Portugal, Sines: Porto Covo                            | 37°51'15.41"N            | 8°47'43.64"W |
|                       | Portugal, Odemira: Cabo Sardão                         | 37°35'49.93"N            | 8°49'3.90"W  |
| <i>L. multiflorum</i> | Portugal, Lourinhã: Vale de Frades                     | 39°16'35.42"N            | 9°20'9.02"W  |
|                       | Portugal, Mafra: Foz do Lizandro                       | 38°56'29.51"N            | 9°24'54.84"W |
|                       | Portugal, Cascais: Cabo Raso                           | 38°42'34.33"N            | 9°29'9.77"W  |