Strategies for Conservation of Rare and Endemic Species: Characterization of Genetic and Epigenetic Variation and Unusual Reproductive Biology of Coastal Species from *Limonium ovalifolium* and *Limonium binervosum* Complexes (*Plumbaginaceae*)

TESE APRESENTADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM BIOLOGIA

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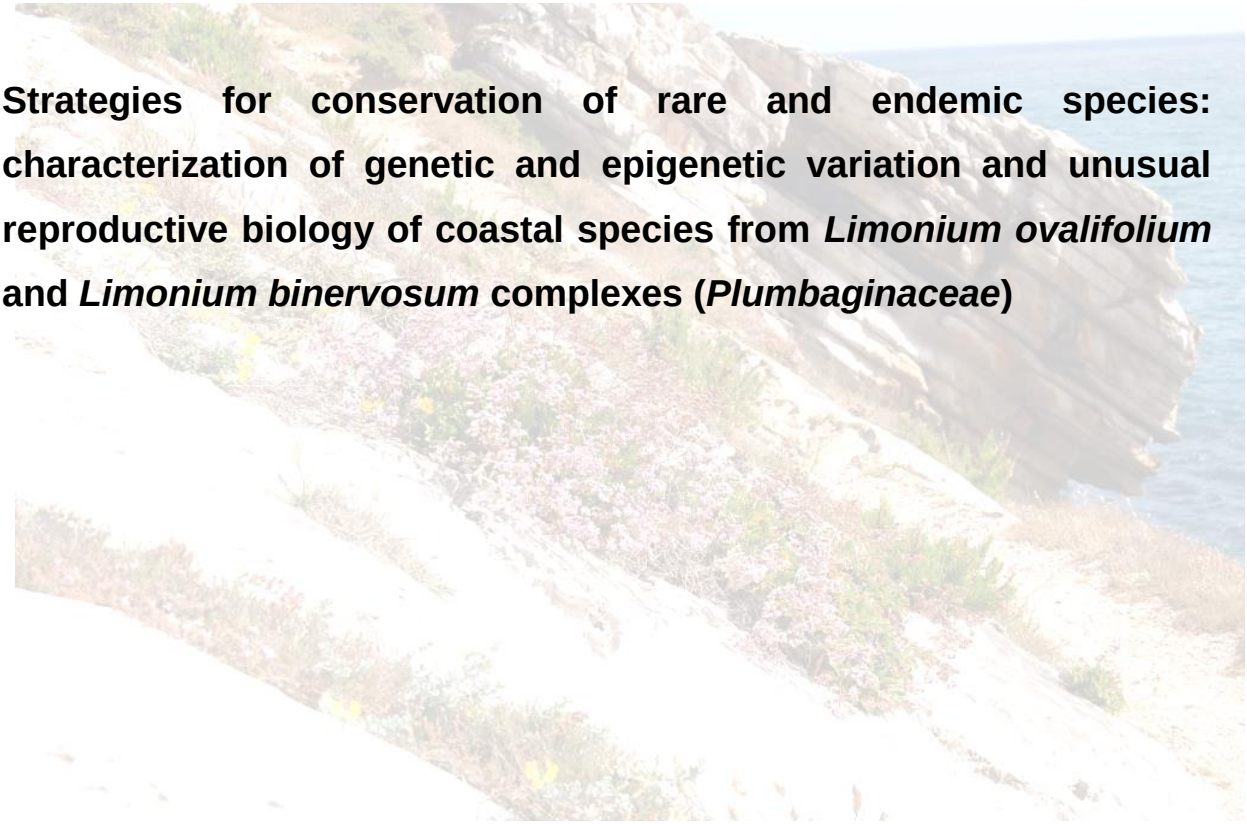
Ao meu eterno avô Zé…



Strategies for conservation of rare and endemic species: characterization of genetic and epigenetic variation and unusual reproductive biology of coastal species from *Limonium ovalifolium* and *Limonium binervosum* complexes (*Plumbaginaceae*)

"We do not even in the least know the final cause of sexuality; why new beings should be produced by the union of the two sexual elements...

The whole subject is as yet hidden in darkness."

Charles Darwin (1862)

The research presented in this thesis was carried out at the research group of Genetics Section, Centro de Botânica Aplicada a Agricultura, Instituto Superior de Agronomia (ISA), University of Lisbon, Portugal; in the Apomixis group at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, Germany; and in the Institute of Biological Sciences, Edward Llwyd Building, at the University of Wales Aberystwyth, United Kingdom.

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ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
ALFA	Associação Alfa de Fitossociologia
AMOVA	Analysis of Molecular Variance
APA	The Environmental Protection Agency of Portugal
bp	base pair
BSA	Bovine Serum Albumin
CBAA	Centro de Botânica Aplicada à Agricultura
CDA	Canonical Discriminant Analysis
CEC	Commission of the European Communities
CH	Casein Hydrolysate
cm	centimeters
cpDNA	Cloroplast DNA
CpG	Phosphodiester bond between the cytosine and the guanine
DAPI	4',6-diamidino-2-phenylindole
DIC	Differential Interference Contrast microscopy
DNA	Deoxyribonucleic acid
EB	Enzyme buffer
EcoRI	Restriction endonuclease *Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
FAA	Formaldehyde – Acetic acid – ethanol
FAM	Fluorescein
FCSS	Flow Cytometric Seed Screen
FDA	Fluorescent diacetat
FM	Fluorescent microscopy
GBIF	Global Biodiversity Information Facility
Gst	Genetic differentiation
h	hour
H	Haplotype
H(%)	Haplotype percentage
Hd	Haplotype diversity
Hn	Haplotype number
He	Expected genetic variability
^{HemiMe}CpCpG	HemiMethylated CpCpG residues
Hj	Expected heterozigosity
HpaII	Restriction endonuclease from *Haemophilus* sp
ICN	Instituto da Conservação da Natureza
ICNF	Instituto da Conservação da Natureza e das Florestas
IPK	Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung
ISA	Instituto Superior de Agronomia
IUCN	International Union for Conservation of Nature
JBA	Jardim Botânico da Ajuda
km	kilometers
^{Me}CpG	Methylated CpG residues
M	Molar
m	minute
µm	micrometer
µl	microliter
mm	Milimeter
MspI	Restriction endonuclease from *Moraxella* sp
OLM	Optical Light Microscopy
PCoA	Principal Coordinate Euclidean Analysis
PCR	Polymerase Chain Reaction
pg	picogram
PhiPT	Population pairwise value
PL (%)	Percentage of Polymorphic loci
Pop	Population
RAPD	Random Amplified Polymorphic DNA
rbcl	ribulose-bisphosphate carboxylase gene
ROX	6-Carboxyl-X-Rhodamine
s	second
SCI	Site of Community Importance
sect.	Section
SEM	Scanning Electron Microscopy
ShI	Shannon's Index
SNP	Single Nucleotide Polymorphism
spp.	some or all of the species in a genus
SSR	Simple Sequence Repeats
subg	Subgenus
subgen	Subgenus
subsp	Subspecies
trnL	chloroplast *trnL* (UAA) intron
trnL-F	region of transfer of chloroplast genome between the 3`-end of 5`*trnL* (UAA) exon and the 5`-end of the *trnF* (GAA) exon
TAE	Tris-acetate-EDTA
T4	DNA-Ligase from T4 phage
var	variety
π	nucleotide diversity

PUBLICATIONS AND PRESENTATIONS (DURING PhD STUDY)

Parts of the PhD study hereto were published elsewhere or were presented on conferences, meetings and symposia.

Published publications

- **Róis AS**, López CMR, Cortinhas AL, Erben M, Espírito-Santo D, Wilkinson M, Caperta AD (2013) Epigenetic rather than genetic factors may explain phenotypic divergence between coastal populations of diploid and tetraploid *Limonium* spp. (*Plumbaginaceae*) in Portugal. BMC Plant Biology 13:205. doi:10.1186/1471-2229-13-205.

- **Róis AS**, Teixeira G, Sharbel TF, Fuchs J, Martins S, Espírito-Santo D, Caperta AD (2012) Male fertility versus sterility, cytotype, and DNA quantitative variation in seed production in diploid and tetraploid sea-lavenders (*Limonium* sp., *Plumbaginaceae*) reveal diversity in reproduction modes. Sexual Plant Reproduction 25(4):305-318. doi:10.1007/s00497-012-0199-y.

Submitted/In preparation

- Caperta AC Espírito-Santo MD, Silva V, Ferreira A, Paes AP, **Róis AS**, Costa JC and Arsénio P (2014) Habitat specificity assessment of the rare and endemic cliff-dwelling halophyte *Limonium multiflorum* Erben in the Atlantic coast. (Submitted to AoB Plants).

- **Róis AS**, Sádio F, Teixeira G, Paes AP, Espírito-Santo D, Sharbel TF, Caperta AD (2014) Sex and apomixis shape chloroplast DNA variation patterns in diploid and tetraploid *Limonium* spp. (*Plumbaginaceae*). (in preparation).

Oral presentations

- **Róis AS**, Sádio F, Teixeira G, Paes AP, Espírito-Santo D, Sharbel T, Caperta AC. Sex and apomixis shape chloroplast DNA variation patterns in diploid and tetraploid *Limonium* spp.. 23rd International Congress on Sexual Plant Reproduction, Porto, Portugal, July - 13th to 18th, 2014. (submitted)

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Poster presentations

- **Róis AS**, Sádio F, Teixeira G, Paes AP, Espírito-Santo D, Caperta AC. Diploids and tetraploids *Limonium* spp. halophytes show contrasting reproduction modes. COST Action FA0901, Final Meeting, University of Coimbra, Portugal, April - 9th to 10th, 2014, Abstract Book pp. 36. http://sequoia.bot.uc.pt/costFA0901/files/abstracts_book.pdf

- Cortinhas AL, Caperta AD, Espírito-Santo MD, Silva V, Ferreira A, Paes AP, **Róis AS**, Costa JC, Arsénio P. Cliff-dwelling halophyte *Limonium multiflorum* Erben morphological and habitat assessment. COST Action FA0901, Final Meeting, University of Coimbra, Portugal, April - 9th to 10th, 2014, Abstract Book P. pp. 32. http://sequoia.bot.uc.pt/costFA0901/files/abstracts_book.pdf

- **Róis AS**, Cortinhas AL, Erben M, Santo DE, Sharbel TF, Caperta AD (2013) High intra- and low inter-populational levels of genetic variation in the coastal *Limonium* diploid and tetraploid species of Portugal. XXXVIII Jornadas Portuguesas de Genética, Porto, Portugal, June - 4th to 5th, 2013, http://www.ibmc.up.pt/genetica2013/programme.html

- **Róis AS**, Teixeira G, Sharbel T, Martins S, Espírito-Santo D, Caperta AD. "Male sporogenesis and gametogenesis in *Limonium* species (*Plumbaginaceae*)". Symposium - Everything you want to know about Plant Sex but were afraid to ask, European Plant Reproduction Research Network Harnessing Plant Reproduction for crop improvement (HAPRECI), Porto, Portugal, July - 9th to 11th, 2012, Abstract Book WG2. 11 pp. 42. http://www.hapreci.org

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ABSTRACT

Limonium Mill. (*Plumbaginaceae*) is among the best represented genus in coastal habitats. In Continental Portugal, two taxonomically complex groups, diploid *Limonium ovalifolium* and tetraploid *Limonium binervosum* complexes are present, and species within these complexes present morphological similarities. These species are threatened as a result of negative anthropic impacts in coastal areas.

The aims of the study presented in here were to collect information on chorology, karyology, natural population genetic and epigenetic variation, and reproductive biology of those species. A comparison of populations' chromosome numbers revealed the presence of aneuploid individuals in species of both complexes. Male micro- sporogenesis and gametogenesis analyses showed regular meiosis and viable pollen grains formation in diploids as opposed to tetraploids. Floral heteromorphisms studies, pollination experiments and cytoembryological analyses demonstrated facultative apomixis in diploids and obligate apomixis in tetraploids. Analysis of methylation sensitive amplification polymorphisms revealed modest genetic and epigenetic differentiation among species populations'. Phylogeographic studies using chloroplast DNA marker sequences demonstrated a large amount of haplotype sharing indicating hybridization among species.

This thesis offers deeper insights into these taxonomic complexes for better design conservation strategies *in situ* and *ex situ*, and a basis for ongoing and future research projects dealing with the expression of apomixis as well as genome evolution in *Limonium* spp..

Key-Words: Apomixis, conservation, karyological polymorphisms, *Limonium*, population genetics and epigenetics.

RESUMO

O género *Limonium* Mill. (*Plumbaginaceae*) está entre géneros melhor representados nos habitats costeiros. Em Portugal Continental, dois grupos de espécies taxonomicamente complexos, o complexo diplóide *Limonium ovalifolium* e complexo tetraplóide, *Limonium binervosum*, estão presentes. As espécies de cada complexo apresentam semelhanças morfológicas. Estas espécies estão ameaçadas em resultado de impactos antrópicos negativos nas áreas costeiras.

Os objetivos do estudo aqui presente foram recolher informação sobre corologia, cariologia, variação genética e epigenética de populações naturais e biologia reprodutiva daquelas espécies. A comparação do número de cromossomas revelou a presença de indivíduos aneuploides em espécies de ambos os complexos. Análises da micro- esporogénese e gametogénese masculina mostraram que, nos diplóides a meiose é regular e os grãos de pólen são viáveis, contrariamente ao que é observado nos tetraplóides. Estudos de heteromorfismos florais, testes de polinização e análises citoembriológicas demonstraram que os diplóides são apomíticos facultativos, e os tetraplóides são apomíticos obrigatórios. A análise de polimorfismos amplificados sensíveis à metilação revelou uma modesta diferenciação genética e epigenética entre espécies e populações. Estudos filogeográficos usando sequências de DNA do cloroplasto demonstraram a partilha de haplótipos entre espécies de ambos os complexos, indicando hibridação entre as mesmas.

Esta tese oferece uma perceção mais profunda destes complexos taxonómicos para desenhar melhores estratégias de conservação *in situ* e *ex situ*, e uma base para projetos científicos em curso e futuros relacionados com a expressão de apomixia, bem como a evolução do genoma em *Limonium* spp..

Palavras-chave: Apomixia, conservação, polimorfismos cariológicos, *Limonium*, genética e epigenética de populações.

CHAPTER I.

GENERAL INTRODUCTION AND LITERATURE REVIEW

1. *Limonium* Mill. genus

1.1. General description

Limonium Mill. comprises annuals and perennial herbs showing preferences for saline and/or coastal habitats (halophytes). These flowering plants (angiosperms) have leafless and underground woody stems and present leaves usually in basal rosettes, and most show tanniniferous roots (Kubitzki 1993). Flowers are clustered in panicles with terminal one-sided spikes, or clustered into racemes along few-branched inflorescences, sometimes green and more or less winged. Each flower shows a funnelform to tubular petaloid calyx and an often less conspicuous corolla, with petals united only at the base and with a one-seeded capsule enclosed by the calyx (Erben 1993). Some species, such as *Limonium brasiliensis* A. Heller and *L. sinuatum* (L.) Mill. are valuable ornamentals (Igawa *et al.* 2002), while others are used as drugs (dried flowers, stems, roots) (e.g. *Limonium bicolor* (Bunge) Kuntze) (Ding *et al.* 2012) or to feed animals (fresh leaves) (e.g. *Limonium lilacinum* (Boiss. & Bal.) Wagenitz) (Furtana *et al.* 2013).

The genus *Limonium* belongs to *Plumbaginaceae* A.L. Jussieu, a typical coastal cosmopolitan family well-represented in temperate zones of the northern Hemisphere, especially in the Mediterranean and Irano-Turanion regions, and consists of 27 genera and about 650 species (Kubitzki 1993). This family shows two distinct well-differentiated groups based upon morphological, chemical and molecular characters, corresponding to the subfamilies *Plumbaginoideae* and *Staticoideae*. The *Plumbaginoideae* comprises shrubs, climbers and rarely herbs usually with pantropical distribution and includes four genera, of which *Plumbago*, with approximately 20 species, is the largest. The *Staticoideae* comprises herbs, mostly Mediterranean and Irano-Turanian in origin but also spreading into other regions with a Mediterranean climate. This subfamily is morphologically more diverse than *Plumbaginoideae* and more than 85% of the species are in the three genera, *Limonium*, *Armeria* Willd. and *Acantholimon* Boiss. (Kubitzki 1993; Lledó *et al.* 2005). About 350 to 400 species are described, distributed all over the world, with two main centers of diversification, the Asian steppes and the coastal habitats of the western Mediterranean (Erben 1978, 1979).

Baker (1948, 1953ab, 1966) has been influential in the development of evolutionary and biogeographical hypotheses for *Plumbaginaceae*, and has used all available information (morphology, breeding systems, chorology and karyology) to

clarify relationships among genera and tribes in the family. Baker (1948) (Figure 1.A) first discribed the approximate distribution boundaries of the genus *Limonium* throughout the world, and its present distribution has remained more or less the same (Figure 1.B). This author studied pollen-stigma dimorphisms and heterostyly, two mechanisms that affect self-incompatibility and which control the breeding behavior of plants in both *Plumbaginaceae* subfamilies. While heterostyly is observed in *Plumbaginoideae* but is rare in *Staticoideae*, pollen dimorphism (A and B *Armeria* type pollen grain) is present in all *Staticoideae* except *Aegilitis*, which is monomorphic with *Plumbago*-type pollen (Nowicke and Skvarla 1977). Stigma dimorphisms (*cob*-like or papillate) are only present in the genera *Limonium*, *Armeria* and *Limoniastrum* Heist. ex Fabr. (Lledó *et al.* 2001). These floral morphisms favor selfing, outcrossing and/or asexual reproduction through seeds (apomixis).

1.2. Taxonomic complexity

Limonium is by far the largest genus in *Plumbaginaceae* in terms of number of species, and is taxonomically (at both infra- and supraspecific) and phylogenetically the most complicated (Lledó *et al.* 2005). The earliest comprehensive accounts for the genus were published by Boissier, and later on other authors followed his system with only small alterations (Lledó *et al.* 2005). Boissier divided *Limonium* (former *Statice, nom. rej. vs. Armeria*; Greuter *et al.* 2000) into 13 sections and two main groups: *corolla polypetala* (many-petalled) with eight sections, and *corolla gamopetala* (petals united or joined, forming a tube) with four sections plus others published later (Table 1). Only two sections of the original *corolla gamopetala*, *Limonium* sect. *Polyarthrion* and *Limonium* sect. *Siphonantha*, are still included in *Limonium* (Lledó *et al.* 2005). Within the *corolla polypetala* group, only *Limonium* sect. *Circinaria* is currently separated from *Limonium* as the genus *Afrolimon*. Pignatti (1971, 1972) made an attempt to simplify the taxonomy of this genus and arranged it for *Flora Europaea* dividing the European *taxa* into three subgenera: *Limonium* subgenus *Pteroclados*, from corolla polypetala group, with approximately 18 species, mostly endemic from the Canary Islands; *Limonium* subgenus *Myriolepis*, created to include three sections (*L.* sects. *Myriolepis*, *Siphonantha*, *Polyarthrion*), all of them members of Boissier's *corolla gamopetala* group; and finally the remainder of the European sections in a large unstructured *L.* subgenus *Limonium*, leaving African, American and Asiatic species out of this system. An example of the difficulty in

classifying *Limonium* species is shown in Figure 2, where C. E. Salmon, a well-known botanist, tried to solve the taxonomic problems of the *taxon Limonium binervosum* (G.E.Sm.) C.E. Salmon in which a number of morphological variants is found. Homotypic and heterotypic descriptions, in addition to the accepted nomenclature, are described in Appendix.

Taxometric analysis has been undertaken to find the closest relatives of some *Limonium* species, based upon studies of the genus and an exhaustive review of species in southwestern Europe in which 59 species (13 new to science) are fully described (Erben 1978). Qualitative descriptions were converted to multistate characters by ranking them on a simple scale, and converted into coded characters with complete comparability between species (Ingrouille 1984) to encompass the whole range of variation of this genus in western Europe (Ingrouille 1984; Ingrouille and Stace 1985). Characters were taken from all parts of the plant, including the woody stem and basal rosette to floral characters. The majority of these characters were quantitative measurements, such as for example leaf lamina length and petal width (Ingrouille and Stace 1985) as shown in Figure 3. In the analysis of western European *Limonium,* Ingrouille (1984) reported that sexual and apomictic *taxa* had a tendency to cluster separately and suggested that the apomictic species evolved independently from the sexual ones. Nevertheless, the results of Lledó *et al.* (2005) did not support this hypothesis since apomictic and sexual species are mixed in phylogenetic trees, suggesting that both groups evolved interdependently.

In Continental Portugal about 15 species have been recognized (Erben 1978, 1993, 1999; Franco 1984), namely *Limonium algarvense* Erben (Iberian endemism), *Limonium auriculae-ursifolium* (Pourr.) Druce, *L. binervosum*, *Limonium daveaui* Erben (Lusitania endemism), *Limonium dodartii* (Girard) Kuntze, *Limonium echioides* (L.) Mill., *Limonium lanceolatum* (Hoffmanns. and Link) Franco (Iberian endemism, protected by law – Habitats Directive 92/43/CEE, B-II and B-IV), *Limonium laxiusculum* Franco (Lusitania endemism), *Limonium multiflorum* Erben (Lusitania endemism, protected by law – Habitats Directive 92/43/CEE, B-II and B-IV), *Limonium nydeggeri* Erben (Lusitania endemism), *Limonium ovalifolium* (Poir.) Kuntze, *Limonium plurisquamatum* Erben (Lusitania endemism), *L. sinuatum, Limonium virgatum* (Willd.) Fourr., and *Limonium vulgare* Mill.. In the Madeira Island *Limonium lowei* R. Jardim, M. Seq., Capelo, J.C. Costa and Rivas Mart. (endemic from Porto Santo Island, Madeira) (Jardim *et al*. 2007) and *Limonium papillatum*

(Webb and Berthel.) Kuntze var. *callibotryum* Svent. (endemic from Ilhas Selvagens, Madeira) are also present (Hansen A and Sunding P 1993). In the Azorean islands, *L. vulgare* is found (Borges *et al.* 2005). *L. multiflorum*, *L. laxiusculum*, *L. daveaui* and *L. plurisquamatum* are considered crop wild relatives from mainland Portugal (Magos-Brehm *et al.* 2008). According to Erben (1993) there are at least ten hybrids described in Portugal. For example, hybrids between *L. algarvense* x *L. ovalifolium*; *L. binervosum* x *L. dodartii*; *L. multiflorum* x *L. ovaliolium*; *L. dodartii* x *L. multiflorum*; or *L. narbonense* x *L. vulgare* are observed.

Some of these species are part of apomictic complexes often treated as informal groups such as aggregates (Stace 1998) or simply called “complexes” (Grant 1981). An example is the *L. ovalifolium* complex composed by three species, *L. ovalifolium*, *L. nydeggeri* and *L. lanceolatum*, which show marked morphological similarities (Erben 1999). *L. ovalifolium* has a coastal Atlantic-Mediterranean distribution from France till Morroco, in sea-cliffs and salt-marshes, and in Portugal is found in the west (Estremadura), southwest Alentejo and Algarve sea-cliffs (Erben 1993; Lahondère and Bioret 1995). *L. nydeggeri* and *L. lanceolatum* have more restricted distributions, the former being restricted to west and southwest Atlantic calcareous sea-cliffs (Erben 1999) whereas the latter is found in the salt-marshes of west, southwest and Algarve provinces (Franco 1984; Erben 1993; Espírito-Santo *et al.* 2012).

Another example is the *L. binervosum* complex, which includes a group of Atlantic coastal microspecies or agamospecies, i.e., a minimally differentiated series of populations derived from uniparental reproduction which are morphologically very similar (Ingrouille and Stace 1985). This group is entirely coastal, from the southwest of Scotland and Ireland (Pignatii 1971), absent from the southwest coast of France but some of its species are observed in Portugal and Spain coastlines (Lahondère and Bioret 1995). The species of this group grow on cliffs, on exposed rock most often found in limestones, on drier edges of salt-marshes and on eroded dunes. Three closely nested species *L. binervosum*, *L. dodartii* and *L. multiflorum* are part of this group and shared an Atlantic coastal distribution (Erben 1978).

The inter- and intraspecific relationships of the genus *Limonium* are remarkably difficult to interpret, and the efforts made so far to define the taxonomy of its species using only qualitative morphological characters have not presented suitable results.

1.3. Karyological polymorphisms

Diverse works have tried to understand the huge cytological diversity of *Limonium* species which are characterized by a great range of ploidy levels. Diploid (2*n*=2x=16, 18 chromosomes), triploid (2*n*=3x=24, 25, 27), tetraploid (2*n*=4x=32, 35, 36), pentaploid (2*n*=5x=43), hexaploid (2*n*=6x=51, 54, 56) and octoploid (2*n*=8x=72) species have been described (Erben 1978, 1993; Brullo and Pavone 1981; Arrigoni and Diana 1993; Georgakopoulou *et al.* 2006; Castro and Rosseló 2007).

Two basic chromosome numbers, x=8 and x=9, appear to be the basis of the genus (Erben 1978). Diploid species (2*n*=16 or 2*n*=18) seem apparently stable whereas tetraploids are the most abundant class among the higher-ploidy *taxa* (Erben 1993; Castro and Rosselló 2007). However, in both diploid and polyploid species different chromosome numbers have been shown within the same species, population or even in the same specimen (Dolcher and Pignatti 1967, 1971; Diana 1995; Castro and Rosseló 2007). The most extreme karyological polymorphism was reported in the Corsican endemic *Limonium bonifaciense* Arrigoni et Diana, where in more than 50% of the seedlings mixoploidy occurred (Diana 1995).

Erben (1979) hypothesized that polyploid species originated from interspecific hybridization of the x=8 and x=9 genomes through the fusion of reduced and unreduced gametes. Triploid *taxa* seem to be highly concentrated in the west Mediterranean region, while tetraploid *taxa* and higher ploidy levels are found on the Atlantic coasts and in the east Mediterranean region (Erben 1978, 1979, 1993; Ingrouille and Stace 1985; Dawson 1990). In the Portuguese coasts diploid (e.g. *L. lanceolatum*), triploid (e.g. *L. virgatum*) and tetraploid (e.g. *L. multiflorum*) species have been reported (Erben 1978, 1993, 1999).

1.4. Floral heteromorphies and mating systems

The great cytological diversity seems to be associated with various breeding systems observed in species from the genus. *Limonium* species are known to have a heteromorphic sporophytic self-incompatibility (SI) system (Baker 1966) linked with pollen-stigma dimorphism. In this system A-pollen type grains germinate on papillate stigmas and B-pollen type germinate on *cob*-like stigmas, while the complementary combinations produce no successful fertilization (Baker 1948, 1953a, 1966) (Figure 4.A). Four pollen stigma combinations occur: *cob*-like stigmata and coarsely

reticulate pollen grain surface (A); papillate stigmata and finely reticulate pollen grain surface (B); *cob*-like stigmata and finely reticulate pollen grain surface (C); papillate stigmata and coarsely reticulate pollen grains (D). A and B combinations are self-incompatible (plants self-sterile) while C and D combinations are self-compatible (plants self-fertile). Species presenting both A and B combinations in each population reproduce sexually (outcrossers) whereas those with only A or B combinations are apomictic. Species with the combinations C or D are selfers. An example of floral heteromorphism can be seen in *L. vulgare* Mill. (Figure 4.B). For example, Georgakopoulou *et al.* (2006) reported that *Limonium palmare* (Sm.) Rech.f. populations are found to be dimorphic or monomorphic with respect to their pollen-stigma combinations, and thus this species seems to reproduce through facultative apomixis, i.e., the co-existence of apomictic and sexual seed production in the same plant (Curtis and Grossniklaus 2007). In contrast, populations of *Limonium narbonense* Mill. were found to be dimorphic with the self-incompatible combinations A and B, suggesting that it reproduces only sexually. In general, the presence of pollen-stigma dimorphisms, as well as higher pollen fertility have been associated with sexual reproduction and are typical of diploid *taxa*. Conversely, monomorphic, self-incompatible pollen-stigma combinations within populations and low pollen fertility have been linked with apomixis (Erben 1978, 1979; Cowan *et al.* 1998). Whereas pollen stainability appears to be high in sexual diploids, in polyploids low to high pollen fertility have been reported (Erben 1978, 1979; Ingrouille and Stace 1985).

According to indirect inferences, using floral heteromorphism determinations, facultative apomixis is common in *Limonium* (e.g. *L. palmare* and *L. virgatum*), whereas truly sexual species are infrequent (Cowan *et al.* 1998; Lledó *et al.* 2005; Georgakopoulou *et al.* 2006). More than 150 putative agamospermous (i.e. asexual reproduction through seeds, apomicts) microspecies have been described (Cowan *et al.* 1998; Lledó *et al.* 2005). Also, a high number of natural hybrids have been reported in *Limonium* (Erben 1993). For example, Baker (1953ab), hypothesized that different variants of the *L. auriculae-ursifolium* group had arisen as hybrids between the related diploid sexual species *L. ovalifolium* (2*n*=2x=16) and triploid or tetraploid members of the agamospermous *L. binervosum* group (2*n*=3x=27 or 2n=4x=35). According to this author the 2*n*=3x=25 chromosomes of *L. auriculae-ursifolium* was obtained by adding a haploid *L. ovalifolium* complement to a haploid *L. binervosum*

complement (8+17=25). These hybrids are fertile and have the ability of producing seeds by apomixis (Baker 1953ab, 1966; Erben 1978). Also, other studies have attempted to produce artificially interspecific hybrids between *Limonium* species. For example, Morgan *et al.* (1995) developed a hybridization program between *Limonium peregrinum* (P.J. Bergius) R.A. Dyer and *Limonium purpuratum* (L.) F.T. Hubb. *ex* L.H. Bailey*,* both pollen-stigma monomorphic, leading to the development of new cultivars with an introgression of desirable new traits, (e.g. long flower stemmed plant with an inflorescence similar in form to that of *L. peregrinum*).

1.5. Genetic diversity and differentiation

Molecular phylogenetic studies have tried to resolve the taxonomic complexity within *Limonium* in a global perspective using nuclear DNA sequence information (Palacios *et al.* 1999, 2000) and plastid DNA (cpDNA) (Lledó *et al.* 1998, 2001, 2005). Studies using cpDNA regions: the *rbcL* (which has demonstrated variability at the species level in the *Caryophyllid* clade (Lledó *et al.* 1998, 2001; Crespo and Lledó 2000); the *trnL* intron; and the intergenic spacer of the *trnL-trnF* (Taberlet *et al.* 1991; Crespo and Lledó 2000), supported division of two well-supported clades, corresponding to *L.* sect. *Pteroclados* and the rest of the genus. These two groups can be accepted as *L.* subgenus *Pteroclados* and *L.* subgenus *Limonium* in a system similar to the one proposed by Pignatti (1971). Also, using cpDNA data, Lledó *et al.* (2005) obtained a phylogenetic tree where apomictic triploids and sexual diploids are interspersed.

At the species level, studies using Amplified Fragment Length Polymorphisms (AFLPs) and Random Amplified Polymorphic DNAs (RAPDs) showed that substantial genetic variation and differentiation occurs within and among populations of triploids *Limonium dufourii* (Girad) Kuntze (Palacios *et al.* 1999). And in *Limonium cavanillesii* Erben also using AFLP, low levels of genetic variability were detected, which has been attributed either by the effects of the apomictic reproductive system of the species, or the passage through a severe recent bottleneck, after which there was no chance for mutation to restore significant genetic variation (Palacios *et al.* 1999). Nevertheless, it is not yet demonstrated wheter selfing and/or apomixis occurs in these species.

Palacios *et al.* (2000) attempted to amplify microsatellite (SSR) loci in closely related species, although no polymorphisms were detected and low success rate for

the across species transferability of SSR primers from tetraploid *L. narbonense* to most other species from the Section *Limonium* was observed. Palop-Esteban and González-Candelas (2002) designed primers for the amplification of SSRs in *L. dufourii* and in *L. narbonense* which did not amplify in other *Limonium* species. The developed primers were used in *L. narbonense* by Palop-Esteban *et al.* (2011) and reveal high levels of genetic diversity.

Other studies, for example in the diploid *Limonium dendroides* Svent., both reproductive data and electrophoretic analyses of nine allozymes were combined to assess the levels and structuring of genetic variation (Suárez-Garcia *et al.* 2009). This study shows moderate gene flow among some subpopulations and therefore, despite radical habitat fragmentation and extremely small size, some subpopulations seem to have enough genetic variation to compensate for the influence of drift on their genetic divergence (Suárez-García *et al.* 2009). These studies of population genetics, such as this one in *L. dendroides*, have been identified since Holsinger and Gottlieb (1991), as one of the main priorities for *in situ* and *ex situ* conservation of endemic and endangered species, such as is the case of some *Limonium* species.

Nevertheless, the genetic differentiation of most *Limonium* microspecies has not been studied yet. Also, some of these *taxa*, after more detailed studies, could finally be shown to be mere phenotypically plastic forms of others. Genetic markers provide tools for elucidating the evolutionary processes generating taxonomic biodiversity and together with the biology of the species can identify the causes leading to a species decline. Hence, such analyses are extremely important to establish a conservation strategy for rare and endemic species.

Hybridization, polyploidy and apomixis are putative key factors in the radiation of *Limonium* in areas such as the western Mediterranean basin.This evolutionary model has been used to explain multiple series of complex aggregates of sexual diploids species and asexual polyploid hybrids which are perpetuated through apomixis (Lledó *et al.* 2005).

2. Sexual reproduction *versus* apomixis

2.1. Tetraspory and apomixis

The reduced eight-celled female monosporic gametophyte (*Polygonum*-type) characterizes more than 70% of flowering plants (e.g. *Brassicaceae*, *Poaceae*, *Fabaceae* and *Solanaceae*) and is considered ancestral (Maheshwari 1950; Rudall 2006; Sundaresan and Alandete-Saez 2010). Although this *Polygonum* embryo sac type is the most frequent, there are a great variety of other configurations, ranging from four- to sixteen-nucleated cells (Maheshwari 1950). There are other types of embryo sacs depending on the number of haploid megaspore nuclei which participate in the development of the female gametophyte, namely bisporic (*Allium*-type) and tetrasporic (e.g. *Penaea*, *Adoxa*, *Drusa* and *Fritillaria*-type) embryo sacs (Figure 5). Polyspory, i.e., formation of embryo sacs out of more than one spore (bispory and tetraspory) Carman (1997), is probably derived from monospory and occurs occasionally in 88 flowering plant families (Carman 1997). According to Carman (1997) polyspory and polyembryony, i.e., individual seeds with more than one embryo, are evolutionarily-linked with apomixis, since polysporic and polyembrionic species occurs in families that expressed apomixis, such as *Poaceae* and *Asteraceae*. Monospory, bispory and tetraspory are observed in different ovules of the same plant in polysporic species, and all combinations of these forms of reproduction are expressed in many species (Hjelmqvist and Grazi 1964; Johri *et al.* 1992).

According to Savidan (2007) apomixis is found in about 31 of 63 orders (Carman 1997; APG III 2009) and is reported in only 223 genera (of about 14,000), 41 of which belong to the *Poaceae*. Apomixis and sexuality are not mutually exclusive processes, i.e., occurring simultaneously in some plants or even in the same ovule (Nogler 1984). Agamic complexes (Stebbins 1950), derived from the term agamospermy, is used for species with sexual populations and apomictic clones (van DijK *et al.* 2009). The main difference between sexual and apomictic reproduction is generally based on meiotic *versus* apomeiotic development (Hörandl and Hojsgaard 2012). Many apomicts also belong to genera in which sexual members predominantly exhibit physiological self-incompatibility, dioecy, or heterostyly (Asker and Jerling 1992). Apomixis occurs almost exclusively in polyploids, with only very rare reports in diploid species, e.g. *Boechera holboellii* (Hornem.) Á.Löve & D.Löve (Böcher 1951) and *Paspalum* L. (Sienna *et al.* 2008).

The crucial elements of apomixis include the escaping of reductional meiosis, (apomeiosis), parthenogenetic development of the unfertilized egg and development of the endosperm with (i.e. pseudogamy) or without (i.e. autonomous) fertilization (Richards 2003). Asexual seed production includes sporophytic apomixis in which embryos are formed directly from maternal sporophytic cells and gametophytic apomixis where embryos result from the parthenogenic development of an egg in a non-reduced embryo sac (Figure 6) (Spielman 2003). This later reproductive mode can involve apospory or diplospory (Vijverberg and van Dijk 2007). In gametophytic apomixis, the megaspore mother cell (MMC) tries to begin meiosis but aborts (apomeiosis) and undergoes three mitotic divisions until it produces an unreduced (2n) gametophyte (diplospory). Alternatively, a diploid ovular cell, other than MMC, can go through three mitotic cycles to form an unreduced embryo sac (apospory) (Ranganath 2003). This unreduced egg may then form an embryo without fertilization (i.e., parthenogenesis) (Grossniklaus *et al.* 2001). Autonomous endosperm production is common in diplospory, mainly in the *Asteraceae* (Koltunow 1993). In diplosporic grasses (e.g., genus *Tripsacum* L.), pollination is required for endosperm production (Bashaw and Hanna 1990). Pseudogamy is characterized as the selective fusion of an unreduced polar nucleus with a sperm nucleus to initiate endosperm production for fertile seed set. In some apomictic species, meiotic abnormalities are also observed in male reproductive cells. Unreduced pollen grain formation has been reported in the genus *Boechera* A. Löve & D. Löve (Schranz *et al.* 2006) and *Tripsacum* (Grimanelli *et al.* 2003), suggesting that male and female reproductive pathways are affected in similar ways.

Investigations of the origin of the embryo sac in *Plumbaginaceae* have shown it to be tetrasporic (D'Amato 1940; Maheswari 1946). In some genera of the *Staticeae* (e.g. *Armeria*) the *Fritillaria*-type occurs, while in other genera (e.g. *Plumbagella*), a very different type of embryo sac which results in the formation of an embryo sac lacking in synergidae and antipodal cells occurs (Baker 1948; Hjelmqvist and Grazi 1964). The discovery of obliged and facultative apomictic species in *Limonium* is attributed to D'Amato (1949), who reported the existence of *Erigeon*-type embryo sac with non-haploid eggs and haploid eggs of *Adoxa*-type in triploid (2*n*=3x=27) *Statice oleaefolia* var. *confusa*. Hjelmqvist and Grazi (1964) and Ingrouille (1982) reported diplospory and tetraspory followed by parthenogenesis in triploid and tetraploid agamospermous species of the obligate apomictic (i.e., giving

100% maternal progenies) *L. binervosum* complex, although and as in nearly all apomictic genera it also contains sexual species and apomictic facultative ones (Stace 1998).

2.2. Origins of apomixis

The origin of apomixis have been explained by different evolutionary models which propose either hybridization between sexual ancestors (Stebbins 1950; Carman 1997), or genetic mutation within a single lineage that then spreads via hybridization among related species (Mogie 1992; Savidan 2007). Carman (1997) hypothesized that some polysporic species evolved from apomictic ones since polyspory occurs in at least 31 of 33 apomictic genera, and it is related with high chromosome numbers. As a result, neosexual plants are likely to occur either as polyploids, with no changes in chromosome base number, or in paleopolyploids groups, generally with higher number (transitional-phase hypothesis). According to this idea apomixis seldomly provides long-term reproductive stability for the evolution of species with novel genome modifications, like genome restructuring, and reproductive behaviors (Carman 1997).

Apomixis is heritable in angiosperms but the basis of its genetic regulatory mechanism is very complex. Apomeiosis and parthenogenesis are, according to Ozias-Akins and van Dijk (2007) under different genetic control although other authors hypothesized that it could result rather from epigenetic control (Grimanelli 2012). Contrasting with mutations, epigenetic changes can occur rapidly and may be particularly frequent as a result of hybridization and polyploidization, providing the epigenetic raw material necessary to form an apomict (Koltunow and Grossniklaus 2003).

Recent studies (Grimanelli 2012; Rodriguez-Leal and Vielle-Calzada 2012) of the regulation of sexual reproduction in plants suggest that the genetic basis of female gametogenesis is complemented by epigenetic mechanisms (e.g. DNA methylation) that are crucial to control events that distinguish sexual from apomictic development. Other studies advanced the crucial role of chromatin-based regulation in vegetative developmental transitions (Poethig 2003), suggesting that transitions during reproduction and early seed development are epigenetically regulated through dynamic changes in chromatin state (Curtis and Grossniklaus 2008). For example, Garcia-Aguilar *et al.* (2010) showed that the expression of *dmt*102 and *dmt*103 in the

ovule is found in a restricted domain in and around the germ cells, indicating that a DNA methylation pathway active during reproduction is essential for gametophyte development and likely plays a critical role in the differentiation between apomictic and sexual reproduction. It has been proposed (Carman 1997, 2007; Bicknell and Koltunow 2004) that apomixis could result from a temporal or spatial deregulation of the transcriptional programs that regulate sexual reproduction. Sharbel *et al.* (2009, 2010) have also been trying to understand some of these effects by comparing sexual and apomictic species of the genus *Boechera,* and have shown downregulation of expression in apomictic ovules compared with sexual ones early in development followed by upregulation relative to sexual ovules at later stages.

3. Conservation issues

Genetic diversity is critical for adaptation to environmental changes and for long-term survival of a species. Knowledge of the level and distribution of genetic diversity within and among populations is a prerequisite for the establishment of effective and efficient conservation practices (Jian *et al.* 2006). Furthermore, genetic diversity may be affected by geographic distribution, and widespread species may have higher levels of genetic variability than narrowly distributed ones (Hamrick and Godt 1996; Ding *et al.* 2012). Epigenetic variation may be generated at a higher rate than equivalent genetic variation, especially in fast changing environmental conditions, when organisms must respond by producing alternative phenotypes (Angers *et al.* 2010). Therefore, natural epigenetic processes seem to play a significant role in adaptation and natural selection, so it may be taken into account in conservation strategies (Paun *et al.* 2010).

Also, knowledge about reproductive ecology and breeding systems has potential impact upon recruitment in the species for the successful management of existing populations and the restoration of extirpated ones (Khan *et al.* 2012). Apomixis, present in the genus *Limonium* generates a genetically diverse mixture of related individuals, often at more than one ploidy level, whose biological diversity defies simple classification into discrete species (Ennos *et al.* 2005). Moreover, *Limonium* species grow in coastal cliffs and salt-marshes which have been endured increasing threats due to the development of human activities such as industry, agriculture, tourism and increased pollution (Gedan *et al.* 2009). For example, *Limonium girardianum* (Guss.) Fourr. a rare, endemic and endangered species to

Mediterranean salt-marshes have been subjected to strong human-driven alterations (Baumberger *et al.* 2012a). Because endemism is rare, it is highly valued, and endemic *taxa* are ideal flagships for popularizing and generating support for local conservation initiatives, like the ones developed by Caperta *et al.* (2013) with some Lusitania *Limonium* endemics, namely, *L. multiflorum* and *L. nydeggeri*.

Recovery plans for endangered species like these of *Limonium* often require the creation of new self-sustaining populations within their historic range and habitat (Pavlik *et al.* 1993; Khan *et al.* 2012). Creating such populations with the genetic and ecological characteristics of the natural ones remains a daunting task with many questions to be answered, namely, what factors determine the viability of those populations, which life stage is most critical for the viability of the population and which management strategy offers the greatest chances for facilitating the survival of the populations (Heywood and Iriondo 2003; Khan *et al.* 2012). For example, a recovery plan was initiated in 2007 by the local government creating a study site for propagation and research of *Limonium barceloi* Gil & L. Llorens in the Canary islands, where plants were grown from seed taken from natural populations (Khan *et al.* 2012).

Also, Baumberger *et al.* (2012b) have studied the survival and growth capacities of seedlings under long flood duration compared with juveniles, in addition to the effects of submersion on final production of above and below-ground dry biomass for seedlings and juveniles of *L. girardianum*. This species is strictly protected in France, and these studies were performed in order to develop efficient conservation measures in the species' disturbed areas, protecting the existing populations by the maintenance and/or restoration of suitable hydrological regimes.

New strategies are required that focus on conserving evolutionary processes, including hybridization and introgression, that are responsible for the generation of taxonomic diversity rather than preserving the individual taxonomic entities within groups (Ennos *et al.* 2005). Implementing conservation strategies in *Limonium* to maintain the evolutionary processes generating diversity will present major challenges. Although *in situ* conservation emerges as top priority, for each situation, we will need to obtain a complete ecological, reproductive and evolutionary understanding of the ways in which taxonomic diversification occurs before appropriate management can be recommended.

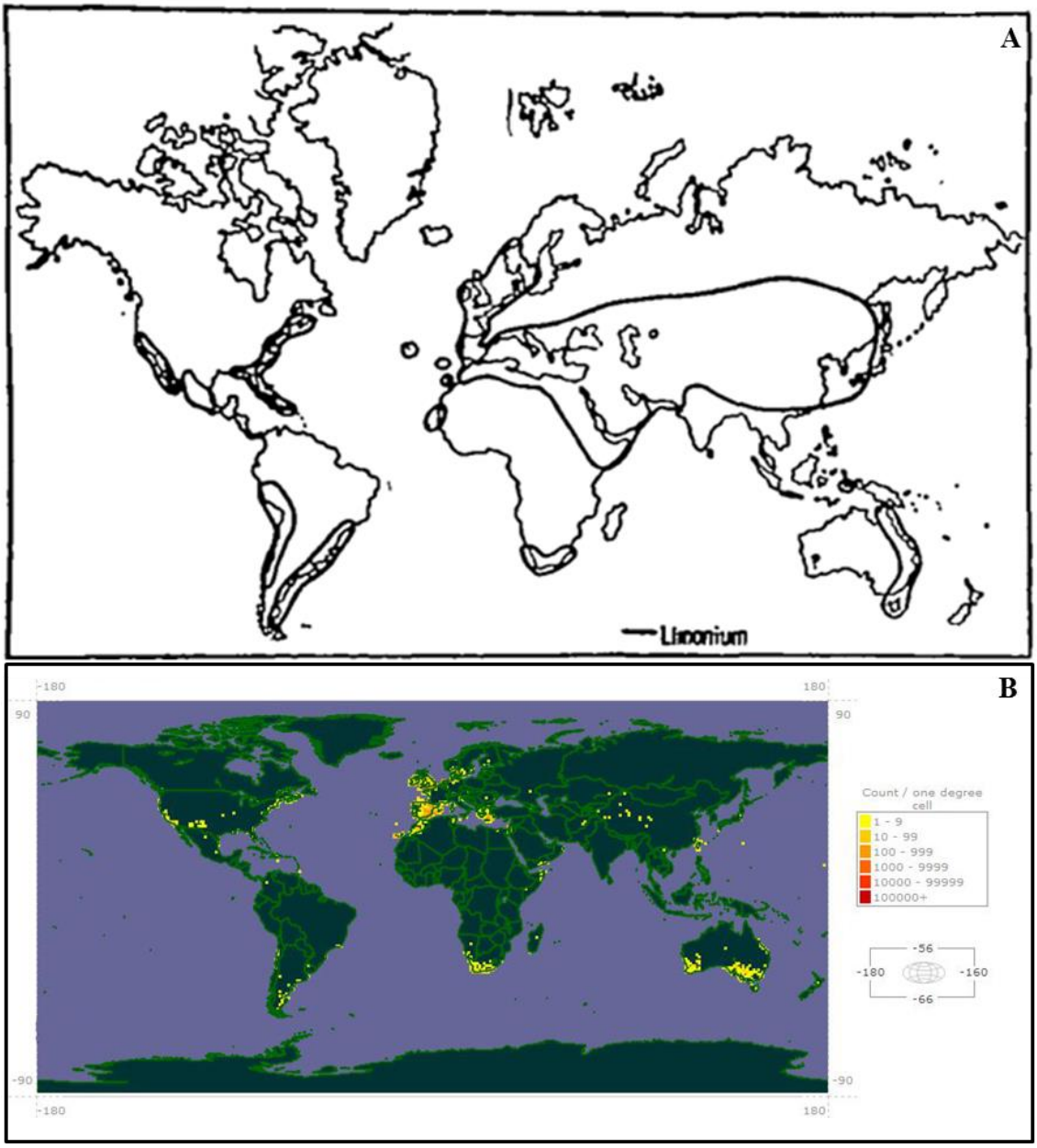

Figure 1. Approximate boundaries of *Limonium* genus distribution areas in the world. A. In 1948 (adapted from Baker, 1948). B. Present distribution of the genus, according to GBIF network (GBIF 2014).

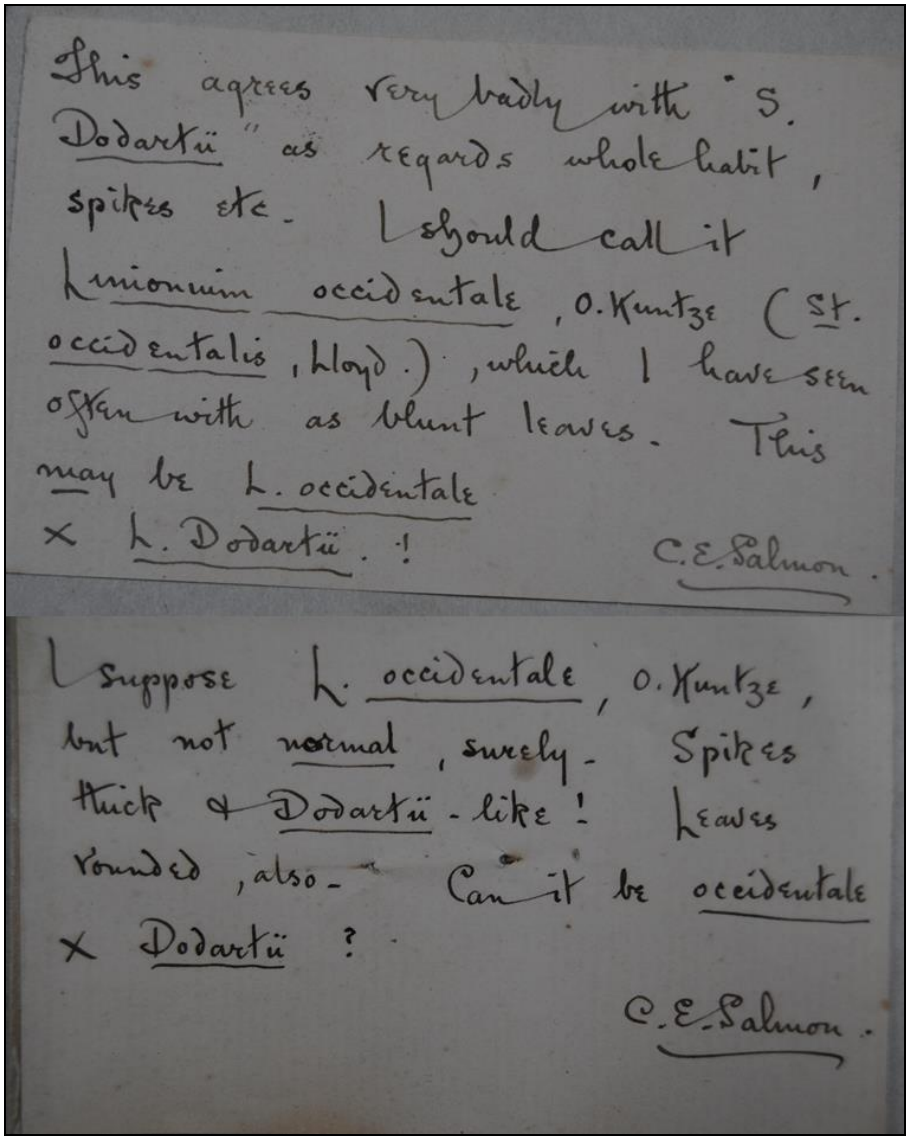

Figure 2. Annotations in a herbarium specimen deposited at Coimbra Herbarium (COI). Inconclusive annotations made by C.E. Salmon in relation to *L. binervosum* (G. E. Sm.) Salmon, collected in Cabo Mondego, in 1977 (photograph by Sérgio Martins).

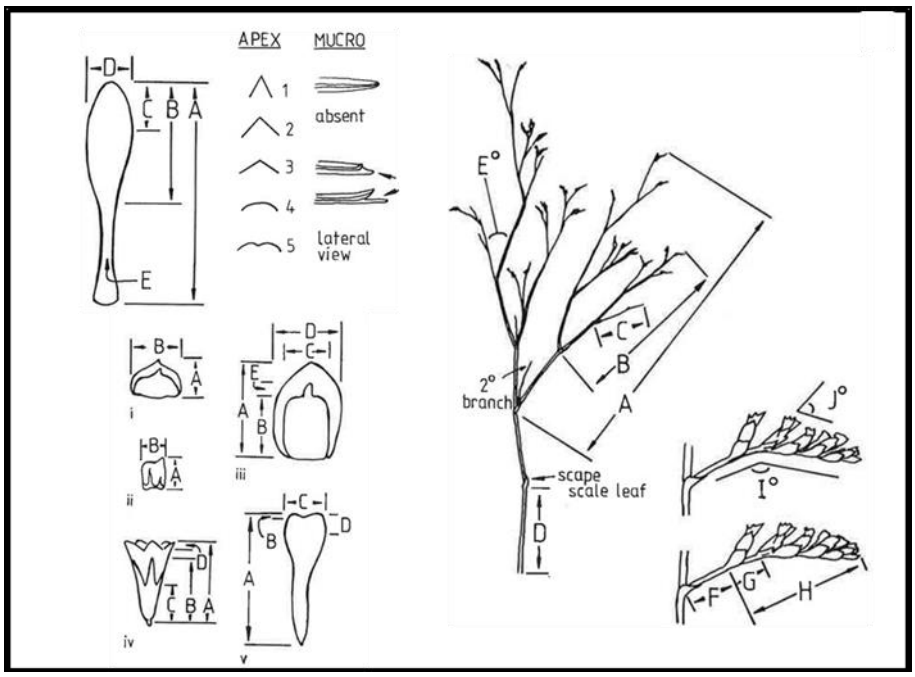

Figure 3. Taxometric characters in *Limonium* spp.. Leaf, scape and spikelet measurements (adapted from Ingrouille and Stace 1985).

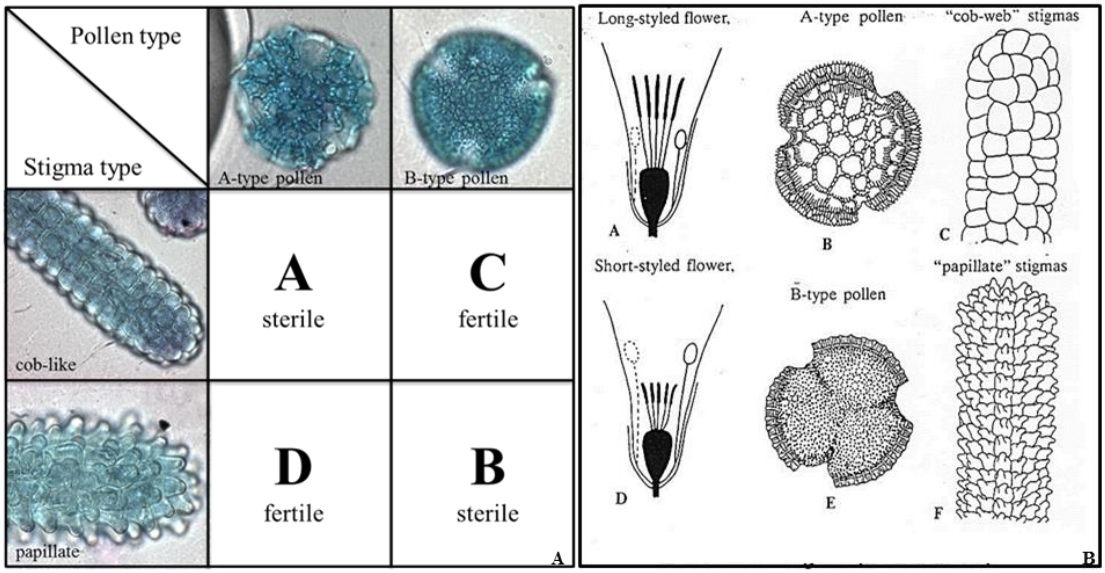

Figure 4. Pollen-stigma combinations and heterostyly in *Limonium* genus. A. Combinations A (*cob*-like stigma and A-pollen); B (*papillate* stigma and B-pollen); C (*cob*-like stigma and C-pollen); D (*papillate* stigma and A-pollen). B. Heterostyly and pollen-stigma dimorphism in *Limonium vulgare* (adapted from Baker 1966).

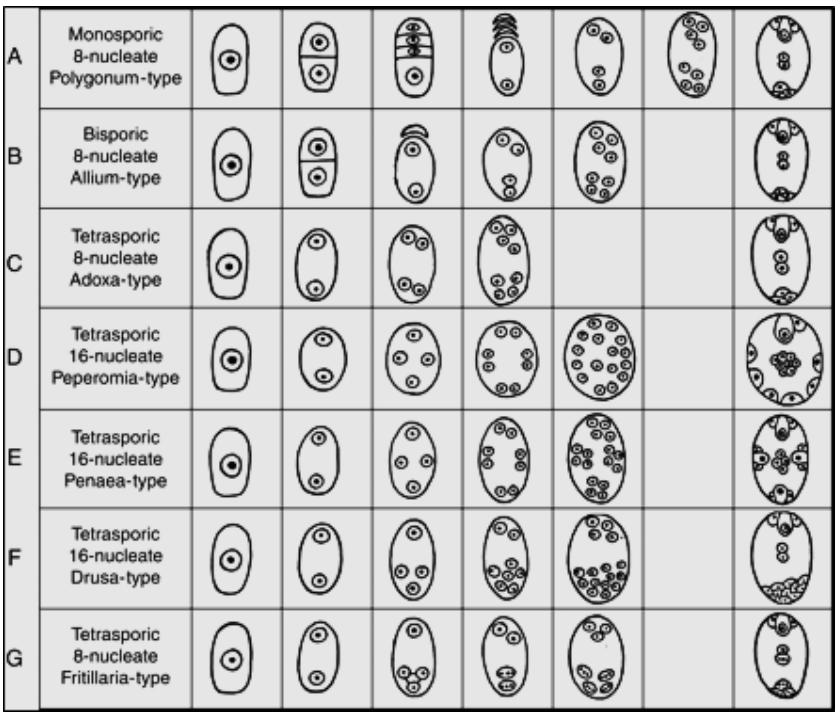

Figure 5. Female gametophyte development in angiosperms. Monosporic (A), bisporic (B) and tetrasporic (C-G) embryo sac formation (adapted from Maheshwari 1950).

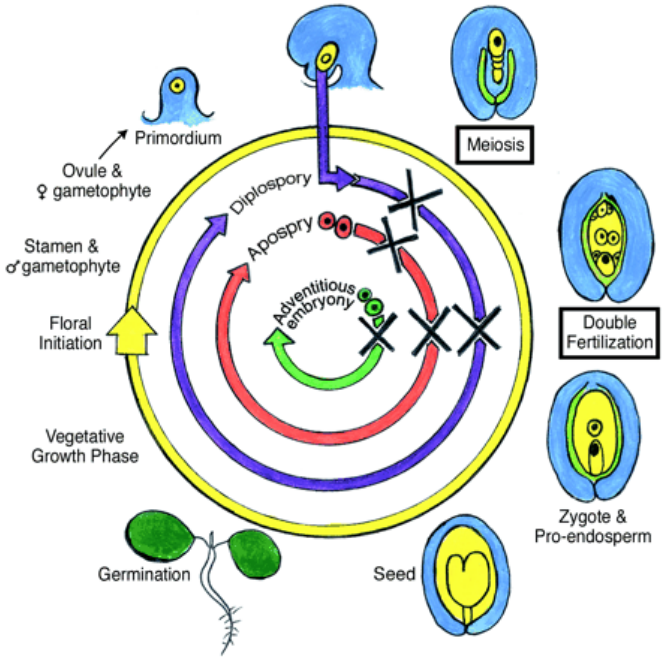

Figure 6. Apomictic mechanisms relative to events in the sexual life cycle of angiosperms (adapted from Bicknell and Koltunow 2004).

Table1. Infrageneric classification of *Limonium*. First classification according to Boissier plus latter additions (adapted from Lledó *et al.* 2005).

Section	Subsection	Other taxonomic proposals	Species sa	Rank accented
			Corolla Polypetala	
Pteroclados	*Odontolepideae*	Subg. *Pteroclados*	*L. beaumieranum* (Maire) Maire	Subgen. *Pteroclados*
			L. lobatum (L.f.) Kuntze	
			L. mouretii (Pitard) Maire	
			L. sinuatum (L.) Mill.	
	Nobiles		*L. arborescens* (Brouss.) Kuntze	Subgen. *Pteroclados*
			L. fruticans (Webb ex Boiss.) Kuntze	
			L. macrophyllum Kuntze	
			L. spectabile (Svent.) Kunkel & Sunding	
			L. sventenii A. Santos & M. Fernández	
Ctenostachys			*L. pectinatum* (Aiton) Kuntze	Subgen. *Limonium*
Jovibarba			*L. jovibarba* (Webb ex Boiss.) Kuntze	Subgen. *Limonium*
Plathymenium	*Rhodanthae*		*L. tenellum* (Turcz.) Kuntze	Subgen. *Limonium*
	Chrysanthae		*L. sinense* (Girard) Kuntze	
			L. tetragonum (Thunb.) Bullock	
Circinaria		Gen. *Afrolimon*	*A. peregrinum* (Berg.) Lincz.	Subgen. *Limonium*
			A. purpuratum (L.) Lincz.	
Schizhymenium			*L. echioides* (L.) Mill.	Subgen. *Limonium*
Sphaerostachys			*L. globuliferum* (Boiss.) Kuntze	Subgen. *Limonium*
Limoniodendron			*L. dendroides* Svent.	Subgen. *Limonium*
Limonium	*Densiflorae*	Subg. *Limonium*	*L. dufourii* (Girard) Kuntze	Subgen. *Limonium*
	Dissitiflorae		*L. bellidifolium* (Gouan) Dumont	Subgen. *Limonium*
			L. delicatulum (Girard) Kuntze	
	Steirocladae		*L. furfuraceum* (Lag.) Kuntze	Subgen. *Limonium*
	Hyalolepideae		*L. tuberculatum* (Boiss.) Kuntze	Subgen. *Limonium*
	Sarcophyllae		*L. axillare* (Forssk.) Kuntze	Subgen. *Limonium*
			L. carnosum Kuntze	
			L. cylindrifolium (Forssk.) Kuntze	
			L. somalorum (Vierh.) Hutch. & E. A. Bruce	
			L. stocksii (Boiss.) Kuntze	
	Genuinae		*L. narbonense* Mill.	Subgen. *Limonium*
			L. vulgare Mill.	
	not assigned to subsection		*L. biflorum* (Pignatti) Pignatti	Subgen. *Limonium*
			L. binervosum (G. E. Sm.) C. E. Salmon	
			L. carthaginense (Rouy) C. E. Hubb. & Sandwith	
			L. cossonianum Kuntze	
			L. densissimum (Pignatti) Pignatti	
			L. agg. *densissimum*	
			L. estevei Fern. Casas	
			L. girardianum (Guss.) Fourr.	
			L. mansanetianum M. B. Crespo & Lledó	
			L. parvibracteatum Pignatti	
			L. perplexum L. Sáez & Rosselló	
			L. rigualii M. B. Crespo & Erben	
			L. santapolense Erben	
			L. thiniense Erben	
			Corolla Gamopetala	
Siphonantha		Subg. *Myriolepis*	*L. tubiflorum* (Dedile) Kuntze	Subgen. *Limonium*
Polyarthrion		Subg. *Myriolepis*	*L. caesium* (Lag.) Kuntze	Subgen. *Limonium*
			L. insigne (Coss.) Kuntze	
Myriolepis		Subg. *Myriolepis*	*M. ferulaceum* (L.) Lledó, Erben & M. B. Crespo	Gen. *Myriolimon*
Pterolimon		Gen. *Bakerolimon*	*B. plumosum* (F. Phil.) Lincz.	Gen. *Bakerolimon*
Arthrolimon		Gen. *Muellerolimon*	*M. salicorniaceum* (F. Mull.) Lincz.	Gen. *Muellerolimon*
Psylliostachys		Gen. *Psylliostachys*	*P. suworouwii* (Regel) Rossklk.	Gen. *Psylliostachys*

4. Aims of the dissertation

The main aim of this dissertation is a comprehensive analysis of the reproductive biology, and genetic and epigenetic variation of the *L. ovalifolium* species' complex, namely *L. ovalifolium*, *L. nydeggeri* and *L. lanceolatum*, and *L. binervosum* species' complex, *L. multiflorum*, *L. dodartii* and *L. binervosum*, that could have a direct impact on generating diversity in their distribution areas in Portugal coast. We expect that the overall outcomes of this work will provide a number of important insights into the genetic and epigenetic variation and associated diversity in reproductive biology, in order to delineate better conservation strategies to preserve rare and endemic *Limonium* populations under strong anthropogenic pressure.

In this study, chorological, cytological, cytometric, cytoembryological, reproductive and molecular tools were used to:

- determine the present distribution areas of the above species (Chapter III);
- investigate chromosome polymorphisms and ploidy variation to get insights on the coexistence of different cytotypes within and among natural populations (Chapter IV);
- analyze female and male sporogenesis and gametogenesis to identify pollen and ovule development patterns, determine floral morphisms, test breeding system and seed production to determine reproduction modes within each species complex (Chapter IV and VI);
- examine natural genetic and epigenetic variation in each species group through global DNA methylation profiles using Methylation Sensitive Amplified Polymorphism (MSAP) markers (Chapter V);
- evaluate the levels and distribution of cpDNA haplotype diversity within and among populations, using cpDNA regions (intron *trnL* and intergenic spacer *trnL-trnF*) (Chapter VI);

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CHAPTER II.

GENERAL MATERIALS AND METHODS

In this chapter the methodologies used in the study described in this dissertation, including morphometric, cytological, cytometric, cytoembriological, cytohistological and molecular techniques, will be presented.

1. Ethics statements

Part of the field work for this study was conducted in protected land under permits issued by Instituto da Conservação da Natureza e das Florestas (ICNF), Portugal.

2. Field methods

2.1. Study species and population sampling

Three diploid species *L. ovalifolium*, *L. nydeggeri* and *L. lanceolatum*, and also those of the tetraploids, *L. binervosum, L. dodartii, L. multiflorum* (Franco 1984; Erben 1978, 1993, 1999) were studied. In some chapters *L. vulgare* was also subject of study. A brief description of these species as well as detailed information on nomenclature and on habitat is given in chapter III. These perennial species are found near the sea on a variety of substrates, on sand, as well as on limestone rocks, greywacke and salt-marshes. Most of them are included in the Habitats Directive listed habitat 1240 "Vegetated sea-cliffs of the Mediterranean coasts with endemic *Limonium* spp." (CEC 2006), comprising sea-cliffs and salt meadows presented in endured increasing threats due to human activities (Gedan *et al.* 2009; IUCN 2013).

Most individuals sampled for this work were selected from populations within designated Portuguese Sites of Community Interest (SCI) for the Mediterranean biogeographical region. Site designation is officially approved by the European Commission as a site which, “in the biogeographical region or regions to which it belongs, contributes significantly to the maintenance or restoration at a favourable conservation status of a natural habitat type in Annex I or of a species in Annex II and may also contribute significantly to the coherence of Natura 2000 and/or contributes significantly to the maintenance of biological diversity within the biogeographic region or regions concerned” (EEA 2013).

All individuals are recorded in this area and a match is made in relation to the total prospected area, a much more reliable method than just observation. Populations were sampled specifically in SCI Peniche/Sta Cruz (PTCON0056), SCI Sintra/Cascais (PTCON0008), SCI Costa Sudoeste (PTCON0012) and SCI Ria

Formosa/Castro Marim (PTCON0013) (CEC 2006). SCI PTCON0008 includes the Sintra-Cascais Natural Park, namely the conservation of natural habitats and wild flora and fauna of the Habitat Directive, earlier designated as Protected Landscape in 1981, and Natural Park in 1994; SCI PTCON0012 includes Sudoeste Alentejano e Costa Vicentina Natural Park, designated Protected Landscape in 1988 and Natural Park in 1995; and SCI PTCON0013 refers to Ria Formosa Natural Park, designated in 1978 as Natural Reserve, and in 1987 as Natural Park (ICNF 2013). Natural populations spanned the distribution range of the species studied, in Portugal coasts in Beira Litoral (west), Estremadura (west), Alentejo (southwest) and Algarve (south) based on previous information from herbaria data (see details in chapter III). The selected populations were recorded using a Global Positioning System, Google Earth 6.0.2 used for georeferencing and geographic mapping was performed using ArcGIS Desktop 10 (ESRI).

3. Laboratory methods

3.1. Plant materials and growth conditions

The species were identified using diagnostic characters and dichotomous keys from Erben (1993) and through comparison with herbarium specimens deposited in the Herbarium João de Carvalho e Vasconcellos (LISI - Instituto Superior de Agronomia, ISA). Also, from all populations at least one specimen was collected, prepared and deposited at LISI herbarium for additional studies.

Mature capsules were collected from up to twenty specimens of each natural population. These seeds were left to air dry at room temperature. Then, part of these seeds were used to establish controlled experimental collections and another part were preserved in appropriate containers with silica gel for *ex-situ* conservation at João de Amaral Seed Bank (JBA, Ajuda Botanical Garden, ISA). In the lab, seeds were placed on moist filter paper in petri dishes and then transferred to a growth chamber (Rumed) for germination with controlled light and temperature with a photoperiod of 18 h light and 6 h dark at 25ºC until germination. Then, the germinating seeds were transplanted into plastic pots containing 3 parts of commercial substrate: 1 part of sand river and grown in a greenhouse at ISA. Species identifications were confirmed after 8-9 months in flowering plants with particular emphasis placed on leaf, inflorescence, and flower morphology according

to Erben (1993). Plants growing in these experimental collections were the basis of cytological and reproduction studies and thus were used in subsequent tasks.

4. Morphometric analysis

Ten morphometric traits (Table 2) were scored in approximately twenty herbarium specimens representative from each species previously referred to, and deposited in LISI herbarium. The diagnostic characters were chosen based on previous taxometric studies in the *Limonium* genus by several authors (Erben 1978, 1993; Ingrouille 1984; Ingrouille and Stace 1985; Dawson and Ingrouille 1995). Morphometric measurements were obtained for each individual in the lab. Statistical analysis was performed with the program SPSS 20 (IBM SPSS, 2010) for Windows.

5. Cytometric analysis

5.1. Genome size estimations

Flow cytometry is commonly used for plant genome size estimations and uses a rapid procedure for nuclei isolation from intact plant tissues (Galbraith *et al.* 1983, 1989). This technique may be characterized as a kind of dynamic fluorescence microphotometry, with a focused stream of suspended particles (nuclei) instead of a microscope slide. The measurements of relative fluorescence intensity of stained nuclei are performed on a linear scale and typically 5000–20000 nuclei are analysed for each sample in a flow cytometer (Galbraith *et al.* 1998). The absolute DNA amount of a sample is calculated based on the values of the G_1 peak means: Sample 2C DNA content = [(sample G_1 peak mean) / (standard G_1 peak mean)] x standard 2C DNA content (pg DNA). Absolute DNA amounts are traditionally reported in pg DNA in terms of the number of base pairs (Doležel and Bartos 2005).

To estimate genome size in leaves, roughly 10 mm^2 of leaf tissue from 2 weeks old plantlets of *L. nydeggeri*, *L. ovalifolium* and *L. multiflorum* were used for flow cytometric genome size estimations. This study was developed only with the available plantlets grown in petri dishes during my internship at IPK, Germany (for details, please see Chapter IV).

Flow cytometry analyses of genome size and relative embryo and endosperm ploidy levels of mature seeds provide information about the reproductive behavior of plants (FCSS; Matzk *et al.* 2000). This technique allows the identification of different reproductive pathways in flowering plants based on the proportion of DNA content

(C-value) of embryo and endosperm nuclei, whose relative values enable the determination of the formation of reduced or unreduced megaspores, zygotic or parthenogenetic embryos, autonomous or pseudogamous endosperm and reduced or unreduced male gametes. Thus this method allows determination of the routes of seed formation of different species (Matzk *et al.* 2000). Flow cytometry may be applied to a suspention prepared from dry seed chooped or ground with fine sand paper where peaks from embryo and endosperm nuclei are observed (Matzk 2007).

To determine the routes of seed formation, sexual or apomixis, seeds from fructifing individuals (bearing capsulas) of *L. nydeggeri*, *L ovalifolium* and *L. multiflorum* plants were collected in the wild, and fifty mature seeds per plant were used for flow cytometric seed screening (for details, please see Chapter IV).

6. Cytological, cytoembryological and cytohistological studies

6.1. Karyotyping

For help in the identification of plant species and to determine each species chromosome numbers, five to seven distinct plants from experimental collections growing in the greenhouse were randomly chosen. Root tips were excised and chemical treatments (8-hydroxyquinoline) were used to induce c-metaphases. Mitotic squashes were prepared after enzymatic digestion in 1 x EB (40 ml 0.1 M citric acid-1-hydrate and 60 ml of 0.1 M sodium citrate dihydrate; pH 4.8) as described in Caperta *et al.* (2008). Slides were analyzed using a Zeiss Axioskop2 Fluorescence Microscope (FM). Images were collected with an AxioCam MRc5 digital camera (Zeiss) and further processed using Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA). (For details please see Chapter IV).

6.2. Reproductive biology analyses

Comparisons of male and female sporogenesis and gametogenesis between sexual and apomictic species enabled the identification of major phenotypic differences in meiocyte formation, pollen tube germination, and embryo sac formation, in addition to complex fertilization scenarios entailing reduced and unreduced male and female gametes (Galla *et al.* 2011). The cytological basis of both sporogenesis and gametogenesis of *Limonium* spp. is extremely important to understand irregularities during chromosome segregation and the origin of aneuploidy. Also the angiosperm female gametophyte is critical for plant

reproduction, being required for sexual and asexual seed development in angiosperms (Koltunow and Grossniklaus 2003). It is therefore important to understand if the embryo sacs are monosporic or polysporic (meiotic) or diplosporic (apomictic) in origin.

6.2.1. Male sporogenesis and gametogenesis

To study microsporogenesis and male gametophyte formation, flower buds of distinct floral stages were collected in *L. ovalifolium* and *L. multiflorum* plants from experimental collections before anthesis. Male meiosis was studied using meiocytes in different stages of development with a FM as above referred. Pollen grains were observed and analyzed using Optical Light Microscopy (OLM), Leitz hm-lux 3, and Scanning Electron Microscope (SEM), (JSM-5220 LV) equipped with a direct image acquisition system. Images were collected with an AxioCam MRc5 digital camera (Zeiss) and further processed using Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA), and pollen tubes observed and analyzed using a FM.

To estimate pollen morphology and dimensions based on Erdtman (1952), randomly chosen grains were measured from SEM micrographs. Polen viability was estimated by two methods, namely Alexander's staining test and the fluorescent diacetat (FDA) test (Alexander 1969). The first test yields either pale turquoise blue (aborted) or dark blue or purple (viable) pollen. It contains malachite green, that stains cellulose in pollen walls and acid fuchsin that stains the pollen protoplasm. The FDA test stains pollen grains for fluorescein diacetate as the vital indicator of membrane integrity, and only fertile grains fluoresce under microscopic examination (Heslop-Harrison and Heslop-Harrison 1970). Pollen grains which fluoresced brightly were scored as viable.

Limonium in vitro pollen germination followed the method developed by Zhang *et al.* (1997). Pollen grains were considered germinated when pollen tubes had a tube length equal or greater than the diameter of the pollen grains. Germinated pollen grains were measured on micrographs recorded with a FM microscope using the Axiovision 4.0 (Zeiss) (for details see Chapter IV).

6.2.2. Female sporogenesis and gametogenesis

Contrasting male sporogenesis and gametogenesis, observations of female meiosis (megasporogenesis) and embryo sac development (megagametogenesis) is

demanding because the cells are enclosed within the nucellus and ovule tissues of the female flower. This is particularly hard in *Limonium* since each mature capsule only show one seed within the ovary.

To analyze female gametophyte development, flower buds of distinct floral stages from *L. ovalifolium* and *L. multiflorum* plants were collected, and pistils dissected to subsequent staining procedures. Ovules were mounted under a coverslip and observed using a FM under Differential Interference Contrast microscopy (DIC) optics (see details in Chapter VI).

6.3. Seed analysis

Studies on the morphology and anatomy of seeds contribute to the knowledge of taxonomy, evolution, and ecology of angiosperms, also increasing the knowledge of the reproductive biology of the species.

To show the cellular structure of the different tissues of *L. ovalifolium* and *L. multiflorum* mature seeds, longitudinal extremely thin sections were cut on a microtome and stained for starch localization (Johansen 1940)**.** Cytohistological observations of mature seeds were made with an OLM Nikon Labophot 2 and a SEM (see details in Chapter IV).

7. Floral heteromorphies analyses, pollen exclusion tests and crossing experiments

Floral hermaphroditism results in sexual conflicts and compromises the parental roles of plants during pollination and mating (Barrett 2002). Major classes of floral polymorphisms have been observed, including pollen-stigma dimorphism (A and B pollen, papillate and *cob*-stigma; see Introduction, Chapter I); heterostyly, where plant populations are composed of two or three different morphs; style-stamen polymorphism, usually accompanied by a sporophytic self-incompatibility system; and dicogamy in which temporal separation of pollen and stigma receptivity is observed (Barrett et al 1999).

For floral morphs determinations, 5 to 10 flowers from *L. ovalifolium* and *L. multiflorum* plants were used, and stigma and pollen types were verified (A/B pollen and *cob*-like/ papillate stigmas) and pollen-stigma combinations were determined (A, B, C and D) (Erben 1978; Suaréz-Garcia *et al.* 2009). Pollen and stigma preparations were observed using an OLM (see details in Chapter VI).

Plants were subjected to pollen exclusion experiments to ascertain the level of apomixis and to examine if apomixis contributes towards seed set, as described in Khan *et al.* (2012). Pollen exclusion and crossing experiments were made using bags that exclude pollinators or pollen delivery systems. Opened control flowers, without bags, could be visited by all type of animals while bagged flowers prevented access to all visitors (see details in Chapter VI).

8. Molecular analysis

8.1. Epigenetic assessment: the MSAP technique

Several methods have been developed for the detection of cytosine methylation changes in animal and plants. Among these, MSAP (Methylation-Sensitive Amplified Polymorphism) (Xiong *et al.* 1999) represents a simple, efficient and realible technique for the detection of methylation alterarions in both level and pattern from a genome-wide perspective (Ochogavía *et al.* 2009).

The MSAP approach is similar to standard AFLP (Amplified Fragment Length Polymorphism) (Vos *et al.* 1995) but uses two methylation-sensitive isoschizomers (*MspI* and *HpaII*) as frequent cutters, each in combination with the same rare cutter (*EcoRI*) in parallel batches (Baurens *et al.* 2003). The two isoschizomers recognize the same sequence (5'-CCGG) but differ in their sensitivity to DNA methylation. Comparison of the two profiles for each individual allowed the assessment of the methylation state of the restriction sites (Figure 7; Table 3).

Epigenetic characterization of *Limonium* plants was achieved by means of MSAP analyses, modified from the protocol of Reyna-Lopez *et al.* (1997). MSAP assays were performed on DNA samples of both diploid (*L. lanceolatum*; *L. nydeggeri* and *L. ovalifolium*) and tetraploid species (*L. dodartii*, *L. multiflorum* and *L. vulgare*) to reveal global variability in CG methylation patterns. For every individual and particular fragment, it was first determined whether the fragment was (i) present in both *EcoRI-HpaII* and *EcoRI-MspI* products (denoting a nonmethylated state); (ii) absent from both *EcoRI-HpaII* and *EcoRI-MspI* products (corresponding to a methylated state); (iii) present only in either *EcoRI-HpaII* or *EcoRI-MspI* products (uninformative, can be attributed to either fragment absence or hypermethylation (Cervera *et al.* 2002). This technique have the advantage of sampling sequences across a genome without prior sequence knowledge and are well suited for non-

model species with little or no genomic information, such as in the case of species in this study (Richards 2011) (for details, please see Chapter V).

8.2. Genetic analysis: cpDNA marker analysis

CpDNA markers, namely the *trnL-F* region, has become one of the most widely used markers for phylogenetic and phylogeographic analysis in plants (Quandt *et al.* 2004). Using these markers is extremely suitable for measuring the genetic effects of colonization due to maternal inheritance, and its low effective population size makes it more sensitive to genetic drift than the nuclear genome (Clegg *et al.* 1994). The potential of using noncoding regions of the chloropast genome was recognized for low-level (intergeneric, interspecific, and intraspecific) studies (Taberlet *et al.* 1991). Regions such as the *trnL-F* region comprising the *trnL* intron and intergenic spacers often display more variation on a per site basis than coding regions (Piñeiro *et al.* 2011). These two plastid DNA regions have been previously used to study phylogenetic relationships within the *Plumbaginaceae*, and are variable enough to elucidate species level relationships (Lledó *et al.* 1998, 2001, 2005; Crespo and Lledó 2000).

For cpDNA analysis, total genomic DNA of all species from the *L. ovalifolium* (*L. lanceolatum*, *L. nydeggeri* and *L. ovalifolium*) and *L. binervosum* complexes (*L. binervosum*, *L. dodartii* and *L. multiflorum*) were extracted and the two regions (*trnL* intron and *trnL-trnF* intergenic spacer) amplified using polymerase chain reaction and sequenced using plant universal primers of Taberlet *et al.* (1991) (Figure 8) (see details in Chapter VI).

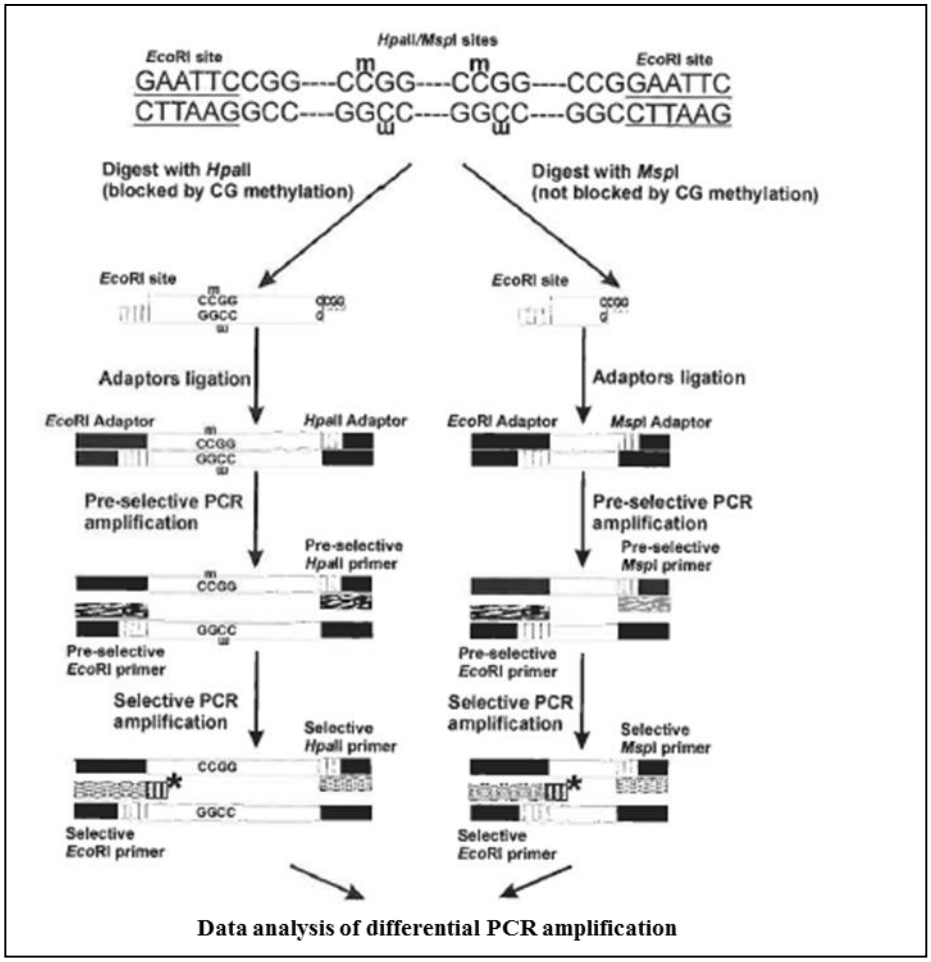

Figure 7. Schematic representation of the MSAP technique (adapted from Yaish *et al.* 2014).

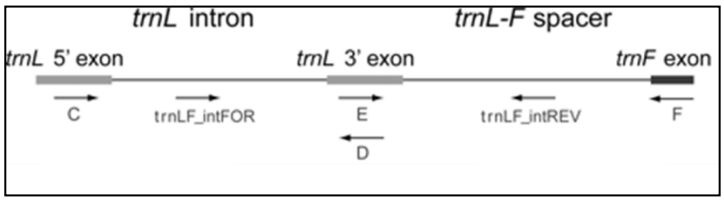

Figure 8. Scheme of primers used to amplify cpDNA *trnL* intron and *trnLF* intergenic spacer (adapted from Pirie *et al.* 2007).

Table 2. Morphometric characters measured for morphometric analysis.

Diagnostic Characters	Code
Maximum Spike Lenght	MSL
Maximum Number of Spikelets per Cm	MNSC
Maximun Number of Florets per Spikelet	MNFS
Maximum Outer Bract Lenght	MOBL
Maximum Outer Bract Width	MOBW
Maximum Middle Bract Lenght	MMBL
Maximum Middle Bract Width	MMBW
Maximum Inner Bract Lenght	MIBL
Maximum Inner Bract Width	MIBW
Maximum Calyx Lenght	MCL

Table 3. Oligonucleotides used for MSAP analysis. Nucleotides are indicated as +XYZ in the primer code column. Enzyme column indicates the restriction enzyme site associated with each primer. * FAM labeled selective primers.

Primer code	Sequence	Enzyme
Adaptor*EcoR*I Forward	CTCGTAGACTGCGTACC	*EcoR*I
Adaptor*EcoR*I Reverse	AATTGGTACGCAGTCTAC	*EcoR*I
Adaptor*Hpa*II/*Msp*I Reverse	CGCTCAGGACTCAT	*Hpa*II/*Msp*I
Adaptor*Hpa*II/*Msp*I Forward	GACGATGAGTCCTGAG	*Hpa*II/*Msp*I
Preamp*EcoR*I (+A)	GACTGCGTACCAATTCA	*EcoR*I
Preamp*Hpa*II/*Msp*I (+C)	GATGAGTCCTGAGCGGC	*Hpa*II/*Msp*I
E1 (+AAA)	GACTGCGTACCAATTCAAA	*EcoR*I
H1 (+CA) *	GATGAGTCCTGAGCGGCA	*Hpa*II/*Msp*I
H3 (+CT) *	GATGAGTCCTGAGCGGCT	*Hpa*II/*Msp*I

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CHAPTER III.

SPECIES DISTRIBUTION IN CONTINENTAL PORTUGAL

Part of the results mentioned in this chapter were compiled in a manuscript submitted for publication in AoB Plants: **Caperta AC, Espírito-Santo MD, Silva V, Ferreira A, Paes AP, Róis AS, Costa JC and Arsénio P (2014) Habitat specificity assessment of the rare and endemic cliff-dwelling halophyte *Limonium multiflorum* Erben in the Atlantic coast.**

1. Abstract

The genus *Limonium* Mill. (sea-lavenders; *Plumbaginaceae*) comprises species of the *L. ovalifolium* and *L. binervosum* complexes, recognized across coastal regions. Some of these species are located in Portuguese NATURA 2000 Sites of Community Importance, being mainly found in habitat 1240 "Vegetated sea-cliffs of the Mediterranean coasts with endemic *Limonium* spp.". However, information on their present geographic distribution in Continental Portugal and population's size is lacking. In this study, field prospecting was based on herbarium data and was carried out along the Portuguese coast. Descriptive morphological and morphometric characters were used to identify the species of both complexes. Population sizes were estimated using a grid of 10 x 10 m quadrats which was overlaid over the aerial photographs of the site and later transferred to the site using Global Positioning System receivers, and all individuals within the grid were counted. Our results confirm that the species studied were distributed in the same Provinces as documented at the Herbarium LISI, although an increase in specimens and localities was recorded. Furthermore, most of the prospected areas showed populations with small size. Moreover, sympatric, populations of species from both complexes were found, such as *L. multiflorum* and *L. nydeggeri*. This chapter provides deeper insights into chorology of these taxonomic complexes and a basis for ongoing and future research projects on ecology in *Limonium*.

Keywords: Chorology, conservation, *Limonium,* population size.

2. Introduction

Despite the fact that are few studies on *Limonium* spp. from the Portuguese Coast, the country has exceptional conditions for their presence, given that they inhabit rocky coastal slopes, cliffs, sandy beaches and salt-marshes. Taxonomic (Pignatti 1971; Franco 1984; Erben 1993, 1999; Jardim *et al*. 2007) and vegetation (Costa *et al.* 1998, 2000, 2001; ALFA 2004) studies on some of these species are documented but the actual distribution and biology of species with conservation interest in the coast of Portugal is limited.

Within the European continent, the Iberian Peninsula is the area which presents the highest floristic richness. The number of vascular plants including

subspecies is around 7000 (Castroviejo 1997; Aedo *et al.* 2013). Such richness and endemicity is favored by the great diversity of habitats, the result of high climatic variability superimposed on the lithologic variability. According to the thermotypes of Rivas-Martínez (2007) and ombrotypes of Monteiro-Henriques (2010), the Portuguese coastal climate ranges from Upper thermomediterranean to humid, Lower mesomediterranean in Minho province; Lower mesomediterranean, Lower humid in Douro Litoral, Lower mesomediterranean sub-humid in Beira Litoral, Upper thermomediterranean, sub-humid, in Estremadura; Upper thermomediterranean, Upper dry in Baixo Alentejo, and Lower thermomediterranean, Lower dry in Algarve. In terms of biogeographical typology and following Costa *et al.* (1999) and Rivas-Martínez *et al.* (2002, 2007), the Coastal Portuguese District is included in Dividing Portuguese Sector, Sadensean-Dividing Portuguese Subprovince, Coastal Lusitan-Andalusian Province, western Mediterranean Subregion, Mediterranean Region. The frontier between the Mediterranean and the Eurosiberian regions converge on the Portuguese northwest coastline. This terrestrial border was proposed by Costa *et al.* (2009) to be the Tagus River salt marsh.

Among representative species of the Portuguese coast are those of *Limonium*. Hybridization and apomixis, geographic isolation, micro-habitats and populations with low size in *Limonium* species lead to a great diversity of communities (Costa *et al.* 1998). Plant communities' distribution in the soutwestern Iberian Peninsula, namely those from coastal habitat have been influenced by both the depth and salt content of the phreatic sheet and the sea water submersion period (Costa *et al.* 1996) as well as by strong winds laden with salt from the Atlantic ocean (Costa *et al.* 2001). As reported in Costa *et al.* (2012) *L. virgatum* is one of the characteristic species of the association *Crithmo Maritimi-Limonietae* (Br.-Bl. in Br.-Bl., Roussine and Nègre 1952 nom. mut.), found in sea-cliffs leptosols with a Mediterranean and European Atlantic distribution. The association *Crithmo maritimi-Daucion halophili* (Rivas Martínez, Lousã, T.E. Díaz, Fernández-Gonzáles and J. C. Costa 1990) includes the thermomediterranean communites of coastal Lusitanian-Andalusian cliff-dwelling species, *L. laxiusculum*, *L. multiflorum*, *L. nydggeri* and *L. plurisquamatum*. Two other species included in the present study, *L. dodartii* and *L. ovalifolium*, belong to Cantabrian-Atlantic halophilic communities of the *Limonio ovalifolii-Frankenion laevis* association (Arbesú, Bueno, Prieto F 2002) in northern Spain and France. The lower salinity tolerant association, *Juncetea maritimi* (Br.-Bl. in Br.-Bl., Roussine and Nègre

1952) contains the Atantic and Mediterranean *L. auriculatae-ursifolium*. *L. vulgare* is a characteristic species of two different associations, *Glauco-Puccinellietalia* (Beeftink and Westhoff in Befftink 1962) with a temperate, continental or coastal Cantabrian-Atlantic and Central European distribution, together with *L. binervosum*; and *Sarcocornienion perennis* (Rivas Martínez in Rivas Martínez, Costa, Castroviejo and Valdés 1980 nom. mut.) with a Coastal Lusitanian-Andalusian and Cantabrian-Atlantic distribution, from the southwest of England to Morocco. *Arthrocnemion macrostachyi* (Rivas Martínez and Costa 1984 nom. mut) include *L. algarvense* as a characteristic species of the association, in Mediterranean and Galician-Portuguese communities. The same species is part of the *Limonion algarvensi-lanceolati* (JC Costa, Neto, Monteiro-Henriques, Arsénio, Portela-Perreira, Caperta and Izco all. nova hoc loco) association in coastal Lusitanian-Andalusian communities, as well as *L. daveaui* and *L. lanceolatum* (Costa *et al.* 2012, 2014). Other species such as the annual *L. echioides* has a Mediterranean and southern Eurosiberian distribution and is found in *Brachypodietalia distachyi* association (Costa *et al.* 2012).

Some endemic *Limonium* spp. are included in natural habitat types of community interest whose conservation requires the designation of special areas of conservation, existing in coastal and halophytic habitats, sea-cliffs and shingle or stony beaches (CEC 2006), with the code 1240 - “Vegetated sea-cliffs of the Mediterranean coasts with endemic *Limonium* spp.” (APA 2011). The IUCN Red List Categories and Criteria are intended to be an easily and widely understood system for classifying species at high risk of global extinction (Barreto-Caldas 2011). The only *Limonium* species from the Portuguese coast referred to in this list is *L. multiflorum*, which is reported to occupy an area of 900 km², although there is no available information on population sizes or trends (Barreto-Caldas 2011). According to this author, even if it is assessed as ‘Least Concern’, trampling by tourists is the main threat to this species, which could lead to a severe population decline in the near future (ICN 2006). Species from the *L. ovalifolium* complex inhabit coastal communities of salt marsh and sea-cliffs throughout the Portuguese shorelines, whereas those from *L. binervosum* complex, such as *L. multiflorum*, inhabit in sea-cliffs from west Portugal (Espírito-Santo *et al.* 2012).

The studied area of this work was the Continental Portuguese coastline, with around 1230 km, located at the confluence of the Atlantic Ocean and the Mediterranean Sea, comprising the Mediterranean and thermo-Atlantic halophilous

habitats. In 6 of the 11 provinces of the Portuguese coastal area, respectively Minho, Douro Litoral, Beira Litoral, Estremadura, Baixo Alentejo and Algarve, sea-cliffs and saltmarshes with transitional tides flow and consequently with a change in salinity are found (ALFA 2004). The main target of this chapter was the collection of information on chorology (population distribution and size), for the species of *L. ovalifolium* and *L. multiflorum* complexes, hybrids between them, and of *L. vulgare*.

3. Material and methods

3.1. Study species

Prospecting studies were based in previous examination of herbarium species in the following herbaria: Herbarium João de Carvalho e Vasconcellos of Instituto Superior de Agronomia-LISI, Herbarium of Museu Nacional de História Natural-LISU, Herbarium of Estação Agronómica Nacional-LISE, Herbarium of Coimbra University-COI and Herbarium of Porto University-PO (abbreviations according to Holmgren *et al.* 1990).

Field prospectings were carried out during the flowering season (April-August) for collecting whole specimens, leaves and seeds, and during fruit season (July-September) for seed collection. *L. binervosum*, *L. dodartii*, *L. multiflorum L. nydeggeri* and *L. ovalifolium* bloomed earlier (April to July) than *L. lanceolatum* and *L. vulgare* (July to October). For estimations of population sizes we used 10x10 m quadrats (Figure 9) to score the presence of individuals of the selected species, and all individuals within the grid were counted. Only populations having more than 15 individuals were sampled and whole specimens were collected, including leaves and seeds. All populations were georeferenced with a GPS model Geoexplorer II, Trimble. The data were processed using GPS Google Earth 6.1.0 and their mapping was done with ArcGIS Desktop 10.0.

Plants were identified in the lab using the dichotomous keys from the Flora Iberica (Erben 1993; Erben 1999). When analyzing species we deliberated that *L. dodartii* subsp. *lusitanica* and *L. multiflorum* were synonyms, the same way that Erben (1993) considered. Collected specimens were then prepared and deposited in the LISI herbarium and part of the collected seeds were preserved in appropriate containers with silica gel in João do Amaral Franco seed bank (JBA) (Figure 10).

Morphological analysis of leaves and flowers included about 10-20 individuals of each species. The descriptive morphological and morphometric characters shown

in Table 4 were used to discriminate the species. Morphometric parameters were analyzed using a stereoscopic microscope and a ruler for measurements.

3.2. Studied area

The prospected locations along the Portuguese coast were in Minho, Douro Litoral, Beira Litoral, Estremadura, Ribatejo, Alentejo and Algarve provinces. Some populations were included in Sites of Community Importance (SCI) biogeographical regions, each with its own characteristic blend of vegetation, climate and geology. For each biogeographical region a list of SCI are designated at the national level as Special Areas of Conservation (Off J Eur Union 2012), namely in Estremadura, Peniche SCI PTCON0056; Cascais SCI PTCON0008; Alentejo: Odemira SCI PTCON0012; Algarve: Vila do Bispo SCI PTCON0012 and Tavira SIC PTCON0013. Some other populations, not included in a SCI, were also prospected in Figueira da Foz (Cabo Mondego); Marinha Grande (Praia da Concha); Caldas da Rainha (Salir do Porto) and Lagos (Praia da Luz).

3.3. Plant material

Due to the extended underground woody stem resulting from vegetative reproduction, collections were made very carefully to guarantee that different individuals were collected. Therefore, individuals were collected at least 50 cm apart from each other. For all the species, collected leaves were washed with distilled water and dried. Mature capsules were collected from up to twenty specimens of each natural population. These seeds were left to air dry at room temperature. Then, part of these seeds was used to establish controlled experimental collections.

Seeds were germinated in petri dishes on moist filter. Germinated seedlings were then grown in a growth chamber (Rumed) in controlled light, 18 h light and 6 h dark, and temperature of 25ºC. Plantlets were transplanted into plastic pots and maintained in a greenhouse at ISA (Figure 11).

4. Results

Although the prospection work was carried out in all the coastal area, where it is supposed to find a sudden *Limonium* spp. due to its preference to a particular kind of habitat, we could only observe the studied species as well as hybrids between them in some of the prospected places (Table 5; Figure 12). It was not possible to

prospect some localities, as initially planned according to data present in the consulted herbaria, due mainly to the fact that many populations were located in private properties or in unreachable places. However, this work revealed an increase in the distribution area and the number of populations of the studied species. As a result of this prospection work we sampled 203 specimens from *L. binervosum*, *L. ovalifolium* and *L. vulgare*, as well as hybrids between them, all of which were deposited in the Herbarium (LISI) (Figure 13).

Only specimens from *L. vulgare* were observed in the Minho and in Douro Litoral provinces. This is in sharp contrast with other coastal provinces where different, sometimes sympatric, populations of distinct species from both complexes were found such as *L. multiflorum* and *L. nydeggeri* in Cabo Raso (Cascais, Estremadura province), which shared the same cliff-dwelling habitat. In the case of *L. multiflorum* individuals in Praia dos Belgas and in Óbidos (both in Estremadura province) they were for the first time recorded in these sites. Also, in Vila Nova de Milfontes (Odemira, Alentejo province) *L. lanceolatum* and *L. vulgare* share the same salt-marsh but *L. vulgare* prefers partial inundation, while *L. lanceolatum* is located in drier positions only inundated in the high tide.

Some of the studied species are included in different plant communities. *L. multiflorum*, *L. dodartii* and *L. ovalifolium* belong to the *Limonio ovalifolii-Frankenion laevis* (Arbesú, Bueno and Prieto F 2002) association; *L. lanceolatum* to the *Limonion algarvensi-lanceolati* (JC Costa, Neto, Monteiro-Henriques, Arsénio, Portela-Perreira, Caperta and Izco all. nova hoc loco); *L. vulgare* together with *L. binervosum* belong to *Glanco-Puccinellietalia* (Beeftink and Westhoff in Befftink 1962), and *L. vulgare* to *Sarcocornienion perennis* (Rivas Martínez in Rivas Martínez, Costa, Castroviejo and Valdés 1980 nom. mut.).

As most sites prospected showed low numbers of individuals (less than 10), in this study only some natural populations surveyed from the Portuguese provinces of Beira Litoral, Estremadura, Alentejo and Algarve (Table 6, Figure 14 and 15) were chosen. These include six *L. nydeggeri* populations from Ilha do Baleal, Nossa Srª dos Remédios, Papoa, Cabo Raso, Pontal da Carrapateira and Cabo de São Vicente; two *L. ovalifolium* populations from Cabo de Sagres and Praia da Luz; two *L. lanceolatum* populations from Vila Nova de Milfontes and Praia do Barril; three *L. binervosum* populations from Cabo Mondego, Praia da Concha and Salir do Porto; two *L. dodartii* population from Cabo Sardão and Porto Covo; four *L. multiflorum*

populations from Cabo Raso, Foz do Lizandro, Vale dos Frades and Papoa and one population of *L. vulgare* from Vila Nova de Milfontes. In this study only one population of this latter species was selected due to the fact that it was the only one that appeared to be composed mostly of *L. vulgare* individuals and not a mixed population of *L. narbonense* and *L. vulgare*, which is often the case in Portuguese populations (Cortinhas 2012). At least one *L. vulgare* population was selected because it was described by Baker (1953) as a sexual tetraploid, and this would help us in ongoing studies subsequent to this dissertation.

L. binervosum was observed, collected and registered from Figueira da Foz (Cabo Mondego), Marinha Grande (Praia da Concha) and Caldas da Rainha (Salir do Porto), in the Beira Litoral province. However, this species' populations were not included in a SCI or in Natural parks as in the case of some of the other species. *L. dodartii*, a close relative of *L. binervosum* was observed in the Alentejo province of Odemira (Cabo Sardão and Porto Covo). Considering the species of the *L. ovalifolium* complex, *L. ovalifolium sensu stricto* grew from Peniche (Estremadura) to Tavira (Algarve); *L. nydeggeri* from Peniche (Estremadura) to Sagres (Algarve); and *L. lanceolatum* from southwest Troia to Tavira (Algarve). The only species observed in salt-marshes, was *L. lanceolatum*, (e.g. Vila Nova de Milfontes and Tróia, Alentejo province) and *L. vulgare* (e.g. Vila Nova de Milfontes, Alentejo province, and Sapal do Barril in Algarve province). Except for these two latter species, which grew in salt-marshes, all other species' populations vegetated on limestone sea-cliffs, in crevices within exposed rocks or on shallow soil above the rock strata and on scree slopes where competition with other species was very low (Figure 15).

All species had small population sizes of about 15 to 50 individuals, except for *L. multiflorum* from Cabo Raso with around 1000 individuals. *L. multiflorum* from Papoa (Estremadura Province) and *L. vulgare* from Vila Nova de Milfontes (Alentejo Province) revealed even smaller population sizes of about 15 individuals each. In fact, in some prospected places we found populations with less than 15 individuals, such as in *L. multiflorum* from Praia das Maçãs or Praia dos Belgas (both in Estremadura Province).

5. Discussion

Our prospection work resulted in an increase (n= 203) of *Limonium* herbarium specimens of *L. binervosum*, *L. ovalifolium* and *L. vulgare* in relation to what was recorded so far till 2009 (n= 323). Comparing the described distributions areas of these species from the GBIF database (GBIF 2014) with our data, we have both increased information regarding known distribution areas in Portugal in addition to having added new localities. However, the species studied were distributed in the same Provinces as documented at the Herbarium LISI.

In relation to *L. binervosum* species' complex, *L. binervosum sensu stricto* was for the first time recorded from Figueira da Foz to Caldas da Rainha (Beira Litoral) in sea-cliffs. According to Lahondère *et al.* (1991), *L. binervosum* and *L. dodartii* are the subject of taxonomic confusion in terms of their distribution area due to similar morphologies. *L. binervosum* has an Atlantic distribution in the southwest of Scotland and Ireland, but it is absent from southwest France. As for the other species of this complex, *L. dodartii* is a south Atlantic-Mediterranean species, reported from Spain and Portugal (Beira Litoral). It is most mentioned in *Flora Europaea* (Pignatti 1971) perhaps due to the confusion with *L. binervosum*. *L. dodartii* is restricted to the Alentejo region in the southwest coast. Unidentified specimens at the Herbarium LISI from Alentejo Province collected before 2009 were attributed to *L. dodartii* based on diagnostic keys from Erben (1993). However these specimens appear to be representative of a slightly different morphotype, and ongoing molecular studies seem to confirm this tendency (unpublished data). *L. multiflorum* has a very restrict distribution area, limited to the west (Estremadura), from Lagoa de Óbidos to Cascais in sea-cliffs and salt-marshes.

As for the *L. ovalifolium* species' complex, *L. ovalifolium sensu stricto* is described as an Atlantic-Mediterranean species that stretches from northern Morocco to the Rance estuary, in France, the northern limit of the species distribution (Lahondère *et al.* 1991). According to Costa *et al.* (2001), *L. ovalifolium* also have an Atlantic-Mediterranean distribution in sea-cliffs from Cabo Mondego to Cabo Carvoeiro (Beira Litoral), Cabo Raso (Estremadura) and from Ponta de Sagres to Ferragudo (Algarve), as shown in the current study. *L. nydeggeri* also has an Atlantic-Mediterranean distribution observed in sea-cliffs of Estremadura and Algarve, while *L. lanceolatum* is restricted to salt-marshes of Alentejo (e.g Sado and

Mira estuaries) and Algarve (Ria Formosa coastal lagoon) provinces as reported in Costa *et al.* (2014).

L. vulgare which has a European and Atlantic distribution (Dawson and Ingrouille 1995) is found from Douro Litoral to Algarve provinces (Costa *et al.* 1996; GBIF 2014). The present study also confirm this distribution area in salt-marshes of Lima, Aveiro, Tejo, Sado and Mira estuaries, and Ria de Aveiro and Ria Formosa coastal lagoons although the majority of these populations are mixed, i.e. composed by *L. vulgare* and *L. narbonense* individuals (Cortinhas 2012). In this study, we also recorded the presence of the hybrids *L. lanceolatum* x *L. ovalifolium* in Alentejo Province, *L. multiflorum* x *L. ovalifolium* in Estremadura Province and *L. ovalifolium* x *L. algarvense* in Algarve Province, as already reported by Erben (1993). Although *L. ovalifolium*, *L. binervosum* and *L. dodartii* have been described as existing both in salt-marshes (Lahondère *et al.* 1991), and in sea-cliffs, in this study, we only observed these species in rocky cliffs. Conversely, *L. multiflorum* was observed for the first time in salt-marshes.

The information presented in this chapter also contributes to the estimation of population size in some species, which has not been reported so far. The ICNF (2013) only mentions abundance data for *L. multiflorum* and *L. dodartii* as rare or uncommon, respectively. According to our study all *Limonium* species prospected in this work have a patchy distribution across southwestern Atlantic Iberia with small population sizes of about 15-50 individuals each, and in most cases, fragmented into subpopulations. Also, in most cases the prospected sites showed less than 10 individuals. Nevertheless the observed populations appear to be bigger than they really are because of abundant vegetative reproduction. This is related with the age of a plant (Hegazy 1992) since dead rosettes and leaves can remain attached for many years. Leaf appearance rate is higher in juvenile plants rather than in adults. Therefore, the prospected populations may be composed by older individuals rather than younger ones. However, in this study we have not assessed nor estimated population age.

A study case for *in situ* conservation purposes in Raso cape (Cascais, Estremadura province) focused in *L. nydeggeri*, *L. ovalifolium* and *L. multiflorum* populations (Caperta *et al.* 2013; Caperta *et al.* 2014, submitted). Raso is a cape formed by limestone rocks, with a belt of bio-erosive rock pools with a characteristic sharp miniature relief developed along the surf line (Scheffers and Kelletat 2005),

and is under the influence of strong salty winds (i.e. splashed by marine salt spray). A multidisciplinary approach is thus necessary for the rehabilitation of these species and control of invasive plants. The ecological assessment demonstrated narrow habitat specificity of *L. multiflorum*, exposed to strong anthropogenic pressure mainly from urbanization in coastal areas and trampling by tourism (Caperta *et al.* 2013). An interdisciplinary integration of multiple demographic, genetic, reproductive, and ecological data is thus necessary for the effective conservation of this rare species. To ensure an effective protection of the studied population, the authors suggested the creation of a plant micro-reserve in Raso cape (Caperta *et al.* 2014, submitted).

Although the vegetation of the Portuguese coast is extremely rich in endemics (Costa *et al.* 1998, 2001; Aedo *et al.* 2013) and some are included in Rede Natura 2000 as belonging to protected habitat in some locations, this biodiversity seems to be threatened in some sites, mainly due to human activity. It is tremendously important to combat this human pressure to avoid species extinction. However, till now studies within coastal plants in general, and halophytes designation in particular, were based on limited sampling of *taxa* and on classical morphological studies. Furthermore, knowledge on their reproductive modes is important for ensuring their survival and for efficient restoration of natural population. In addition to the legal tools needed for the conservation of some of these species, apart from the above mentioned *in situ* conservation study there exist no other conservation works for *Limonium* in Portugal, which is tremendously needed.

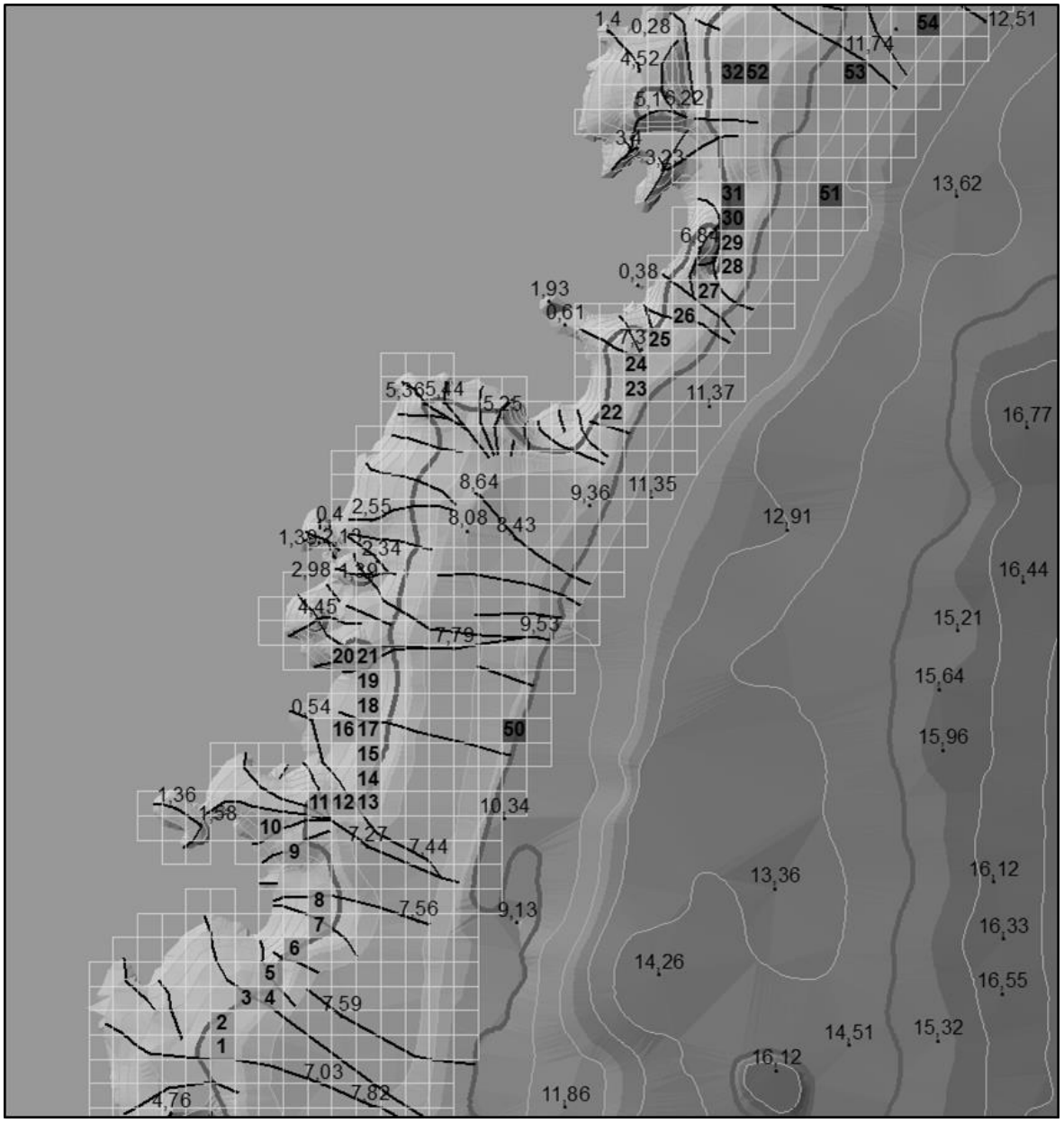

Figure 9. Example of a schematic drawing of the quadrats sampling of *Limonium multiflorum* in Raso cape coast.

Figure 10. Silica-gel sorbent tubes with *Limonium* spp. seeds. Seeds were preserved *ex-situ* in João do Amaral Franco seed bank (Jardim Botânico da Ajuda, JBA).

Figure 11. Experimental *ex situ Limonium* spp. collections. Plants are maintained in a greenhouse at Instituto Superior de Agronomia (ISA).

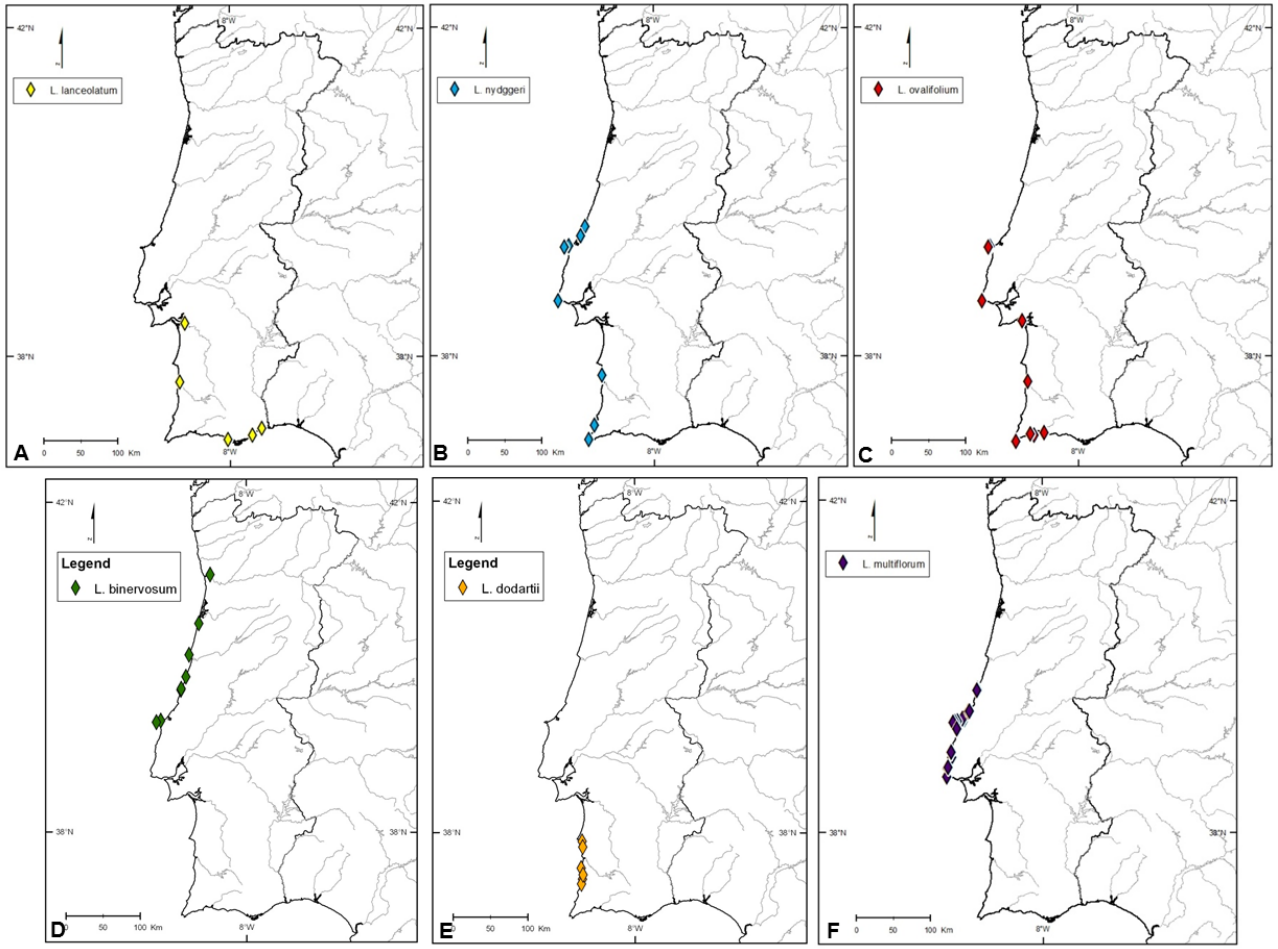

Figure 12. **Geographic distribution of *Limonium ovalifolium* and *L. binervosum* complexes in Continental Portugal.** A. *L. ovalifolium*; B. *L. nydeggeri*; C. *L. lanceolatum*; D. *L. binervosum*; E. *L. dodartii*; F. *L. multiflorum*.

Figure 13. ***Limonium*** **herbarium specimens deposited at herbarium João de Carvalho e Vasconcellos (LISI).** A. *L. ovalifolium*; B. *L. lanceolatum*; C. *L. nydeggeri*; D. *L. multiflorum*; E. *L. dodartii*; F. *L. binervosum*; G. *L. vulgare*; H. voucher of one herbarium specimen, *L. multiflorum*, deposited in the LISI.

Figure 14. **Species from *Limonium ovalifolium* and *L. binervosum* complexes in their natural habitat**. A. *L. lanceolatum* in Vila Nova de Milfontes; B. *L. nydeggeri* in Srª dos Remédios; C. *L. ovalifolium* in Lagos; D. *L. vulgare* in Barril; E. *L. dodartii* in Porto Covo; F. *L. mutiflorum* in Vale dos Frades; G. *L. binervosum* in Cabo Mondego.

Figure 15. ***Limonium* spp. natural habitats**. Sea-cliffs (A, B, D and E) and salt-marshes (C and F). A. Baleal, *L. nydeggeri*; B. Lagos, *L. ovalifolium*; C. Praia do Barril salt-marsh, *L. lanceolatum* and *L. vulgare*; D. Foz do Lizandro, *L. multiflorum*; E. Porto Covo, *L. dodartii*; and F. São Martinho do Porto, *L. binervosum*.

Table 4. **Descriptive morphological and morphometric characters used to identify *Limonium* spp.** (adapted from Franco 1984; Erben 1993).

			L. binervosum	*L. dodartii*	*L. lanceolatum*	*L. multiflorum*	*L. nydeggeri*	*L. ovalifolium*	*L. vulgare*
Leaves	**Lamina shape**	elliptic to oblong-obovate							X
		spatulate		X					
		oblanceolate to spatulate		X					
		oblanceolate	X		X		X	X	
		oblanceolate to lanceolate				X			
	Vein number	1-3	X			X	X		
		3							
		3-5		X				X	
		5-7			X				
		pinatinerve							X
	Petiole length in relation to the lamina	very short					X		
		1/2 to 3/4	X	X				X	
		4/4 to 5/4			X				
		178				X			
		non superior 1/2 lamina							X

Table 4. Continued.

Inflorescence	**Scape orientation**	erect	X	X	X	X	X		X
		erect to zig-zag	X					X	
	Presence of sterile branches	none	X	X	X	X	X	X	X
		few	X	X					X
	Inflorescence type	A	X	X		X			
		B							
		C			X	X		X	
		D	X			X			
		F							X
		G						X	X
		subglobose					X		
	Corolla color	reddish-violet	X		X	X	X	X	X
		pale blue-violet		X					

Table 4. Continued.

Spike lenght	**Relative spikelets length (mm)**	4-5						X	
		4,5-5				X	X		
		5-6,5			X				X
		6-7		X					
		6,5-7,5	X						
	Number of spikelets per cm	4-7	X		X				X
		7-9		X					
		9-12				X			
		9-13						X	
		10-15					X		
	Number of flowers per spikelet	2							X
		2-3		X	X				
		2-4	X				X		
		3-6				X			
		3-8						X	
	Inner bracts length X width (mm)	1,2-1,8 × 1,2-2					X		
		2,7-3,6 × 3,1-4,1			X			X	
		3,7-5,3 × 2,6-3,4						X	X
		4,4-5,3 × 3,4-4,1	X	X					
		5-6 × 4-4,8				X			

Table 5. **Collected *Limonium* specimens deposited at the Herbarium João de Carvalho e Vasconcellos (LISI).** Species are ordered by collection date.

Species	Prospection Site/Population Name	Province	Colector(s)	Collection date	Voucher nº
L. multiflorum Erben	Cascais, Cabo Raso	Estremadura	P Arsénio & A Caperta	20-III-2009	586/2009
L. multiflorum Erben	Lourinhã, Praia de Vale dos Frades	Estremadura	A Caperta	28-VI-2009	350/2010
L. multiflorum Erben x *L. ovalifolium* (Poiret) O. Kuntze	Cascais, Cabo Raso	Estremadura	A Caperta	9-VII-2009	598/2010
L. nydeggeri Erben	Cascais, Cabo Raso	Estremadura	A Caperta & AR Antunes	9-VII-2009	252/2010
L. nydeggeri Erben	Peniche, Srª dos Remédios	Estremadura	A Caperta & AR Antunes	4-VII-2009	265/2010
L. nydeggeri Erben	Vila do Bispo, Cabo de São Vicente	Algarve	A Caperta & AR Antunes	23-VIII-2009	266/2010
L. nydeggeri Erben	Tavira, Pedras del Rei	Algarve	A Caperta & AR Antunes	17-X-2009	267/2010
L. nydeggeri Erben	Tavira, Pedras del Rei	Algarve	A Caperta & AR Antunes	17-X-2009	268/2010
L. nydeggeri Erben	Alzejur, Pontal da Carrapateira	Algarve	A Caperta & AR Antunes	24-VIII-2009	355/2010
L. ovalifolium (Poiret) O. Kuntze	Lagos, Praia da Luz	Algarve	A Caperta & AR Antunes	16-X-2009	339/2010
L. ovalifolium (Poiret) O. Kuntze	Tavira, Praia do Barril	Algarve	A Caperta & AR Antunes	21-VIII-2009	340/2010
L. ovalifolium (Poiret) O. Kuntze	Vila do Bispo, Ponta de Sagres	Algarve	A Caperta & AR Antunes	23-VIII-2009	341/2010
L. ovalifolium (Poiret) O. Kuntze	Tavira, Praia do Barril	Algarve	A Caperta & D Espírito-Santo	21-VIII-2009	342/2010
L. ovalifolium (Poiret) O. Kuntze	Lagos, Praia da Luz	Algarve	A Caperta & AR Antunes	16-X-2009	343/2010
L. ovalifolium (Poiret) O. Kuntze	Lagoa, Ferragudo, Ponta do Altar	Algarve	A Caperta & AR Antunes	23-VIII-2009	344/2010
L. vulgare Mill.	Faro, Praia do Ancão	Algarve	A Caperta & AR Antunes	17-X-2009	348/2010
L. vulgare Mill.	Vila Nova de Milfontes, Sapal do Mira	Alentejo	A Caperta & AP Paes	22-V-2009	349/2010
L. binervosum (G.E.Sm.) Salmon	Figueira da Foz, Cabo Mondego	Beira Litoral	A Caperta, ASRóis & AP Paes	30-IV-2010	271/2010
L. binervosum (G.E.Sm.) Salmon	Figueira da Foz, Cabo Mondego	Beira Litoral	A Caperta, ASRóis & AP Paes	30-IV-2010	345/2010

Table 5. Continued.

L. dodartii (Girard.) O. Kuntze	Odemira, Cavaleiro, Cabo Sardão	Alentejo	A Caperta, AP Paes & AS Róis	7-VII-2010	569/2010
L. dodartii (Girard.) O. Kuntze	Odemira, Cavaleiro, Cabo Sardão	Alentejo	A Caperta, AP Paes & AS Róis	7-VII-2010	570/2010
L. dodartii (Girard.) O. Kuntze	Odemira, Cavaleiro, Cabo Sardão	Alentejo	A Caperta, AP Paes & AS Róis	7-VII-2010	571/2010
L. dodartii (Girard.) O. Kuntze	Sines, Porto Covo, Praia Vieirinha	Alentejo	A Caperta, AP Paes & AS Róis	7-VII-2010	573/2010
L. dodartii (Girard.) O. Kuntze	Porto Covo, Ponta do Farol, Praia Pequena	Alentejo	A Caperta, AP Paes & AS Róis	7-VII-2010	574/2010
L. dodartii (Girard.) O. Kuntze	Mafra, Ericeira, Foz do Lizandro	Estremadura	A Caperta, AP Paes & AS Róis	23-IV-2010	260/2010
L. lanceolatum (Hoffmanns & Link) Franco	Aljezur, Praia da Amoreira	Algarve	A Caperta, AP Paes & AS Róis	7-VII-2010	575/2010
L. lanceolatum (Hoffmanns & Link) Franco	Aljezur, Praia da Amoreira	Algarve	A Caperta, AP Paes & AS Róis	7-VII-2010	576/2010
L. lanceolatum (Hoffmanns & Link) Franco	Aljezur, Praia da Amoreira	Algarve	A Caperta, AP Paes & AS Róis	7-VII-2010	577/2010
L. lanceolatum (Hoffmanns & Link) Franco	Aljezur, Praia da Amoreira	Algarve	A Caperta, AP Paes & AS Róis	7-VII-2010	578/2010
L. lanceolatum (Hoffmanns & Link) Franco	Odemira, Vila Nova Milfontes, Sapal do Rio Mira	Alentejo	A Caperta, AP Paes & AS Róis	7-VII-2010	272/2012
L. lanceolatum (Hoffmanns & Link) Franco	Odemira, Rio Mira, sapal	Alentejo	A Caperta & AP Paes	22-V-2010	593/2010
L. lanceolatum (Hoffmanns & Link) Franco	Odemira, Sapal do Rio Mira	Alentejo	A Caperta & AP Paes	22-V-2010	601/2010
L. lanceolatum (Hoffmanns & Link) Franco *x L. ovalifolium* (Poiret) O. Kuntze	Odemira, Sapal do Rio Mira	Alentejo	A Caperta, AP Paes & AS Róis	7-VII-2010	558/2010
L. lanceolatum (Hoffmanns & Link) Franco *x L. ovalifolium* (Poiret) O. Kuntze	Odemira, Sapal do Rio Mira	Alentejo	A Caperta, AP Paes & AS Róis	7-VII-2010	559/2010
L. lanceolatum (Hoffmanns & Link) Franco *x L. ovalifolium* (Poiret) O. Kuntze	Odemira, Sapal do Rio Mira	Alentejo	A Caperta, AP Paes & AS Róis	7-VII-2010	560/2010
L. lanceolatum (Hoffmanns & Link) Franco *x L. ovalifolium* (Poiret) O. Kuntze	Odemira, Sapal do Rio Mira	Alentejo	A Caperta, AP Paes & AS Róis	7-VII-2010	561/2010

Table 5. Continued.

L. ovalifolium (Poiret) O. Kuntze	Lagos, Praia da Luz	Algarve	A Caperta & AS Róis	15-V-2010	263/2010
L. ovalifolium (Poiret) O. Kuntze	Lagoa, Ferragudo, Farol da Ponta do Altar	Algarve	A Caperta, AP Paes & AS Róis	6-VII-2010	544/2010
L. ovalifolium (Poiret) O. Kuntze	Lagoa, Ferragudo, Farol da Ponta do Altar	Algarve	A Caperta, AP Paes & AS Róis	6-VII-2010	545/2010
L. ovalifolium (Poiret) O. Kuntze	Lagoa, Ferragudo, Farol da Ponta do Altar	Algarve	A Caperta, AP Paes & AS Róis	6-VII-2010	546/2010
L. ovalifolium (Poiret) O. Kuntze	Lagoa, Ferragudo, Farol da Ponta do Altar	Algarve	A Caperta, AP Paes & AS Róis	6-VII-2010	547/2010
L. ovalifolium (Poiret) O. Kuntze	Cascais, Cabo Raso	Estremadura	A Caperta, AP Paes & AS Róis	23-VI-2010	582/2010
L. ovalifolium (Poiret) O. Kuntze	Cascais, Cabo Raso	Estremadura	A Caperta, AP Paes & AS Róis	23-VI-2010	583/2010
L. nydeggeri Erben	Vila do Bispo, Cabo de São Vicente	Algarve	A Caperta & AS Róis	15-V-2010	254/2010
L. nydeggeri Erben	Cascais, Cabo Raso	Estremadura	A Caperta, AP Paes & V Alegria	28-V-2010	269/2010
L. nydeggeri Erben	Peniche, Baleal	Estremadura	A Caperta	30-V-2010	354/2010
L. multiflorum Erben	Cascais, Cabo Raso	Estremadura	A Caperta, AP Paes & AS Róis	23-VI-2010	584/2010
L. multiflorum Erben	Cascais, Cabo Raso	Estremadura	A Caperta, AP Paes & AS Róis	26-V-2010	595/2010
L. multiflorum Erben	Cascais, Cabo Raso	Estremadura	A Caperta, AP Paes & AS Róis	28-V-2010	596/2010
L. multiflorum Erben	Cascais, Cabo Raso	Estremadura	A Caperta, AP Paes & V Alegria	28-V-2010	261/2010
L. multiflorum Erben	Cascais, Cabo Raso	Estremadura	A Caperta, AP Paes & V Alegria	28-V-2010	262/2010
L. multiflorum Erben	Cascais, Cabo Raso	Estremadura	A Caperta, AP Paes & AS Róis	28-V-2010	351/2010
L. multiflorum Erben	Cascais, Cabo Raso	Estremadura	A Caperta, AP Paes & V Alegria	28-V-2010	352/2010

Table 5. Continued.

L. multiflorum Erben	Cascais, Cabo Raso	Estremadura	A Caperta, AP Paes & AS Róis	28-V-2010	353/2010
L. multiflorum Erben	Óbidos, Lagoa de Óbidos, Foz	Estremadura	A Caperta, AP Paes & AS Róis	14-VIII-2010	814/2010
L. multiflorum Erben x *L. ovalifolium* (Poiret) O. Kuntze	Cascais, Cabo Raso	Estremadura	A Caperta, AP Paes & AS Róis	12-V-2010	597/2010
L. ovalifolium (Poiret) O. Kuntze	Tavira, Pedras del Rei	Algarve	A Caperta & AS Róis	16-V-2010	594/2010
L. vulgare Mill.	Tavira, Pedras del Rei	Algarve	A Caperta & AS Róis	16-V-2010	259/2010
L. vulgare Mill.	Tavira, Praia do Barril	Algarve	A Caperta, AP Paes & AS Róis	5-VII-2010	552/2010
L. vulgare Mill.	Tavira, Praia do Barril	Algarve	A Caperta, AP Paes & AS Róis	5-VII-2010	553/2010
L. vulgare Mill.	Tavira, Praia do Barril	Algarve	A Caperta, AP Paes & AS Róis	5-VII-2010	554/2010
L. vulgare Mill.	Odemira, Sapal do Rio Mira	Alentejo	A Caperta, AP Paes & AS Róis	7-VII-2010	556/2010
L. vulgare Mill.	Odemira, Sapal do Rio Mira	Alentejo	A Caperta, AP Paes & AS Róis	7-VII-2010	557/2010
L. vulgare Mill.	Tavira, Luz, Praia do Barril	Algarve	A Caperta, AP Paes & AS Róis	5-VII-2010	813/2010
L. multiflorum Erben	Lourinhã, Ponta de Vale Frades	Estremadura	A Caperta, AP Paes, AS Róis & A Cortinhas	13-VII-2011	162/2011
L. multiflorum Erben	Lourinhã, Ponta de Vale Frades	Estremadura	A Caperta, AP Paes, AS Róis & A Cortinhas	13-VII-2011	163/2011
L. multiflorum Erben	Peniche, Ilhéu da Papoa	Estremadura	A Caperta, AP Paes, AS Róis & A Cortinhas	13-VII-2011	152/2011
L. nydeggeri Erben	Odemira, Praia do Malhão	Alentejo	A Caperta, AP Paes, AS Róis & A Cortinhas	12-VII-2011	156/2011
L. nydeggeri Erben	Odemira, Praia do Malhão	Alentejo	A Caperta, A P Paes, AS Róis & A Cortinhas	12-VII-2011	157/2011

Table 5. Continued.

L. nydeggeri Erben	Peniche, (Ajuda) Remédios	Estremadura	A Caperta, AP Paes, AS Róis & A Cortinhas	13-VII-2011	169/2011
L. ovalifolium (Poiret) O. Kuntze	Cascais, Cabo Raso	Estremadura	A. Caperta, A. P. Paes & J.C.Costa	26-V-2011	174/2011
L. vulgare Mill.	Grândola, Carvalhal, Tróia	Alentejo	A Caperta, AP Paes, AS Róis & A Cortinhas	5-IX-2011	172/2011
L. binervosum (G.E.Sm.) Salmon	Marinha Grande, S. Pedro Moel, Praia Velha	Beira Litoral	S Murra, A Caperta, AS Róis & AP Paes	14-VI-2012	994/2013
L. binervosum (G.E.Sm.) Salmon	Marinha Grande, S. Pedro Moel, Praia Velha	Beira Litoral	S Murra, A Caperta, AS Róis & AP Paes	14-VI-2012	995/2013
L. binervosum (G.E.Sm.) Salmon	Marinha Grande, S. Pedro Moel, Praia Velha	Beira Litoral	S Murra, A Caperta, AS Róis & AP Paes	14-VI-2012	996/2013
L. binervosum (G.E.Sm.) Salmon	Marinha Grande, S. Pedro Moel, Praia Velha	Beira Litoral	S Murra, A Caperta, AS Róis & AP Paes	14-VI-2012	997/2013
L. binervosum (G.E.Sm.) Salmon	Marinha Grande, S. Pedro Moel, Praia Velha	Beira Litoral	S Murra, A Caperta, AS Róis & AP Paes	14-VI-2012	998/2013
L. binervosum (G.E.Sm.) Salmon	Marinha Grande, S. Pedro Moel, Praia Velha	Beira Litoral	S Murra, A Caperta, AS Róis & AP Paes	14-VI-2012	999/2013
L. binervosum (G.E.Sm.) Salmon	Marinha Grande, S. Pedro Moel, Praia Concha	Beira Litoral	S Murra, A Caperta, AS Róis & AP Paes	14-VI-2012	1000/2013
L. binervosum (G.E.Sm.) Salmon	Marinha Grande, S. Pedro Moel, Praia Concha	Beira Litoral	S Murra, A Caperta, AS Róis & AP Paes	14-VI-2012	1001/2013
L. dodartii (Girard.) O. Kuntze	Odemira: S. Teotónio, Cabo Sardão	Alentejo	A Caperta	20-VI-2012	1010/2013
L. dodartii (Girard.) O. Kuntze	Odemira: S. Teotónio, Cabo Sardão	Alentejo	A Caperta & AS Róis	20-VI-2012	1011/2013
L. dodartii (Girard.) O. Kuntze	Sines, S. Teotónio, Porto Covo	Alentejo	A Caperta & AS Róis	20-VI-2012	1012/2013
L. dodartii (Girard.) O. Kuntze	Sines, Praia da Oliveirinha	Alentejo	S Murra, A Caperta, AS Róis & AP Paes	2-VI-2012	1017/2013
L. dodartii (Girard.) O. Kuntze	Odemira, Zambujeira do Mar	Alentejo	S Murra, A Caperta, AS Róis & AP Paes	2-VI-2012	1018/2013
L. dodartii (Girard.) O. Kuntze	Odemira, S. Teotónio, Cabo Sardão	Alentejo	A Caperta	20-VI-2012	1019/2013

Table 5. Continued.

L. dodartii (Girard.) O. Kuntze	Odemira, Zambujeira do Mar	Alentejo	S Murra, A Caperta, AS Róis & AP Paes	2-VI-2012	897/2012
L. lanceolatum (Hoffmanns & Link) Franco	Tavira, Luz, Pedras Del Rei, Sapal do Barril	Algarve	A Caperta, D Espírito-Santo, JC Costa & AP Paes	31-VII-2012	886/2012
L. lanceolatum (Hoffmanns & Link) Franco	Tavira, Luz, Pedras Del Rei, Sapal do Barril	Algarve	A Caperta, D Espírito-Santo, JC Costa & AP Paes	31-VII-2012	888/2012
L. lanceolatum (Hoffmanns & Link) Franco	Tavira, Luz, Pedras Del Rei, Sapal do Barril	Algarve	A Caperta, D Espírito-Santo, JC Costa & AP Paes	31-VII-2012	889/2012
L. lanceolatum (Hoffmanns & Link) Franco	Tavira, Luz, Pedras Del Rei, Sapal do Barril	Algarve	A Caperta, D Espírito-Santo, JC Costa & AP Paes	31-VII-2012	890/2012
L. multiflorum Erben	Cascais, Cabo Raso	Estremadura	A Caperta & APPaes	12-VI-2012	1003/2013
L. multiflorum Erben	Cascais, Cabo Raso	Estremadura	A Caperta & APPaes	12-VI-2012	1004/2013
L. multiflorum Erben	Lourinhã, Praia da Peralta	Estremadura	A Caperta	8-VIII-2012	895/2012
L. multiflorum Erben	Peniche, Atouguia da Baleia, Praia da Consolação	Estremadura	A Caperta	8-VIII-2012	895/2012
L. multiflorum Erben	Cascais, Cabo Raso	Estremadura	A Caperta & APPaes	12-V-2012	1082/2012
L. nydeggeri Erben	Caldas da Rainha, Salir do Porto	Beira Litoral	S Murra, A Caperta, AS Róis & AP Paes	14-VI-2012	873/2012
L. nydeggeri Erben	Aljezur, Bordeira, Pontal da Carrapateira	Algarve	S Murra, A Caperta, AS Róis & AP Paes	4-VI-2012	274/2012
L. nydeggeri Erben	Caldas da Rainha, Salir do Porto	Beira Litoral	S Murra, A Caperta & AP Paes	14-VI-2012	884/2012
L. nydeggeri Erben	Peniche, Baleal	Estremadura	A Caperta & APPaes	27-VI-2012	891/2012
L. nydeggeri Erben	Cascais, Cabo Raso	Estremadura	A Caperta & APPaes	12-V-2012	1081/2012
L. ovalifolium (Poirret) O. Kuntze	Sines, Porto Covo, Praia do Malhão	Estremadura	S Murra, A Caperta, AS Róis & AP Paes	2-VI-2012	271/2012

Table 5. Continued.

L. ovalifolium (Poirret) O. Kuntze x *L. algarvense* Erben	Tavira, Luz, Torre de Ares, Sapal	Algarve	A Caperta, D Espírito-Santo, JC Costa & AP Paes	31-VI-2012	892/2012
L. ovalifolium (Poirret) O. Kuntze x *L. algarvense* Erben	Olhão, Fuzeta, Sapal da Ilha do Coco, Bias	Algarve	A Caperta, D Espírito-Santo, JC Costa & AP Paes	31-VI-2012	893/2012
L. vulgare Mill.	Tavira, Luz, Pedras Del Rei, Sapal do Barril	Algarve	A Caperta, D Espírito-Santo, JC Costa & AP Paes	31-VII-2012	885/2012
L. vulgare Mill.	Tavira, Luz, Pedras Del Rei, Sapal do Barril	Algarve	A Caperta, D Espírito-Santo, JC Costa & AP Paes	31-VII-2012	887/2012
L. binervosum (G.E.Sm.) Salmon	Aveiro, Vagos, Gafanha da Boa Hora	Beira Litoral	S Murra, A Caperta, AP Paes & P Arsénio	26-VII-2013	1173/2013
L. binervosum (G.E.Sm.) Salmon	Aveiro, Vagos, Gafanha da Boa Hora	Beira Litoral	S Murra, A Caperta, AP Paes & P Arsénio	26-VII-2013	1174/2013
L. binervosum (G.E.Sm.) Salmon	Aveiro, Dunas de São Jacinto	Beira Litoral	S Murra, A Caperta, AP Paes & P Arsénio	26-VII-2013	1175/2013
L. vulgare Mill.	Viana do Castelo, Rio Lima, Cabedelo	Minho	A Caperta	24-VIII-2013	1002/2013
L. vulgare Mill.	Viana do Castelo, Rio Lima, Cabedelo	Minho	A Caperta	24-VIII-2013	1005/2013

Table 6. **Selected natural *Limonium* populations in Portuguese Provinces.**

Species	Habitat	Site Location/ Collectors*/ Province	Geographical coordinates	Population Size
L. lanceolatum	Salt-marsh	Odemira, Vila Nova de Milfontes ADC, APP, ASR/Alentejo	37.727756/-8.770931	25
		Tavira, Sapal do Barril ADC,APP, ASR/Algarve	37.0861361/-7.662772	50
L. nydeggeri	Sea-cliffs	Peniche, Ilha do Baleal ADC, APP, ASR/Estremadura	39.378919/-9.340983	25
		Peniche, Nossa Srª dos Remédios ADC, APP, ASR/Estremadura	39.369906/-9.395731	25
		Cascais, Cabo Raso ADC, APP, ASR/Estremadura	38.710039/-9.485883	50
		Aljezur, Pontal da Carrapateira ADC, APP, ASR/Algarve	37.195039/-8.911103	25
		Vila do Bispo, Cabo de São Vicente ADC, ASR/Algarve	37.022611/-8.996564	25
L. ovalifolium	Sea-cliffs	Lagos, Praia da Luz ADC, ASR/Algarve	37.087442/-8.729094	25
		Vila do Bispo, Cabo de Sagres ADC, APP, ASR/Algarve	36.994242/-8.948756	30
L. binervosum	Sea-cliffs	Cabo Mondego, Figueira da Foz ADC, APP, ASR, SM/Beira Litoral	40.191111/-8.905278	25
		Praia da Concha, Marinha Grande ADC, APP, ASR, SM/Beira Litoral	39.755278/-9.032281	20
		Salir do Porto, Caldas da Rainha ADC, APP, ASR, SM/Beira Litoral	39.49843055/-9.17065	50
L. dodartii	Sea-cliffs	Odemira, Cabo Sardão ADC, APP, ASR, SM, PA/Alentejo	37.598631/-8.816056	50
		Odemira, Porto Covo ADC, APP, ASR, SM, PA/Alentejo	37.851667/-8.791944	50

Table 6. Continued.

L. multiflorum	Sea-cliffs	Cascais Cabo Raso ADC, APP, ASR/Estremadura	38.710039/-9.485883	1000
		Lourinhã, Vale dos Frades ADC, APP, ASR/Estremadura	39.276506/-9.335839	20
		Mafra, Foz do Lizandro ADC, APP, ASR/Estremadura	38.941531/-9.415233	50
		Peniche, Península da Papoa ADC, APP, ASR/ Pop18/Estremadura	39.222687/-9.223874	25
L. vulgare	Salt-marsh	Odemira, Vila Nova de Milfontes ADC, APP, ASR /Alentejo	37.727756/-8.770931	15

*Abbreviations of collectors: ADC, AD Caperta; APP, Ana Paula Paes; ASR, AS Róis; PA, P Arsénio; SM, S Martins; DES, Dalila Espírito-Santo. Centro de Botânica Aplicada à Agricultura, Instituto Superior de Agronomia.

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CHAPTER IV.

MALE FERTILITY VERSUS STERILITY, CYTOTYPE, AND SEED PRODUCTION IN DIPLOID AND TETRAPLOID *LIMONIUM* REVEAL DIVERSITY IN REPRODUCTION MODES

The data presented in this chapter were published in **Róis AS, Teixeira G, Sharbel TF, Fuchs J, Martins S, Espírito-Santo D, Caperta AD (2012) Male fertility versus sterility, cytotype, and DNA quantitative variation in seed production in diploid and tetraploid sea-lavenders (*Limonium* sp.; *Plumbaginaceae*) reveal diversity in reproduction modes. Sex Plant Rep, 25(4):305–318. doi:10.1007/s00497-012-0199-y**

1. Abstract

The genus *Limonium* Mill., a complex taxonomic group, comprises annuals and perennials that can produce sexual and/or asexual seeds (apomixis). In the study presented in this chapter, we used diverse cytogenetic and cytometric approaches to analyze male sporogenesis and gametogenesis for characterizing male reproductive output on seed production in *L. ovalifolium* and *L. multiflorum*. We showed here that the first species is mostly composed of diploid cytotypes with 2*n*=2x=16 chromosomes and the latter species by tetraploid cytotypes with 2*n*= 32, 34, 35, 36 chromosomes and had a genome roughly twice as big as the former one. In both species, euploid and aneuploid cytotypes with large metacentric chromosomes with interstitial decondensed sites were found within and among populations, possibly involved in chromosomal reconstructions. *L.ovalifolium* diploids showed regular meiosis resulting in normal tetrads, while diverse chromosome pairing and segregation irregularities leading to the formation of abnormal meiotic products are found in balanced and non-balanced *L. multiflorum* tetraploids. Before anther dehiscence the characteristic unicellular, bicellular or tricellular pollen grains showing the typical *Limonium* micro- or macro-reticulate exine ornamentation patterns were observed in *L. ovalifolium* using SEM. Most of these grains were viable and able to produce pollen tubes *in vitro*. Instead, in both balanced and unbalanced *L. multiflorum* tetraploids microspores only developed until the "ring-vacuolate stage" presented a collapsed morphology without the typical exine patterns, pointing to a sporophytic defect. These microspores were unviable and therefore never germinated *in vitro*. *L. ovalifolium* individuals presented larger pollen grains than those of *L. multiflorum*, indicating that pollen size and ploidy levels are not correlated in the *Limonium* system. Cytohistological studies in mature seeds from both species revealed that an embryo and a residual endosperm were present in each seed. Flow cytometric seed screens using such mature seeds showed quantitative variations in seeds ploidy level. It is concluded that male function seems to play an important role in the reproduction modes of *Limonium* diploids and tetraploids.

Keywords: apomixis, genome size, *Limonium,* male sporogenesis, male gametogenesis, polyploidy

2. Introduction

The genus *Limonium* Mill. (sea lavenders) is the most species-rich and widespread of the *Plumbaginaceae* and comprises halophytes inhabiting sea shores, salt-marshes and salt steppes (Erben 1993). For this genus, a reticulate diversification model involving changes in ploidy levels has been proposed, and is thought to be responsible for its high taxonomic complexity (Palacios *et al.* 2000; Lledó *et al.* 2005). Several systematic and phylogenetic studies tried to clarify the taxonomic complexity of this genus in a global perspective (Baker 1948; Pignatti 1971; Karis 2004; Lledó *et al.* 2005) or specifically on particular geographic areas (Ingrouille 1984; Artelari 1989, 2002). A large number of microspecies have been described especially in the Mediterranean basin (Erben 1993). Among them, it has been accepted that truly sexual species are infrequent, whereas it is assumed that facultative apomictic species account for a large proportion of the species (Erben 1978; Cowan *et al.* 1998), but it is yet to be confirmed.

Taxonomic complexity has been linked to a sporophyte self-incompatibility pollen-stigma dimorphism system (Baker 1966) and the polyploid hybrids ability of producing seeds by apomixis (asexual reproduction by seed) (d'Amato 1949; Baker 1953ab, 1966; Erben 1978). In members of the *Limonium* genus reduced and unreduced embryo sacs have been described. In addition, to tetrasporic 8-nucleate *Fritillaria*-type or *Adoxa*-type, or tetrasporic 16-nucleate *Penea*-type female gametophytes (Dahlgren 1916; D'Amato 1940), *Ixeris*-type embryo sacs with non-haploid eggs are found in triploid (2*n*=3x=27) *Statice oleaefolia* var. *confusa* (the present genus *Limonium* was known as *Statice*; *Statice*, *nom. rej*. vs. *Armeria*; Greuter *et al.*, 2000) (D'Amato 1949). In this last type of embryo sacs, the first meiotic division ends with the formation of a restitution nucleus followed by a second meiotic division not accompanied by cytokinesis (meiotic diplospory), followed by two further mitotic divisions of the unreduced nuclei to result in 8-nucleate embryo sacs.

Various studies have explored the cytological variability of *Limonium* species from several regions and have reported diploid (2*n*=2x=16, 18), triploid (2*n*=3x= 24, 25, 27), tetraploid (2*n*=4x=32, 35, 36), pentaploid (2*n*=5x= 43) and hexaploid (2*n*=6x=51, 54, 56) species (Erben 1978; Brullo and Pavone 1981; Arrigoni and Diana 1993; Castro and Rosseló 2007). Diploid species (2*n*=16 or 2*n*=18) seem to be apparently stable, with typically two basic chromosome numbers, x=8 and x=9 (Erben 1978). It has been hypothesized that triploid *Limonium* species are predominant in the genus, presumably arisen through hybridization (allopolyploids) of two diploid types with basic chromosome numbers x=8 and x=9 which supply the resulting hybrids with reduced and unreduced gametes (Erben 1978, 1979). In polyploid species different chromosome numbers have been shown within the same species, population or even in the same specimen (Dolcher and Pignatti 1967, 1971; Diana 1995; Castro and Rosseló 2007). The most extreme karyological polymorphism was reported in the Corsican endemic *L. bonifaciense*, where in more than 50% of the seedlings mixoploidy occurred (Diana 1995). However, knowledge on interspecific, intraspecific and population level variability in species of the south-west Atlantic Coast is limited.

Palynological studies in *Limonium* have revealed the two *Armeria* types pollen grains with an exine surface coarsely prominently reticulate (A-pollen) or finely reticulate (B-pollen), and pollen sizes between 40 – 100 µm (Erdtman 1952; Nowicke and Skvarla 1977). In *Limonium* the sporophytic self-incompatibility system is linked with pollen-stigma dimorphism, A-pollen type grains germinate on papillose stigmas and B-pollen type germinate in *cob*-like stigmas, while the complementary combinations produce no successful fertilization (Baker 1953ab; Richards 1997). In general, pollen stainability appears to be high in sexual diploid, while in polyploids low to high fertility has been reported (Erben 1978, 1979). For example, in the hexaploid *Limonium humile* Mill. (2*n*=6x=54), a high percentage of fertile pollen grains is found (95%) (Dawson and Ingrouille 1995), while in the triploid *Limonium viciosoi* (Pau) Erben (2*n*=3x=27) pollen was either not produced or with very low stainability (2-13%) (Erben 1978). Although a few studies exist in male gametophyte development *in vitro* in *Limonium* horticultural valuable crops (e.g.

L. perezii) (Zhang *et al.* 1997), to our knowledge detailed studies on male sporogenesis and gametogenesis are lacking.

In the Continental Portugal about 15 *Limonium* species have been recognized (Erben 1993, 1999). Among them, the species of the *L. ovalifolium* complex group which show marked morphological similarities and have been described as sexual diploids (2*n*=2x=16), are represented by *L. ovalifolium*, *L. nydeggeri* and *L. lanceolatum* (Erben 1993, 1999). And, the Lusitania highly endemic aneuploid tetraploid *L. multiflorum* (2*n*=4x=35), presumably an apomict, is considered as a crop wild relative from mainland Portugal (Magos-Brehm *et al.* 2008). This species belongs to the *L. binervosum* complex group which also includes *L. binervosum* and *L. dodartii* (Erben 1978). However, till now studies within these groups were based on limited sampling of *taxa* and on morphological studies and knowledge in their reproductive modes is lacking.

The aim of the present study is to characterize male sporogenesis and gametogenesis to determine male reproductive output in seed production in diploid *L. ovalifolium* and polyploid *L. multiflorum*. To this end, we first studied the ploidy levels through genome size measurements and chromosome counts in plants from both species. Then, we analyzed male sporogenesis and gametogenesis using OLM and SEM. To reconstruct the mode of reproduction within these groups we analyzed mature seeds through a FCSS procedure, cytohistology and SEM. Our results show contrasting male fertility *versus* sterility in both species, likely associated with distinct reproductive strategies.

3. Materials and methods

3.1. Plant materials and growth conditions

The species were identified using keys from Erben (1993) and herbarium specimens from the Herbarium João de Carvalho e Vasconcellos, (LISI), were examined to confirm species identifications. For each species seeds were collected in the wild from up to twenty specimens each population. In this work we will refer to *L. ovalifolium* in *sensu lato* to the sampled populations from Baleal (B) (Estremadura: Peniche), Papoa (P) (Estremadura: Peniche), Srª Remédios (SR) (Estremadura: Peniche), Cabo Raso (CR) (Estremadura: Cascais) and Cabo de Sagres (CS) (Algarve: Sagres). *L. multiflorum*

populations from Vale dos Frades (VF) (Estremadura: Lourinhã), Foz do Lizandro (FL) (Estremadura: Ericeira) and Cabo Raso (Estremadura: Cascais) were also sampled (Figure 16). All populations were tagged with Global Positioning System and mapping was made using ArcGIS Desktop 10 (ESRI).

To establish controlled experimental populations from both species, about fifty seeds per population from five individuals randomly selected were placed on moist filter paper in petri dishes and then transferred to a growth chamber (Rumed) for germination with controlled light and temperature with a photoperiod of 18 h light and 6 h dark at 25ºC until germination. The germinating seeds were transplanted into plastic pots with substrate and grown under greenhouse conditions, prior to use in cytological studies. Species identifications were confirmed with particular emphasis placed on leaf, inflorescence, and flower morphology on eight month old plants.

3.2. Chromosome preparation and karyotyping

Seven distinct plants from each species population growing in the greenhouse were randomly chosen and analyzed. Root tips were excised and then treated with a 2 mM 8-hydroxyquinoline solution for 2 h at 4ºC in the dark and subsequently for 2 h at room temperature to induce metaphases. Then, root-tips were fixed in a fresh absolute ethanol:glacial acetic acid (3:1) solution overnight and stored at -20ºC until used. Next, root tips were digested with a 2% cellulase, 2% cellulase “Onozuka R-10” and 2% pectinase enzyme solution in 1 x EB (40 ml 0.1 M citric acid-1-hydrate and 60 ml of 0.1 M sodium citrate dihydrate; pH 4.8) for 3 h at 37ºC as described in Caperta *et al.* (2008). Squashes were made in 45% acetic acid, and preparations were counterstained with 4’,6-diamidino-2-phenylindole hydrochloride (DAPI) (1 mg/ml) diluted in Citifluor (Agar).

3.3. Genome size estimations

For flow cytometric genome size estimations roughly 10 mm^2 of leaf tissue from individuals of *Limonium* populations were chopped with a sharp razor blade together with leaf material of either *Pisum sativum* L. subsp. *sativum* convar. *sativum* var. *ponderosum* Alef., Sorte Viktoria, Kifejtö Borsó,

(2C=9.07 pg) (Genebank Gatersleben accession number: PIS 630) or *Secale cereale* L. subsp. *cereale*, (2C=16.55 pg) (Genebank Gatersleben accession number: Sortiments-Nr. R 737) as internal reference standards in a Petri dish containing 1 ml nuclei isolation buffer (Galbraith *et al.* 1983) supplemented with 1% PVP-25, 0.1% Triton X-100, DNase-free RNase (50 µg/ml) and propidium iodide (50 µg/ml). The nuclei suspension was filtered through a 35 µm mesh cell strainer cap and stored on ice until measurement. The relative fluorescence intensities of stained nuclei were measured using a FACStar^{PLUS} (BD Biosciences, New Jersey, USA) flow sorter equipped with an argon ion laser INNOVA 90C (Coherent, Palo Alto, CA, USA). Usually, 10,000 nuclei per sample were analyzed. The absolute DNA amounts of samples were calculated based on the values of the G_1 peak means.

3.4. Cytological analyses of microsporogenesis

Flower buds of distinct floral stages were collected before anthesis from three diploid *2n*=16 *L. ovalifolium* individuals and three polyploid *L. multiflorum* individuals, respectively 2*n*=32, 35 and 36 growing in the greenhouse. Anthers were dissected and fixed in a fresh absolute ethanol: glacial acetic acid (3:1) solution. The material was digested with 2% cellulase, 2% cellulase “Onozuka R-10” and 2% pectinase enzyme solution in 1 x EB for 45 min at 37ºC. Squashes were made in 45% glacial acetic acid according to Caperta *et al.* (2008). The preparations were counterstained with DAPI.

3.5. *In vitro* pollen viability tests

The fluorescent diacetat (FDA) test procedure was used to determine pollen viability as described in Heslop-Harrison and Heslop-Harrison (1970), with some modifications. In brief, FDA was made up as a stock solution acetone at 2 mg/ml. Immediately before use, dilutions were prepared by adding drops of the stock to 2 ml of a sucrose solution. The working solution prepared according to this procedure gave a concentration of $6x10^{(-5)}$ M FDA in 0.5 M sucrose (Pinillos and Cuevas 2008). All pollen grains, which fluoresced brightly, were scored as viable. Total viability estimates were performed by one person using one to seven flowers per plant and counted under 20X magnification. For

L.ovalifolium 500 pollen grains per flower were recorded while for *L. multiflorum* 0-250 pollen grains per flower were counted.

3.6. *In vitro* pollen germination

Three flowers (five anthers each flower) per plant in five replicates were used to analyze pollen tube growth. The pollen grains were collected from plants soon after anther dehiscence and were then cultured in a media containing 20 mM boric acid, 6 mM calcium nitrate, 0.1% CH (Casein Hydrolysate) and 7% sucrose (Zhang *et al.* 1997). A dialysis tubing and filter paper support combined with using 23% polyethylene glycol -20,000 as an osmoticum in the medium provided appropriate physical conditions for pollen germination. Pollen grains were incubated at 37ºC during 48 h or 72 h in the dark. Pollen grains were considered germinated when they had a tube length that was equal or greater than the diameter of the pollen grain. For measurement of tube length, 40 pollen tubes were selected randomly from each treatment and measured on micrographs recorded with a 63X objective using the Axiovision 4.0 (Zeiss).

3.7. Optical microscopy analysis and imaging

Cell preparations, meiocytes, pollen grains and pollen tubes were observed using a FM. Images were collected with an AxioCam MRc5 digital camera (Zeiss) and further processed using Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA). Well spread chromosome complements were measured on micrographs recorded with a 63x objective using the Axiovision 4.0 (Zeiss).

3.8. Electron microscopy pollen and seed analyses

Flower and seed samples of both species were fixed in a 2.5% glutaraldehyde solution in 0.1 M sodium phosphate buffer, pH 7.2, for 5 h at 4°C as described in Hayat (1981), with some modifications. Then, the material was dehydrated in a graded ethanol series, from 30, 50, 75 and 100% ethanol/water for 30 min each. Flowers were then dried at a critical point on a Critical Point Polaron BioRad E3500, and coated with a thin layer of gold on a Jeol JFC-

1200. Observations were carried out on a SEM JSM-5220 LV equipped with a direct image acquisition system. To estimate pollen morphology and dimensions randomly chosen grains were measured from SEM micrographs. Pollen terminology and dimensions followed that of Erdtman (1952). Pollen grain dimensions were based on the length of the longest pollen grain axis, and four dimension classes were considered, including very small (x≤10 µm), small (10>x≤25 µm), medium (25>x≤50 µm), and large (50>x≤100 µm). Mean, standard deviation and standard error of the mean were estimated.

3.9. Cytohistological analysis of mature seeds

To show the cellular structure of the different tissues of mature seeds, they were fixed as described above for flower samples and embedded in paraffin following standard methods (Ruzin 1999). Longitudinal sections were then cut on a Leitz 1512 Minot microtome, at 10-12 µm and stained with Lugol's solution for starch localization (Johansen 1940). Cytohistological observations were made with a Nikon Labophot 2 OLM using 10x and 40x objectives, and images were obtained with a Nikon FX-35W camera.

3.10. Flow cytometric screening of seeds

For flow cytometric seed screening (FCSS, Matzk *et al.* 2000), at least seven specimens per population collected in the wild, and fifty mature seeds per plant were used. Single seeds were ground individually with three 2.3 mm stainless steel beads in each well of a 96-deep well plate containing 80 µl isolation buffer (OTTO1) using a Geno-Grinder 2000 (SPEX CertiPrep) at a rate of 50 strokes/min for 2 min (Matzk 2000). Afterwards 200 µl of isolation buffer was added to recover enough volume for filtration (30 µm mesh). 50 µl of the resultant suspension was stained by adding 150 µl of staining buffer (OTTO2 + 1-2 µg/ml DAPI) and incubated on ice for 10 min before flow cytometric analysis. All sample plates were analyzed at 4ºC on a temperature regulated Robby-well auto-sampler hooked up to a Partec PAII flow cytometer (Partec GmbH, Munster, Germany). To validate the data, five seeds per plant per accession were analyzed with a conventional razor-chopping method according to (Matzk *et al.* 2000). Histograms representing the quantity of nuclei with

distinct DNA content were plotted using a linear scale (x-axis), giving all peaks of the histogram the same width.

4. Results

In both *L. ovalifolium* and *L. multiflorum* estimations of seed set per inflorescence from different individuals of the analyzed populations were higher than a hundred seeds. The overall germination rate was slightly higher in *L. multiflorum* (mean 68%) than in *L. ovalifolium* (mean 63%), although intraspecific differences between populations were detected. In *L. ovalifolium* the germination rate ranged from 30% in P to 77% in CS populations, while in *L. multiflorum* it varied from 70% in CR to 80% in FL populations.

The absolute genome size of diploid *L. ovalifolium* and tetraploid *L. multiflorum* was found to be 3.58 ± 0.03 pg/2C and 7.72 ± 0.01 pg/2C, respectively. While the values of genome size in distinct populations were quite similar in *L. multiflorum* (FL: 7.71 ± 0.05 pg/2C; VF: 7.73 ± 0.11 pg/2C; CR: 7.73 ± 0.06 pg/2C) they showed a slightly higher variability in *L. ovalifolium* and ranged from 3.54 pg/2C in the B population to 3.61 pg/2C in the CS and CR populations (Figure 17.A - a,b). However, co-processing of individuals from B together with individuals of either CS or CR populations did not result in double peaks as it would be expected to confirm a distinct intraspecific genome size variation.

Karyotype analysis and chromosome counts were made on microphotographs of mitotic metaphase spreads of five *L. ovalifolium* and ten *L. multiflorum* specimens from each population. In *L. ovalifolium* most specimens showed 2*n*=16 chromosomes (Figure 17.B - d,e), with a pair of large metacentric chromosomes and four pairs of small metacentric chromosomes that measured 5.5–7 µm and 1.4–2.5 µm, respectively; and three pairs of submetacentric chromosomes that measured 2.5–4 µm. Nonetheless, aneuploid cytotypes with 2*n*=15 (Figure 17.B - c) or 2*n*=17 chromosomes also appeared especially in the B population. With regard to *L. multiflorum*, in all populations we found individuals with 2*n*=32, 34, 35 or 36 chromosomes (Figure 17.B - f-h). This karyological diversity was also present within cells of a few specimens rendering a precise count virtually impossible. With regard to

chromosome types we observed one or two large metacentric chromosomes measuring 5–9 µm, four medium metacentric chromosome pairs ranging 3–8 µm, three large submetacentric chromosome pairs measuring 6–10 µm, seven medium submetacentric chromosome pairs 3–5.9 µm and variable numbers of small metacentric or telocentric chromosomes which measured less than 2.9 µm. Occasionally three to five interstitial constrictions could be observed on the large metacentric chromosomes (Figure 17.C - i-j).

Calculations of total chromosome lengths (µm) in ten cells with approximately the same degree of condensation revealed for both species that metaphase cells with a higher chromosome numbers measured also higher chromosome length. For example, in *L. multiflorum* total chromosome length in cells with 2*n*=34 chromosomes was 104.5 µm while those with 2*n*=35 was 114.0 µm and with 2*n*=36 was 194.0 µm. Furthermore, measurements of chromosome length of the large metacentric chromosomes in 25 metaphase cells from each species showed that in *L. ovalifolium,* two large metacentric chromosomes were always present whereas in *L. multiflorum* one or two large metacentric chromosomes could be found. Nine metaphase cells displayed size differences between the large metaphase chromosomes of around 2 µm which corresponds to the average size of a telocentric chromosome. Sixteen metaphase cells showed only one large metacentric chromosome. However, independent on the presence of one or two large metaphase chromosomes we detected cells with 2*n*= 34, 35 or 36 chromosomes. Although this demonstrates that at least in *L. multiflorum* the large metacentric chromosome might be involved in chromosomal reconstruction it does not explain all variable cytotypes.

Next, we analyzed male meiosis in both species, although it was difficult to select anthers in identical developmental stages for comparative investigation since flower and anther dehiscence appears to occur earlier in *L. multiflorum* than in *L. ovalifolium*. In this last species *homologous* chromosome pairing at pachytene seemed to be complete (Figure 18.A - a), contrasting to that of the tetraploid *L. multiflorum* where some chromosomes were unpaired (Figure 18.A - b). In *L. ovalifolium*, eight sets of homologues were visible at diplotene and congressed during diakinesis/metaphase I transition (Figure 18.A - c). In *L.*

multiflorum metaphase I plates showed bivalent and univalent chromosomes (Figure 18.A - d), which either exhibited an apparent balanced segregation at anaphase I (Figure 18.A - e) resulting in well-formed dyads (Figure 18.A - h) or presented several segregation anomalies such as peculiar chromosome bridges (Figure 18.A - f) or anaphase lag chromosomes (Figure 18.A - g). In some anaphase I cells from both species, we were able to detect the large chromosome with interstitial constrictions (Figure 18.A - j). In both species second division meiocytes were found (Figure 18.A - i-j) and at the end of telophase II normal tetrads or triads were visible in *L. ovalifolium* (Figure 18.A - k), whereas normal and abnormal tetrads, triads or polyads were seen in *L.multiflorum* (Figure 18.A - l). Before anther dehiscence, unicellular pollen, bicellular or tricellular pollen grains were observed in *L. ovalifolium*, the latter with the characteristic vegetative nucleus and two sperm nuclei (Figure 18.B - m). In this species about 93% of the pollen grains were viable and germinated *in vitro* forming pollen tubes with approximately 33 – 274 µm in length (Figure 18.B - n). Instead, in both balanced and unbalanced *L. multiflorum* tetraploids the majority of pollen grains only attained the “ring-vacuolate” microspore stage (Stage 8; Owen and Makaroff 1995) in which microspores exhibited a large vacuole causing the characteristic “signet-ring” appearance (Figure 18.B - o). Although, about 37% of pollen grains were viable in this last species pollen tubes were never observed, even not after 72 h of incubation in the germination medium.

Examination of SEM micrographs of anthers and pollen grains revealed totally distinct patterns between species. In general, *L. ovalifolium* pollen grains could still be seen within the anthers (about 100 each anther) (Figure 19.A - a). These grains were spheroidal in the polar view while in the equatorial outline they tended to be ellipsoid, isopolar, radiosymetric and tricolpate, with usually long *colpi,* acute in the apices, and with a smooth membrane (Figure 19.A - b-c). Two ornamental patterns in exine surface could be seen, macroreticulate (Figure 19.A - c-d) or microreticulate (Figure 19.A - b). In the first type, a large and irregular *reticulum* with spinose side walls (*muri* spinulose) (Figure 19.A - e) and and regular *columellae* fused distally into an incomplete *tectum* was observed (Figure 19.A - d-e). Pollen grains with microreticulate exine surface

with a tighter *reticulum* and less spinulose *muri* were only observed in plants from CS population (Figure 19.A - b; f). Most plants showed pollen grains of medium size (Table 7) and, except for B population, a certain percentage of large pollen grains (Table 7).

In contrast, *L. multiflorum* SEM micrographs revealed several empty anthers (Figure 19.B - g) or presented few pollen grains (<100), with diverse morphology and sizes. Most of the pollen grains were collapsed in morphology among different flowers of the same plant. A very small proportion, however, appeared regular in form (Figure 19.B - h), although lacking the typical exine ornamentation described for the *Limonium* genus (Erdtman 1952; Nowicke and Skvarla 1977). Whenever it was possible to determine pollen grain symmetry, isopolar, radiosymetric (Figure 19.B - h) and bilateral grains were detected (Figure 19.B - i-l). A highly variable exine surface, almost smooth or psilate (Figure 19.B - h-i), perforate or retipilate, with no prominent spines (Figure 19.B - j; l), verrucate or warty (Figure 19.B - k-l) was found, and the *colpi* were not visible but their position could be denounced (Figure 19.B - k). On average, pollen grains from *L. multiflorum* were smaller than those of *L.ovalifolium* (Table 6). CR individuals showed the highest variability in pollen grains size, from very small to large grains (Table 7). Instead, VF and FL individuals only showed small and medium size grains (Table 7).

Small mature capsules of both species contained only a single seed of approximately 1.8 mm (± 0.3) in length and 0.4 mm (± 0.1) in width. The estimation of embryo and residual endosperm nuclear DNA contents by FCSS (Matzk *et al.*, 2000) showed that in *L. ovalifolium* populations, only histograms with a single DNA peak, 2C (channel 50) or 3C (channel 75) were found representing diploid (more than 75%) or triploid (about 25%) seeds, respectively (Table 8). Neither a 3C endosperm peak, nor secondary peaks of (endo-) replicated endosperm nuclei were observed. Also, in *L. multiflorum* only a single DNA peak was observed for each seed, although variation in ploidy levels was obtained within the populations studied. For example, in *L. multiflorum* CR population, most specimens produced triploid or tetraploid seeds although other ploidy levels were found as well (Table 8).

Cytohistological and SEM studies of mature seeds from both species showed both an embryo and a residual endosperm, the latter being about 0.01 mm thick at the widest part in each seed (Figure 20.A). In both species, the embryo was enveloped by an endosperm tissue that no longer contained nuclei, and was formed exclusively by polygonal starch grains with a well-defined hilum in the center, often with radiating clefts (Figure 20.B-D). The embryo was surrounded by an aleurone layer (7.0 μm (± 0.2) thick) (Figure 20.B) and a basal transfer layer (45 μm (± 8)) (Figure 20.C) surrounded by the seed coat. Therefore, it seems that the individual ploidy peaks observed in each seed corresponded to the embryo ploidy levels, since the residual endosperm did not contain any nuclei.

5. Discussion

In this study on ploidy and chromosome variation, male sporogenesis and gametogenesis, pollen and seed analyses of *L. ovalifolium* and *L. multiflorum*, we have found differences in genome size and chromosome numbers between and within species, meiotic dysfunction, pollen variability in terms of morphology, size and fertility and DNA quantitative variation in seed ploidy from single mother plants.

Genome size measurements in leaf material confirmed a diploid genome for *L. ovalifolium* and a tetraploid genome for *L. multiflorum* with a size roughly twice that of *L. ovalifolium*. Also, other species of *L. ovalifolium* group respectively *L. nydeggeri* and *L. lanceolatum* shows a diploid genome (Erben 1999; AS Róis and A Caperta, personal observations). In contrast *L. multiflorum* presents similar genome size to the other tetraploid species of *L. binervosum* group, namely *L. binervosum* and *L. dodartii* (Erben 1978; data not shown).

No severe differences in genome size were detectable within populations of each species. Previous data on *L. ovalifolium* chromosome numbers have shown that this *taxon* is composed of diploid cytotypes with 2*n*=16 chromosomes, (Erben 1978; Erben 1993), which is largely confirmed by the analysis of our samples; although we found a few aneuploid cytotypes. Also, we show here that both diploid and aneuploid diploid *L. ovalifolium* individuals present a peculiar, large metacentric chromosome pair which may present

interstitial constrictions. Erben (1978) designated these chromosomes as marker chromosomes and showed that they were absent in species with 2*n*=18 chromosomes. Conversely, in *L. multiflorum* specimens, in addition to unbalanced aneuploid tetraploids with 2*n*=35 chromosomes (Erben 1978), we found also balanced cytotypes with 2*n*=32, 34 and 36 chromosomes of different types. In both tetraploids cytotypes, one or two large metacentric chromosomes were present, in which three to five interstitial constrictions were occasionally visible. Although our results provide evidence that the large metacentric chromosome could be involved in some kind of chromosomal reconstructions, it is not responsible for all aneuploidies observed. For example, in cytotypes with only one metacentric chromosome, we don't observe necessarily an increased number of small chromosomes as one could expect it. Also, karyological studies in other *Limonium* species have revealed more than a single chromosome number and sometimes ploidy level for each species, population, or even within the same individual (Dolcher and Pignatti 1967, 1971; Arrigoni and Diana 1993; Diana 1995; Castro and Rosseló 2007). This was particularly emphasized for the polyploid *L. humile* in which different cytotypes were found among and within four populations (2*n*=36, 38, 48, 49, 50, 51, 52, 54) (Dawson and Ingrouille 1995), and in the polyploid *Limonium carvalhoi* Rosselló & L.Sáez from the Balearic Islands in which three cytotypes were detected (2*n*=24, 25 and 26) (Rosseló *et al.* 1998). This situation is also commonly observed in species-rich apomictic genera, for example in *Boechera*, in which intraspecific ploidy polymorphisms have been reported for geographically widespread species (Kantama *et al.* 2007).

Cytological investigations of microsporogenesis in diploid (*2n*=2x=16) *L. ovalifolium* and in balanced (2*n*=4x=32 or 36) or unbalanced (2*n*=4x=35) *L. multiflorum* tetraploids have shown striking differences between species. In *L. ovalifolium* meiosis essentially follows the regular course described for higher plants (Bhatt *et al.* 2001), whereby meiocytes at first prophase feature full pairing and normal chiasma formation with 8 bivalents at diakinesis and first metaphase followed by the reductional division of the homologous chromosomes. Meiotic figures characteristic for the equational division such as tetrads are also observed, indicating that reduced gametes are formed.

Conversely, in *L. multiflorum* tetraploids, during the first prophase at pachytene incomplete pairing is visible and sometimes at the first metaphase, bivalents and univalents are seen. In the first anaphase, disturbances in segregating chromosomes such as chromosome bridges and/or laggard chromosomes are detected. In consequence, triads, normal and abnormal tetrads and polyads are produced, likely originating abnormal microspores. In *Taraxacum officinale* Wiggers and *Boechera holboelli* apomicts, incomplete pairing, chromosome association through stickiness, and unbalanced meiotic division have also been observed (Van Baarlen *et al.* 2000; Kantama *et al.* 2007).

In *L. ovalifolium*, after the tetrad stage, microspores undergo two mitotic divisions before anther dehiscence and pollination, resulting in the formation of tricellular pollen grains with a vegetative cell and two smaller sperm cells (McCue *et al.* 2011). SEM analyses reveal *Armeria* types A- and B- pollen, where the exine shows a distinct *reticulum* and pollen sizes between 40 – 100 µm (Erdtman 1952; Nowicke and Skvarla 1977). Most of these grains were viable as revealed by the FDA procedure tests and give rise to pollen tubes after germinating *in vitro* (Figure 21.A). Contrastingly, in *L. multiflorum* after the second meiotic division only unicellular pollen grains are observed even in dehisced anthers. These microspores only attained the stages 6-8 of pollen development, and some of them show the characteristic “signet-ring” appearance, in which the cytoplasm and nucleus are peripheral (Stage 8 of Owen and Makaroff 1995) (Figure 21.B). Furthermore, these grains which reveal a collapsed morphology and lack the typical exine patterns for *Limonium* species, never germinate *in vitro*. Thus, in addition to meiotic disturbances in pairing and segregation, we observed defects in pollen development and in exine patterning in post-tetrad stages. Various studies of pollen fertility in *Limonium* polyploids, like for example, in the triploid species *Limonium supinum* (Girard) Pignatti (2*n*=3x=26) and *L. viciosoi* (2*n*=3x=27) reported low pollen fertility (respectively, 3-27% and 2-13%) (Erben 1978). While in balanced tetraploids such as *L. vulgare* (2*n*=4x= 36) or in the hexaploid *L. humile* (2*n*=6x=54), a high percentage of fertile pollen grains was detected (respectively, 96% and 95%) (Dawson and Ingrouille 1995). In our case, however, both balanced and unbalanced tetraploids showed male sterility.

Moreover, our results indicate for the first time that pollen size and ploidy are not correlated in the *Limonium* system, since in tetraploids most grains are smaller than in diploids. Considering that natural selection pressure on male gamete formation may be relaxed relative to female gamete formation in asexual *taxa* (Maynard-Smith 1978; Voigt *et al.* 2007), the variability in pollen formation measured here and the absence of pollen tube formation *in vitro* is consistent with degeneration of the male function, possibly due to mutation accumulation.

FCSS investigations have demonstrated the potential of this methodology to determine the routes of seed formation (Matzk *et al.* 2000). However, in both species we only find one DNA peak each time instead of two which we interpret to be the embryo peak, since mature only show one embryo and a well-developed starchy endosperm without nuclei. At earlier stage of seed development cells with nuclei might have been present. Nevertheless, it was not possible to get enough immature seeds for FCSS experiments due to their small size and the various layers of involucres in which they are enclosed (ovary, calyx, inner, medium and outer bracts). Even if by hypothesis embryo sac development would result in only one nucleus in the central cell and thus FCSS could not discriminate fertilized endosperm from embryo peak, at this point we are not able to refute this argument. In fact, nothing is known on female development for the species here analyzed. Furthermore, in previous older works of other *Limonium* species female gametophytes of *Fritillaria*-type, *Adoxa*-type and *Penea*-type have been described (Dahlgren 1916; D'Amato 1940), and also non-reduced embryo sacs of *Ixeris*-type (D'Amato 1949). Moreover, we find quantitative variation of seeds ploidy. If we attribute the single DNA peak to the embryo peak, this variation could be either due to fertilization of rare fertile pollen grains with varying ploidy. Nevertheless, after having tried to germinate *L. multiflorum* pollen grains several times, pollen tubes were never obtained. Alternatively, tetraploids could show a partial apomixis via an uncoupling of apomeiosis and parthenogenesis in the seed material. Hence, ploidy shifts could be derived from female meiosis with parthenogenesis resulting in embryos with lower ploidies (polyhaploids) and/or apomeiosis combined with fertilization revealing higher ploidies, as in the so-called B_{III}

hybrids (Matzk *et al.* 2000). Diploids from *Ranunculus kuepferi* Greuter & Burdet *taxon* resist introgression of apomixis while among polyploids only tetraploid apomicts form stable populations; the other cytotypes that arise by partial apomixis fail to establish (Cosendai and Hörandl 2010). Interestingly, even if a moderate to high germination ratio is observed in *L. ovalifolium*, none of the karyotyped seeds were triploid. Also, in *L. multiflorum* only tetraploid seeds gave rise to plantlets, probably due to cytotype disadvantages.

In the sporophytic self-incompatibility system earlier described for *Limonium* A-pollen type grains germinate on papillose stigmas and B-pollen type germinate in *cob*-like stigmas, while complementary combinations do not produce successful fertilization (Baker 1953ab; Richards 1997). In *L. ovalifolium,* meiocytes generally follow a regular course of meiosis leading to the formation of A- and B type pollen grains, likely involved in the production of seeds. Since in most populations analyzed, both pollen and stigma types are present (data not shown), cross fertilization may be favored. Nevertheless, since we identified a significant frequency of seeds with triploid embryos, they could be derived from unreduced male gametes, as most populations produce low frequencies of large grains. Alternatively, we cannot exclude that low levels of facultative apomixis could have occurred in the individuals studied. In *Limonium* triploids, *Ixeris*-type embryo sacs with non-reduced gametes are observed (D'Amato 1949). Although, the present dataset do not prove the hypothesis of *L. multiflorum* tetraploids reproduction via apomixis, they tend to suggest that high seed set formation in the male sterile individuals could be achieved through this mode of reproduction. Detailed female sporogenesis and gametogenesis and seed development studies would confirm possible diversity of reproductive modes.

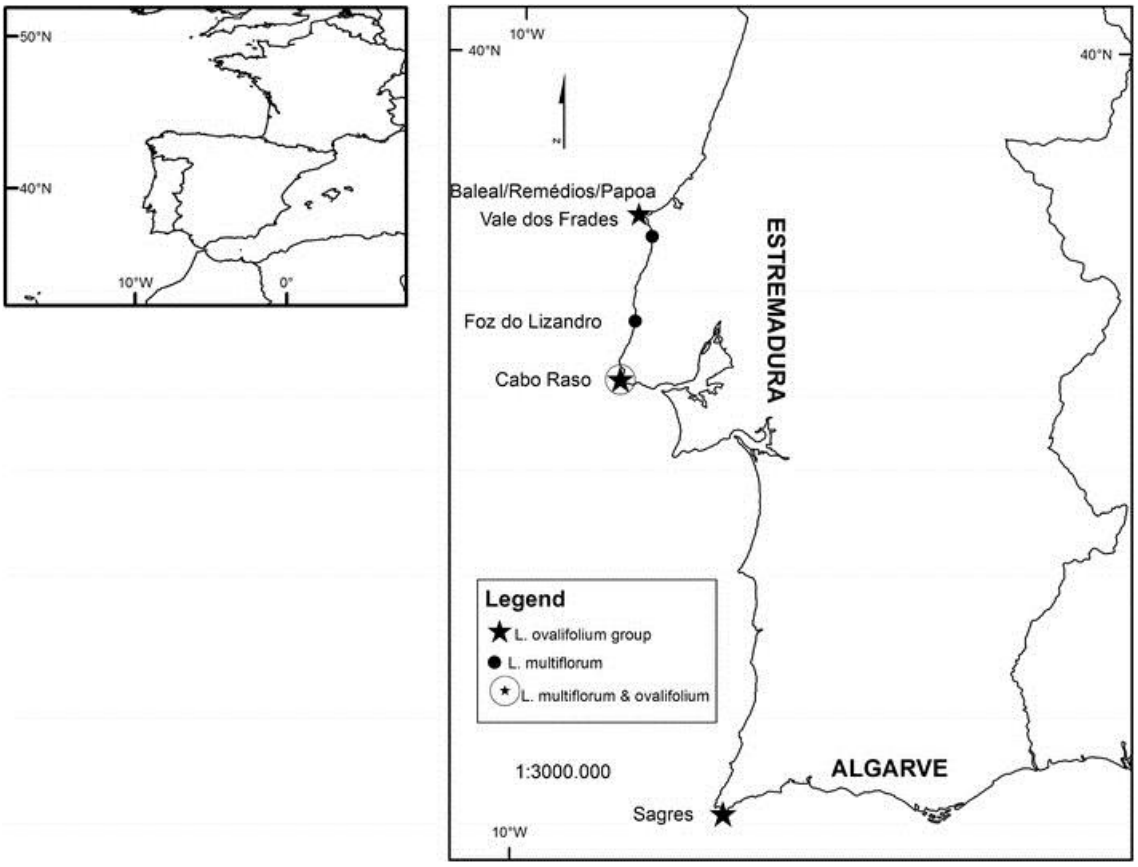

Figure 16. *Limonium ovalifolium* and *L. multiflorum* populations sampled in Continental Portugal. Populations were surveyed in Estremadura (Baleal, Papoa, Sra dos Remédios, Vale dos Frades, Foz do Lizandro, Cabo Raso) and Algarve (Sagres) provinces.

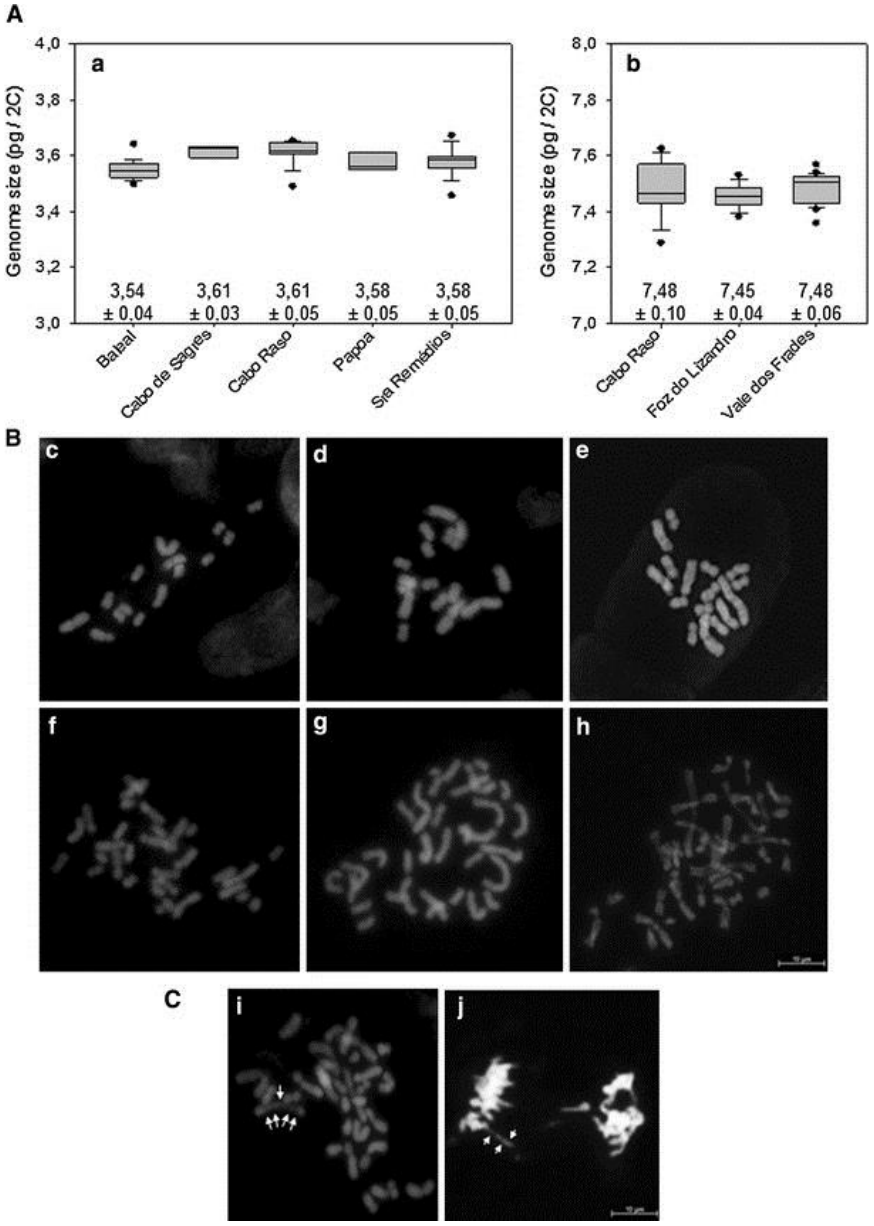

Figure 17. Ploidy levels and chromosome polymorphisms in *Limonium ovalifolium* and *L. multiflorum*. A. Boxplots showing technical replicate distribution of genome size measurements for different localities of *L. ovalifolium* (a) and *L. multiflorum* (b). For each locality three to eighteen individuals were analyzed. B. Mitotic metaphase plates of DAPI-stained chromosome spreads from seven distinct individuals of different *Limonium ovalifolium* (c-e) and *L. multiflorum* (f-h) populations c. Baleal, 2n=15; d. Cabo Raso, 2n=16; e. Sagres, 2n=16; f. Cabo Raso 2n=32; g. Vale Frades 2n=35; h. Foz do Lizandro 2n=36; C. Cabo Raso 2n=36 mitotic cells showing a large metacentric chromosome with insterstitial decondensation sites (arrowed) in metaphase (i) or anaphase (j). The fluorescent images have been digitally inverted.

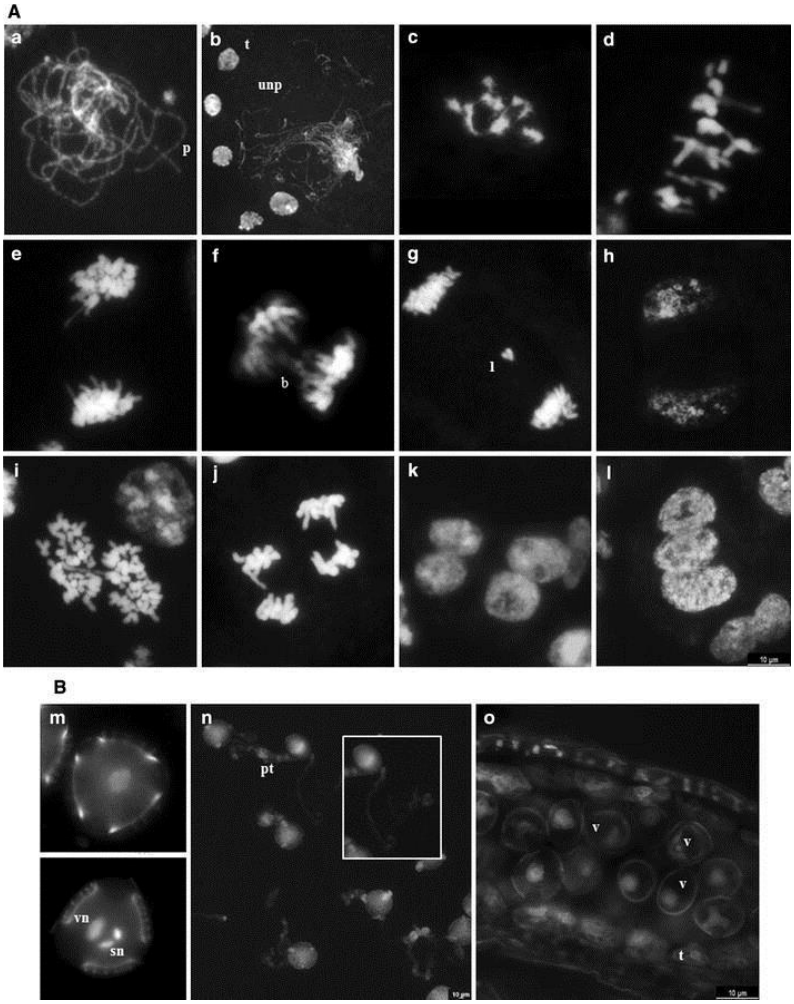

Figure 18. Chromosome pairing and segregation in DAPI-stained male sporocytes and male gametogenesis in *Limonium ovalifolium* and *L. multiflorum*. A. Full pairing (p) of chromosomes at pachytene in *L. ovalifolium* (a); Incomplete pairing (unp) of chromosomes at pachytene in *L. multiflorum*; compare meiocytes with small *tapetum* cells (t) (b).Diakinesis/ metaphase I transition in *L. ovalifolium* showing bivalents (c); Metaphase I in *L. multiflorum* with univalents and bivalents (d); e-g. *L. multiflorum* anaphase I meiocytes showing balanced segregation (e), (f) chromosome bridges (b) and lagged chromosomes (l) (g); h. Balanced telophase I in *L. multiflorum*; i. *L. multiflorum* meiocytes in metaphase/anaphase transition during second meiotic division; j. *L. ovalifolium* meiocytes in anaphase II; k *L. ovalifolium* normal tetrad; l. *L.multiflorum* triad; B. *L. ovalifolium* unicellular pollen (m, upper image) and tricellular pollen grain with a vegetative nucleus (vn) and two sperm nuclei (sn) (m; lower image); n. *L. ovalifolium* pollen tubes (pt) germinated *in vitro*; the inset exhibits a detailed pollen tube; o. Longitudinal section of a non-dehisced anther from *L. multiflorum* showing unicellular microspores exhibiting a large vacuole (v) causing the characteristic "signet-ring" appearance; the *tapetum* cells are in the periphery of the anther (t). The fluorescent images have been digitally inverted.

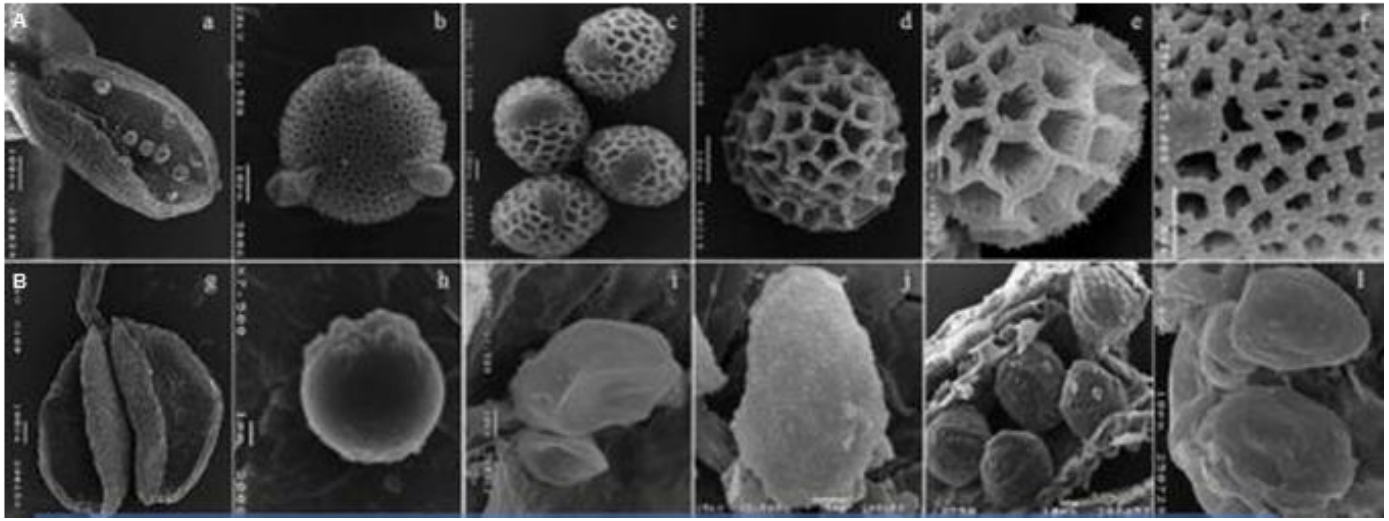

Figure 19. Scanning electron microscopy photographs of *Limonium ovalifolium* and *L. multiflorum* pollen grains. *Limonium ovalifolium* (A: a-f) a. Dehisced anther with a few pollen grains still inside; b. Spheroid tricolpate pollen grain with microreticulate exine surface (polar view); c. Ellipsoid grains with macroreticulate exine surface (diverse equatorial views); d. Pollen grain with coarsely reticulate exine surface with a large *reticulum* (polar view); d. Detail of the irregular macroreticulate exine net with spinose side walls; f. Detail of the *reticulum* with strait spaces in microreticulate exine surface; g. Dehisced anther without pollen grains; h. Radiosymetric pollen grain with psilate exine ornamentation; i. Bilateral pollen grains with distinct dimensions; j. Bilateral pollen grains showing perforate exine surface with no prominent spines; k. Pollen grains with verrucate or warty exine surface with *colpi* position denounced; l. Pollen grain with perforate exine surface and with *colpi* position denounced.

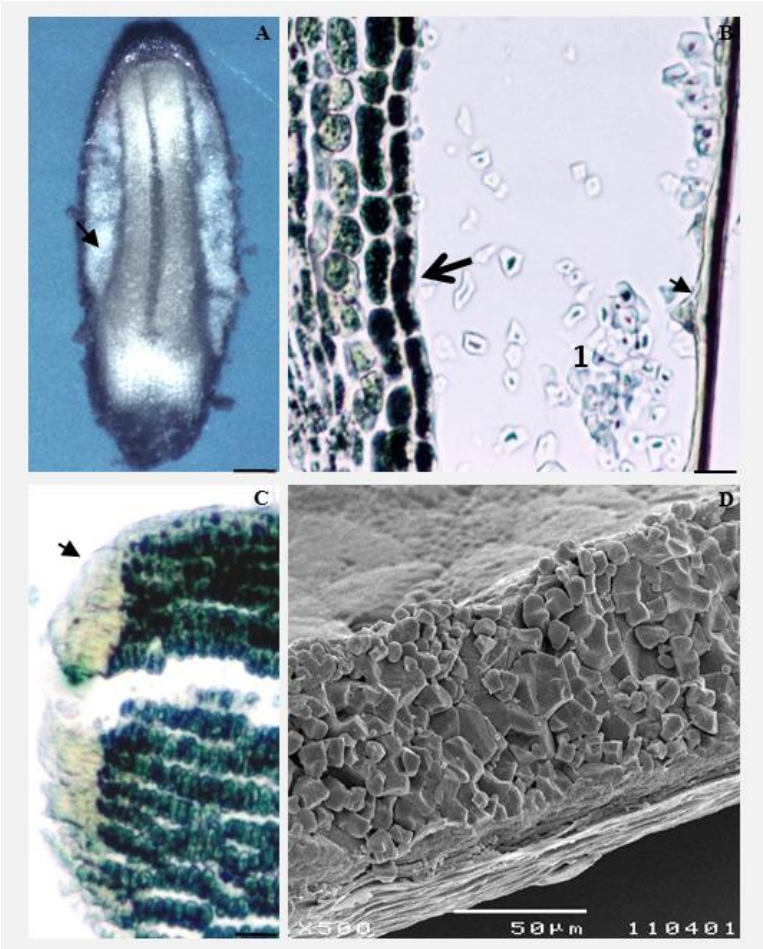

Figure 20. Structure and composition of *Limonium ovalifolium* and *L. multiflorum* of mature seeds. a-b. *L. multiflorum*, LM micrographs. A. View of a seed embedded in a paraffin block (scale bar 2 µm). B. Detail of a longitudinal section, embryo tissue (large arrow) enveloped by a starchy endosperm (1) and a single aleurone layer (small arrow) covered by the seed coat is observed (scale bar 20 µm); C. *L. ovalifolium*, LM micrograph showing the embryo tissue with a basal transfer layer (arrow) (scale bar 30 µm). D. *L. ovalifolium*, SEM micrograph demonstrating a starchy endosperm with polygonal grains.

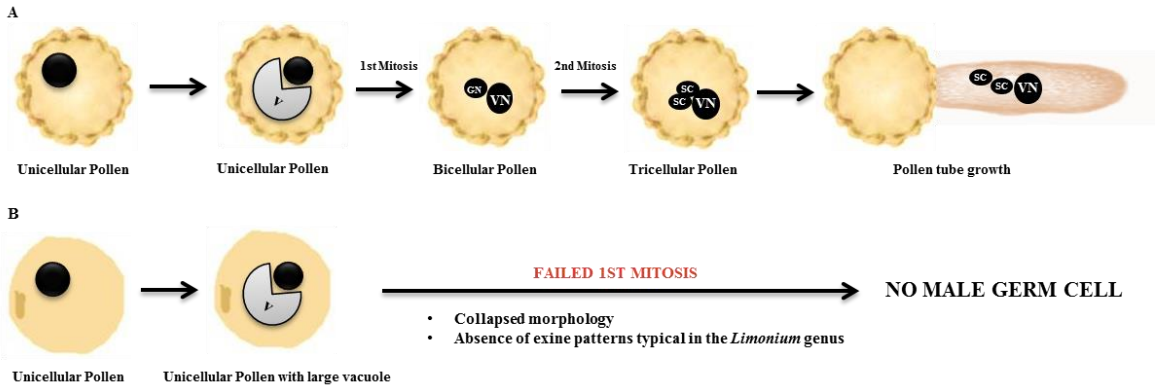

Figure 21. Diagrams for explaining pollen grain development in *L. ovalifolium* and *L. multiflorum*. A. In *L. ovalifolium* pollen grains follow a normal, first asymmetric mitotic division producing a generative cell (GC) within the vegetative pollen grain cell (VN) in the binucleate pollen stage. The second pollen division occurs before anther dehiscence and pollen germination originates tricellular pollen with two sperm cells (SCs). At this stage microspores are surrounded by the differentiated exine characteristic of the *Limonium* genus. When pollen germination occurs, the pollen tube growth is achieved. B. In *L. multiflorum* unicellular pollen never undergoes first mitosis. Even after anther dehiscence, released pollen only attains the "ring-vacuolate" microspore with a large vacuole causing the characteristic "signet-ring" appearance (following the system of Owen and Makaroff, 1995). The male germ unit is not produced. Key: VN – vegetative nucleus; GN - generative nucleus; SC – sperm cells; V – vacuole.

Table 7. *Limonium ovalifolium* and *L. multiflorum* pollen grain dimensions (µm). Classes were defined according to Erdtman (1952).

Species	Population	Mean	stdev*	% Grains in Dimension Classes**				Number of grains analyzed
				≤10	10>x≤24	25>x≤49	50>x≤100	
L. ovalifolium	Baleal	40.43	3.01	0	0	100	0	35
	Cabo Raso	46.79	6.18	0	0	86	14	44
	Cabo Sagres	42.69	5.29	0	0	92	8	71
	Papoa	45.14	3.51	0	0	95	5	39
	Sra. Remédios	45.65	3.14	0	0	92	8	36
L. multiflorum	Cabo Raso	30.6	11.2	8	16	73	3	38
	Foz do Lizandro	21.77	8.39	0	27	73	0	30
	Vale dos Frades	29.34	4.28	0	11	89	0	36

* stdev Standard deviation
** Dimension Classes (µm) according to Erdtman (1952): very small (x ≤ 10); small (10 > x ≤24); medium (25 > x ≤49); large (50 > x ≤100)

Table 8. Summary of flow cytometric data with the observed ploidy levels in *L. ovalifolium* and *L. multiflorum* seeds.

Species	Population	% of seeds C values							Number of individuals analyzed
		2C	3C	4C	5C	6C	7C	8C	
L. ovalifolium	Baleal	23	65	12	-	-	-	-	14
	Cabo Raso	78	22	-	-	-	-	-	12
	Cabo Sagres	79	20	-	-	-	-	-	16
	Lagos	-	-	-	-	-	-	-	0
	Papoa	-	-	-	-	-	-	-	0
	Sra. Remédios	54	44	2	-	-	-	-	14
L. multiflorum	Cabo Raso	6	50	30	-	6	7	1	13
	Foz do Lizandro	15	15	29	4	17	16	4	18
	Vale dos Frades	28	43	11	4	13	-	-	7

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CHAPTER V.

EPIGENETIC RATHER THAN GENETIC FACTORS MAY EXPLAIN PHENOTYPIC DIVERGENCE BETWEEN DIPLOID AND TETRAPLOID *LIMONIUM*

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1. Abstract

The genus *Limonium* Mill. comprises annual and perennial halophytes that can produce sexual and/or asexual seeds. In this chapter, genetic and epigenetic (DNA methylation) variation patterns were investigated in populations of three phenotypically similar putative sexual diploid species (*L. nydeggeri, L. ovalifolium*, *L. lanceolatum*), one sexual tetraploid species (*L. vulgare*) and two apomict tetraploid species thought to be related (*L. dodartii, L. multiflorum*). The extent of morphological differentiation between these species was assessed using ten diagnostic morphometric characters. A discriminant analysis using the morphometric variables reliably assign individuals into their respective species groups. We found that only modest genetic and epigenetic differentiation was revealed between species by Methylation Sensitive Amplification Polymorphism (MSAP). However, whilst there was little separation possible between ploidy levels on the basis of genetic profiles, there was clear and pronounced interploidy discrimination on the basis of epigenetic profiles. We infer that epigenetic variation probably drives the observed phenotypic divergence between diploid and tetraploid *taxa* and discuss that intergenome silencing offers the most plausible mechanistic explanation for the observed phenotypic divergence between these microspecies. We suggest epigenetic profiling offer an additional tool to infer ploidy level in herbarium or stored specimens and that stable epigenetic change may play an important role in apomict evolution and species recognition.

Keywords: Epigenetic variation, genetic variation, *Limonium*, MSAPs, ploidy diagnosis, polyploidy

2. Introduction

DNA sequence divergence clearly plays a leading role in shaping the phenotypic variation observed between most *taxa* e.g. (Whitham *et al.* 2006; Johnson and Stinchcombe 2007) but does not explain all forms of adaptive phenotypic differentiation (Cubas *et al.* 1999; Manning *et al.* 2006; Tricker *et al.* 2012). Epigenetic modifications of DNA and histones, the core components of

chromatin are able to influence the expression of the underlying genes and so phenotype (Law and Jacobsen 2010). Of these epigenetic mechanisms, the best studied and the one to show more examples of transgenerational stability is cytosine DNA methylation (Law and Jacobsen 2010; Margueron and Reinberg 2010). Evidence has also been found suggesting that heritable phenotypic variation observed in natural populations can be due to stable epigenetic variation, even in the absence of genetic variation and could play a role in plant adaptation and evolution (Tricker *et al.* 2012; Rapp and Wendel 2005; Bossdorf *et al.* 2008; Richards 2011). The scope for epigenetic mechanisms driving morphological differentiation is perhaps best illustrated when genetic variation is lacking. Understanding the relative importance of genetic and epigenetic sources of phenotypic variation where both systems are operating is therefore attracting increasing interest. For example, *Laguncularia racemosa* (L.) C.F. Gaertn. is a mangrove plant species that shows low genetic variability but in populations from distinct, nearby habitats, cytosine methylation variation among individuals correlates more closely with environmental variation than does genetic variation (Lira-Medeiros *et al.* 2010). In the perennial species *Viola cazorlensis* Gand., cytosine methylation patterns were found to be partitioned and positively correlated with adaptive genetic variation (Herrera and Bazaga 2010). Also, in populations of individuals with reduced or negligible genetic variation such as those of triploid asexual dandelion lineages (apomixis; diplospory), changes in genomic methylation patterns are found between individuals (Verhoeven *et al.* 2010).

The genus *Limonium* Mill. (sea-lavenders; *Plumbaginaceae*) has long been recognized to have a history of recurrent hybridization and polyploidization, and comprises 150 (Erben 1978) to 350 *taxa* recognized across coastal, steppe and desert regions (e.g. Erben 1993; Kubitzki 1993). This wide range is due to the description of new *taxa*, mainly microspecies from geographically restricted areas. In this genus, a sporophytic self-incompatibility system is linked with pollen-stigma dimorphisms, A-pollen type grains germinate on papillose stigmas and B-pollen type germinate in *cob*-like stigmas, while the complementary combinations produce no successful fertilization (Baker 1953ab). Most sexual species of *Limonium* usually have a dimorphic self-

incompatibility system (both pollen and stigmas are dimorphic) while agamospermous species are generally monomorphic and have monomorphic populations (Erben 1978; Baker 1966). Determination of these characters in individuals from natural and/or experimental populations has since long been used as an indirect method for estimation of each species reproduction mode (Erben 1978; Baker 1953ab). The high number of polyploid *taxa* has been explained to be a consequence of this self-incompatibility system and the ability of polyploid hybrids to produce seeds asexually via apomixis (agamospermy; asexual seed formation) (Erben 1978; Baker 1953ab, 1966; D'Amato 1949). *Ixeris*-type embryo sacs with non-haploid eggs are found in triploid (2*n*=3x=27) *Statice oleaefolia* var. *confusa* (D'Amato 1949). In triploid and tetraploid agamospermous species of the *L. binervosum* group diplospory followed by parthenogenesis is reported (Hjelmqvist H and Grazi E 1964; Ingrouille 1982). Molecular phylogenetic studies have tried to resolve the taxonomic complexity within this genus in a global perspective using nuclear DNA sequence information (Palacios *et al.* 2000) and plastid DNA (Lledó *et al.* 1998, 2001, 2005)

In Continental Portugal about 15 *Limonium* species have been recognized with ecological importance for plant communities of the Atlantic and Mediterranean coastlines (Erben 1993; Espírito-Santo *et al.* 2012). Among these, the *L. ovalifolium* complex consists a group of three sexual diploids (2*n*=2x=16): *L. ovalifolium*, *L. nydeggeri* and *L. lanceolatum* (Erben 1999). The first species appears has a broader distribution including several Sites of Community Importance (SCI) for the Mediterranean biogeographical regions (CEC 2006) in the west (Estremadura), southwest Alentejo and Algarve coastlines (Franco 1984; Erben 1993). Conversely, the Lusitania endemic *L. nydeggeri* and *L. lanceolatum* have more restricted distributions; the former is restricted to west and southwest Atlantic sea-cliffs whereas the latter is found in the southwest and south coastlines (Franco 1984; Erben 1999). *Limoniun* tetraploid *taxa* include among others, the Lusitania endemic apomict, *L. multiflorum* Erben (Erben 1978, 1993) which exhibits both tetraploid and aneuploid tetraploid cytotypes (2*n*=4x=35 (Erben 1978); 2*n*=4x=32, 34, 35, 36 (Rois *et al.* 2012) and the aneuploid tetraploid apomict *L. dodartii* (2*n*=4x=35)

which most frequently grow on maritime cliffs in the province of Estremadura. A third tetraploid, *L. vulgare* (2*n*=4x=36), a sexual species (Baker 1948, 1966; Dawson and Ingrouille 1995), grows in salt-marshes (Franco 1984; Erben 1993).

Dominant genetic markers, such as those generated by Amplified Fragment Length Polymorphism (AFLP), are valuable for assessing genetic diversity within and between populations (Kremer *et al.* 2005) and for inferring *taxon* differentiation (Garcia-Verdugo *et al.* 2010), especially in species for which codominant markers are unavailable. Some recent publications have added data on natural epigenetic variation in animal and plant species by sampling cytosine methylation using Methylation-Sensitive Amplification Polymorphism (MSAP) technology (Reyna-Lopez *et al.* 1997; Morán and Pérez-Figueroa 2011; Paun and Schönswetter 2012), a modification to the original AFLP protocol that compares product profiles generated by methylation-sensitive/insensitive isoschizomers. Central to the technique is the differential behavior of the two isoschizomer restriction enzymes (*Hpa*II and *Msp*I) in the presence of cytosine methylation in the CCGG context. *Hpa*II is inactive if one or both cytosines are methylated at both DNA strands, but cleaves when one or both cytosines are methylated in only one strand. *Msp*I, by contrast, cleaves C5mCGG but not 5mCCGG. Comparison of the profiles generated by each enzyme from each individual allows the assessment of the methylation state of the restriction sites and so provides a relative comparison of genetic and epigenetic variability. Several reports suggest that only methylation marks in the CG context can be transmitted between generations and so have potential for stable adaptive significance (Mathieu *et al.* 2007), furthermore, recent data shows that although non-CG methylation can be inherited, only inherited CG methylation is inversely correlated with gene expression (Tricker *et al.* 2013). Since only *Hpa*II is affected by methylation of this kind, for simplicity, in this study we refer to profiles from this enzyme as epigenetic (meaning potentially transgenerationally stable and epigenetic) whereas *Msp*I is insensitive in this sense and so can only detect transgenerationally relevant genetic variation. The focus of this study was to therefore to use MSAP analysis as the primary tool in comparing the extent to which genetic and epigenetic diversity in natural

populations of diploid and tetraploid *Limonium* species correlate with species identity and ploidy.

3. Materials and methods

3.1. Study species

Natural populations of the three diploid species from the *L. ovalifolium* complex (*L. ovalifolium*, *L. nydeggeri*, *L. lanceolatum*) (Franco 1984; Erben 1999) and three tetraploids species (*L. dodartii*, *L. multiflorum*, *L. vulgare*) (Erben 1978, 1993) were surveyed in the three Portuguese provinces of Estremadura (west), Alentejo (south-west) and Algarve (south). With the exception of *L. lanceolatum* and *L. vulgare* (which grow in salt-marshes). Google Earth 6.0.2 was used for georeferencing and to estimate geographic distances between populations. Geographic mapping of the populations was performed using ArcGIS Desktop 10 (ESRI).

Three leaves per individual were sampled from approximately ten individuals for each population, with a total of 125 plants included in this study (Table 9). Three leaves from plants at the same phenological stage were sampled from all sites during 2010 and kept on silica gel. In this way, variation in DNA methylation profiles attributable to developmental or storage conditions differences was minimized.

3.2. Morphometric analysis

Morphometric analyses were performed in approximately twenty herbarium specimens from each species deposited in the herbaria João de Carvalho e Vasconcellos (LISI; ISA), Portugal. These specimens were previously collected in the same populations selected for MSAP analysis and identified on the basis of species descriptions, diagnostic keys, and locations already described (Erben 1978), and by comparison with other herbarium specimens present in Portuguese herbaria. The following diagnostic characters were measured: maximum spike length (MSL), maximum number of spikelets per cm (MNSC), maximum number of florets per spikelet (MNFS), maximum outer bract length (MOBL), maximum outer bract width (MOBW), maximum middle bract length (MMBL), maximum middle bract width (MMBW), maximum

inner bract length (MIBL), maximum inner bract width (MIBW), and maximum calyx length (MCL). All traits were measured in the lab, after removal of flower parts of each individual. Statistical evaluations were performed with the program SPSS 20 (IBM SPSS, 2010) for Windows. The morphometric variables were tested for deviations from a normal distribution using a Kolmogorov-Smirnov test, and then these variables were log transformed. CDA were conducted to give indication of the degree to which the species were distinguishable from each other and to determine which characters contributed to this discrimination. The box-plots showing the medians and interquartile ranges were produced for each significant character for each species.

3.3. DNA isolation

Three replicate DNA extractions from leaves of each sample were performed from *c*. 0.05 g silica gel dried leaf material using the using the DNeasy 96 Plant Kit (Qiagen, UK) and the Mixer Mill MM 300 (Retsch, Haan, Germany) according to the manufacturers' instructions. DNA quality and quantity were verified using the nanodrop 2000 spectrophotometer (ThermoScientific, Wilmington, USA). Isolated DNA was diluted in nanopure water to produce working stocks of approximately 10 ng μl^{-1}.

3.4. Genetic/Epigenetic analyses - MSAP procedure

We used a modification of the MSAP protocol to reveal global variability in CG methylation patterns between samples of the different specimens studied (Reyna-Lopez *et al.* 1997). For each individual, 50 ng of DNA was first digested and ligated using 5U of EcoRI and 1U of *Msp*I or *Hpa*II (New England Biolabs), 0.45 µM EcoRI adaptor, 4.5 µM *Hpa*II adaptor and 1U of T4 DNA ligase (Sigma) in 11 µL total volume of 1X T4 DNA ligase buffer (Sigma), 1 µL of 0.5 M NaCl, supplemented with 0.5 µL at 1 mg/ml of BSA for 2 h at 37°C. The enzymes were then inactivated by heating to 75°C for 15 min. Following restriction and adaptor ligation, there followed two successive rounds of PCR amplification. For pre-selective amplification, 0.3 µL of the restriction/ligation products described above were incubated in 12.5 µL volumes containing 1X Biomix (Bioline, London, UK) with 0.05 µL of PreampEcoRI primer and 0.25 µL

Preamp*Hpa*II/*Msp*I (both primers at 10 lM) supplemented with 0.1 µL at 1 mg/ml of BSA. PCR conditions were 2 min at 72°C followed by 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 2 min with a final extension step of 10 min at 72°C. Selective PCRs were then performed using 0.3 µL of pre-selective PCR products and the same reagents as deployed for the pre-selective reactions but using FAM labeled selective primers Cycling conditions for selective PCR were as follows: 94°C for 2 min, 13 cycles of 94°C for 30 s, 65°C (decreasing by 0.7°C each cycle) for 30 s, and 72°C for 2 min, followed by 24 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, ending with 72°C for 10 min. Initially, eight selective primer combinations were evaluated for their ability to detect of inter-specific variation and to generate informative and consistent MSAP profiles using two replicated samples from six different populations (data not shown). Two primer combinations (E1/H1 and E1/H3) were chosen for the comparative selective amplification.

Fluorescently labelled MSAP products were diluted 1:10 in nanopure sterile water and 1 µL was combined with 1 µL of ROX/HiDi mix (50 µL ROX plus 1 ml of HiDi formamide, Applied Biosystems, USA). Samples were heat-denatured at 95°C for 3–5 min and snap-cooled on ice for 2 min. Samples were fractionated on an ABI PRISM 3100 at 3 kV for 22 s and at 15 kV for 45 min.

3.5. Data analyses

MSAP profiles were analysed using Genemapper 4.0 software (Applera Corporation, Norwalk, Connecticut, USA). For analysis of the genetic/epigenetic variability between samples revealed using MSAP, reproducible product peaks were scored as present (1) or absent (0) to form a raw data matrix. In order to minimize the occurrence of fragment size homoplasy (Vekemans *et al.* 2002) only fragments with lengths between 100 and 500 bp were considered for the analysis. All monomorphic fragments and any fragments present/absent in all but one individual were considered uninformative and removed from all data sets (Bonin *et al.* 2004). Reproducibility was estimated calculating the proportion of dimorphic markers between the replicates of the selected samples (Bonin *et al.* 2004). For biological error rates, we compared paired MSAP profiles from two leaves of one plant of six populations using each primer

combination. Technical reproducibility of the MSAP technique was assessed through the direct comparison of profiles derived from single DNA extractions from ten representative genotypes.

3.6. Analysis of Genetic/Epigenetic Variance

Genetic and epigenetic similarity between tested samples was determined by PCoA (Gower 1966) based on the MSAP profiles obtained from primer combinations E1/H1 and E1/H3 using GenAlex (v.6.4). Different components of variability were obtained using GenAlex (6.4) software by grouping the samples in two different levels (Populations (i.e. a group of samples) and Regions (i.e. a group of populations). To calculate distances between natural populations, samples from the same natural population restricted with each enzyme were grouped by Populations (this generated two populations from each natural population, one restricted with *Msp*I and one with *Hpa*II) and then at higher level they were grouped into two Regions (all samples restricted with each enzyme). To calculate distances between species, samples from the same *taxa* restricted with each enzyme were considered and grouped as one Population (this generated two populations from each original species, one restricted with *Msp*I and one with *Hpa*II) and then at higher level they were grouped into two Regions (all samples restricted with each enzyme). We then used AMOVA (Excoffier *et al.* 1992) to evaluate the structure and degree of epigenetic diversity among and between populations, and between species. Pairwise PhiPT (Excoffier *et al.* 1992) comparisons (an analogue of the Fst fixation index, that measures differential connectivity/genetic diversity among populations) between samples restricted with *Msp*I or *Hpa*II was used to infer their overall level of genetic or epigenetic divergence respectively. AMOVA was subsequently calculated using GenAlex (v.6.4) to test the significance of PhiPT between populations and species (Michalakis and Excoffier 1966) with the probability of non-differentiation (PhiPT = 0) being estimated over 9,999 permutations. We then calculated genetic diversity estimates (expected heterozygosity, *Hj*) and the actual genetic diversity for each of the groups above, by using Shannon's index (ShI) (Abbott *et al.* 2007). Finally, pairwise genetic or epigenetic and geographical distance (kilometres) matrices were

analysed between populations within species and among species by means of a Mantel test. The level of significance was assigned after 1000 permutation tests, as implemented in Genalex 6 (Peakall and Smouse 2006).

4. Results

4.1. Morphological differentiation between diploid and tetraploid species

Herbarium specimens of the diploid species *L. ovalifolium*, *L. nydeggeri* and *L. lanceolatum* and of the tetraploid species *L. dodartii*, *L. multiflorum* and *L. vulgare* obtained from individuals sampled in natural populations were used for morphometric measurements. Ten diagnostic characters were selected based on an exhaustive review of *Limonium* species in southwest Europe by Erben (1978, 1993), and on previous biometric studies in the *Limonium* genus (Ingrouille 1984; Ingrouille and Stace 1985; Dawson and Ingrouille 1995).

Only one of the ten morphological variables measured from representatives of the six species fitted a normal distribution, Maximum inner bract length (MIBL), whilst the other nine failed to do so, even after a logarithmic transformation. The remaining analyses were therefore performed using the original (untransformed) values. Canonical discriminant analysis (CDA) of the morphological variables accounted for most of the variation (82.2% in the first two dimensions, comprising 61.9 and 20.3%, respectively) and correctly assigned individuals to species in 92.7% of cases (n=110; Figure 22; Tables 10 and 11). However, a small number of intermediate or ambivalent specimens were encountered (Wilks' lambda = 0.002, $\chi^2 = 650.259 > \chi^2_{0.05;50} = 67.505$, $P < 0.001$). The first axis distinguished diploid from tetraploid species through the following characters: Maximum outer bract length (MOBL), Maximum calyx length (MCL), Maximum middle bract length (MMBL) and MIBL (Table 12; Figure 23). The second axis separated *L. nydeggeri* from the other diploid species and *L. multiflorum* from the other tetraploid species by the following characters: Maximum middle bract width (MMBW), Maximum outer bract width (MOBW) and Maximum inner bract width (MIBW). Thus, these features were largely responsible for separation of species sharing the same ploidy level.

4.2. MSAP profiles analysis

Genetic-epigenetic analyses were performed on 125 individuals selected from natural populations within designated SCI(s) (Table 9). The two MSAP primer combinations applied to all samples yielded 835 scorable fragments comprising 792 from *Msp*I and 778 from *Hpa*II, 92.78% and 95.36% respectively, were polymorphic (i.e. not present in all the analysed samples/replicates when restricted with one of the isoschizomers). Overall reproducibility between biological replicates was 83% and 85% for primer combinations E1/H1 and E1/H3 respectively. The methylation insensitive (genetic variation) profiles showed only very slightly higher concordance among replicates (83.6% and 85.7%) than did the methylation-sensitive (epigenetic variation) profiles (82.4% and 84.3%). Technical reproducibility of the MSAP technique revealed between 92-95% band concordances (data not shown), indicating that the higher variability between independent DNA extractions probably arises from variation between tissues and cell mixtures used in the DNA extraction. Profiles from the tetraploid species (i.e. *L. multiflorum, L. dodartii, L. vulgare*) included a higher number of *Msp*I fragments (genetic profiles) per individual for both primer combinations (Table 13). Conversely, these species contained a lower number of *Hpa*II-generated (methylation-sensitive epigenetic profiles) and fewer fragments per individual than the three diploid species (i.e. *L. nydeggeri, L. ovalifolium, L. lanceolatum*), implying a higher level of genome-wide methylation among tetraploids.

4.3. Genetic/epigenetic divergence of diploid and tetraploid species

Principal Coordinate Euclidean Analysis (PCoA) was used to provide an overview of the genetic/epigenetic variability and structure of the studied *taxa*. Overall, epigenetic profiles (*Hpa*II) provided imperfect but slightly better separation of the *taxa* than did the genetic profiles (*Msp*I) (Figure 24A). Genetic data (*Msp*I) alone also largely failed to discriminate within the diploid species and the tetraploid species (Figure 24B). More remarkably, PCoA plots of the epigenetic *Hpa*II profiles revealed clear separation between the diploid and tetraploid *taxa* (Figure 24C). On average, genetic and epigenetic distances

between the three diploid species were significantly lower than comparable distances between the tetraploid species (T-test, Genetic distance $p < 0.023$; epigenetic distance $p < 0.0006$ (two-tailed test); Table 14). Furthermore, calculated epigenetic distances between individuals from different diploid species and between tetraploid species were higher than genetic distances between the same pairings (T-test, $p < 0.04$ (between diploid species); $p < 0.15$ (between tetraploid species) (two-tailed test); Table 14). Analysis of genetic/epigenetic variability using Analysis of Molecular Variance (AMOVA) showed that 24% of the total observed variability can be attributed to differences between epigenetic (*Hpa*II) and genetic (*Msp*I) sources of variability (Figure 24A). Independent analysis of results generated by each enzyme type revealed that differences between diploid and tetraploid samples accounted for 6-8% of the genetic variability and for 3-6% of the epigenetic variability. This compared with a much higher level of within-species variation, which comprised 79-83% and 74-78% of the genetic and epigenetic variability respectively (Figure 24B-C). Surprisingly, while genetic differences between species accounted for 9-15%, epigenetic differences accounted for 19-20%, suggesting that characterisation of these very closely related species is best served by considering both genetic and epigenetic information rather than genetic information only (Table 15).

4.4. Tetraploid species compensate lower genetic variability with higher epigenetic variability

Closer consideration of the genetic and epigenetic variability revealed that all species had more epigenetic than genetic variability. And, the diploid species were more variable (both at genetic and epigenetic level) than were the tetraploid ones (Table 15). However, the difference between genetic and epigenetic variability was higher among the tetraploid species despite containing lower levels of variability overall (Table 15). PCoA of the genetic profiles revealed no or only weak co-clustering of individuals according to population origin, with the best example being seen among *L. nydeggeri* samples (Figure 25 A-B). No co-clustering was evident from the epigenetic profiles (data not shown). A lack of structuring according to population origin

was further supported by AMOVA, with the main component of both genetic (90-97%) and epigenetic variance (86-95%) residing within populations.

We next sought structuring across a geographic scale. Mantel test analysis revealed a correlation between genetic distances and geographic separation among conspecific populations for *L. nydeggeri* (R^2 = 0.784, P < 0.03). This correlation was significant for both primer pairs (H1/E1, R^2 = 0.858, P < 0.02; H1/E3, R^2 = 0.616, P < 0.04) (Figure 26 A-B). Both primers generated a scatterplot showing a positive and monotonic relationship over all geographic distances of separation. In contrast, *L. multiflorum* populations showed no detectable relationship between genetic distance and geographic distance and large variance in estimates of divergence. None of the studied species showed a significant correlation between epigenetic and geographic distances, with extensive scatter between the plotted samples (data not shown).

Finally, Mantel test analysis revealed a strong significant positive correlation between genetic and epigenetic distances among *L. nydeggeri* populations (H1/E1, R^2 = 0.800, P < 0.008; H1/E3, R2 = 0.200, P < 0.07 and R^2 = 0.600, P < 0.005, using the information provided by both primer combinations) (Figure 26 C). Conversely, there was only a weak negative correlation between genetic and epigenetic distances among *L. multiflorum* populations (data not shown).

5. Discussion

The effects of hybridization, polyploidy and apomixis have all combined to shape radiation currently seen in *Limonium* species (Cowan *et al.* 1998; Lledó *et al.* 2005; Rois *et al.* 2012). This evolutionary model has been used to explain multiple series of complex aggregates of sexual diploid species and asexual polyploid hybrids which are perpetuated through gametophytic apomixis (Asker 1992; Carman 1997, 2001, 2007; Paun *et al.* 2006a). In other plant groups, hybridization and polyploidy have combined to generate genetic and phenotypic complexity in the form of classical polyploidy pillar complexes. In these situations species delimitations typically become blurred at the higher ploidy levels and interploidy discrimination can also become difficult for some *taxa*. The many examples of this type of species complex include the *Festuca*

ovina aggregate (Wilkinson and Stace 1991), *Dactylis glomerata* (Bretagnolle and Thompson 2001) and *Knautia arvensis* species complexes (Kolar *et al.* 2012).

The additional and intermittent appearance of facultative or obligatory apomixis, as seen in triploid and tetraploid *Limonium* species (D'Amato 1949; Hjelmqvist H and Grazi E 1964), adds another layer of complexity for species delimitation and diagnosis. In these instances the phenotypic range of *taxa* can be highly variable, as can their morphological distinctiveness and stability (e.g. Nylehn *et al.* 2003). The cumulative effect of these processes is typically manifest in the recognition of a large number of microspecies, with the diagnosis and genetic characterisation of many of the resultant *taxa* often being highly demanding (e.g. *Limonium*, reviewed in (Lledó *et al.* 2005). The publication of a comprehensive revision of *Limonium* species in southwest Europe by Erben (1978) in which 59 species (13 new to science) were fully described allowed for several taxometric studies of geographically related species in this genus to be conducted. For example, various authors used a taxometric approach to study several sexual and agamospermous species of the *L. binervosum* from western Europe (Ingrouille and Stace 1986), and also to examine the closely related *L. vulgare* and *L. humile* species in the British Isles (Dawson and Ingrouille 1995). Morphometric studies have also been used at population level. For instance, some authors studied the taxometric relationships between triploid or aneuploid tetraploid *L. binervosum* agamospermous colonies in the British Isles (Ingrouille and Stace 1985; Dawson and Ingrouille 1995). A similar strategy is applied to describe morphological differentiation patterns between individuals of a *L. dufourii* population from eastern Spain (Rodríguez *et al.* 2003).

In the current study, CDA was applied using ten morphometric traits collected from representative individuals of three diploid and three tetraploid *Limonium* species from Portugal. As previous studies had suggested (Erben 1978; Ingrouille 1984; Ingrouille and Stace 1985; Dawson and Ingrouille 1995), the collective use of these characters in CDA was sufficient to provide clear morphological differentiation between species at each ploidy level. This differentiation is based primarily on the use of seven morphometric variables,

viz: MOBL, MCL, MMBL and MIBL for the first axis and MMBW, MOBW and MIBW on the second. Overall, these analyses not only provided clear separation of all diploid species but also indicated that *L. lanceolatum* and *L. ovalifolium* share a closer phenotypic affinity. Separation of the tetraploid species was also possible but with intermediate individuals blurring the boundaries between *L. vulgare* and *L. dodartii*, and between the latter and *L. multiflorum*. The rather surprising finding, however, lay in the clear phenotypic separation of tetraploid species from the diploids on the basis of the bract and calyx characteristics MCL, MOBL, MMBL and MIBL rather than plant size features more usually associated with ploidy level changes (e.g. see te Beest *et al.* 2012). The relative importance of genetic and epigenetic processes in shaping the observed phenotype structuring among species and ploidy levels in *Limonium* has thus far remained elusive.

Previous works on the analysis of genetic variation and population genetic structure in other *Limonium* species have deployed a wide range of molecular marker systems to infer the importance of genetic structuring in defining interspecies delimitation. There has, nevertheless, been considerable evidence that modest but significant levels of genetic variation does occur within species in the genus. For example, studies on the presumed agamospermous triploid *L. dufourii* have invariably revealed low but substantial inter-individual genetic variation in habitats with significant fragmentation and low population sizes (Rodríguez *et al.* 2003; Palacios *et al.* 1997, 1999; Palop-Esteban *et al.* 2007). Similarly, in diploid *L. dendroides* from Canary Islands, despite radical habitat fragmentation and small population size, some subpopulations of have enough genetic variation to compensate for the influence of drift (Suárez-García *et al.* 2009). However, in plant populations with low genetic variability, epigenetic variation can also act as an important source of potentially adaptive phenotypic variability (Lira-Medeiros *et al.* 2010; Herrera and Bazaga 2010; Verhoeven *et al.* 2010). Nevertheless, the extent and importance of epigenetic variation in natural populations of sexual and agamospermous *Limonium* species is still largely unexplored (Kalisz *et al.* 2004; Bossdorf *et al.* 2008; Richards 2011).

Data generated in the current study unsurprisingly reveals that tetraploids have a higher level of methylation than diploids. In part this may be accounted for the increased genome size, with scope for intergenome heterozysity for methylation marks adding to that expected between homologous loci. There are also additional indirect processes that can contribute to an expected increased incidence in methylation across polyploid species. In newly formed polyploids, genomic duplications from transposons and duplication of regulatory genes, can result in high levels of cytosine methylation on the new portions of the genome (Holliday 1984; Adams *et al.* 2003; Doyle *et al.* 2008). Several methylation-based processes have been implicated in the competitive silencing of duplicated gene regions (e.g. Fortune *et al.* 2007), leading to increased methylation relative to the diploid progenitor(s). The same processes also lead to the expectation of differential methylation patterning between the diploid progenitor and the polyploid offspring, partly because of imperfect duplication of genes in the neopolyploid, as was reported for the Waxy gene in species of the genus *Spartina* Schreb. (Fortune *et al.* 2007), but also because of various systems of asymmetric homeolog silencing (e.g. Fortune *et al.* 2007). Given the causal links between methylation and gene regulation (Tricker *et al.* 2012) there is scope for such changes to influence the phenotype of the polyploids relative to their diploid relatives. Principal coordinate and genetic distance analyses performed in the current study yielded greater separation between diploid and tetraploid *taxa* when using epigenetic information than when using only genetic information, suggesting that ploidy levels are better separated using epigenetic information than genetic information alone. Intriguingly, this pattern of variation was mirrored by the more pronounced morphological separation between the diploid and tetraploid *taxa* than that seen between species at either ploidy level. Should this observation apply more broadly to other groups, it implies that epigenetic profiling may provide a useful additional tool to infer ploidy level of preserved specimens.

We found similar trends when making comparisons at the species level. Moreover, the diploid species within *L. ovalifolium* complex have imperfect but reasonable morphological differentiation but genetic co-variation with species identity was relatively modest. Interspecies separation was more strongly

enhanced when analysis was focused on the epigenetic variation encompassed in the *Hpa*II profiles, implying that epigenetic patterning (and associated gene silencing) may play a significant role in species separation of the group and so may have some utility for species diagnosis. In both cases, further research would be required to convert these anonymous profiles into Sequence Tagged Site epigenetic markers for robust diagnostic purposes.

Analysis of genetic/epigenetic variability using AMOVA revealed that tetraploid species present lower levels of genetic variability than diploid species. This observation could be most plausibly explained through consideration of their reproductive biology. Several previous studies of the diploids *L. ovalifolium, L. nydeggeri,* and the tetraploids *L. dodartii*, *L. multiflorum* and *L. vulgare* have provided some insights into their primary reproductive strategies (Erben 1978; Rois *et al.* 2012; Cortinhas 2012). These and other works were based on the determination of flower dimorphisms linked to a sporophytic self-incompatibility system (Baker 1953ab; Erben 1978, 1979, 1999; Dawson and Ingrouille 1995; Richards 1997). In these studies diploids *L. nydeggeri* and *L. ovalifolium* were deemed probable sexual species based on their reproductive characteristics. The same applies to the tetraploid *L. vulgare* (Erben 1978; Cortinhas 2012). Conversely, tetraploids *L. dodartii* and *L. multiflorum* both belonging to the *L. binervosum* group, were considered as agamospermous (Ingrouille and Stace 1985; Ingrouille and Stace 1986; Rois *et al.* 2012). Hence, it seems most likely that the lower level of genetic diversity in the putative agamospermous tetraploids could be best explained by their apomictic reproduction mode. In other polyploid apomictic species, such as in *Ranunculus* spp., genetically uniform populations have been similarly observed as a consequence of this mode of reproduction (Paun *et al.* 2006). This finding contrasts with the relatively high level of epigenetic variability among the tetraploids, leading us to speculate that in apomictic polyploid *Limonium* species, the lack of genetic variability caused by the loss of meiotic segregation could be partially compensated by enhanced epigenetic variation. Several authors have suggested similar heritable phenotypic variation due to stable epigenetic variation in the absence of genetic variation (e.g., *Viola cazorlensis*; *Laguncularia racemosa*, and in triploid asexual dandelion lineages (reviewed in

Rapp and Wendel 2005; Bossdorf *et al.* 2008; Richards 2011). Viewed in this context, the results of the present study add *Limonium* to that list.

The present work failed to support any relationship between genetic or epigenetic distance with geographic distance between populations of each species, except for a positive correlation between genetic and geographic distances among *L. nydeggeri*, consistent with regional equilibrium between gene flow and drift (Hutchison and Templeton 1999). The absence of co-correlation is not unexpected for the apomictic polyploids, and can be explained by restricted gene flow between populations, founder events produced by a limited number of individuals, absence of recombination and spread of single asexual clones within populations (Paun *et al.* 2006). One plausible explanation of these results considered collectively is to propose that genetic information flows between populations but that epigenetic information is mainly induced locally by the environment. Alternatively, it might be that selection pressure on epiloci is higher than on genetic loci, or simply that epiloci are plastic, in the sense that they appear and disappear depending on the environmental cues. Conversely, Mantel test analysis of the correlation between genetic and epigenetic distances between *L. nydeggeri* populations, which presents a regional equilibrium at genetic level, show a strong significant positive correlation between genetic and epigenetic distances. While, genetic and epigenetic distances between *L. multiflorum* populations, which does not present genetic regional equilibrium, showed a weak negative correlation. Again, this might suggest that on the absence of a strong gene flow between populations, environmental conditions exert a higher pressure on the fixation of epigenetic loci that cannot be masked by genetic variability introduced by sexual reproduction.

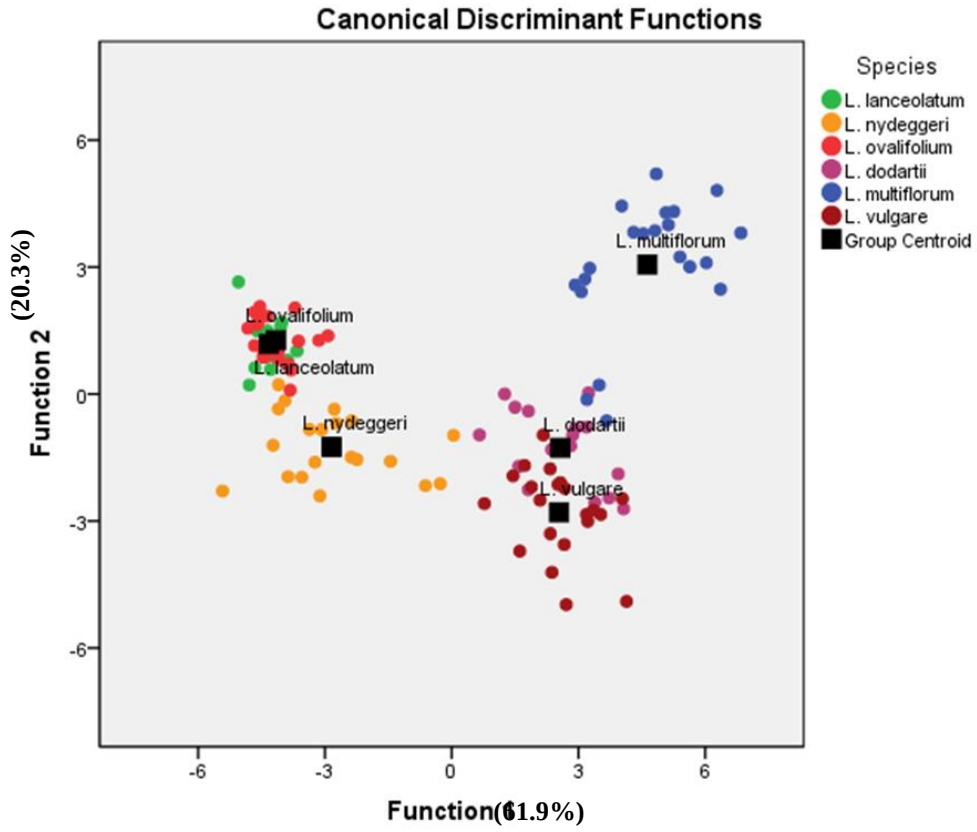


Figure 22. Discriminant function analyses of morphometric data with predefined *Limonium* species. Individuals from each diploid (*L. lanceolatum, L. nydeggeri, L. ovalifolium*) or tetraploid (*L. dodartii, L. multiflorum* and *L. vulgare*) species are represented by colour symbols. Each species centroid is represented by filled squares. Percentages of total variance explained by the functions are given in parentheses.

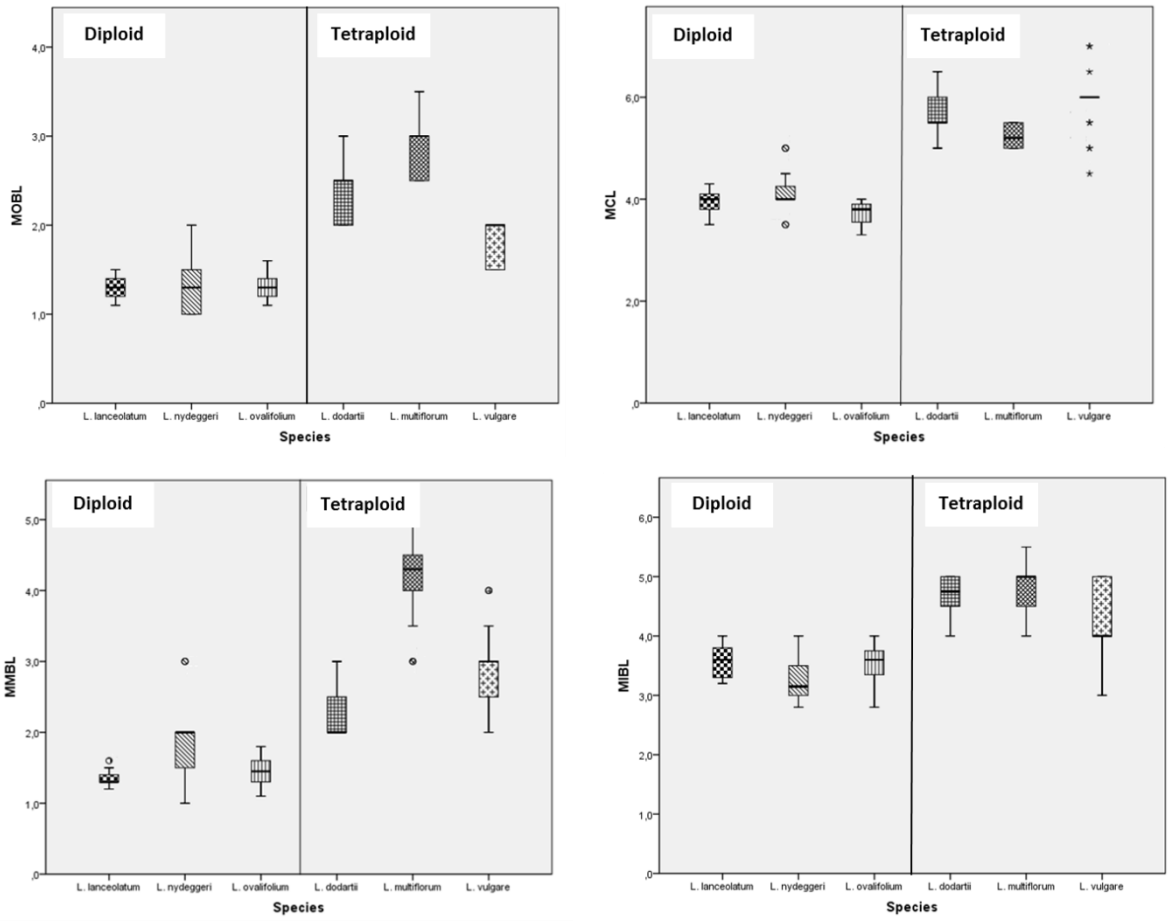
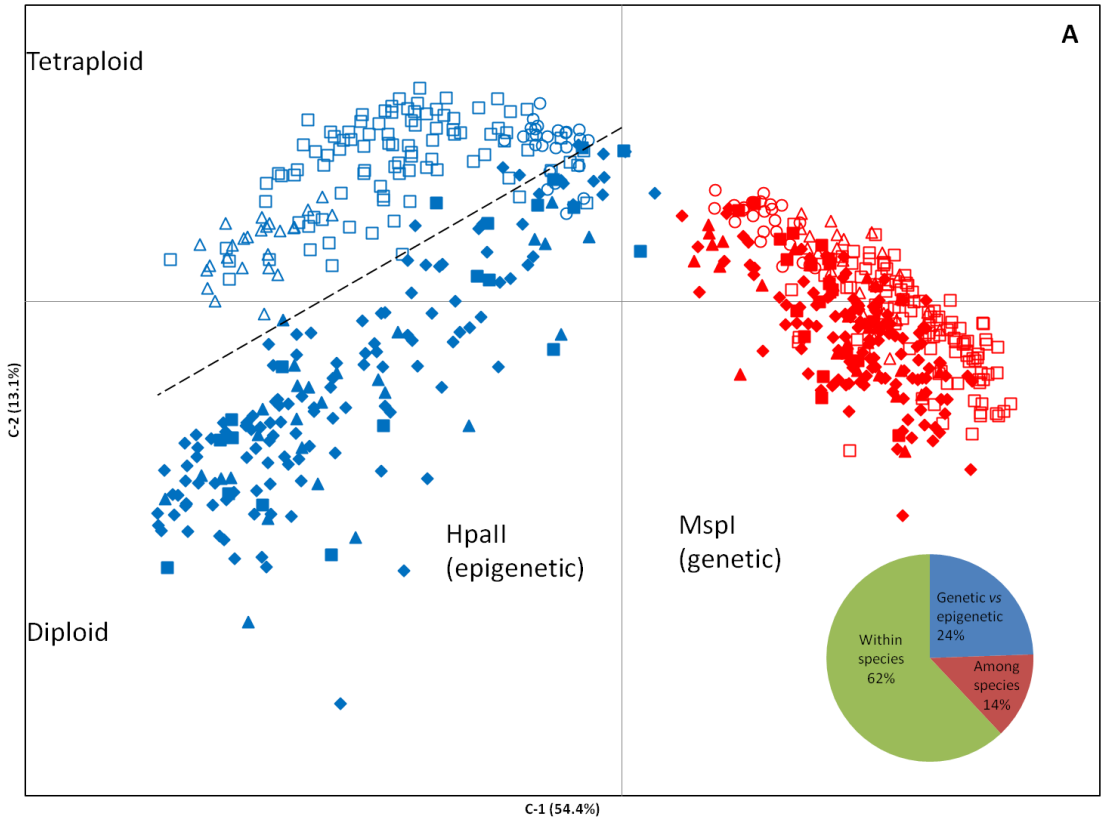
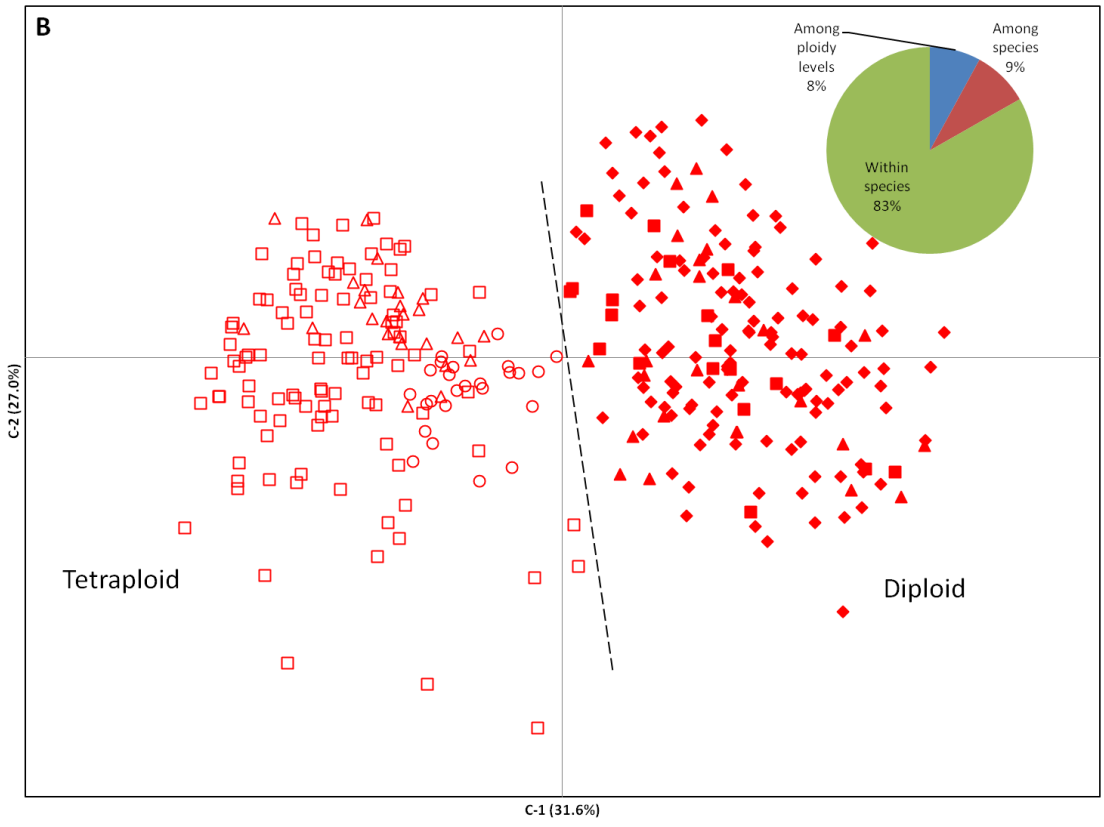


Figure 23. Box plots of significant characters that discriminate diploid from tetraploid *Limonium* species. The box from diploids *L. nydeggeri, L. ovalifolium*, *L. lanceolatum*, and tetraploids *L. dodartii, L. multiflorum*, *L. vulgare* show the twenty-fifth and seventy-fifth percentile ranges and the median; circles and asterisks are outliers (cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box). MOBL - Maximum outer bract length; MCL - Maximum calyx length; MMBL - Maximum middle bract length; and MIBL - Maximum inner bract length.


A
Tetraploid
Diploid
HpaII
(epigenetic)
MspI
(genetic)
Genetic *vs* epigenetic 24%
Among species 14%
Within species 62%
C-2 (13.1%)
C-1 (54.4%)


B
Among ploidy levels 8%
Among species 9%
Within species 83%
Tetraploid
Diploid
C-2 (27.0%)
C-1 (31.6%)

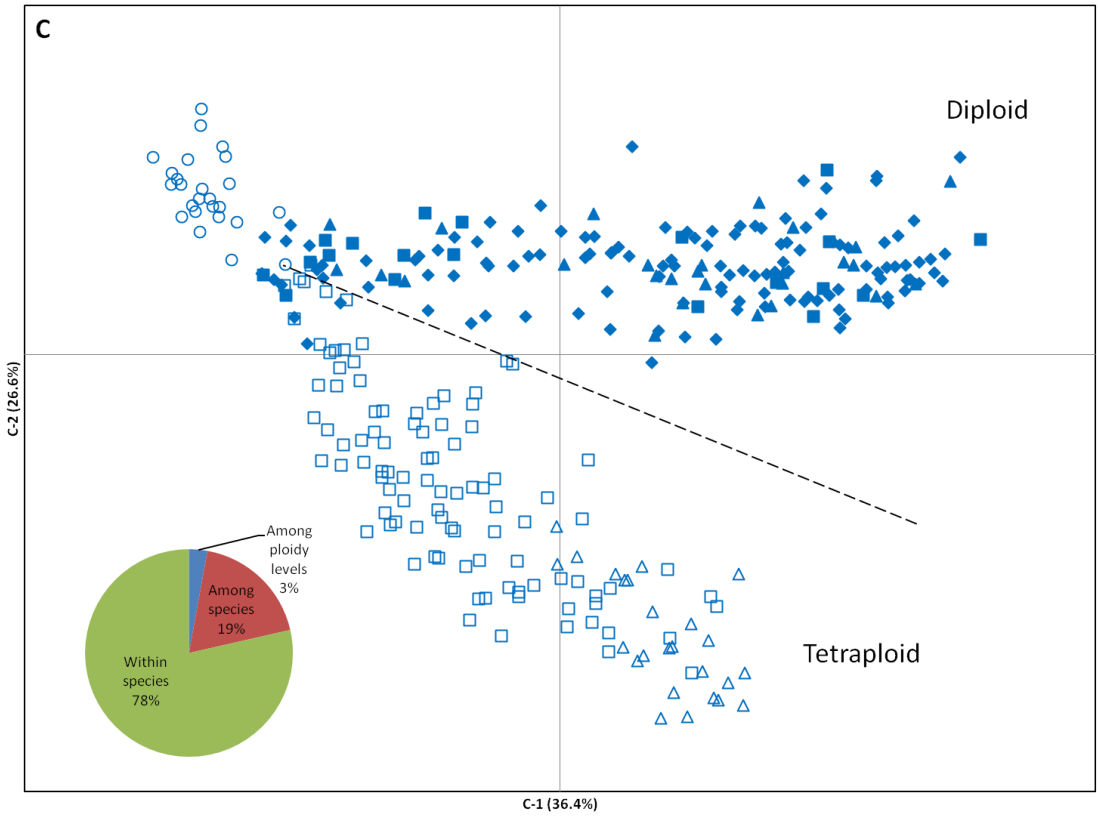


Figure 24. Principal Coordinate Analysis (PCoA) representing genetic and epigenetic variability in diploid and tetraploid *Limonium* species. PCoA was based on presence/absence scores of 488 polymorphic loci obtained from MSAP profiles using isoschizomers *Msp*I (methylation insensitive - red symbols in A and B) or *Hpa*II (methylation sensitive - blue symbols in A and C) as frequent cutters and amplified with primers (E1/H3). The first two coordinates were extracted and plotted against each other. Percentage of the variability shown by each coordinate is indicated between parentheses. Diploid species are represented by solid symbols (*L. lanceolatum*, triangles; *L. nydeggeri*, rhomboids; *L. ovalifolium*, rectangles) and tetraploid species are represented by empty symbols (*L. dodartii*, triangles; *L. multiflorum*, rectangles; *L. vulgare*, circles).

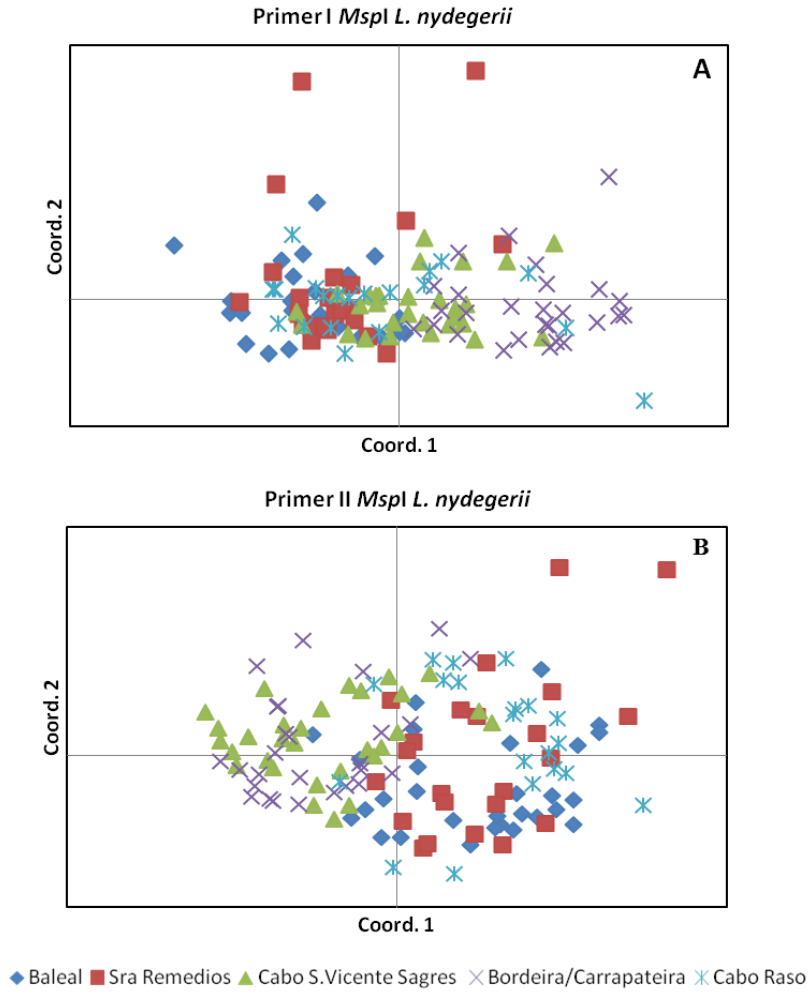


Figure 25. Principal Coordinate Analysis (PCoA) representing genetic variability in *Limonium nydeggeri* populations. PCoA was based on presence/absence scores of 488 (primer I, E1/H1) (A) and 347 (primer II, E1/H3) (B) polymorphic loci obtained from MSAP profiles using isoschizomers *Msp*I (methylation insensitive) or *Hpa*II (methylation sensitive) as frequent cutters. The first two coordinates were extracted and plotted against each other.

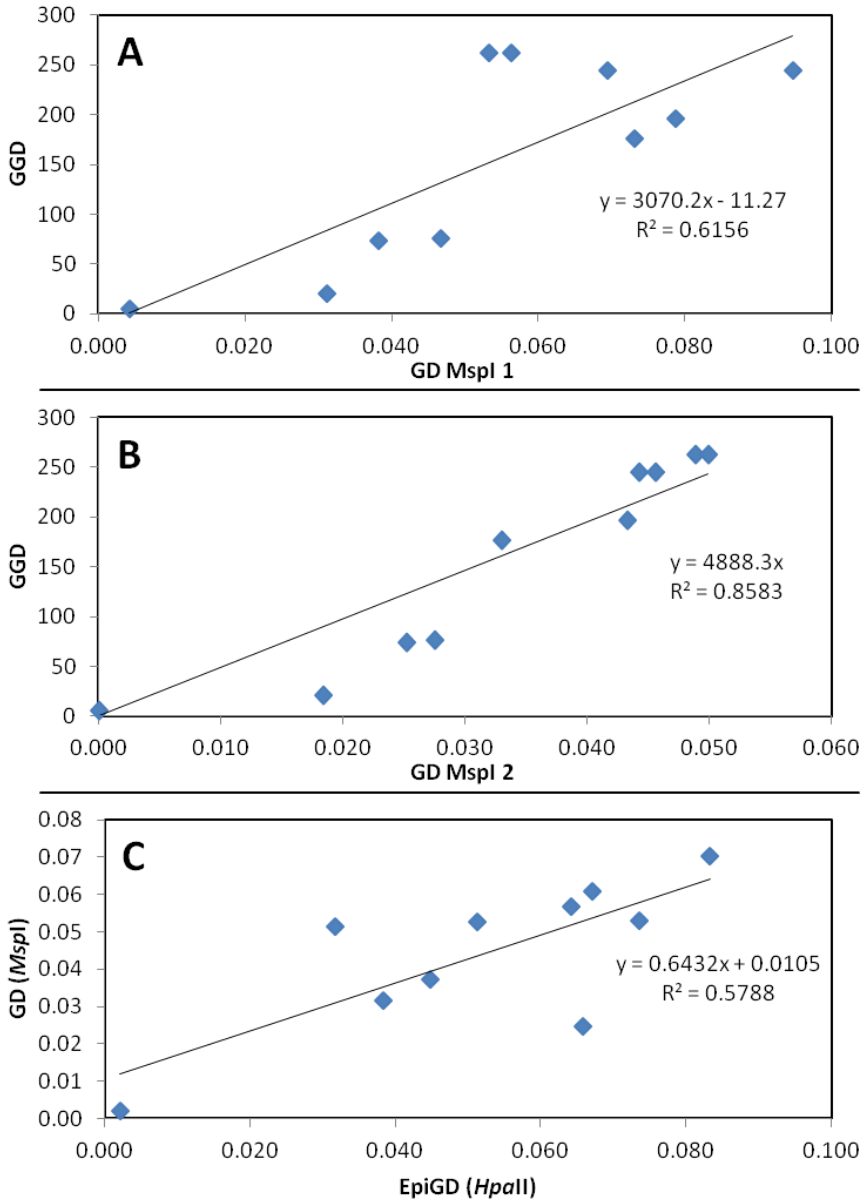


Figure 26. Correlation between pairwise genetic differentiation and geographical distance and between pairwise genetic and epigenetic differentiation between *Limonium nydeggeri* populations. Mantle tests were based on MSAP data obtained using *HpaII* and primer combinations primer I (E1/H1) (A) and primer II (E1/H3) (B) and *HpaII* and *MspI* and both primers (C). Shown equations are the linear functions and R^2 values for each Mantel test. Analysis using 1000 permutation tests showed a significant correlations (A: $P < 0.04$; B: $P < 0.02$; C: $P < 0.03$). GD, PhiPT - pairwise genetic differentiation; GGD, in Km - geographical distance; GD and EpiGD, respectively - genetic and epigenetic differentiation.

Table 9. Collection data of fourteen populations of *Limonium*.

Ploidy level	Species	Site location collector*	Geographical Coordinates	N
Diploid	***L. lanceolatum***	Odemira, Vila Nova de Milfontes, ADC, APP, ASR	37.727756/-8.770931	10
		Peniche, Ilha do Baleal, ADC, APP, ASR	39.378919/-9.340983	9
		Peniche, Nossa Sr[a] dos Remedios, ADC, APP, ASR	39.369906/-9.395731	7
	L. nydeggeri	Cascais, Cabo Raso, ADC, APP, ASR	38.710039/-9.485883	7
		Aljezur, Pontal de Carrapateira, ADC, APP, ASR	37.195039/-8.911103	10
		Vila do Bispo, Cabo de São Vicente, ADC ASR	37.002611/-8.996564	10
	L. ovalifolium	Vila do Bispo, Cabo de Sagres, ADC, APP, ASR	36.994242/-8.948756	6
		Lagos, Praia da Luz ADC, ASR	37.087442/-8.729094	10
Tetraploid	***L. dodartii***	Odemira, Cabo, Sardão, ADC, APP, ASR	37.598344/-8.818272	10
	L. multiflorum	Peniche, Península da Papoa, ADC, APP, ASR	39.374131/-9.377428	6
		Lourinhã, Vale dos Frades, ADC, APP, ASR	39.276506/-9.335839	7
		Mafra, Foz do Lizandro, ADC, APP, ASR	38.941531/-9.415223	9
		Cascais, Cabo Raso, ADC, APP, ASR	38.710039/-9.485883	14
	L. vulgare	Odemira, Vila Nova Milfontes, ADC, APP, ASR	37.727756/-8.770931	10

Geographical coordinates of each population and sampling size (*N*; approximately 10 individuals per population) are included.

*Abbreviations of collectors: ADC, AD Caperta: APP AP Paes; ASR, AS Rois; AC, Ana Cortinhas. Centro de Botânica Aplicada Agricultura, Instituto Superior de Agronomia, Lisboa, Portugal.

Table 10. Summary of the discriminant analysis of six predefined diploid and tetraploid *Limonium* species.

Species			Predicted Group Membership-Classification Results						Total
			L. lanceolatum	*L. nydeggeri*	*L. ovalifolium*	*L. dodartii*	*L. multiflorum*	*L. vulgare*	
Original	Count	*L. lanceolatum*	10	0	1	0	0	0	11
		L. nydeggeri	0	20	0	0	0	0	20
		L. ovalifolium	3	0	18	0	0	0	21
		L. dodartii	0	0	0	16	0	0	16
		L. multiflorum	0	0	0	1	18	2	21
		L. vulgare	0	0	0	1	0	20	21
	%	*L. lanceolatum*	90.9	0	9.1	0	0	0	100
		L. nydeggeri	0	100	0	0	0	0	100
		L. ovalifolium	14.3	0	85.7	0	0	0	100
		L. dodartii	0	0	0	100	0	0	100
		L. multiflorum	0	0	0	4.8	85.7	9.5	100
		L. vulgare	0	0	0	4.8	0	95.2	100

Table 11. Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions of morphological characters.

	Function				
	1	**2**	**3**	**4**	**5**
MOBL[1]	0.586*	0.414	-0.047	0.37	0.311
MCL	0.569*	-0.44	-0.203	-0.165	-0.123
MMBL	0.512*	0.298	0.336	-0.434	0.032
MIBL	0.350*	0.144	-0.203	0.193	0.021
MMBW	0.405	0.537*	0.306	-0.348	0.151
MOBW	0.256	0.527*	-0.045	0.318	0.343
MIBW	-0.02	0.442*	0.01	0.223	-0139
MNSC	-0.334	0.288	-0.603	-0.372	0.418*
MSL	-0.304	-0.08	0.39	0.195	0.656*
MNFS	-0.005	0.174	0.117	0.075	-0.493

Variables were ordered by absolute size of correlation within function.
[1]MSL, Maximum spike length ; MNSC, Maximum number of spikelets per cm; MNFS, Maximum number of florets per spikelet; MOBL, Maximum outer bract length; MOBW, Maximum outer bract width; MMBL, Maximum middle bract length; MMBW, Maximum middle bract width; MIBW, Maximum inner bract width; MCL,Maximum calyx length.
*Largest absolute correlation between each variable in respective function.

Table 12. Mean values of morphometric characters in diploid and tetraploid species. Diploid *L. lanceolatum*, *L. nydeggeri*, *L. ovalifolium* and tetraploid *L. dodartii*, *L. multiflorum* and *L. vulgare* species are considered.

Ploidy	Species	Morphometric Characters[1]									
		MNSC	MSL	MNFS	MOBL	MOBW	MMBL	MMBW	MIBL	MIBW	MCL
Diploid	*L. lanceolatum*	10.82	4.87	2.24	1.29	1.54	1.34	1.16	3.58	3.44	3.93
		(0.98)	(0.09)	(0.56)	(0.12)	(0.15)	(0.12)	(0.07)	(0.28)	(0.28)	(0.25)
	L. nydeggeri	5.35	8.4	2.55	1.33	1.29	1.9	1.3	3.31	3.15	4.13
		(1.31)	(4.33)	(0.51)	(0.31)	(0.29)	(0.56)	(0.35)	(0.38)	(0.37)	(0.46)
	L. ovalifolium	8.38	4.86	3.09	1.33	1.52	1.44	1.19	3.5	3.73	3.73
		(0.92)	(0.31)	(1.38)	(0.15)	(0.15)	(0.19)	(0.13)	(0.33)	(0.34)	(0.21)
Tetraploid	*L. dodartii*	6.31	1.29	2.19	2.31	1.88	2.23	1.43	4.66	3.17	5.68
		(1.08)	(0.30)	(0.66)	(0.31)	(0.22)	(0.36)	(0.35)	(0.40)	(0.24)	(0.37)
	L. multiflorum	5.24	1.24	3	2.93	2.58	4.18	3.43	4.83	3.97	5.22
		(0.83)	(0.36)	(0.32)	(0.36)	(0.53)	(0.58)	(0.78)	(0.40)	(0.72)	(0.24)
	L. vulgare	5	1.91	2.24	1.86	1.31	2.81	1.67	4.17	2.64	5.91
		(1.26)	(1.55)	(0.62)	(0.23)	(0.33)	(0.66)	(0.37)	(0.66)	(0.65)	(0.58)

[1]MSL, Maximum spike length ; MNSC, Maximum number of spikelets per cm; MNFS, Maximum number of florets per spikelet; MOBL, Maximum outer bract length; MOBW, Maximum outer bract width; MMBL, Maximum middle bract length; MMBW, Maximum middle bract width; MIBW, Maximum inner bract width; MCL,Maximum calyx length.

Table 13. MSAP fragment number analysis.

Ploidy level	Species	E1/H1		E1/H3		Total	
		***Hpa*II**	***Msp*I**	***Hpa*II**	***Msp*I**	***Hpa*II**	***Msp*I**
Diploid	***L lanceolatum***	52	52	47	38	99	90
	L. nydeggeri	50	48	43	33	93	81
	L ovalifolium	48	43	42	28	90	71
Tetraploid	***L. dodartii***	37	77	24	56	61	133
	L multiflorum	33	59	30	35	63	94
	L. vulgare	26	46	20	39	46	85

Average number of MSAP fragments per diploid (*L. lanceolatum, L. nydeggeri, L. ovalifolium*) and tetraploid (*L. dodartii, L. multiflorum* and *L. vulgare*) species obtained using isoschizomers enzymes *Hpa*II (methylation sensitive) and *Msp*I (methylation insensitive) and primer combinations E1/H1 and E1/H3.

Table 14. Estimated genetic and epigenetic distances between and within diploid and tetraploid species. Diploid (*L. lanceolatum*, *L. nydeggeri*, *L. ovalifolium*) and tetraploid (*L. dodartii*, *L. multiflorum* and *L. vulgare*) species are considered.

Primer		GD diploid *vs* tetraploid	GD amongst diploid species			GD amongst tetraploid species		
			L. nyd. vs L. oval.	*L. nyd. vs L. lanc.*	*L. oval. vs L. lanc.*	*L. vulg. vs L. mult.*	*L. dod. vs L. mult.*	*L. dod. vs L. vulg.*
E1/H1	**PhiPT**	0.138	0.017	0.021	0.022	0.380	0.139	0.302
	Nei GD	0.015	0.004	0.004	0.005	0.033	0.015	0.022
E1/H3	**PhiPT**	0.125	0.025	0.014	0.020	0.253	0.086	0.226
	Nei GD	0.006	0.002	0.001	0.002	0.011	0.004	0.007
Average	**PhiPT**	0.131	0.021	0.018	0.021	0.316	0.113	0.264
	Nei GD	0.011	0.003	0.003	0.004	0.029	0.009	0.015

Primer		GD between diploid and tetraploid species								
		L. nyd. vs L. mult.	*L. oval. vs L. mult.*	*L. lanc. vs L. mult.*	*L. nyd. vs L. dod.*	*L. oval. vs L. dod.*	*L. lanc. vs L. dod.*	*L. nyd. vs L. vulg.*	*L. oval. vs L. vulg.*	*L. lanc. vs L. vulg.*
E1/H1	**PhiPT**	0.209	0.225	0.218	0.158	0.134	0.141	0.237	0.231	0.213
	Nei GD	0.016	0.015	0.021	0.019	0.014	0.017	0.024	0.018	0.018
E1/H3	**PhiPT**	0.155	0.177	0.154	0.149	0.141	0.131	0.227	0.227	0.200
	Nei GD	0.008	0.010	0.009	0.008	0.007	0.008	0.011	0.009	0.010
Average	**PhiPT**	0.182	0.201	0.186	0.153	0.138	0.136	0.232	0.229	0.206
	Nei GD	0.012	0.012	0.015	0.013	0.010	0.013	0.018	0.013	0.014

Table 14. Continued.

Primer		EpiGD diploid *vs* tetraploid	EpiGD amongst diploid species			EpiGD amongst tetraploid species		
			L. nyd. vs L. oval.	*L. nyd. vs L. lanc.*	*L. oval. vs L. lanc.*	*L. vulg. vs L. mult.*	*L. dod. vs L. mult.*	*L. dod. vs L. vulg.*
E1/H1	**PhiPT**	0.156	0.024	0.024	0.026	0.388	0.372	0.500
	Nei GD	0.015	0.004	0.003	0.006	0.047	0.049	0.091
E1/H3	**PhiPT**	0.121	0.014	0.019	0.029	0.405	0.295	0.498
	Nei GD	0.006	0.002	0.002	0.004	0.019	0.018	0.044
Average	**PhiPT**	0.138	0.019	0.022	0.027	0.396	0.333	0.499
	Nei GD	0.011	0.003	0.003	0.005	0.019	0.034	0.068

Primer		EpiGD between diploid and tetraploid species								
		L. nyd. vs L. mult.	*L. oval. vs L. mult.*	*L. lanc. vs L. mult.*	*L. nyd. vs L. dod.*	*L. oval. vs L. dod.*	*L. lanc. vs L. dod.*	*L. nyd. vs L. vulg.*	*L. oval. vs L. vulg.*	*L. lanc. vs L. vulg.*
E1/H1	**PhiPT**	0.209	0.225	0.288	0.332	0.319	0.347	0.325	0.330	0.378
	Nei GD	0.029	0.030	0.032	0.062	0.063	0.065	0.042	0.040	0.048
E1/H3	**PhiPT**	0.161	0.166	0.213	0.266	0.293	0.271	0.308	0.290	0.333
	Nei GD	0.007	0.006	0.008	0.024	0.027	0.023	0.020	0.017	0.022
Average	**PhiPT**	0.185	0.196	0.251	0.299	0.306	0.309	0.316	0.310	0.355
	Nei GD	0.018	0.018	0.020	0.043	0.045	0.044	0.031	0.029	0.035

Table 15. Genetic and epigenetic variability within diploid and tetraploid *Limonium* species.

Primer		Ploidy level: Diploid/Tetraploid		
		%PL	Sh I	He
E1/H1	Genetic	74.45/52.93%	0.183/0.116	0.106/0.066
	Epigenetic	82.23/58.79/%	0.204/0.098	0.118/0.110
E1/H1	Genetic	63.32/31.21%	0.104/0.062	0.054/0.035
	Epigenetic	69.67/42.89%	0.128/0.098	0.071/0.058
Average	Genetic	68.88/42.072%	0.143/0.089	0.095/0.084
	Epigenetic	75.95/50.84%	0.166/0.139	0.095/0.084

Primer		*L. nydeggeri*			*L. ovalifolium*			*L. lanceolatum*		
		%PL			Sh I			He		
E1/H1	Genetic	90.29%	0.191	0.107	51.71%	0.132	0.073	80.29%	0.222	0.127
	Epigenetic	90.29%	0.194	0.112	61.14%	0.188	0.113	64.86%	0.194	0.118
E1/H3	Genetic	85.25%	0.103	0.052	34.63%	0.075	0.040	65.16%	0.124	0.064
	Epigenetic	85.66%	0.130	0.071	48.16%	0.119	0.066	59.43%	0.132	0.074
Average	Genetic	87.77%	0.147	0.079	43.17%	0.103	0.056	72.72%	0.173	0.095
	Epigenetic	87.97%	0.162	0.092	54.65%	0.154	0.090	62.14%	0.163	0.096

Primer		*L.multiflorum*			*l. dodartii*			*L. vulgare*		
		%PL			Sh I			He		
E1/H1	Genetic	76.86%	0.186	0.110	47.14%	0.130	0.075	35.14%	0.089	0.051
	Epigenetic	66.29%	0.119	0.067	42.86%	0.186	0.120	38.29%	0.131	0.082
E1/H3	Genetic	65.57%	0.094	0.051	22.95%	0.060	0.034	20.90%	0.048	0.027
	Epigenetic	49.18%	0.077	0.043	27.05%	0.104	0.067	32.99%	0.092	0.056
Average	Genetic	71.22%	0.140	0.081	35.05%	0.095	0.055	28.02%	0.069	0.039
	Epigenetic	57.73%	0.098	0.055	34.95%	0.145	0.093	35.64%	0.112	0.069

Calculated Percentage of Polymorphic Loci (%PL), Shannon diversity index (ShI) and Expected Variability (He) – 2*p*q) for diploid (*L. lanceolatum, L. nydeggeri, L. ovalifolium*) and tetraploid (*L. dodartii, L. multiflorum* and *L. vulgare*) species. All values obtained using GenAlex (v.6.4).

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CHAPTER VI.

SEX AND APOMIXIS SHAPE CHLOROPLAST DNA VARIATION PATTERNS IN DIPLOID AND TETRAPLOID *LIMONIUM*

The data presented in this chapter is in preparation to be submitted to Annals of Botany: **Róis AS, Sádio F, Teixeira G, Paes AP, Espírito-Santo D, Sharbel T and Caperta AD (2014) Sex and apomixis shape chloroplast DNA variation patterns in diploid and tetraploid *Limonium* spp. (*Plumbaginaceae*).**

CHAPTER VI – SEX AND APOMIXIS SHAPE CHLOROPLAST DNA VARIATION PATTERNS IN DIPLOID AND TETRAPLOID *LIMONIUM*

1. Abstract

The genus *Limonium* Mill. (sea-lavenders; *Plumbaginaceae*) has long been recognized to have sexual and apomictic (asexual seed formation) modes of reproduction and a history of recurrent hybridization and polyploidization. Herein we aimed at elucidating reproductive modes and genetic cpDNA variation patterns in populations of three putative sexual diploid species with morphological affinities (*L. nydeggeri*, *L. ovalifolium*, *L. lanceolatum*), and three apomict tetraploid species thought to be related (*L. binervosum*, *L. dodartii*, *L. multiflorum*). Inter- and intraspecific levels of cpDNA haplotype variation and differentiation underlying natural populations of these diploid and tetraploid species were investigated using two chloroplast sequence regions (*trnL* intron and *trnL-trnF* intergenic spacer). Floral heteromophies, ovule cytoembryological analyses, pollination and crossing tests were performed in representative species of each ploidy group using plants from greenhouse collections. Our findings show higher cpDNA haplotype variation in diploid compared to tetraploid species. Network analysis revealed no correlation between cpDNA haplotype and geographic distribution, but cpDNA haplotype sharing within and among *Limonium* species with distinct ploidy levels. Furthermore, our results provide the first direct evidence of facultative apomixis in diploid species, and obligate apomixis in tetraploid ones. In addition, pollen-independent (i.e. autonomous) endosperm formation occurs in these biological systems. Collectively, our data give further insights for probable reticulate evolution in *Limonium* spp. Due to the maternal inheritance of plastids, potential reticulation is not detected, but it nonetheless appears that apomicts have arisen repeatedly from sexual *taxa*.

Keywords: Apomixis, cpDNA, female gametophyte, *Limonium* spp., self-incompatibility

2. Introduction

Patterns of genetic variation across a species' range is influenced by many factors. Of these, mating systems are among main agents shaping gene

flow patterns within and between populations of flowering plants (angiosperms) (Charlesworth and Wright 2001; Charlesworth 2006). Two major pathways of reproduction are known: sex (outcrossing and selfing) and apomixis (agamospermy; i.e. asexually formed seeds) (Asker and Jerling 1992). In flowering plants, this latter mode of reproduction occurs in diploids, polyploids or polyhaploids (Carman 1997; Hörandl and Hojsgaard 2012). The sexual reproductive pathway is distinguished from the apomictic pathway by three developmental steps: (i) apomeiosis, the development of an unreduced embryo sac from a somatic cell of the nucellus (apospory) or from an unreduced megaspore mother cell (diplospory); (ii) parthenogenetic development of an embryo without fertilization; and (iii) balanced endosperm formation with (pseudogamous) or without (autonomous) fertilization of the central cell (e.g. Asker and Jerling 1992).

In species within the same genus with different breeding systems, patterns of DNA sequence polymorphism has revealed lower variability in selfers compared to outcrossers (reviewed in Glémin *et al.* 2006). Both selfing and apomixis result in the reduction of genotypic diversity within populations, but apomixis fixes heterozygosity in the offspring, which maintains the maternal genotype (Hörandl 2010). Nevertheless, in several apomictic plant populations clonal diversity is extensive (Ellstrand and Roose 1987), and many multilocus genotypes co-occur (e.g. Noyes and Soltis 1996; van der Hulst *et al.* 2000; Lovell *et al.* 2013). For example, isoenzyme studies in sexual and apomictic *taxa* of the *Ranunculus auricomus* complex showed that levels of observed heterozygosity in apomicts markedly exceeded the values of their sexual relatives (Hörandl *et al.* 2001). In triploid asexual dandelion lineages reduced or negligible genetic variation is observed, even between localities, although some populations are composed of several genotypes (e.g. van der Hulst *et al.* 2003; van Dijk 2003).

Limonium Mill. (sea-lavenders; *Plumbaginaceae*) is a cosmopolitan species rich genus of annuals and perennial herbs, shrubs and lianas, often adapted to extreme coastal environments (Kubitzki, 1993). A wide range of species have been identified, mainly microspecies from geographically restricted areas (Erben 1978, 1993; Kubitzki 1993; Cowan *et al.* 1998).

Together, hybridization, polyploidy and apomixis have shaped the extant radiation of *Limonium*, in particular in the well-studied areas such as the Mediterranean basin (Baker 1966; Erben 1978; Lledó *et al.* 2005). Floral heteromorphies, like heterostyly and pollen-stigma dimorphisms linked to a sporophytic self-incompatibility system, prevent mating between individuals of the same floral morph but allow mating between morphs (Baker 1948, 1953ab, 1966). In this system, dimorphic pollen-stigma populations, with plants featuring flowers with distinct pollen grains types (coarsely reticulate, A-type; or finely reticulate, B-type) and distinct stigma types (polygonal or papillate cells) seem to be outcrossers. In contrast, monomorphic self-incompatible populations showing only one pollen-stigma combination appear to form seeds through apomixis (Baker 1966; Cowan *et al.* 1998).

Various types of tetrasporous (meiotic) reduced embryo sacs have been reported in distinct *Limonium* spp., including *Adoxa*-, *Fritillaria*-, *Drusa*- and *Penea*-types (Dahlgren 1916; D´ Amato 1940, 1949; Hjelmqvist and Grazi 1964). Also, in polyploid *L. vulgare* (known as *Statice limonium* L.; *Statice*, nom. rej. vs. *Armeria*; Greuter *et al.* 2000) *Fritillaria*-type female gametophyte development occurs (D'Amato 1940). Furthermore, facultative apomicts like triploid (2*n*=3x=27) *Statice oleaefolia* var. *confusa* form meiotic, tetrasporous reduced *Adoxa*- type embryo sacs in parallel with apomictic, unreduced, diplosporous *Erigeron*-type embryo sacs (D'Amato 1949).

Diploid and tetraploid *Limonium* species are present in Portugal in Sites of Community Importance (SCI) for the Mediterranean biogeographical region (CEC 2006). Among them, three diploid (2*n*=2x=16) species of the *L. ovalifolium* complex are found: *L. ovalifolium*, the Lusitania endemic *L. nydeggeri* and *L. lanceolatum* (Franco 1984; Erben 1993; Erben 1999). The tetraploid species from the *L. binervosum* complex (Erben 1978) include, among others: *L. binervosum* (2*n*=4x=35), *L. dodartii* (2*n*=4x=35) and the Lusitania endemic *L. multiflorum* with both tetraploid and aneuploid cytotypes (2*n*=4x=35 – Erben 1993; 2*n*=4x=32, 34, 35, 36 – Róis *et al.* 2012). Inferences into the primary reproductive strategies of these species have been based upon analyses of flower heteromorphies (Erben 1978, 1999; Ingrouille and Stace 1985, 1986), which have shown that *L. nydeggeri* and *L. ovalifolium* are likely

sexual whereas tetraploids are all putative agamospermous species (Ingrouille and Stace 1985, 1986; Cowan *et al.* 1998). Flow cytometric seed screening, which has the potential to determine the routes of seed formation based embryo to endosperm ploidies (Matzk *et al.* 2000), failed in the elucidation of *L. ovalifolium* and *L. multiflorum* reproductive modes using mature seeds (Róis *et al.* 2012). Thus, even if sexuality and/or agamospermy is suspected in these species groups, it has never been directly tested.

Notably, the species within the *L. ovalifolium* complex have imperfect but distinguishable morphological differentiation and genetic and epigenetic co-variation, as revealed by biometric studies and Methylation Sensitive Amplification Polymorphism (MSAP) analysis (Róis *et al.* 2013). Conversely, tetraploid species of the related *L. binervosum* complex contain even lower levels of overall genetic diversity, but higher levels of epigenetic variability (Róis *et al.* 2013). Here, we investigated (1) the levels and distribution of cpDNA haplotype diversity within and among populations of diploid and tetraploid *Limonium* spp. using the maternally-inherited chloroplast sequences *trnL* intron and *trnL-trnF* intergenic spacers since these data can provide clues at the intraspecific level of variation in *taxa* with morphological affinities because of the slower rate of evolution of cpDNA compared to nuclear DNA in plants (Petit *et al.* 2003); (2) directly evaluate the influence of their reproduction modes on inter- and intraspecific genetic differentiation of these species.

3. Materials and methods

3.1. Study species and population sampling

Populations were surveyed along the Portugal coast in Beira Litoral (west), Estremadura (west), Alentejo (southwest) and Algarve (south). The populations sampled spanned the distribution range of three diploid (*L. ovalifolium, L. nydeggeri* and *L. lanceolatum*) and three tetraploid species (*L. binervosum*, *L. dodartii L. multiflorum*. All species have patchy distributions across southwestern Atlantic Iberia (Espírito-Santo *et al.* 2012) and small population sizes (20-30 individuals), albeit one *L. multiflorum* population (Cabo Raso) has more than 1000 individuals (Caperta *et al.* 2014, submitted). Except for *L. lanceolatum* and *L. binervosum* most species grow on limestone Atlantic

sea-cliffs, in crevices within exposed rocks or on shallow soil above the rock strata, and on scree slopes where competition with other species is very low. All populations were recorded using a global positioning system, Google Earth 6.0.2 was used for georeferencing, and geographic mapping of the populations was performed using ArcGIS Desktop 10 (ESRI).

A total of 115 plants, with 2-10 individuals per population, were included in this study (Table 16). Most individuals were selected from populations within designated Portuguese Sites of Community Interest (SCI): SCI Peniche/Sta Cruz (PTCON0056), SCI Sintra/Cascais (PTCON0008), SCI Costa Sudoeste (PTCON0012) and SCI Ria Formosa/Castro Marim (PTCON0013) (CEC 2006).

3.2. DNA isolation, PCR amplification and sequencing

Three young leaves per individual were sampled from all plants at the same developmental stage. Leaves were cut, placed in paper envelopes and dried immediately at room temperature in sealed containers with abundant silica gel. Total genomic DNA was extracted using an Agencourt Chloropure System kit of Beckman Coulter, Inc. Two regions of the chloroplast genome (*trnL* intron and *trnL-trnF* intergenic spacer; hereafter the *trnL-F* region; Clegg *et al.* 1994) were amplified and sequenced using the universal PCR primers of Taberlet *et al.* (1991; combinations c/d and e/f). 10 µl PCR reactions were performed containing 50 ng of template DNA, 10x NH4 PCR buffer, 50 mM MgCl2, 100 mM dNTPs, 10 mM of each primer and 5U/µl Taq DNA polymerase (Bioline GmbH). PCR amplifications began with an initial cycle of 5 min at 95ºC, 30 cycles of 30 s at 95ºC, 30 s at 55ºC and 1.40 min at 72ºC, and a final step of 10 min at 72ºC. 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 BigDye Ready Reaction Terminator Cycle Sequencing Kit on an ABI 3700 genetic analyser. Sequences were generated from at least two separate PCR amplifications per individual, and both strands of all fragments were sequenced.

3.3. Data analyses

The intron *trnL* and intergenic spacer *trnL - trnLF* sequences were aligned and using BioEdit 7.1.3.0 (Hall 1999) and concatenated using

Concatenator V 1.1.0 (Pina-Martins and Paulo 2008). Chloroplast haplotypes 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 (weights = 10 and epsilon = 0). Genetic diversity parameters such as haplotype diversity (Hd) and nucleotide diversity (π), genetic differentiation (Gst) as well as neutrality tests (Tajima's D) were calculated using the program DnaSP4.0 (Rozas *et al.* 2003). Three analyses of molecular variance (AMOVA) were performed to determine how cpDNA haplotype variation is distributed within and among collection sites between the resultant genetic groups using Arlequin version 3.1 (Excoffier *et al.* 2005), and fixation indices were calculated according to Excoffier *et al.* (2005).

3.4. Pollen exclusion and crossing experiments

A total of 10 fresh flowers were used for floral heteromorphism determinations in 12 *L. ovalifolium* and 6 *L. multiflorum* plants growing in experimental collections at Instituto Superior de Agronomia. From these plants, stigma and pollen types (A/B pollen and *cob*-like/papillate stigmas; Baker 1948; Erben 1978) were determined. These organs were dissected, covered with a drop of water, and stigma preparations were observed using OLM (Leitz hm-lux 3) with 40X magnification. Pollen viability was scored using the Alexander's stain test (Alexander 1969). Total pollen grains and pollen viability estimates were performed by one person using c. 20 flowers per plant and counted under an OLM (Leitz hm-lux 3) under 20X magnification.

From these plant collections, 12 self-incompatible *L. ovalifolium* plants (either with A or B combination) and 6 *L. multiflorum* male-sterile plants were selected (Róis *et al.* 2012) and subjected to pollen exclusion experiments to ascertain if apomixis contributes towards seed set, as described in Khan *et al.* (2012), with some modifications. As the plants were self-incompatible or male-sterile, no flower emasculation prior to anther dehiscence was necessary. One of the flowering stems (scape) was left unbagged (natural pollination) while the another scape was bagged with vegetal cellulose paper that did not allow the passage of pollen or floral visitors, the idea being that any seeds produced from bagged scapes would have originated without pollination (i.e. apomixis). In

addition, 3 self-incompatible A combination plants and 3 self-incompatible B combination plants were used for manual cross-pollinations soon after anther dehiscence. All cross-pollinations were performed by bagging scapes from individual plants together for 9 weeks, after which bags were removed and the number of produced seeds counted. Seeds were then germinated as described in (Róis *et al.* 2012). Germination was considered to have occurred on emergence of radicles, and the number of seeds that germinated after 1 week was recorded.

3.5. Electron microscopy analyses

Stigma, pollen and ovules were analyzed using flowers from the above *L. ovalifolium* and *L. multiflorum* plants. Flowers were fixed in a 2.5% glutaraldehyde solution in 0.1 M sodium phosphate buffer, pH 7.2, for 5 h at 4°C as described in Hayat (1981). The material was then dehydrated in a graded ethanol series (30, 50, 75 to 100% ethanol for 30 min each). Flowers were then dried on a Critical Point Polaron BioRad E3500, and coated with a thin layer of gold on a Jeol JFC-1200. Observations were carried out on a JSM-5220 LV SEM equipped with a direct image acquisition system.

3.6. Cytohistological analyses of mega- sporogenesis and gametogenesis

Flower buds at distinct floral stages ranging from 2 mm to 5 mm length were collected from the above-mentioned *L. ovalifolium* and *L. multiflorum* plants. Pistils were then dissected from flower buds, followed by clearing and staining following the protocol of Stelly *et al.* (1984), with minor modifications. Briefly, the tissues were fixed in FAA (3.7% formalin, 5% acetic acid, 70% ethanol) overnight at 4ºC and then hydrated in 50, 75 and 100% progressive water/ethanol solutions for 30 min each. Samples were stained with pure Mayer's hemalum for 48 h and placed in chloral hydrate. Ovules were then digested with a 2 % cellulase, 2 % cellulase ''Onozuka R-10'', and 2 % pectinase enzyme solution in 1xEB for 20m at 37 ºC as described in Caperta *et al.* (2008). Ovules were mounted under a coverslip in one drop of chloral hydrate. Cytohistological observations were made using a Zeiss Axioskop 2 FM using DIC optics, under 20X and 100X magnification.

4. Results

4.1. Intra- and interpopulation cpDNA haplotype distribution and diversity

The sequencing of the intron *trnL* and intergenic spacer *trnL-F* resulted in a final matrix of 122 individuals for a 646 bp concatenated fragment (Table 17). Fifty five SNP and four indels were detected.

A total of 24 (H1-H24) haplotypes were identified in the 17 populations (Tables 17 and 18; Figure 27). Diploid species show higher numbers of haplotypes (n=16) than tetraploid species (n=8) and most diploid species had populations with two or three haplotypes. A common haplotype (H9) was found in at least one population of each species. Furthermore, a unique haplotype (H24) was shown in a *L. lanceolatum* population (Pop2; south, Algarve). In diploids, moderate to high levels of haplotype (Hd) and nucleotide diversity (π) were detected in *L. nydeggeri* and *L. ovalifolium*. In tetraploids, *L. multiflorum* had higher numbers of haplotypes than *L. binervosum* and *L. dodartii,* the latter of which whose populations showed lower cpDNA haplotype variability. One haplotype (H3) was exclusive to *L. dodartii* (Pop13 and Pop14; southwest Alentejo) and just one haplotype (either H1 or H2) was found in each of the *L. binervosum* populations (Pop10, Pop11, Pop12; west, Estremadura). Haplotype H1 was detected in all species of *L. binervosum* complex (albeit not in all populations) in addition to being present in some populations of the *L. ovalifolium* species complex (i.e., *L. ovalifolium* and *L. nydeggeri*).

4.2. Network relationships among haplotypes and genetic differentiation among complexes

Median joining analysis of species from both complexes evidenced that there was no haplotype specific to species, ploidy level and/or geographic distribution (Figure 28). No clustering was observed when considering both species complexes together although some haplotypes were more frequent (e.g. H1, H9 and H11). The genetic differentiation coefficient (Gst) among populations was 0.4431.

The AMOVA (Table 19) demonstrated that most of the variation (54.58%) was explained by within population cpDNA variation. Notwithstanding we tested

two different groupings, and for both cluster 1 (defining different complexes) and cluster 2 (grouping by species), F_{CT} values were low. For the first grouping F_{CT} was 0.17187, 17.19% of the variation was explained among groups, 32.99% among populations within groups, and 49.82% within populations. For the second grouping F_{CT} was 0.2033, 20.34% of variation was explained among groups, 26.85% among populations within groups, and 52.82% within populations. Moreover, Tajima's D neutrality tests were non-significant -1.46795 ($P < 0.10$).

4.3. Reproductive studies in diploids and tetraploids

In both *L. ovalifolium* and *L. multiflorum*, regular, hypogynous and pentamerous flowers were found. Each flower presented a synsepalous calyx, a sympetalous corolla, five antepetalous stamens and a superior pentacarpellary unilocular gynoecium. There were differences between species in terms of pollen-stigma morphs (Figure 29). In *L. ovalifolium* plants featuring flowers showing stigmas with polygonal cells (*cob*) and pollen with macroreticulate exine (self- incompatible A combination – Baker 1948; Figure 29.A and 29.C), or stigmas with papillate (*pap*) cells and pollen with microreticulate exine (self-incompatible B combination; Figure 29.B and 29.D) were detected. In contrast, in *L. multiflorum* stigmas with *cob* or *pap* cells were present (Figure 29.E-F) but pollen grains exhibited a collapsed morphology without the typical exine patterns (Figure 29.G-H). Therefore, in this later species, we were unable to attribute a specific pollen-stigma morph. Furthermore, in *L. ovalifolium*, empty anthers were seldom found, whereas *L. multiflorum* had many flowers with empty anthers (2-3) and sometimes flowers with no pollen at all were detected. *L. ovalifolium* pollen grains showed very high viability (about 97%), but only low to moderate levels of pollen grains viability (0-69%) were exhibited for *L. multiflorum* plants.

Pollination experiments in both self-incompatible A-type and B-type *L. ovalifolium* plants showed that scapes produced on average a much lower amount of seeds (< 2 seeds, n=6) than *L. multiflorum* (> 100 seeds, n=6) (Table 20). No significant differences in seed production were found between *L. ovalifolium* plant types considering both control and bagged plants (Table 20; p

> 0.929, α < 0.05). However, significant differences between *L. ovalifolium* and *L. multiflorum*, in terms of the amount of seeds produced either in control and bagged scapes were detected (Table 20; p = 0.044, α < 0.05). In both species, the percentage of seed germination was similar (~ 55%).

Cross-pollination experiments between pairs of self-incompatible *L. ovalifolium* plants (A x B combinations) resulted in the production of a small amount of seeds on average (Table 20) with a moderate percentage of germination (62%).

4.4. Ovule development and gamete formation

In both *L. ovalifolium* and *L. multiflorum* unopened flowers with less than 2.5 mm exhibited five common primordia which were initiated simultaneously on a flattened apex after calyx initiation (data not shown). Then, the gynoecial primordia reached continuity and developed subsequently into five distinct styles and stigmata, and thereafter, the remaining apex was transformed into the single ovule primordium (Figure 30.A). During development of the unique basal ovule, a long and slender funicle was developed around it (Figure 30.B-C) and the ovule underwent a curvature of 360 º (ovule circinotropous) (Figure 30.D). In the mature ovule, the micropyle pointed away from the basal placenta towards the top of the ovary and was in close contact to the obturator (Figure 30.E-F). This transmitting tissue protruded from the roof of the ovary into the unilocular superior ovary (data not shown). In general, a single ovary yielded just one single basal ovule but ovaries with two ovules were occasionally found.

During the development of ovules, megasporogenesis and megagametogenesis took place to generate female gametophytes of sexual and/or apomictic origin. For both *L. ovalifolium* and *L. multiflorum* c. 700 ovules were examined in this study, and the first meiosis was always observed in unopened flowers with less than 2.5 mm. In *L. ovalifolium* tetrasporic embryo sac development of the *Gagea ova* – type was the most common (> 85%) (Figure 31). In brief, at the beginning of meiotic interphase, megasporocytes showed nuclei with two nucleoli (Figure 31.A) but soon progressed to prophase I where only one nucleolus was visible (Figure 31.B). Then, female gametophytes exhibited two nuclei in division, not partitioned into separate cells

(Figure 31.C), and following meiosis II, a linear tetrad was formed (Figure 31.D). At the beginning of gametogenesis, the coenomegasporocyte attained a bipolar 1 + 3 nuclear arrangement, with one nucleus in a micropylar-most position, with the other three located towards the chalazal pole (Figure 31.E). At this stage the gametophyte exhibited prominent vacuoles between each nucleus, and no cytokinesis or nucleus degeneration occurred. At the micropylar pole the single megaspore nucleus underwent a nuclear mitotic division, while at the chalazal pole the three megaspore nuclei fused to produce a triploid restitution nucleus which further divided into a secondary tetranucleate embryo sac (Figure 31.F). Thereafter, the nucleus at the micropylar-most pole divided again, originating the cells of the egg apparatus, the synergids and egg cell, respectively, while the chalazal pair of nuclei divided no further. Thus the mature embryo sacs were 6-nucleate (Figure 31.G). Other tetrasporic-origin embryo sacs were also produced, namely of the *Adoxa* – (Figure 31.H-I) and *Drusa*-types (Figure 31.J). Occasionally, unreduced, diplosporic (apomictic) embryo sacs of *Rudbeckia*–type were formed (data not shown).

By contrast, *L. multiflorum* produced diplosporous (apomictic) meiotically-unreduced eggs of the *Rudbeckia*-type. During meiosis I prophase cells showed a single nucleolus (Figure 32.A) and metaphase plates showed bivalent and univalent chromosomes scattered over the spindle (Figure 32.B). After anaphase I, a reconstitution nucleus was formed, irregular in shape, slightly elongated and somewhat constricted in the middle (Figure 32.C). This was followed by progression of the restitution nucleus through a mitotic anaphase, which in most cases formed a tripolar spindle (Figure 32.D). Most embryo sacs at the 2-nucleate stage showed one nucleus with one big nucleolus positioned at the micropylar end and another bi-nucleolated nucleus at the most chalazal end (Figure 32.E). However, other nuclear arrangements within embryo sacs were also found, including for example those with two bi-nucleolated nuclei. After a karyokinetic nuclear division at the 2-nucleated stage, a 4-nucleate gametophyte was produced (Figure 32.F). In the micropylar region, the egg cell apparatus (egg cell; two synergids; upper polar nucleus) was formed after a second mitotic division of the these nuclei (Figure 32.G). The central part of the embryo sac contained more than one protoendospermatic cell (four in the case

illustrated, Figure 32.G), and at the chalazal-most region a variable number of antipodal cells with two nucleoli each were usually present (Figure 32.H). The ultimate development of the egg cell appeared to occur autonomously, as no pollen tubes were observed at the micropyle nor at the extension of the gametophyte, and the first developmental stages of the embryo proceeded with perfectly intact synergids and polar nuclei (Figure 32.I).

5. Discussion

5.1. Phylogeographic relationships

In this study, cpDNA data reveal low genetic differentiation between distinct *Limonium* species' complexes, i.e. *L. ovalifolium s.l.* and tetraploid *L. binervosum s.l.* as discriminated by descriptive morphological data (Erben 1978, 1993) and biometric surveys (Róis *et al.* 2013). Remarkably, these genetic data are concordant with previous genetic studies (MSAP analysis) on these species in which only modest levels of genetic and epigenetic variation and differentiation were found (Róis *et al.* 2013). A genetic comparison among *L. ovalifolium* species *s. l.* shows that *L. nydeggeri* is the most differentiated species of the group, although differentiation between each species' populations is generally low. Comparisons among species revealed that high Hd and low π levels occurred in most *L. ovalifolium, L. nydeggeri* and *L. lanceolatum* populations, except for a population (Pop2) of the latter species, which shows no differentiation at all (Table 18). Noticeably, few species-specific haplotypes but elevated haplotype sharing is also observed among species of this complex. Moreover, the networks obtained for both complexes reveal no indication of grouping according to species ploidy level, or geographic distribution. In fact, three core haplotypes (H1, H9 and H11) are spread throughout almost all the populations from both species complexes. cpDNA haplotype sharing by geographically distant populations of species from the same complex, like *L. nydeggeri* in Estremadura (Pop4) and *L. ovalifolium s. s.* in Algarve (Pop9), suggests that the most representative haplotype (H1) could be ancestral (Figure 27). In apomictic complexes common ancestry followed by sequence divergence has been observed, for example, in the *Boechera holboellii* complex (Sharbel and Mitchell-Olds 2001; Dobeš *et al.* 2004b; Kiefer

et al. 2009) and in the *Ranunculus auricomus* complex (Paun *et al.* 2005; Hörandl *et al.* 2009). Alternatively, some species could be so young that differentiation between haplotypes has not yet occurred. When specifically considering *L. nydeggeri*, which was sampled in all known populations along its distribution range, cpDNA sequences did not provide enough resolution to allow reconstruction of the genealogical relationships among their members.

Genetic comparisons between tetraploid species of *L. binervosum* complex indicate low cpDNA haplotype diversity overall (Table 18). This has been frequently observed for apomictic species whose genetic variability can range from little to none (due to founder effects and to asexual reproduction) to levels that are comparable to diploid sexual species (due to facultative sexuality or backcrossing between apomictic lineages with sexual backgrounds) (Paun *et al.* 2006). The AMOVA data here support the hypothesis that haplotypes aren't geographically structured across the range of the *L. binervosum* species complex. Predictably, most populations share one haplotype (H1), although *L. dodartii* has populations with a distinctive haplotype (H3) not shared by any of other species of the complex. Notwithstanding we tested the significance of genetic variance partitioning resulting from different groupings, our results suggest that genetic variability in both complexes is best explained at intra-population level. Interestingly, cpDNA haplotypes are shared between species with distinct ploidy levels from different complexes, like *L. binervosum* and *L. nydeggeri* (e.g. Pop7 and Pop10), or even sympatric populations of species from distinct complexes, such as *L. nydeggeri* and *L. multiflorum* (Pop5 and Pop16 – in Cabo Raso) (Table 18). These data indicate hybridization between populations, as observed for for *Boechera* and *Ranunculus* spp. (Dobeš *et al.* 2004b; Paun *et al.* 2005, Lovell *et al*. 2013).

5.2. Reproductive findings in diploid and tetraploids

Our results on floral heteromorphies, female gametophyte development and pollination experiments further demonstrate distinct reproduction modes in diploid and tetraploid *Limonium* spp.. Although both *L. ovalifolium* and *L. multiflorum* plants exhibit regular pentamerous flowers commonly found in *Limonium* (de Laet *et al.* 1995), *L. ovalifolium* displayed dimorphic flowers

confirming earlier descriptions (Erben 1978). By contrast, *L. multiflorum* exhibited regular stigmas but collapsed pollen grains without the typical exine patterns, as described previously (Róis *et al.* 2012). Indirect estimates of *Limonium* breeding systems based on pollen-stigma dimorphisms (Baker *et al.* 1953ab; Baker 1966; Richards 1997) show that species presenting self-compatible flower monomorphic populations are regarded as selfers (e.g. *L. dodartii*), dimorphic populations are outcrossers (e.g. *L. ovalifolium*) (Erben 1978), and self-incompatible monomorphic populations are deemed apomictic (e.g. *L. binervosum*) (Baker 1966; Erben 1978; Cowan *et al.* 1998). Here we show for the first time that *L. ovalifolium* mostly forms meiotically reduced tetrasporic embryo sacs of the *Gagea ova*-type (Romanov 1957), although tetrasporous female gametophytes of *Adoxa*- and *Drusa*-types are also found as earlier reported for other *Limonium* species (Dahlgren 1916; D'Amato 1949; Hjelmqvist and Grazi 1964). Moreover, occasionally unreduced, diplosporic (apomictic) embryo sacs of the *Rudbeckia*–type (Battaglia 1946) were formed. These data are consistent with the pollen exclusion tests we realized in *L. ovalifolium*, which form a very small amount of seeds of apomictic origin, demonstrating that it may reproduce through facultative apomixis. However, compared to plants from field origin in which a higher number of seeds per scape are obtained (~ 100; Róis *et al.* 2012), the number of seeds produced in control plants from experimental collections is much lower (this work). Moreover, since in bagged scapes seeds are produced in the absence of self-compatible pollen, the present data set support that pollen-independent (i.e. autonomous) endosperm formation might occur. The other cases of apomixis known at the diploid level include the genus *Boechera* (Koltunow and Grossniklaus 2003) and some species of *Paspalum* (Siena *et al.* 2008), and this study adds *L. ovalifolium* to this list.

Contrastingly, in *L. multiflorum* meiotically-unreduced, diplosporous (apomictic) embryo sacs of the *Rudbeckia*-type (Battaglia 1951) are produced and autonomous endosperm formation is observed. In other polyploid apomicts such as *Statice oleaefolia* var. *confusa*, the formation of reduced embryo sacs of the *Adoxa*-type occurs in parallel with unreduced apomictic embryo sacs of the *Erigeron*–type (facultative apomixis; D´Amato 1949). Also, in polyploid

Limonium transwallianum Pugsley reduced tetrasporous embryo sacs of the *Fritillaria*- and *Drusa*-types, and unreduced diplosporous female gametophytes are formed (Hjelmqvist and Grazi 1964). Moreover, pollen exclusion tests reveal that *L. multiflorum* forms a moderate to high set of viable seeds per scape, even in the presence of pollen grains with various morphological abnormalities. Therefore, we hypothesize that endosperm formation is also pollen-independent.

Interestingly, a common origin between apomixis and tetraspory has been hypothesized based on the identification of similar cytological characteristics during ovule development (Carman *et al.* 1991, 1997; Peel *et al.* 1997ab). For example, both tetrasporic and diplosporic embryo sacs form precociously due to ’precocious gametophytization’ of the megaspore mother cell (MMC) (Battaglia 1989), meaning that MMCs undergo vacuolization which normally occurs later on in the surviving megaspore (Crane and Carman 1987), as we also detected here.

5.3. Reproductive mode influence on cpDNA haplotype patterns in diploid and tetraploids

Here we show that higher levels of cpDNA haplotype variation observed in *L. ovalifolium* compared to *L. multiflorum* natural populations could be explained considering their main reproduction modes. Considering that in *L. ovalifolium* populations, single diploid individuals could have both sexual and asexual seed production (i.e. facultative apomixis), this phenomenon can lead to an increase of genetic diversity by continuous formation of new cytotypes as observed for example, in the *Ranunculus auricomus* complex (Hörandl *et al.* 2009). Remarkably, in *L. ovalifolium* populations, aneuploid diploids (2*n*=2x=15, 16, 17) occur (Róis *et al.* 2012), and this instability of cytotypes might have arisen because of facultative sexual processes or incomplete sexuality. Unfortunately, previous flow cytometric seed screening studies in this species did not allow the determination of their reproduction modes since each mature seed profile was only characterized by a single DNA peak, corresponding to the embryo peak only (Róis *et al.* 2012). Therefore, we were unable to quantify sexual versus apomixis seed production in this species.

In contrast, decreased genotypic variability in natural populations *L. multiflorum* apomicts could be due to apomixis fixation of ancestral genotypes, or frequency-dependent selection for the most fitted genotypes (Dickinson and Campbell 1991; van Dijk 2003). Although individual populations of agamosperms tend to be homogeneous in comparison to those of sexual species, allozyme and DNA marker studies have demonstrated they could be highly polyclonal within their native range (e.g. Ellstrand and Roose 1987; van Dijk 2003). In triploid asexual dandelion lineages (Verhoeven *et al.* 2010) and in the tetraploid *L. binervosum* complex, higher levels of epigenetic variability are found than in diploid *L. ovalifolium* species complex (Róis *et al.* 2013).

Moreover, cpDNA haplotypes shared by *L. ovalifolium s. l.* and *L. binervosum* species (e.g., *L. multiflorum*; e.g. H1) indicate that hybridization between species with facultative sexuality and apomictic lineages could occur. *L. ovalifolium* balanced diploids can produce meiotically-reduced and non-reduced pollen grains which usually display high viability (Róis *et al.* 2012). Instead, *L. multiflorum* forms non-reduced pollen with several abnormalities which lack viability (Róis *et al.* 2012). Thus, as demonstrated in other apomictic complexes (e.g., *Ranunculus* and *Boechera*) (Koch *et al.* 2003; Hörandl and Paun 2007; Kiefer *et al.* 2012), gene flow via pollen between populations could be the most plausible mechanism for *Limonium* new apomictic lineages are arise, either through hybridization or by facultative sexuality.

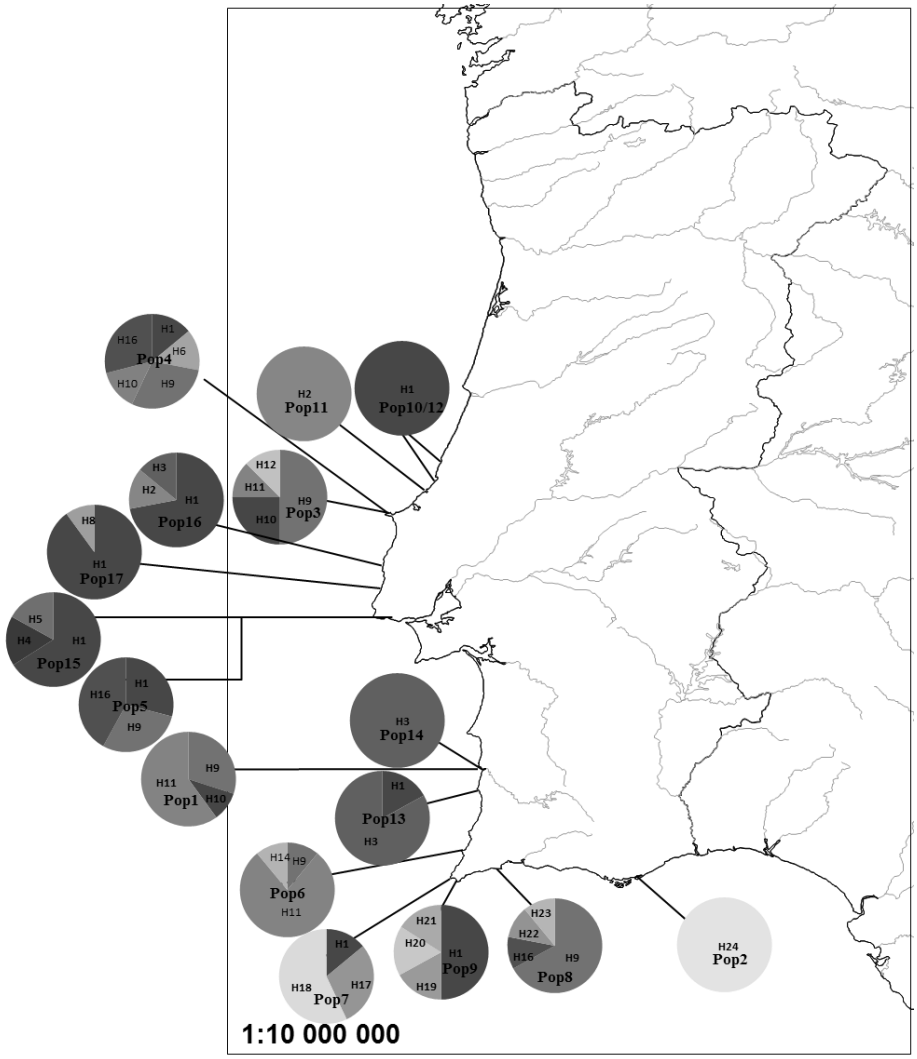

Figure 27. **Geographic distribution of the 24 cpDNA haplotypes in populations of *Limonium ovalifolium* and *L. binervosum* species' complexes.**

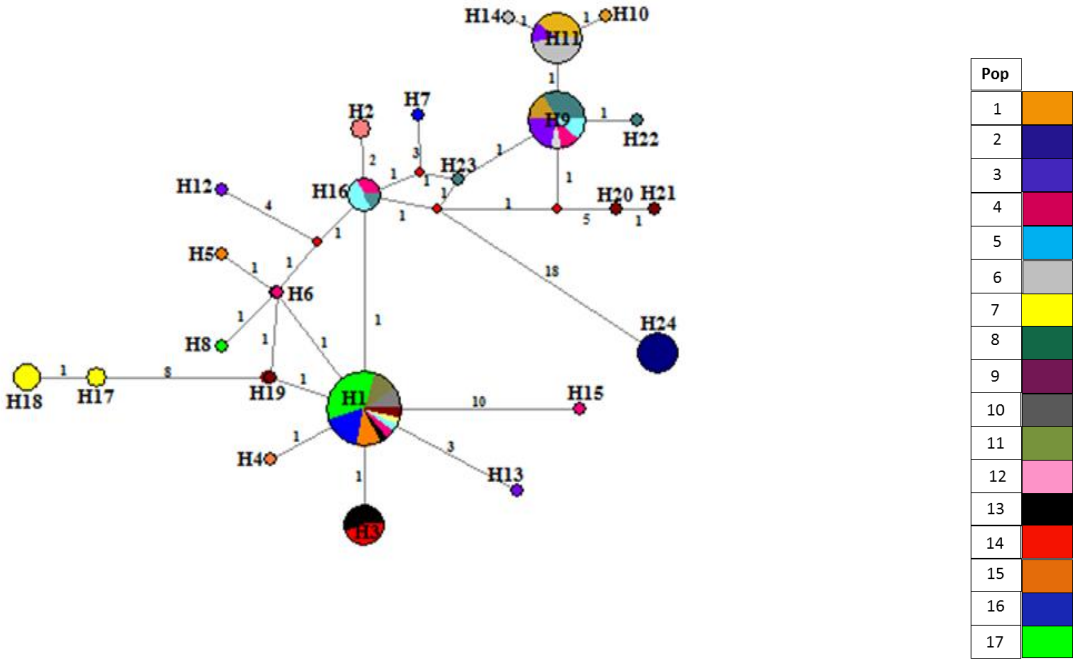

Figure 28. Median-joining network of the two cpDNA sequence regions (intron *trnL* and intergenic spacer *trnL* - *trnLF*). The circles represent populations and circle size is proportional to haplotype frequencies. Each color represents each of the 17 populations. The number of mutations required to explain transitions among haplotypes is indicated along the lines connecting the haplotypes.

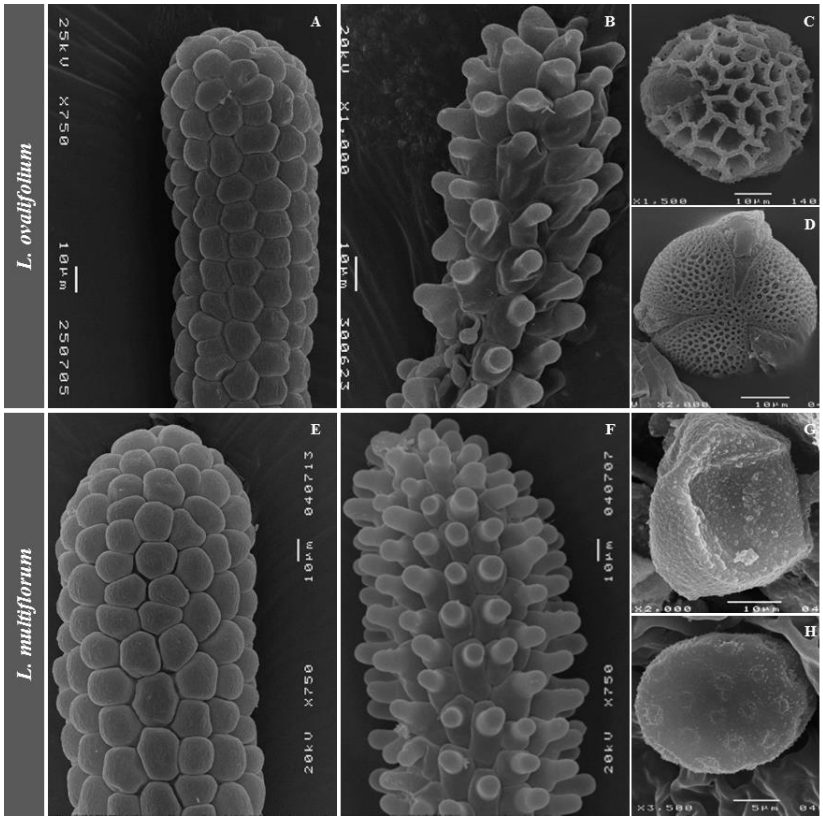

Figure 29. **Scanning electron microscopy (SEM) photographs of *Limonium ovalifolium* and *L. multiflorum* stigmas and pollen grains.** *Limonium ovalifolium* (A–D); A-B Papillate and *cob*-type stigma, respectively; C. Pollen grain with coarsely reticulate exine surface with a large reticulum (polar view); D. Pollen grain with finely reticulate exine surface (polar view); *L. multiflorum* (E–H); E-F Papillate and *cob*-like stigmas, respectively; G. Bilateral pollen grains showing perforate exine surface with *colpi* position denounced; H. Pollen grains with verrucate exine surface.

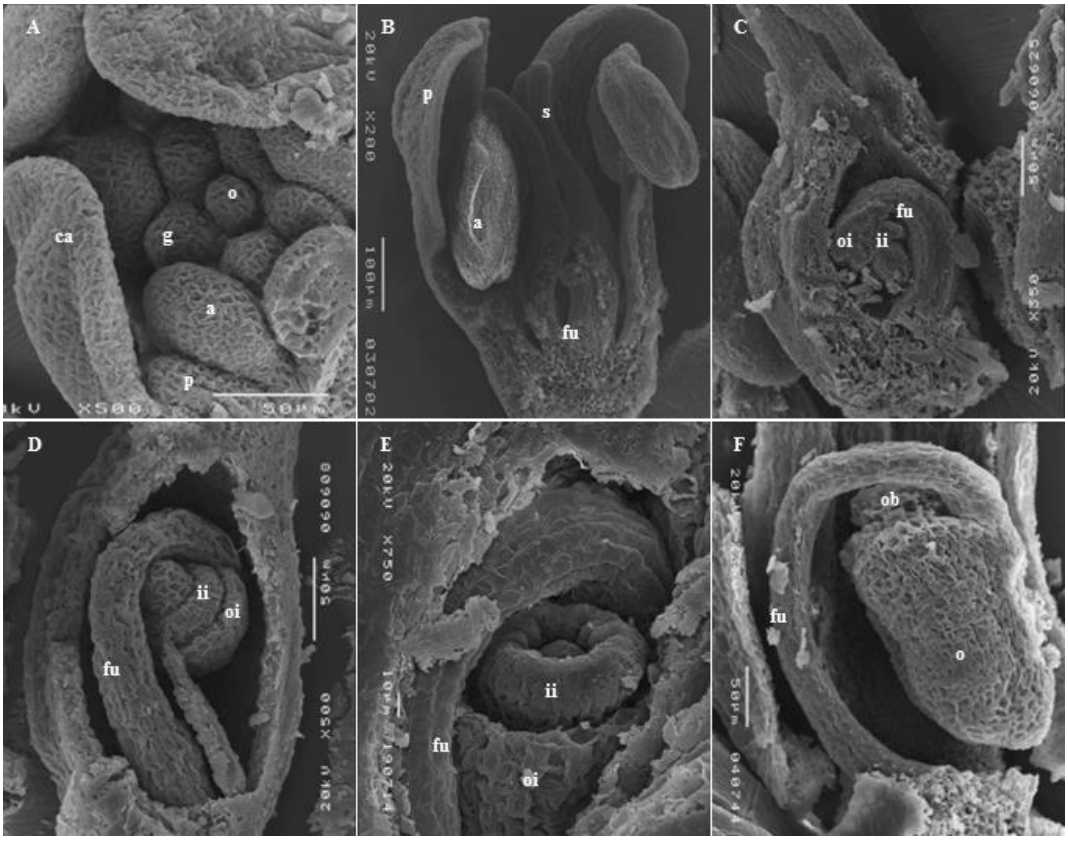

Figure 30. Scanning electron microscopy (SEM) photographs of floral and ovule development in *Limonium multiflorum* and *L. ovalifolium*. A. Five continuous gynoecial primordial and one ovule primordium in *L. multiflorum*; B. Lateral view of a young flower with superior ovary, anthers, filiform stigmas and the single developing *L. multiflorum* ovule; C. Young *L. multiflorum* ovule with a curvature of 180º exhibiting outer integument, inner integument and funicle; D-E Slightly older ovule of *L. ovalifolium* with a curvature of 360º; F. Mature ovule from *L. ovalifolium* with the obturator protuding from the roof of the ovary. *Figure abbreviations*: a – anther; ca – calyx; fu – funicle; g – gynoecial primordium or gynoecium; ii – inner integument; o – ovule; ob – obturator; oi – outer integument; p – petal; s – stigma.

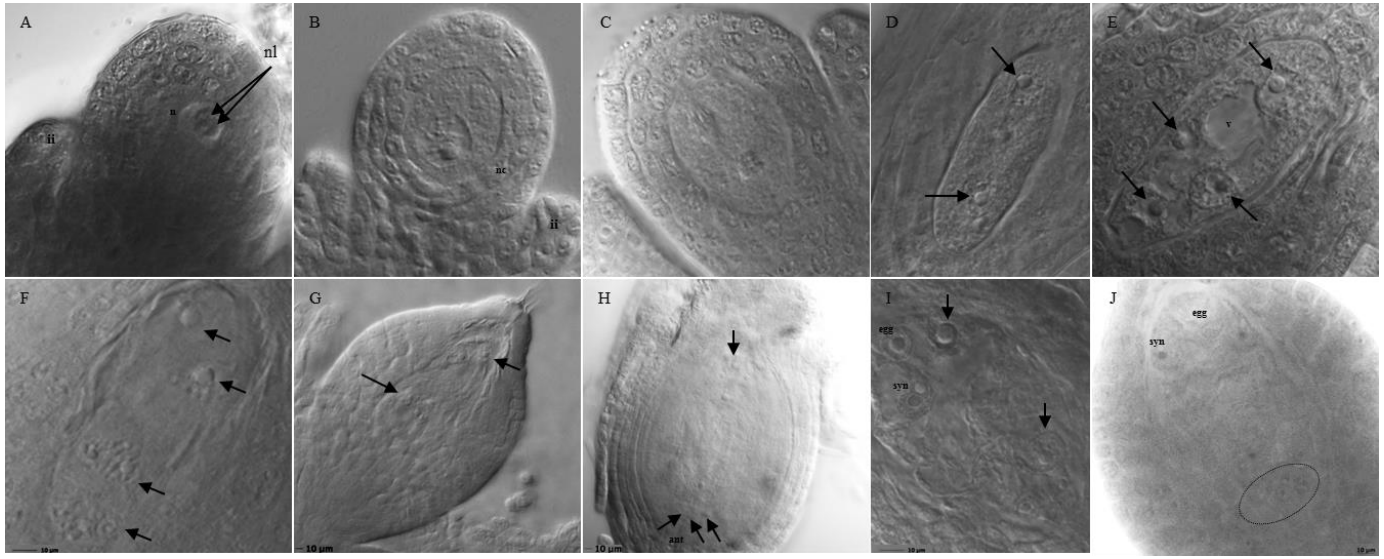

Figure 31. **Megaspore and embryo sac formation in *Limonium ovalifolium* (differential interference contrast; DIC).** A. Megaspore mother cell at interphase with two nucleoli (arrowed); B. Megaspore mother cell in meiosis I (prophase I) showing a single nucleolus at the chalazal pole; C. Two nucleus in division (meiosis II; anaphase II) not partitioned into a separate cell; D. Coenomegaspore with four reduced nuclei, each nucleus with a single nucleolus (arrowed) (4-nucleated stage; tetrasporic development); E. Megaspores in a bipolar 1 + 3 arrangement (arrowed) with the embryo sac showing extended vacuolization (v); F. Embryo sac at secondary tetranucleate stage upon division of the restitution nucleus (mono-nucleolated nuclei – micropylar pole - and tri-nucleolated nuclei – chalazal pole – are arrowed); G. 6-nucleate embryo sac; the lowest nuclei of the secondary tetranucleate stage do not further divide (*Gagea ova*–type); H. Adoxa-type embryo sac (antipodals are arrowed); I. Detail of an embryo sac of Adoxa-type with superior and inferior polar nuclei arrowed; J. Drusa-type embryo sac (the dashed circle point to cells at the chalazal pole). Each image is oriented so that the micropyle is towards the top of the page and the chalaza is towards the bottom. Figures abbreviations: ant – antipodals; egg - egg cell; ii – integuments; n – nucleus; nl – nucleolus; nc – nucellus; syn – synergid; v – vacuole.

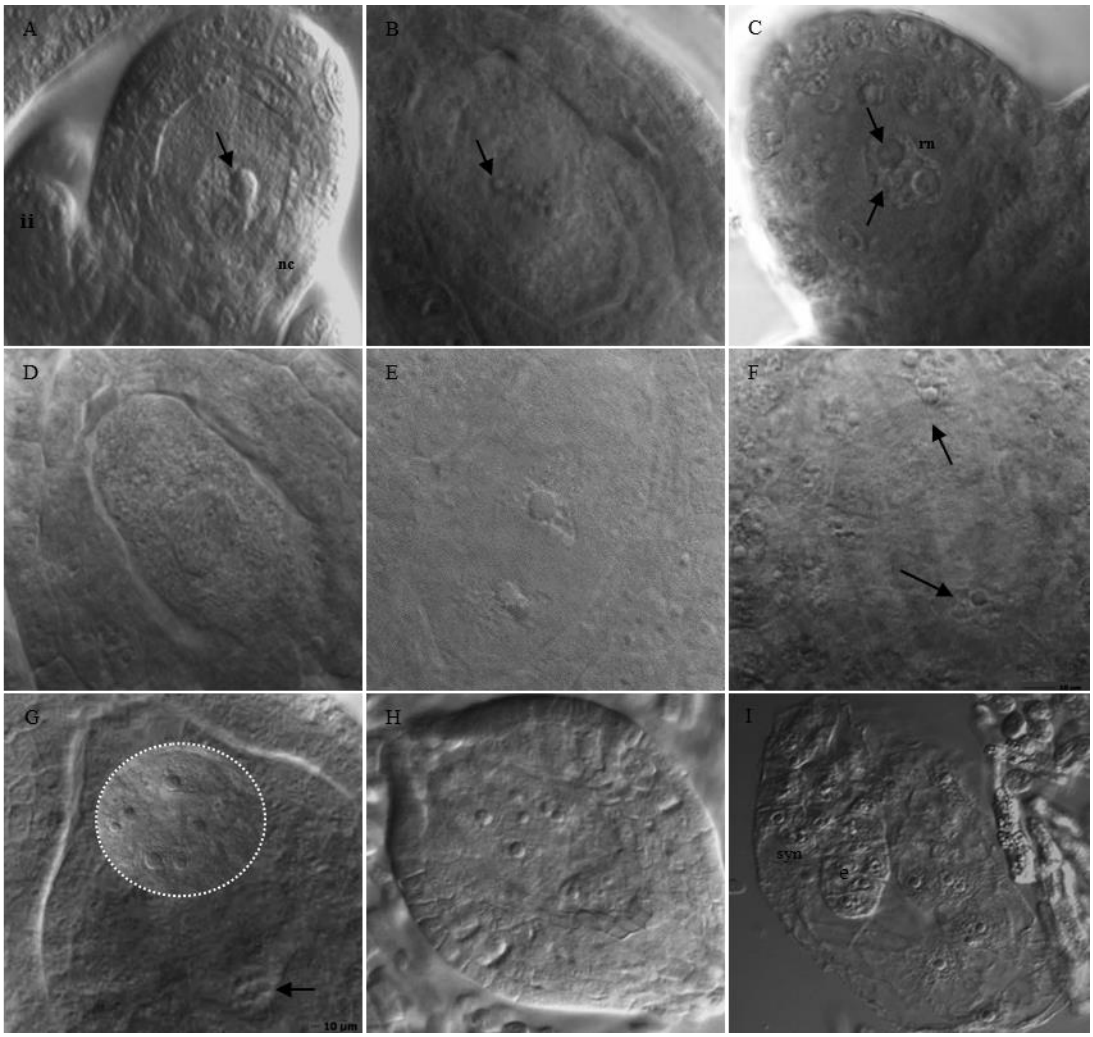

Figure 32. **Megaspore and embryo sac formation in *Limonium multiflorum* (differential interference contrast; DIC).** A. Megasporocyte within ovule showing one nucleolus; B. First metaphase exhibiting lagged chromosomes (arrowed); C. Restitution nuclei after first meiotic division (meiotic diplospory). Nucleoli with distinct dimensions within the same nucleus are arrowed; D. Tripolar spindle after restitution nucleus formation; E. 2-nucleate stage embryo sac resulting from mitotic-like division of megasporocyte showing one nucleus with one nucleolus at the micropylar most position and another nucleus with two nucleoli at the chalazal pole; F. Embryo sac at 4-nucleate stage resulting from mitotic-like division of megasporocyte (spindles are arrowed); G. Detail of the egg cell apparatus (encircled by dashed lines) in a mature embryo sac; antipodal cells is arrowed; H. Mature embryo sac of *Rudbeckia*- type; I. Parthenogenetic embryo formation within a mature sac.

Each image is oriented so that the micropyle is towards the top of the page and the chalaza is towards the bottom. *Figure abbreviations*: e – embryo; egg - egg cell; ii – integuments; ipn – inferior polar nucleus; rn – restitution nucleus; nc – nucellus; syn – synergid.

Table 16. Natural *Limonium* populations surveyed in four Portuguese provinces. Species were sampled in Beira Litoral (west), Estremadura (west), Alentejo (southwest) and Algarve (south).

Ploidy level	Species	Habitat	Site Location/ Collectors*/ Population Number/ Province	Geographic coordinates
Diploid	*L. lanceolatum*	Salt-marshes	Odemira, Vila Nova de Milfontes ADC, APP, ASR / Pop1/Alentejo	37.727756/-8.770931
			Tavira, Sapal do Barril ADC,APP, ASR/ Pop2/Algarve	37.0861361/-7.662772
	L. nydeggeri	Sea-cliffs	Peniche, Ilha do Baleal ADC, APP, ASR/ Pop3/Estremadura	39.378919/-9.340983
			Peniche, Nossa Srª dos Remédios ADC, APP, ASR/ Pop4/Estremadura	39.369906/-9.395731
			Cascais, Cabo Raso ADC, APP, ASR/ Pop5/Estremadura	38.710039/-9.485883
			Aljezur, Pontal da Carrapateira ADC, APP, ASR/ Pop6/Algarve	37.195039/-8.911103
			Vila do Bispo, Cabo de São Vicente ADC, ASR/Pop7/Algarve	37.022611/-8.996564
	L. ovalifolium	Sea-cliffs	Lagos, Praia da Luz ADC, ASR/Pop8/Algarve	37.087442/-8.729094
			Vila do Bispo, Cabo de Sagres ADC, APP, ASR/ Pop9/Algarve	36.994242/-8.948756
Tetraploid	*L. binervosum*	Sea-cliffs	Cabo Mondego, Figueira da Foz ADC, APP, ASR, SM/ Pop10/Beira Litoral	40.191111/-8.905278
			Praia da Concha, Marinha Grande ADC, APP, ASR, SM/ Pop11/Beira Litoral	39.755278/-9.032281
			Salir do Porto, Caldas da Rainha ADC, APP, ASR, SM/ Pop12/Beira Litoral	39.49843055/-9.17065
	L. dodartii	Sea-cliffs	Odemira, Cabo Sardão ADC, APP, ASR, SM, PA/ Pop13/ Alentejo	37.598631/-8.816056
			Odemira, Porto Covo ADC, APP, ASR, SM, PA/Pop14/ Alentejo	37.851667/-8.791944
	L. multiflorum	Sea-cliffs	Cascais Cabo Raso ADC, APP, ASR/ Pop 15/ Estremadura	38.710039/-9.485883
			Lourinhã, Vale dos Frades ADC, APP, ASR/ Pop16/ Estremadura	39.276506/-9.335839
			Mafra, Foz do Lizandro ADC, APP, ASR/ Pop17/ Estremadura	38.941531/-9.415233

*Abbreviations of collectors: ADC, AD Caperta; APP, Ana Paula Paes, CBAA, ISA; AS Róis; P Arsénio, CBAA, ISA; SM, Sérgio Martins, CBAA, ISA.

Table 17. The intron *trnL* and intergenic spacer *trnL – trnLF* sequence haplotypes of *Limonium ovalifolium* and *L. binervosum* species' complexes.

	Nucleotide position																												
Haplotype	16	76	79	88	89	103	116	132	180	222	226	_	246	265	_	282	310	314	320	322	323	334	360	367	390	396	407	415
H1	G	A	A	A	T	G	A	A	G	A	A	_	G	A	_	G	A	A	A	G	A	A	G	T	C	G	A	C
H2	T	.	.	.	.	.	C	.	T	.	.	_	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H3	.	.	.	.	.	.	.	.	.	.	.	_	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H4	.	.	.	.	.	.	.	.	.	.	.	_	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H5	.	.	.	.	.	.	.	.	.	.	.	_	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H6	.	.	.	.	.	.	C	.	.	.	.	_	.	.	_	.	.	.	.	A	.	.	T	.	.	A	.	.
H7	.	.	.	.	.	.	C	.	.	.	.	_	.	.	_	.	.	.	C	A	T	G	.	.	.	.	.	.
H8	.	.	.	.	.	.	.	.	.	.	.	_	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H9	.	.	.	.	.	.	C	.	.	.	.	_	.	.	_	.	.	.	.	A	.	.	T	.	.	A	.	.
H10	.	.	.	.	.	.	.	T	.	.	.	_	.	.	_	.	.	.	.	A	.	.	T	.	.	A	.	.
H11	.	.	.	.	.	.	.	.	.	.	.	_	.	.	_	.	.	.	.	A	.	.	T	.	.	A	.	.
H12	.	.	.	.	.	.	C	.	.	C	C	_	.	.	_	.	.	T	.	.	.	.	.	.	.	.	.	.
H13	.	.	.	.	.	.	.	.	.	C	C	_	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.
H14	.	C	C	.	C	.	.	.	.	.	.	_	.	.	_	.	.	.	.	A	.	.	T	.	.	A	.	.
H15	.	.	.	.	.	C	.	C	.	.	.	_	.	.	_	.	C	.	.	.	.	.	.	.	.	.	.	.
H16	.	.	.	.	.	.	C	.	.	.	.	_	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.
H17	.	.	.	.	.	.	.	.	.	.	.	_	.	G	A	.	C	.	.	.	.	.	.	.	.	.	.	.
H18	.	.	.	.	.	.	C	.	.	.	.	_	.	.	_	.	C	.	.	.	.	.	.	.	.	.	.	.
H19	.	.	.	.	.	.	.	.	.	.	.	_	.	.	_	.	C	.	.	.	.	.	.	.	.	.	.	.
H20	.	.	.	.	.	.	C	.	.	.	.	_	.	.	_	.	.	.	.	.	.	.	T	.	.	A	T	.
H21	.	.	.	.	.	.	.	.	.	.	.	_	.	.	_	.	.	.	.	.	.	.	T	.	.	A	T	.
H22	.	.	.	G	.	.	C	.	.	.	.	_	.	.	_	.	.	.	.	A	.	.	T	.	.	A	.	.
H23	.	.	.	.	.	.	C	.	.	.	.	_	.	.	_	.	.	.	.	A	.	.	T	.	.	.	.	.
H24	.	.	.	.	.	.	C	.	.	.	.	A	A	.	_	C	.	.	.	.	.	.	T	A	A	.	.	T

Table 16. Continued.

	Nucleotide position																														
Haplotype	416	420	421	425	_	427	439	444	460	463	470	475	477	480	483	489	490	491	495	498	504	509	514	517	522	525	538	541	565	619
H1	A	T	C	T	_	A	T	T	C	T	A	T	G	T	G	C	A	T	T	A	A	T	C	T	G	A	A	C	A	A
H2	.	.	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H3	.	.	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
H4	.	.	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
H5	.	.	.	.	_	.	.	.	.	A	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H6	.	.	.	.	_	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H7	.	.	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H8	.	.	.	.	_	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H9	.	.	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H10	.	.	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H11	.	.	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H12	.	.	.	.	_	.	.	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H13	.	.	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H14	.	.	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H15	T	.	.	.	_	.	A	.	.	C	.	A	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H16	.	.	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H17	.	.	.	.	_	C	.	.	.	A	T	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	G	G
H18	.	.	.	.	_	C	.	.	.	A	T	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	G	G
H19	.	.	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H20	T	.	.	A	_	.	.	.	G	.	A	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.
H21	T	.	.	A	_	.	.	.	G	.	A	.	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	.
H22	.	.	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H23	.	.	.	.	_	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H24		G	A		G	.	.	.	.	.	.	.	.	.	.	A	T	G		T		T	C	G	.	.	A	G	.	.

Table 18. cpDNA diversity within populations of *Limonium ovalifolium* and *L. binervosum* species' complexes. Pop – population; N – number individuals analyzed; H (%) - percentage of each haplotype; Hn - number of haplotypes present; Hd (SD) - Haplotype diversity and standard deviation; π - nucleotide diversity.

		Species	Pop	N	H (%)	Hn	Hd (SD)	π
Whitin Populations	Diploids	*L. lanceolatum*	Pop1	10	H9 (30); H10 (10); H11 (60)	3	0.600(0.131)	0.0010
			Pop2	9	H24 (100)	1	0	0
		L. nydeggeri	Pop3	8	H9 (50); H11 (25); H12 (12,5); H13 (12,5)	4	0.750(0.139)	0.0051
			Pop4	7	H1(14); H6 (14); H9 (29); H15 (14); H16 (29)	5	0.905(0.103)	0.0080
			Pop5	7	H1(29); H9 (29); H16 (42)	3	0.762(0.115)	0.0013
			Pop6	9	H9 (11); H11 (78); H14 (11)	3	0.417(0.191)	0.0029
			Pop7	7	H1(14); H17 (29); H18 (57)	3	0.667(0.160)	0.0040
		L. ovalifolium	Pop8	9	H9 (67); H16 (11); H22 (11); H23 (11)	4	0.583(0.183)	0.0016
			Pop9	6	H1 (50); H19 (17); H20 (17); H21 (16)	4	0.800(0.172)	0.0068
	Tetraploids	*L. binervosum*	Pop10	2	H1 (100)	1	0	0
			Pop11	3	H2 (100)	1	0	0
			Pop12	2	H1 (100)	1	0	0
		L. dodartii	Pop13	6	H1 (17); H3 (83)	2	0.333(0.215)	0.0005
			Pop14	4	H3 (100)	1	0	0
		L. multiflorum	Pop15	6	H1(86); H4 (17); H5 (17)	3	0.800(0.172)	0.0031
			Pop16	7	H1(72); H6 (14); H7 (14)	3	0.524(0.209)	0.0021
			Pop17	10	H1 (90); H8 (10)	2	0.200(0.154)	0.0006

Table 19. **Analysis of Molecular Variance (AMOVA) of cpDNA sequences.** n.a. - not applicable.

Source of variation	degrees of freedom	Sum of squares	Variance components	Percentage of variation (%)	Fixation indices
Among populations	16	25.058	0.20258	45.42	F_{ST}= 0.45416
Within population	95	23.130	0.24347	54.58	n.a
Total	111	48.188	0.44605	n.a	n.a
Among groups 1	1	5.693	0.08399	17.19	F_{CT} = 0.17187
Among populations within groups	15	19.365	0.16123	32.99	F_{SC}= 0.39839
Within population	95	23.130	0.24347	49.82	F_{ST}= 0.50179
Total	111	48.188	0.48869	n.a	
Among groups 2	5	13.553	0.09375	20.34	F_{CT} = 0.20338
Among populations within groups	11	11.505	0.12376	26.85	F_{SC}= 0.33701
Within population	95	23.130	0.24347	52.82	F_{ST}= 0.47185
Total	111	48.188	0.46099	n.a	

Table 20. Pollen exclusion tests and crossing experiments in *Limonium ovalifolium* and *L. multiflorum* plants. N – Number of individuals analysed; n.a. – not applicable.

Species	Floral morphs type	Average number seeds per scape		N
		Unbagged	Bagged	
Limonium ovalifolium	A	1.5 ± 2.345	2.50 ± 3.507	6
	B	1.67 ± 1.862	0.5 ± 1.225	6
Limonium ovalifolium	Cross A X B	0	27.333 ± 2.517	6
Limonium multiflorum	n.a.	118.5 ± 119.214	106.67 ± 116.397	6

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CHAPTER VII.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The work outlined in the present dissertation represents different approaches to design better conservation strategies of some rare, endemic and 'taxonomically complex groups' (TCGs) of *Limonium* spp. from Continental Portugal. The targets of this study were two different halophyte species' complexes distributed along the Continental Portugal coast, respectively, *L. ovalifolium* and *L. binervosum*. These complexes include *L. ovalifolium senso lato*, *L. nydeggeri* and *L. lanceolatum*, and *L. binervosum senso lato*, *L. dodartii* and *L. multiflorum*, respectively (Erben 1993, 1999; Ingrouille and Stace 1985). Besides different ploidy levels and reproduction modes suggested so far, these species complexes have different distribution areas. *L. binervosum* and *L. dodartii* have an Atlantic distribution, *L. nydeggeri* and *L. ovalifolium* show an Atlantic-Mediterranean distribution, *L. multiflorum* is endemic from the west Atlantic Portugal whereas *L. lanceolatum* is observed in the southwest Mediterranean in both Portugal and Spain (Franco 1984; Lahondère *et al.* 1991; Erben 1993, 1999). In Portugal, plants from these groups are mostly found in sea-cliffs, sometimes in sympatric populations (e.g. *L. nydeggeri* and *L. multiflorum* in Cabo Raso, Cascais), in geographically restricted areas (e.g. *L. multiflorum*, west coast) or in salt-marshes (e.g. *L. lanceolatum,* Sapal do Barril) (Costa *et al.* 2001; Espírito-Santo *et al.* 2012). Although the general descriptions of these species have been recorded (Erben 1978, 1993, 1999), little to non-information was well-known regarding chorology, karyology or reproduction modes. Also, knowledge on population genetic and epigenetic variation, fundamental information for conservation proposes was lacking. An interdisciplinary integration of multiple chorological, demographic, genetic, reproductive and ecological data is thus necessary for the effective conservation of these rare species.

By prospecting the Continental Portugal coastline areas it was possible to gather data on population sizes and distribution of *L. ovalifolium* and *L. binervosum* species' complexes, resulting in an increase of information on new localities of the studied *taxa* as well as more than 200 new specimens deposited in the LISI herbarium and seeds collected for *ex-situ* preservation at ISA in the João de Amaral Seed Bank (JBA). Most of the prospected areas

showed small populations, some of them with less than 10 individuals. Small populations are more vulnerable to demographic, environmental stochasticity and population extinction (Young *et al.* 1996; Menges 2000). The reduction in the number of individuals might be dramatic, being more susceptible to genetic erosion reducing the adaptative capability of a species (Pluess and Stöcklin 2004). Morphometric studies were conducted to provide clear morphological differentiation between species, and we were able to separate clearly all studied species showing closer phenotypic affinity among complexes.

Differences in genome sizes and chromosome numbers in representative species of each species' complexes were found (Róis *et al.* 2012). *L. ovalifolium* populations are mostly composed of diploid cytotypes with 2*n*=16, although aneuploidy cytotypes were also found. While in *L. multiflorum* tetraploid and aneuploid tetraploids cytotypes with 2*n*=32, 34, 35 and 36 were revealed. Little attention has been paid to the chromosome polymorphism found when extensive studies are made of plant populations (Murray and Young 2001), not mentioning the significance of chromosome variation in the context of plant conservation (Avise and Hamrick 1996; Henry 2006). These polymorphisms (eg. inversions, translocations or aneuploidy) have an impact on plant conservation studies, ensuring that maximum genetic variation is retained in species or that reintroduction programmes will not mix plants with different ploidy levels (Young and Murray 2000). Populations with variable chromosome numbers also pose problems in identifying and establishing species relationships (Pillon and Chase 2007; de Lange *et al.* 2008). If in a reintroduction work different cytotypes were unintentionally mixed, the possibility of segregation would take many generations and slow the rate of population decay (de Lange *et al.* 2008). In this study, we observed single diploid individuals and aneuploid diploid populations of the *L. ovalifolium* complex, with both sexual and facultative apomictic seed production and aneuploidy in tetraploids of *L. binervosum* spp. forming new cytotypes. Althought we were not able to quantify sexual versus apomitic seed production due to the limitations of FCSS in the studied species, we detected diploid and triploid seeds in *L. ovalifolium* and different levels of ploidy, from tetraploid to octaploid seeds, in *L. binervosum* ones. Any reintroduction work using these

Limonium species should take this instability of cytotypes, the consequence of incomplete or facultative sexuality, into account for a successful programe.

Understanding the levels and distribution of genetic diversity within and among populations is also a prerequisite for the adaptation to environmental changes for conservation management and to the long-term survival of species (Hamrick *et al.* 1991; Rodriguez *et al.* 2003; Jian *et al.* 2006). Genetic and epigenetic variation patters were investigated through MSAP techniques. Low levels of both genetic and epigenetic variation within and between populations were observed based on this methodology, and tetraploid species presented lower levels of genetic variability than diploid ones. Low levels of genetic diversity have already been reported for other *Limonium* spp., using other molecular markers (e.g *L. dufourii*, Rodríguez *et al.* 2003) and *L. dendroides* (Suaréz-Garcia *et al.* 2009), emphasizing the importance of genetic structuring in describing interspecies delimitation. A higher correlation was found between morphometric and epigenetic differentiation than between morphometric and genetic differentiation. Apomictic *taxa* present conservation problems, including numerous *taxa* where morphological and genetic differentiation is not always correlated causing taxonomic inflation (Isaac *et al.* 2004; Pillon and Chase 2007). Genetic diversity is more slowly recovered in apomictic *taxa* than in their sexually reproducing diploid relatives, eventually, compromising their long-term survival (Richards 2003).

Although the importance of genetic and epigenetic processes in shaping the phenotype structuring among plant species remains vague and largely unexplored (Kalisz *et al.* 2004; Richards 2011), our results suggested that epigenetic variation could be a driver of the observed phenotypic divergence between the *Limonium taxa* through intergenome silencing. Epigenetic mechanisms interact with genetic, physiological and morphological systems, being an important component of organism-environment interactions (Verhoeven *et al*. 2010). DNA methylation, active in the restructuring of the genome in response to polyploidy caused by hybridization (Salmon *et al.* 2005) or in response to environmental stresses (Verhoeven *et al.* 2010), is a source of inter-individual phenotypic variation that can provide an evolutionary and

ecologically important source of phenotypic variation among individuals (Herrera and Bagaza 2010; Paun *et al.* 2010).

In the present dissertation we hypothesized that the low level of genetic diversity observed in diploid and tetraploid *taxa* could be explained by their reproductive modes. Our findings using cpDNA data revealed low differentiation between and among complexes. Few species-specific haplotypes, but elevated haplotype sharing, was observed between species with distinct ploidy levels from diferent complexes, an indication that hybridization could occur between species with facultative sexuality and apomict lineages. This is also observed for *Boechera* and *Ranunculus* spp., indicating possible hybridization between populations (Sharbel and Mitchell-Olds 2001; Paun *et al.* 2005). In *Limonium*, studies of reproduction have been hindered, in part, to difficulties presented by its morphology. For example, a single ovule is present per ovary, and is covered by thick layers of bracts so it is difficult to reach. Hence, although sexuality and/or agamospermy has been suggested in the past (Dahlgren 1916; D'Amato 1949; Hjelmqvist and Grazi 1964), only very few ovule development studies have been conducted in *Limonium* spp.. Here contrasting reproductive modes in diploid *L. ovalifolium* and polyploid *L. multiflorum* for the first time were revealed. In the first species, both tetrasporous (*Gagea* ova-type) and diplosporous (*Rudbeckia*-type) embryo sacs displaying facultative apomixis were observed while in the latter species only diplosporic embryo sacs (*Rudbeckia*-type) were found, implying obligate apomixis (Róis *et al.* 2014, in preparation).

Floral heteromorphies measurements, pollination and crossing tests and cytoembryological analyses in developing ovules taken from greenhouse collections were also performed. *L. ovalifolium* showed dimorphic self-incompatible combinations, and pollen exclusion tests and crossing experiments indicated that most plants were outcrossers, with some of them reproducing through facultative apomixis (Róis *et al.* 2014, in preparation). *L. binervosum* showed self-incompatible monomorphic combinations and thus are apomictic (Baker 1966; Erben 1978; Cowan *et al.* 1998). In *L. multiflorum*, in contrast to what is observed for other apomictic species (Asker and Jerling 1992; Mogie 1992), fertile, meiotically reduced pollen was not produced (Róis *et*

al. 2012), and a tendency to gynodioecism seems to occur, as supported by the existence of many empty anthers and defective meiosis with diverse morphological abnormalities in pollen grains (Róis *et al.* 2014, in preparation). Conversely, *L. ovalifolium* can produce reduced and non-reduced pollen grains, generally with high viability. It is plausible that the genetic variation within and among plant populations can be caused mainly by gene flow through pollen dissemination and/or seed dispersal, and genetic drift leading to inter-clonal diversity (Kimatu et al. 2012). Therefore, at least gene flow via *L. ovalifolium* pollen between populations could be the most plausible mechanism for *Limonium* apomictic lineages to arise, either through facultative sexuality or by hybridization.

The basic steps in species conservation methodologies of TCGs, such as *Limonium* spp., implies combining data and estimations regarding the rarity of the species, information on changes in distribution and population size and sensitivity to environmental disturbances (van der Maarel and van der Maarel-Versluys 1996; Ennos *et al.* 2005). Conservation in TCGs is best achieved by simplifying the evolutionary interactions among members of TCGs that generate and maintain their taxonomic diversity (i.e. hybridization and introgression). The presence of uniparental reproduction within *Limonium* genus could have profound effects on the organization of its biological diversity, leading to a mixture of sexual outcrossing and uniparental lineages. Therefore, the implementation of conservation strategies to maintain the evolutionary processes generating biodiversity presents major challenges (Ennos *et al.* 2005).

Considering that the crucial goals of conservation are to guarantee the continued survival of populations and to maintain their evolutionary potential (Hamirck and Godt 1996), *in situ* conservation emerges as a top priority. Habitat protection should be a priority to safeguard the *in situ* survival of the studied species. Some populations of *Limonium* CWR, such as *L. multiflorum*, are under threat from habitat alteration and loss in the Euro-Mediterranean region. These coastal habitats suffer from general pressures, for example, development of urban-industrial-recreational facilities; damage resulting from tourism as well as shore erosion. Natural reserves have been established to protect local

species populations from such impacts and could protect not only the endangered or endemic species, but also entire functioning communities, conserving biotic diversity or the maximum number of species (Soulé and Simberloff 1986; van der Maarel and van der Maarel-Versluys 1996). Goméz-Campo (1981) proposed the creation of micro-reserves for small and rich areas, emphasizing the necessity to pay attention to the *in situ* conservation of endemic and/or endangered species. These small-sized places that are protected by law should preserve unique or threatened flora, manage the habitat and develop conservation experiences guided by technical and scientific research (Laguna *et al.* 2001). A pioneer project has been developed in the Valencia region by Laguna *et al.* (2004) where the best viable population of *L. dufourii* is successively protected in a public-private plant micro-reserve network. This network is focused on conservation and research, but also environmental education, and contains a representation of all major habitat types included in the Directive 92/43/ECC presented in the region. In the case of the species here studied, we proposed the creation of a micro-reserve in Raso cape (Cascais: Estremadura), inside the Parque Natural Sintra-Cascais to prevent *L. multiflorum* and *L. ovalifolium* populations decline. In this site, new experimental populations of both species were already created, where 285 individuals of the first species and 376 of the latter species were introduced (Caperta *et al.* 2013).

The total information generated during this dissertation regarding chorology (populations sizes and distribution areas), karyology (ploidy level and chromosomal polymorphism at the intra-population level), genetics and epigenetics (maximizing the among and within-population genetic and epigenetic variability) and reproduction (gaining knowledge of the breeding systems) will contribute to design efficient long-term conservation strategies for ensuring an effective protection of species from *L. ovalifolium* and *L. binervosum* species' complexes in Continental Portugal.

CHAPTER VIII.

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APPENDIX I.

NOMENCLATORIAL REMARKS

Accepted names and synonyms for *Limonium binervosum* and *Limonium ovalifolium* species' complexes published in Flora de Portugal (Franco 1984), Flora Iberica (Erben 1993), and Sendtnera (Erben 1999).

Synonyms key

Bold type = accepted name.
Red = homotypic synonym.
Green = heterotypic synonym.
Black = sensu.

LIMONIUM BINERVOSUM COMPLEX

Limonium binervosum (G.E.Sm.) Salmon, C.E. Salmon in L.Bot.45:24 (1907)
Statice binervosa G.E. Sm. in Sm. and Sowerby, Engl. Bot. Suppl. 1:tab. 2663 (1831)
Statice bayonnensis Gren. in DC., Prod. 12:649 (1848)
Limonium binervosum subsp. *occidentale* (J.Lloyd) P. Fourn., Quatre Fl. France 722 (1937)
Statice bubanii Girard in Ann.Sci. Nat., Bot. ser. 32:326 (1844)
Limonium occidentale (J.Lloyd) Kuntze, Revis. Gen. Pl. 2:395 (1891)
Statice occidentalis J. Lloyd, Fl. Loire-Inf. 212 (1844)
Limonium salmonis (Sennen and Elías) Pignatti in Collect. Bot. (Barcelona) 6:321 (1962)
Statice salmonis Sennen and Elías in Bol.Soc.Ibér.Ci. Nat. 35:30 (1936)

Limonium dodartii (Girard) Kuntze, Revis. Gen. Pl. 2: 395 (1891)
Limonium binervosum subsp. *dodartii* (Girard) P. Fourn., Quatre Fl. France 722 (1937)
Statice dodartii Girard in Ann. Sci. Nat., Bot. ser. 2 17:31 (1842)
Limonium binervosum sensu Pignatti in Tutin and al. (eds.), Fl. Eur. 3:48 (1972)

Limonium multiflorum Erben in Mitt. Bot. Staatssamml. München 14: 497 (1978)
Limonium auriculae-ursifolium subsp. *multiflorum* (Pignatti) Pignatti in Bot. J. Linn. Soc. 64(4):367 (1971), nom. inval.
Limonium binervosum subsp. *multiflorum* Pignatti in Collect. Bot. (Barcelona) 6:320 (1962), nom. inval.
Statice densiflora var. *lusitanica* Daveau in Bol. Soc. Brot. 6:183 (1888)
Limonium dodartii subsp. *lusitanicum* (Daveau) Franco, Nova Fl. Portugal 2:564 (1984)

LIMONIUM OVALIFOLIUM COMPLEX

Limonium lanceolatum (Hoffmanns. & Link) Franco, Nova Fl. Portugal 2: 564 (1984)
Statice lanceolata Edgew. J. Asiat. Soc. Bengal 16(2): 1218. 1847

Limonium nydeggeri Erben, Sendtnera 6:103-107 (1999)

Limonium ovalifolium (Poir.) Kuntze, Revis. Gen. Pl. 2:396 (1891)
Statice ovalifolia Poir. in Lam., Encycl. Suppl. 5: 237 (1817)
Statice hybrida Mont. ex J. Lloyd, Fl. Loire-Inf. 211 (1844)
Statice lanceolata Hoffmanns. and Link, Fl. Portug. 1: 446 (1813)
Limonium lanceolatum (Hoffmanns. and Link) Franco, Nova Fl. Portugal 2: 564 (1984)
Limonium ovalifolium subsp. *lusitanicum* Pignatti in Collect. Bot. (Barcelona) 6: 318 (1962), nom. inval.
Statice globulariifolia sensu Hoffmanns. and Link, Fl. Portug. 1: 445 (1813-20)