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Phylogeography and modes of reproduction in diploid and tetraploid halophytes of *Limonium* species (Plumbaginaceae): evidence for a pattern of geographical parthenogenesis

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• **Background and Aims** The genus *Limonium* (Plumbaginaceae) has long been recognized to have sexual and apomictic (asexual seed formation) modes of reproduction. This study aimed to elucidate phylogeographical patterns and modes of reproduction in diploid and tetraploid *Limonium* species, namely three putative sexual diploid species with morphological affinities (*L. nydeggeri*, *L. ovalifolium*, *L. lanceolatum*) and three related, probably apomict tetraploid species (*L. binervosum*, *L. dodartii*, *L. multiflorum*).
• **Methods** cpDNA diversity and differentiation between natural populations of the species were investigated using two chloroplast sequence regions (*trnL* intron and *trnL–trnF* intergenic spacer). Floral heteromorphies, ovule cytoembryological analyses and pollination and crossing tests were performed in representative species of each ploidy group, namely diploid *L. ovalifolium* and tetraploid *L. multiflorum*, using plants from greenhouse collections.
• **Key Results and Conclusions** Genetic analyses showed that diploid species have a higher haplotype diversity and a higher number of unique (endemic) haplotypes than tetraploid species. Network analysis revealed correlations between cpDNA haplotype distribution and ploidy groups, species groups and geographical origin, and haplotype sharing within and among species with distinct ploidy levels. Reproductive biology analyses showed that diploid *L. ovalifolium* mainly forms meiotically reduced tetrasporic embryo sacs of *Gagea ova*, *Adoxa* and *Drusa* types. *Limonium multiflorum*, however, has only unreduced, diplosporic (apomictic) embryo sacs of *Rudbeckia* type, and autonomous apomictic development seems to occur. Taken together, the findings provide evidence of a pattern of ‘geographical parthenogenesis’ in which quaternary climatic oscillations appear to be involved in the geographical patterns of coastal diploid and tetraploid *Limonium* species.

Key words: Apomixis, cytoplasmic markers, cpDNA, female gametophyte development, floral biology, geographical parthenogenesis, *Limonium*, phylogeography, polyploidy, reproductive modes.

INTRODUCTION

Phylogeographical studies based on cpDNA haplotype variation and diversity have provided a great opportunity to reconstruct major processes in evolutionary history and biogeography in flowering plants (angiosperms) (Avise, 2000; Petit and Vendramin, 2007). Some of these works have helped to clarify the complicated reticulate evolutionary patterns observed in plant genera driven by sexual and/or apomictic reproductive modes (e.g. Hörandl *et al*., 2009; Kiefer *et al*., 2009; Majeský *et al*., 2012). Apomixis (reproduction via asexually formed seed) (Asker and Jerling, 1992) is present in 293 genera (∼2·2 %) of flowering plants and found to be taxonomically widespread with no tendency to occur in specific groups, and occurs with sexuality at all taxonomic levels (Hojsgaard *et al*., 2014). Most apomictic plants are polyploids and exhibit facultative sexuality, but there are cases of apomixis at the diploid level, including some species in the genera *Boechera* (Koltunow and Grossniklaus, 2003) and *Paspalum* (Siena *et al*., 2008). In recent years, biogeographical studies of some apomictic taxa (e.g. *Arnica alpina*, *Paspalum simplex*) and apomictic complexes (e.g. *Taraxacum officinale* agg., *Ranunculus auricomus* agg.) have shown patterns typical of ‘geographical parthenogenesis’, in which apomicts have larger distributions in higher latitudes than their sexual relatives, in addition to having differentially populated previously glaciated areas (Bierzychudek, 1985; Kearney, 2005; Hörandl *et al*., 2008).

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However, other apomictic taxa (e.g. *Boechera*) do not show evidence of geographical parthenogenesis, as recurrent hybridization has led to diffuse geographical patterns (Koch *et al*., 2003; Kiefer *et al*., 2009).

In *Limonium* (Plumbaginaceae), a cosmopolitan species-rich genus of annuals and perennial herbs, shrubs and lianas, often adapted to extreme coastal environments (Kubitzki, 1993), the effects of hybridization, polyploidy and apomixis appear to have all combined to shape their radiation (Lledó *et al*., 2005). Multiple series of complex aggregates of sexual diploid species and asexual polyploid hybrids have been described (Erben, 1978, 1993). Some authors have used various molecular markers to infer the importance of genetic structuring in defining *Limonium* interspecies delimitation (Palacios *et al*., 2000; Palop-Esteban *et al*., 2007; Róis *et al*., 2013). However, the majority of these studies covered parts of the distribution ranges or only a small part of the distribution of the species, and for most apomitic *Limonium* taxa biogeographical analyses have not yet been conducted.

Baker (1948, 1953*a*, *b*, 1966), who was influential in the development of evolutionary and biogeographical hypotheses for Plumbaginaceae, described floral heteromorphies, including heterostyly and pollen–stigma dimorphisms, linked to a sporophytic self-incompatibility system, which prevent mating between individuals of the same floral morph while allowing mating between morphs. 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) appear to be outcrossing. In contrast, monomorphic self-incompatible populations showing only one pollen–stigma combination seem to form seeds through apomixis (Baker, 1966). In *Limonium* spp. various types of tetrasporous (meiotic) reduced embryo sacs have been reported, including *Adoxa*, *Fritillaria*, *Drusa* and *Penea* types (Dahlgren, 1916; D'Amato, 1940, 1949; Hjelmqvist and Grazi, 1964). Also, in polyploid *Limonium vulgare* (also 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 gametophytic apomicts like triploid ($2n = 3x = 27$) *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). Nevertheless, for most *Limonium* apomictic taxa, data on patterns of female gametophyte formation are lacking.

Geographically, the Iberian Atlantic coast is between the North Atlantic shore and Mediterranean Iberia, where *Limonium* species showing a typical European Atlantic distribution meet species distributed in the south-west Mediterranean. This is the case for *L. vulgare* and related species with pinnately arranged leaves (Cortinhas *et al*., 2015), as well as species with parallel-nerved leaves such as diploids of the *Limonium ovalifolium* complex, like *L. ovalifolium* and *Limonium lanceolatum* (Franco, 1984; Erben, 1993; EUNIS, 2014), and tetraploids of the *Limonium binervosum* complex, which includes, among others, *L. binervosum*, *Limonium dodartii* and *Limonium multiflorum* (Erben, 1978; Ingrouille and Stace, 1986). Inferences about 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). Flow cytometric seed screening, which has the potential to determine the routes of seed formation based on the proportional DNA content of embryo and endosperm nuclei (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 in this species sexuality and/or agamospermy is suspected, it has never been directly tested. Notably, members of the *L. ovalifolium* species complex have imperfect but distinguishable morphological differentiation and genetic and epigenetic co-variation, as revealed by methylation-sensitive amplification polymorphism (MSAP) analysis (Róis *et al*. 2013). Conversely, the tetraploid *L. binervosum* species complex contains even lower levels of overall genetic diversity, but higher levels of epigenetic variability (Róis *et al*., 2013).

In this work we investigated (1) the phylogeographical patterns of diploid and tetraploid *Limonium* spp. which grow in sympatry as well as in allopatry using the maternally inherited chloroplast *trnL* intron and *trnL–trnF* intergenic spacers (Petit *et al*., 2003; Petit and Vendramin, 2007); and (2) potential correlations of geographical patterns with species differentiation and reproductive modes.

MATERIALS AND METHODS

Study species and population sampling

Here we studied (1) parallel-veined species of the genus *Limonium*, including three diploid species of the *L. ovalifolium* complex: *L. ovalifolium sensu stricto*, *L. nydeggeri* and *L. lanceolatum* (Erben, 1978, 1993, 1999), with both diploid and aneuploid cytotypes ($2n = 2x = 15, 16$) (Róis *et al*., 2012); and (2) three agamospermous tetraploid species from the agamospermous *L. binervosum* complex: *L. binervosum sensu stricto*, *L. dodartii* and *L. multiflorum* (Erben, 1978, 1993; Ingrouille and Stace, 1986), exhibiting tetraploid and aneuploid cytotypes ($2n = 4x = 35$ [Erben, 1993]; $2n = 4x = 32, 34, 35, 36$ [Róis *et al*., 2012]). The *L. ovalifolium* species complex, which thrives on maritime cliffs and in salt marshes, reaches its northern limits on the Atlantic coast of France in the Rance estuary (Lahondère and Biorét, 1995, 1996) and has its southern limits in Morocco (Erben, 1978, 1993, 1999) (Fig. 1). However, the three diploid species have distinct distribution ranges: *L. ovalifolium* has the largest distribution area, extending from Atlantic France, south-west of the Iberian Peninsula to Morocco (Erben, 1978, 1993); *L. lanceolatum* is found in both the north-west and south-west coasts of Iberian Peninsula (Franco, 1984; EUNIS, 2014); and *L. nydeggeri* is a Lusitanian endemic from the west and south-west coasts of Portugal (Erben, 1999; Espírito-Santo *et al*., 2012). The *L. binervosum* species complex is a group of Atlantic coastal agamospermous microspecies that grow on maritime rocks and saltmarshes (Stace, 2010). Geographically, *L. binervosum sensu stricto* is distributed from Scotland to Portugal (Pignatti, 1971; Erben, 1978; Ingrouille and Stace, 1986), although it is not mentioned for Portugal in *Flora Iberica* (Erben, 1993). *Limonium dodartii* has been recognized on the French Atlantic coast, north-western Spain and western

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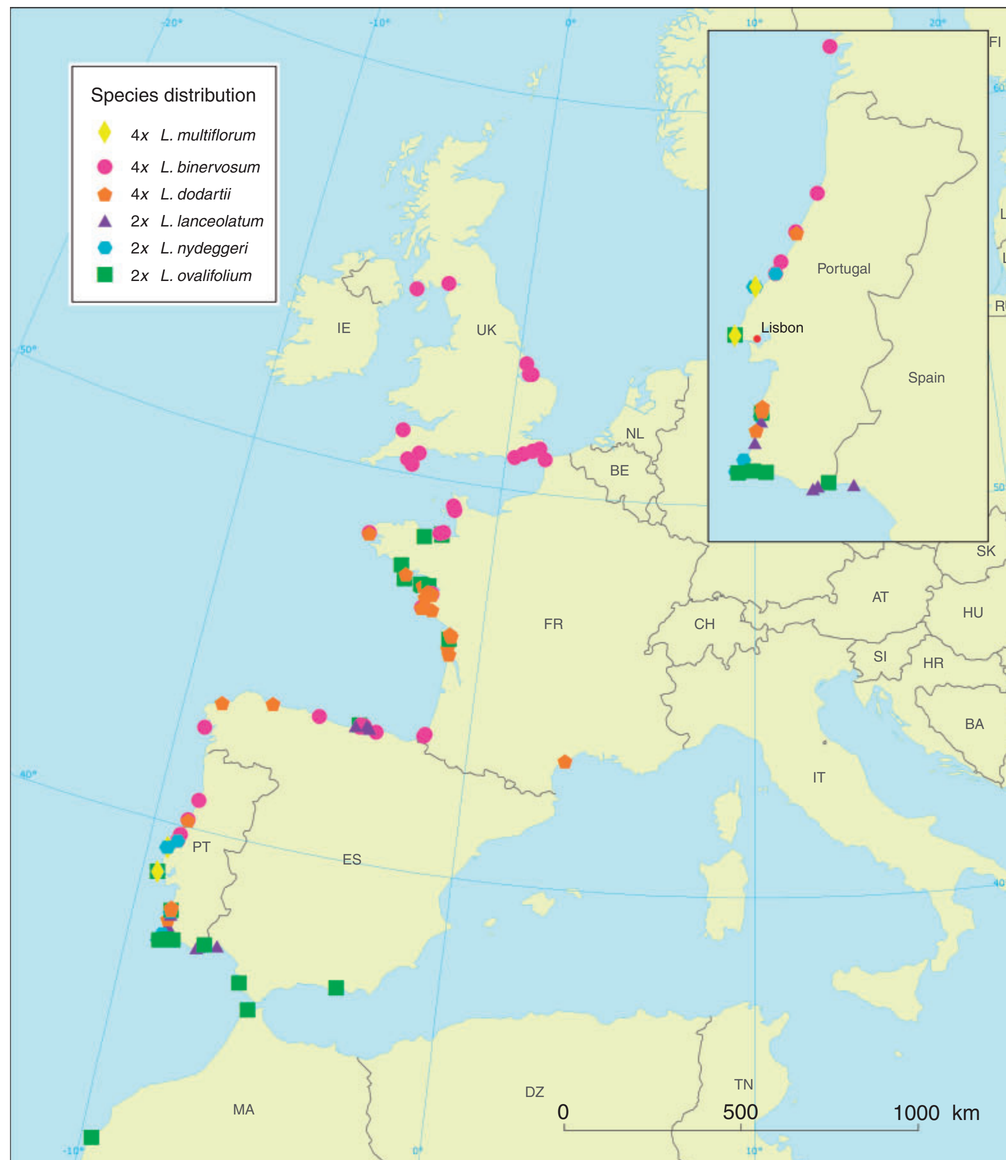

FIG. 1. Distribution of diploid and tetraploid *Limonium* spp. on Atlantic and Mediterranean coasts. Diploid species are *L. lanceolatum*, *L. nydeggeri* and *L. ovalifolium*; and tetraploid species are *L. binervosum*, *L. dodartii*, and *L. multiflorum*.

Atlantic Portugal coasts (Erben, 1993; Lahondère and Biorét, 1995, 1996) as well as on the Atlantic coasts of France (Lahondère and Biorét, 1996). Finally, *L. multiflorum* is a Lusitanian endemic on the western coast of Portugal (Erben, 1978, 1993; Espírito-Santo *et al.*, 2012; Caperta *et al.*, 2014).

Populations were surveyed along the Portuguese coast in Beira Litoral (west), Estremadura (west), Alentejo (south-west) and Algarve (south), where diploid and tetraploid species show overlapping distributions. The two Lusitanian endemic species, *L. nydeggeri* and *L. multiflorum*, were sampled over their whole distribution ranges. Also, an *L. multiflorum* subpopulation of the Cabo Raso (CRm) population was investigated in this study in order to have equivalent numbers of individuals in the populations analysed. All populations were recorded using a global positioning system (Table 1), Google Earth 6·0.2 was used for georeferencing and geographical mapping of the populations was performed using ArcGIS Desktop 10 (ESRI).

DNA isolation, PCR amplification and sequencing

A total of 128 plants were included in this study (Table 2) and 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 kit (Beckman Coulter). Two regions of the chloroplast genome (*trnL* intron and *trnL–trnF* intergenic spacer [Clegg *et al.*, 1994]) were amplified and sequenced using the universal PCR primers of Taberlet *et al.* (1991; combinations c/d and e/f).

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TABLE 1. *Natural* Limonium *populations surveyed in Portuguese provinces. Species were sampled in Beira Litoral (west), Estremadura (west), Alentejo (southwest) and Algarve (south)*

Species	Ploidy level	Habitat	Province/site location	Population acronym	Geographical coordinates	Census
L. lanceolatum	2*x* (A S. Róis & A. D. Caperta, unpubl. res.)	Salt marshes	Alentejo/Odemira, Vila Nova de Milfontes Collectors: ADC, APP, ASR	VNMF	37·7277/–8·7709	25
			Algarve/Tavira, Sapal do Barril Collectors: ADC,APP, ASR	SB	37·0861/–7·6627	50
L. nydeggeri	2*x* (Róis *et al*. 2012)	Sea cliffs	Estremadura/Peniche, Ilha do Baleal Collectors: ADC, APP, ASR	B	39·3789/–9·3409	25
			Estremadura/Peniche, Nossa Sra dos Remédios Collectors: ADC, APP, ASR	NSR	39·3699/–9·3957	25
			Estremadura/Cascais, Cabo Raso Collectors: ADC, APP, ASR	CRn	38·7100/–9·4858	50
	2*x* (A S. Róis & A. D. Caperta, unpubl. res.)		Algarve/Aljezur, Pontal da Carrapateira Collectors: ADC, APP, ASR	C	37·1950/–8·9111	25
			Algarve/Vila do Bispo, Cabo de São Vicente Collectors: ADC, ASR	CSV	37·0226/–8·9965	25
L. ovalifolium	2*x* (Róis *et al*., 2012)	Sea cliffs	Algarve/Lagos, Praia da Luz Collectors: ADC, ASR	PL	37·0874/–8·7290	25
			Algarve/Vila do Bispo, Cabo de Sagres Collectors: ADC, APP, ASR	CS	36·9942/–8·9487	30
L. binervosum	4*x* (A S. Róis & A. D. Caperta, unpubl. res.)	Sea cliffs	Beira Litoral/Cabo Mondego, Figueira da Foz Collectors: ADC, APP, ASR, SM, FS	CM	40·1911/–8·9052	25
			Beira Litoral/Marinha Grande, Praia da Concha Collectors: ADC, APP, ASR, SM	C	39·7552/–9·0322	20
			Beira Litoral/Aveiro, São Jacinto Collectors: ADC, APP, SM	SJ	40·3944/–8·4350	25
L. dodartii	4*x* (A S. Róis & A. D. Caperta, unpubl. res.)	Sea cliffs	Alentejo/Odemira, Cabo Sardão Collectors: ADC, APP, ASR, SM, PA	CSo	37·5986/–8·8160	50
			Alentejo/Odemira, Porto Covo Collectors: ADC, APP, ASR, SM, PA	PCo	37·8516/–8·7919	50
			Alentejo/Sines, Praia da Vieirinha Collectors: ADC, FS, ASR, SM, APP	PV	37·8967/–8·7978	30
L. multiflorum	4*x* (Róis *et al*. 2012)	Sea cliffs	Estremadura/Cascais, Cabo Raso Collectors: ADC, APP, ASR	CRm	38·7100/–9·4858	25
			Estremadura/Lourinhã, Vale dos Frades Collectors: ADC, APP, ASR	VF	39·2765/–9·3358	20
			Estremadura/Mafra, Foz do Lizandro Collectors: ADC, APP, ASR	FL	38·9415/–9·4152	50

Abbreviations of collectors from LEAF, ISA, University of Lisboa: ADC, Ana D. Caperta; APP, Ana P. Paes; ASR, Ana S. Róis; FS, Flávio Sádio; PA, Pedro Arsénio; SM, Sérgio Martins.

PCR reactions 10 µl were performed containing 50 ng of template DNA, 10× NH_4 PCR buffer, 50 mM $MgCl_2$, 100 mM dNTPs, 10 mM of each primer and 5 U/µl Taq DNA polymerase (Bioline). The PCR amplifications began with an initial cycle of 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 1·40 min at 72 °C, and a final step of 10 min at 72 °C. The PCR product was verified on an agarose 1·5 % Tris–acetate–EDTA (TAE) gel, and the products were purified using a Qiagen PCR purification kit and sequenced directly using the ABI Prism™ 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.

Data analyses

The intron *trnL* and intergenic spacer *trnL–trnLF* sequences were aligned 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 ε = 0). Genetic diversity parameters, such as haplotype diversity (Hd) and nucleotide diversity (π) and genetic differentiation (G_{ST}), and neutrality tests (Tajima's *D*) were calculated using the program DnaSP 4·0 (Rozas, *et al*. 2003).

The Bayesian phylogenetic inference was implemented with MrBayes v. 3.2.2 (Ronquist *et al*., 2012). The posterior probabilities of the phylogenetic trees were estimated by a Metropolis-coupled Markov chain Monte Carlo sampling algorithm (MCMCMC). The stationary phase was obtained with a total of 1 500 000 generations that were sampled every 100 generations with a burn-in of 1000. The priors for the model of sequence evolution was estimated with the Akaike information criterion (AIC) using MrModeltest2 (Nylander, 2004).

Pollen exclusion and crossing experiments

A total of ten fresh flowers were used for floral heteromorphism determinations in *L. ovalifolium* and *L. multiflorum* growing in experimental collections at Instituto Superior de

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TABLE 2. *cpDNA haplotype diversity in* Limonium ovalifolium *and* L. binervosum *species complexes*

Species	Diploids									Tetraploids									No. of individuals/ haplotype
	L. lanceolatum		*L. nydeggeri*					*L. ovalifolium*		*L. binervosum*			*L. dodartii*			*L. multiflorum*			
Population	VNMF	SB	B	NSR	CRn	C	CSV	PL	CS	CM	PC	SJ	CSo	PCo	PV	CRm	VF	FL	
H1										5	1	6							12
H2				1	1		1		2		3		1			3	5	9	27
H3													5	4					9
H4															8				8
H5																1			1
H6																1			1
H7																1			1
H8																	1		1
H9																	1		1
H10																		1	1
H11	3		4	2	2	1		6											18
H12	1																		1
H13	6		2			7													15
H14			1																1
H15			1																1
H16						1													1
H17				1															1
H18				1															1
H19				2	3			1											6
H20							2												2
H21							4												4
H22									1										1
H23									1										1
H24									1										1
H25									1										1
H26								1											1
H27								1											1
H28		10																	10
N_i	10	10	8	7	6	9	7	9	6	5	4	6	6	4	8	6	7	10	
Hn	4		11					9		2			3			7			
Hu	2		7					6		1			2			6			
Hd (s.d.)	0·684 (0·070)		0·865 (0·029)					0·829 (0·085)		0			0·111 (0·096)			0·462 (0·128)			
π	0·0156		0·0079					0·0054		0			0·0001			0·002			

H#, haplotype number; N_i, number individuals analysed; Hn, number of haplotypes; Hu, number of unique haplotypes; Hd (s.d.), haplotype diversity and standard deviation; π, nucleotide diversity.

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 and covered with a drop of water, and stigma preparations were observed by optical light microscopy (Leitz HM-LUX 3) with × 40 magnification. Pollen viability was scored using Alexander's stain test (Alexander, 1969). The total number of pollen grains was determined and pollen viability estimates were performed by one person using ~20 flowers per plant; the number was counted under an optical light microscope (Leitz HM-LUX 3) at × 20 magnification.

A total of 18 plants, 12 self-incompatible *L. ovalifolium* individuals (with A or B combination) and six male-sterile *L. multiflorum* individuals (Róis *et al*., 2012) were subjected to pollen exclusion experiments to test for apomixis, as described in Khan *et al*. (2012), with some modifications. No flower emasculation prior to anther dehiscence was performed in inflorescences (scapes) as the plants were self-incompatible or male-sterile.

Pollination treatments included the following: (1) in each plant, an intact inflorescence was left unbagged to test for natural pollination (control); (2) intact inflorescences were bagged with vegetable cellulose paper that did not allow the passage of pollen or floral visitors, to test for apomixis; (3) pollen from conspecifics was used to test for cross-compatibility between three self-incompatible plants (A and B combinations). In the last treatment, manual pollination was performed once for each inflorescence by brushing dehiscing anthers against the receiving stigmas until pollen could be seen on the stigma surface. Pollen exclusion bags were immediately placed over the inflorescences used in each cross-pollination to prevent undesired pollination events. After 9 weeks, bags were removed and the number of produced seeds was counted. The average number of seeds produced per scape (either unbagged or bagged) was calculated from measurements of six plants of each type. Seeds were then germinated as described in Róis *et al*. (2012). Germination was considered to have occurred on the emergence of radicles, and the number of seeds that germinated after 1 week was recorded.

Non-parametric Kruskal–Wallis analyses of variance of data were performed (Sokal and Rohlf, 2012) using SPSS 20 for Windows.

Electron microscopy analyses

Stigma, pollen and ovules were analysed 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

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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 and 100 % ethanol for 30 min each). Flowers were dried on a BioRad Polaron E3500 critical point drier, and coated with a thin layer of gold on a Jeol JFC-1200. Observations were carried out on a JSM-5220 LV scanning electron microscope equipped with a direct image acquisition system.

Cytohistological analyses of megasporogenesis and gametogenesis

Flower buds at distinct floral stages ranging from 2 to 5 mm in length were collected from some of the above-mentioned *L. ovalifolium* and *L. multiflorum* plants. Pistils were dissected from flower buds, followed by clearing and staining following the protocol of Stelly et al. (1984), with some modifications. Briefly, the tissues were fixed in FAA (3·7 % formalin, 5 % acetic acid, 70 % ethanol) overnight at 4 °C and then dehydrated in 50, 75 and 100 % progressive water/ethanol solutions for 30 min each. Then, samples were stained with pure Mayer's haemalum for 48 h, and placed in 0·1 % chloral hydrate (Wallis, 1957). Ovules were digested with 2 % cellulase (Sigma), 2 % cellulase 'Onozuka R-10' (Serva) and 2 % pectinase enzyme solution in Enzyme Buffer (40 mL of 0·1 M citric acid-1-hydrate and 60 mL of 0·1 M sodium citrate dihydrate; pH 4·8) for 20 min at 37 °C, as described in Caperta *et al*. (2008). Finally, ovules were mounted in multi-well slides in a drop of chloral hydrate, under a coverslip. Cytohistological observations were made using a fluorescence microscope (Zeiss Axioskop 2) using differential interference contrast (DIC) optics, under × 20 and × 100 magnification. Images were collected with an AxioCam 289 MRc5 digital camera (Zeiss) and further processed using 290 Adobe Photoshop 5·0 (Adobe Systems, Mountain View, CA, USA).

To test for differences between *L. ovalifolium* and *L. multiflorum*, χ^2 tests of independence were performed (Sokal and Rohlf, 2012) using SPSS 20 for Windows.

RESULTS

cpDNA haplotype distribution and diversity

The sequencing of the intron *trnL* and intergenic spacer *trnL–trnLF* resulted in a final matrix of 128 individuals for a 646-bp concatenated fragment (Supplementary Data Table S1). Sixty-two single-nucleotide polymorphisms and four indels were detected.

A total of 28 (H1–H28) haplotypes were identified in the 18 species populations analysed (Table 2). Diploid species showed a higher number of haplotypes ($n = 18$) than tetraploid species ($n = 10$), and most species presented more than three haplotypes. All species showed populations with unique haplotypes but the number of endemic haplotypes was higher in diploid than in tetraploid species. *Limonium nydeggeri* and *L. multiflorum* had the highest number of haplotypes and the highest number of unique haplotypes of all diploid and tetraploid species respectively.

In both species complexes, populations with just one haplotype were found: haplotype H28 in *L. lanceolatum* SB, haplotype H1 in *L. binervosum* CM and SJ, haplotype H3 in *L. dodartii* CSo and PCo, and haplotype H4 in the PV population. A common haplotype, H11, was detected in all diploid species, albeit not in all their populations. Haplotype H2 was shared by diploid and tetraploid species, as observed in *L. nydeggeri* NSR and CRn, *L. ovalifolium* CS, *L. binervosum* PC, *L. dodartii* CSo and *L. multiflorum* CRM, VF and FL populations. cpDNA haplotype (Hd) and nucleotide diversity (π) was higher in diploid than tetraploid species. In these latter species, cpDNA haplotype diversity ranged from none (*L. binervosum*) to moderate (*L. multiflorum*). Finally, considering all species, the genetic differentiation coefficient (G_{ST}) among populations was 0·5015 and F_{ST} was 0·6103. Moreover, Tajima's D neutrality tests were negative but non-significant (–1·5105; $P > 0·10$).

Network relationships among haplotypes

The network evidenced clustering for ploidy levels in which specific haplotypes were found (Fig. 2A). Apart from haplotype H2, which was shared by both diploid and tetraploid species, diploid (H11–H28) and tetraploid (H1, H3–H10) specific haplotypes were evident. Tetraploid haplotypes seem to cluster around H2, with H2 the putative ancestral (shared with diploid species) and H1, H3, H4, H5, H6, H8 and H10 as derived haplotypes. However, some tetraploid haplotypes that did not cluster with the mentioned group (e.g. H7 and H9) seemed to be derived from haplotypes present in diploid species rather than from tetraploids.

There was also grouping with respect to species (Fig. 2B). Globally, *L. lanceolatum*, *L. nydeggeri* and *L. ovalifolium* had haplotypes that were more interrelated than related to tetraploid species. Diploid species were mainly structured around haplotypes H11, H13 and H19. Nevertheless, in *L. nydeggeri* and *L. ovalifolium* some haplotypes were derived from haplotype H2 (e.g. haplotypes H15 and H17, H18–H21, H25 and H26). *Limonium binervosum*, *L. dodartii* and *L. multiflorum* populations were more homogeneous than those of diploid species (Supplementary Data Figure S1). The first two species were clearly differentiated from *L. multiflorum*, which had two haplotypes, H7 and H9, derived from haplotypes that were frequent in *L. nydegerii* (e.g. H11 and H19).

Although only *L. nydeggeri* and *L. multiflorum* samples were collected considering their total distribution range (both endemic species) (Fig. 1), we identified haplotypes specific to particular geographical regions (Table 2 and Supplementary Data Figure S1). In diploid species, haplotypes H20–H27 (*L. nydeggeri* [CSV]; *L. ovalifolium* [PL and CS]) and haplotype H28 (*L. lanceolatum* [SB]) were only found in southern Portugal. Moreover, a geographical pattern was observed for haplotype H1 in Beira Litoral (*L. binervosum* [CM, PC and SJ]), haplotypes H3 and H4 in south-west Alentejo (*L. dodartii* [CSo, PCo and PV]) and haplotypes H5–H10 on the west coast (*L. multiflorum* [CRm, VF, FL]).

The Bayesian phylogenetic analysis corroborated the results found using the network, with several polytomies resulting from recent divergences (Supplementary Data Figure S2).

Reproductive studies in diploids and tetraploids

In both *L. ovalifolium* and *L. multiflorum*, regular, hypogynous and pentamerous flowers were found. Each flower was

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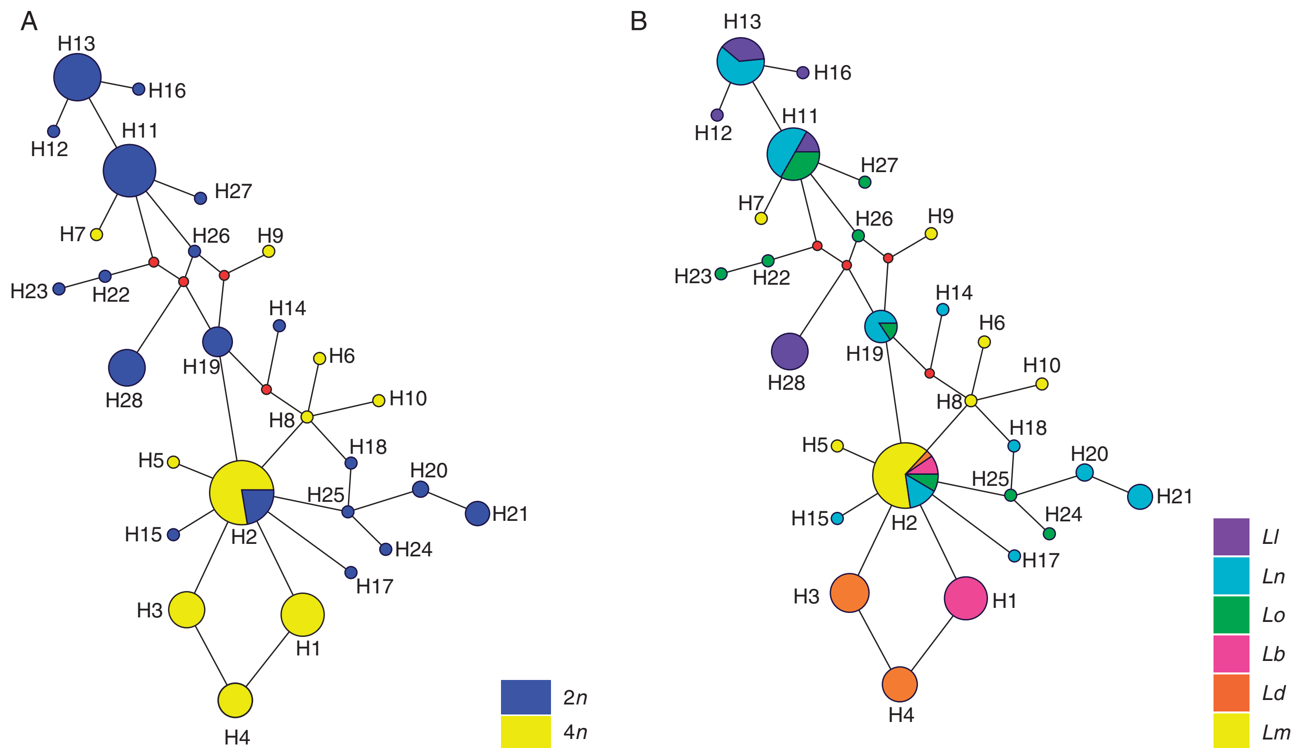

FIG. 2. Median-joining network of the cpDNA sequence regions (intron *trnL* and intergenic spacer *trnL–trnLF*) in *Limonium ovalifolium* and *Limonium binervosum* species complexes. Representation of haplotypes by ploidy level (2*n* or 4*n*) (A) and species (B): *L. lanceolatum*, *L. nydeggeri* and *L. ovalifolium* (diploids) and *L. binervosum*, *L. dodartii* and *L. multiflorum* (tetraploids). Circles represent populations and circle size is proportional to haplotype frequency. Each small red circle represents a (predicted) mutation that was not detected in the individuals sampled.

characterized by 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 (Fig. 3). In *L. ovalifolium* plants featuring flowers showing stigmas with polygonal cells (*cob*) and pollen with macroreticulate exine (self-incompatible A combination [Baker, 1948]; Fig. 3A, C), or stigmas with papillate (*pap*) cells and pollen with microreticulate exine (self-incompatible B combination; Fig. 3B, D) were detected. In contrast, in *L. multiflorum*, stigmas with *cob* or *pap* cells were present (Fig. 3E, F) but pollen grains exhibited an abnormal morphology without the typical exine patterns (Fig. 3G, H). Therefore, in this latter 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 (two or three) and sometimes flowers with no pollen at all were detected. *Limonium ovalifolium* pollen grains showed very high viability (~97 %), but only low to moderate levels of viability (0–69 %) were found for *L. multiflorum* plants.

Pollination experiments in both self-incompatible A-type and B-type *L. ovalifolium* plants showed that scapes produced on average fewer seeds (<2 seeds, $n = 12$ plants) than *L. multiflorum* (>100 seeds, $n = 6$ plants) (Supplementary Data Table S2). No significant differences in seed production were found between *L. ovalifolium* plant types considering both control and bagged plants (Supplementary Data Table S2; $P > 0{\cdot}929$, $\alpha < 0{\cdot}05$) or between *L. multiflorum* bagged and unbagged plants (Supplementary Data Table S2; $P > 0{\cdot}873$, $\alpha < 0{\cdot}05$). However, significant differences between *L. ovalifolium* and *L. multiflorum* for the number of seeds produced either in control or bagged scapes were detected (Supplementary Data Table S2; $P = 0{\cdot}044$, $\alpha < 0{\cdot}05$). The percentage of seed germination was similar (~55 %) in the two species.

Cross-pollination experiments between pairs of self-incompatible *L. ovalifolium* plants (A × B combinations) resulted in the production of a small amount of seeds on average (Supplementary Data Table S2), with a moderate percentage of germination (62 %).

Ovule development and gamete formation

In both *L. ovalifolium* and *L. multiflorum* unopened flowers (<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 attained continuity and developed subsequently into five distinct styles and stigmata, and thereafter the remaining apex was transformed into the single ovule primordium (Supplementary Data Fig. S3A). During development of the unique basal ovule, a long and slender funicle developed around it (Supplementary Data Fig. S3B, C) and the ovule underwent curvature of 360° (ovule circinotropous) (Supplementary Data Fig. S3D). In the mature ovule, the micropyle pointed away from the basal placenta towards the top of the ovary and was in close contact with the obturator (Supplementary Data Fig. S3E, F). This transmitting tissue protruded from the roof of the ovary into the unilocular

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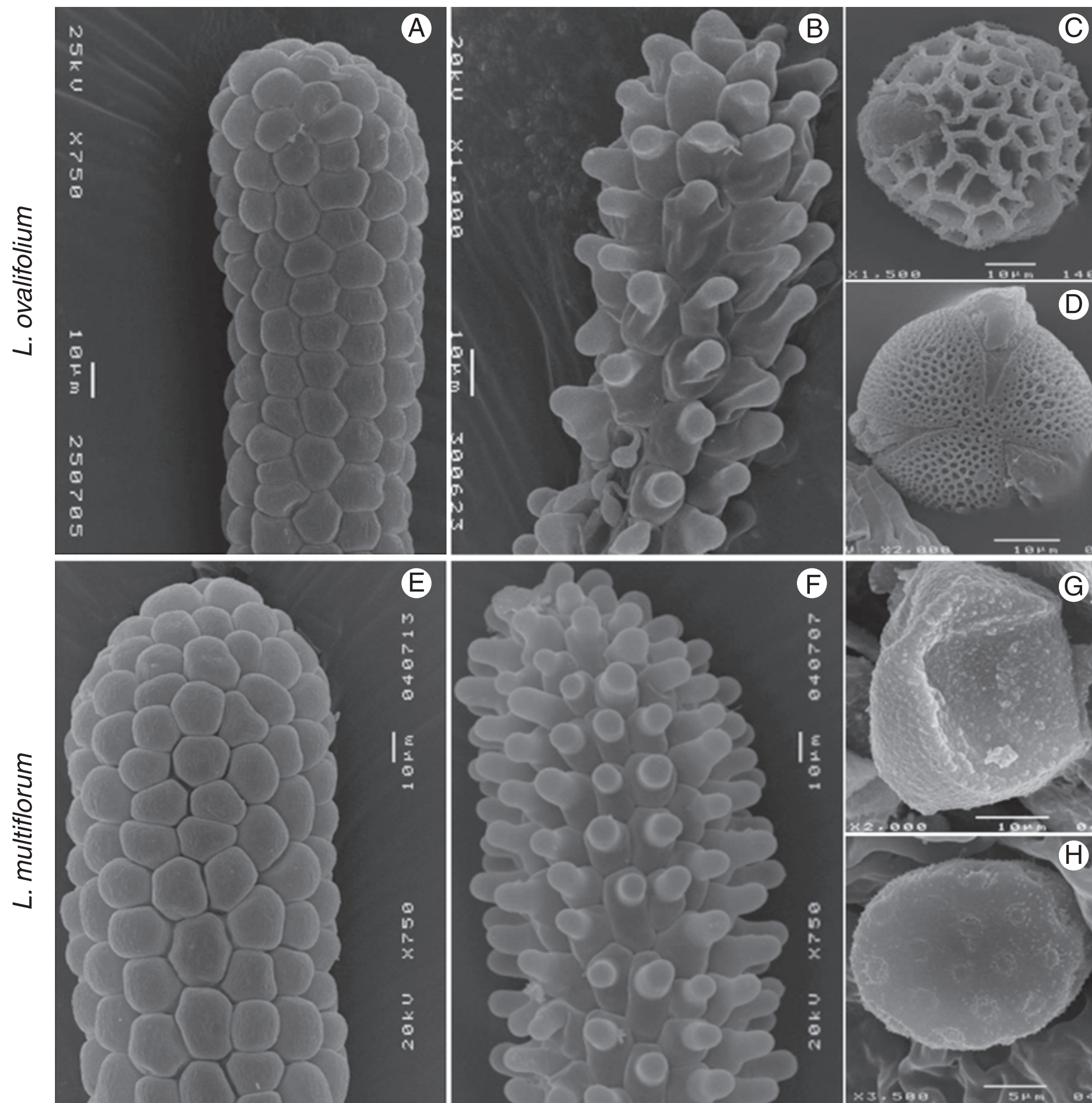

FIG. 3. Scanning electron microscope photographs of *Limonium ovalifolium* and *L. multiflorum* stigmas and pollen grains. Each image is oriented so that the micropyle is towards the top of the page and the chalaza is towards the bottom. (A–D) *Limonium ovalifolium*. (A, B) Papillate and *cob*-type stigma, 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). (E–H) *L. multiflorum*. (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.

superior ovary (data not shown). In general, a single ovary yielded just a 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* ~700 ovules were examined, and there were significant differences between species for the number of ovules of sexual and apomictic origin produced ($\chi^2 = 1270{\cdot}701$; $P < 0{\cdot}05$). The first meiosis was always observed in unopened flowers smaller than 2·5 mm. In *L. ovalifolium*, tetrasporic embryo sac development of the *Gagea ova* type was the most common (>85 %) (Fig. 4; Table 3). In brief, at the beginning of meiotic interphase, megasporocytes showed nuclei with two nucleoli (Fig. 4A) but soon progressed to prophase I, where only one nucleolus was visible (Fig. 4B). Then, female gametophytes exhibited two nuclei in division, not partitioned into separate cells (Fig. 4C), and following meiosis II a linear tetrad was formed (Fig. 4D). 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 (Fig. 4E). 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 (Fig. 4F). Thereafter, the nucleus at the micropylar-most pole divided again, originating the cells of the egg apparatus, the synergids and egg cell, while the chalazal pair of

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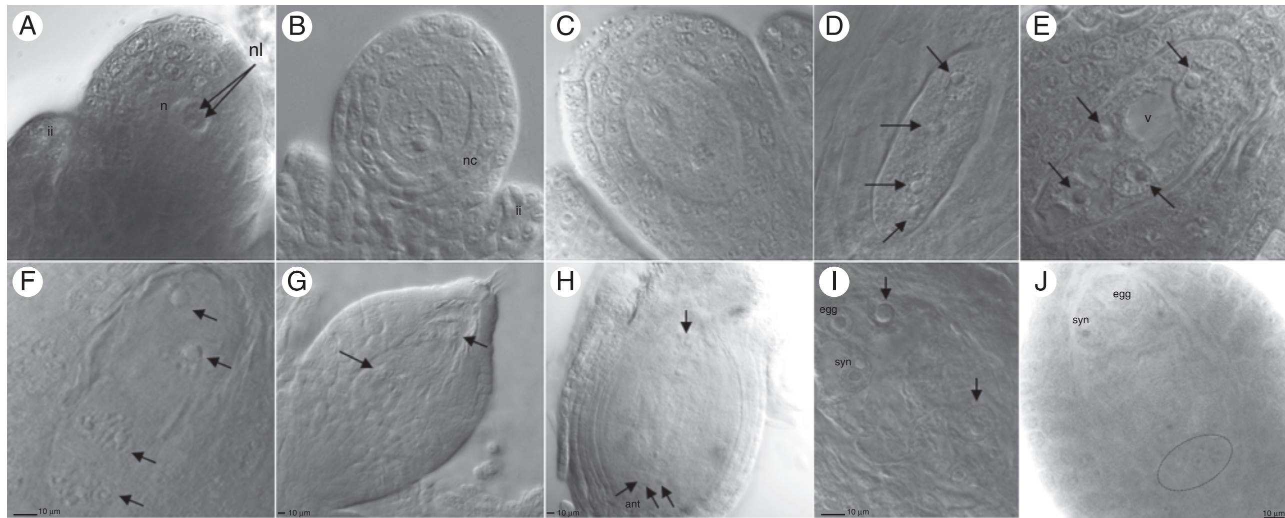

FIG. 4. Megaspore and embryo sac formation in *Limonium ovalifolium* (differential interference contrast). Each image is oriented so that the micropyle is towards the top of the page and the chalaza is towards the bottom. (A–D) Plant with accession number 2009I4SR (Sra Remédios, Peniche). (E, F) Plant 2009I1PL (Praia da Luz, Lagos). (G–I) Plant 2009I2CS (Cabo de Sagres, Vila do Bispo). (J) Plant 2009I4SR (Sra Remédios, Peniche). (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 nuclei in division (meiosis II, anaphase II) not partitioned into separate cells. (D) Coenomegaspore with four reduced nuclei, each nucleus with a single nucleolus (arrowed) (four-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 (arrows indicate mononucleolated nuclei [micropylar pole] and trinucleolated nuclei [chalazal pole]); (G) Six-nucleate embryo sac; the lowest nuclei of the secondary tetranucleate stage do not further divide (*Gagea ova* type). (H) *Adoxa*-type embryo sac. Arrows indicate egg cell and antipodals. (I) Detail of an embryo sac of *Adoxa* type with superior and inferior polar nuclei arrowed. (J) *Drusa*-type embryo sac. Dotted oval indicates 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. Abbreviations: ant, antipodals; egg, egg cell; ii, integuments; n, nucleus; nl, nucleolus; nc, nucellus; syn, synergid; v, vacuole.

TABLE 3. *Ovule development in* Limonium ovalifolium *and* Limonium multiflorum

Taxon	Accession number[a]	Origin[b]	Chromosome number[c]	No. of ovules analysed (%)[d]	No. of embryos (%)[e]	No. of sexual embryo sac types (%)			No. of apomictic, *Rudbeckia*-type embryo sacs (%)
						Gagea ova	*Adoxa*	*Drusa*	
L. ovalifolium	2009I4SR	Sra Remédios, Peniche, Portugal	16	41·7 (304)	6·2 (45)	31·3 (228)	2·2 (16)	1·6 (12)	0·4 (3)
	2009I1PL	Praia da Luz, Lagos, Portugal	16	28 (204)	2·1 (15)	22·1 (161)	2·3 (17)	1·5 (11)	
	2009I2CS	Cabo de Sagres, Vila do Bispo, Portugal	16	30·3 (221)	3·1 (23)	23·3 (170)	1·8 (13)	1 (7)	1·1 (8)
Frequency of ovules (%)[f]					11·4	76·7	6·3	4·1	1·5
Total number of ovules				729	83	559	46	30	11
L. multiflorum	2009I4VF	Vale dos Frades, Lourinhã, Portugal	35	40·1 (298)	3·5 (26)				36·6 (272)
	2009I9CR	Cabo Raso, Cascais, Portugal	32	35·2 (262)	4·2 (31)				31·0 (231)
	2010I5FL	Foz do Lizandro, Mafra, Portugal	36	24·7 (184)	2·5 (19)				22·2 (165)
Frequency of ovules (%)[f]					10·2				89·8
Total number of ovules				744	76				668

[a]Accessions from the ISA collections.
[b]Population origin.
[c]Chromosome numbers were counted from root tip squashes (Róis *et al*., 2012).
[d]Frequency of ovules analysed = number of ovules analysed in each accession/total number of ovules analysed × 100. Numbers in parentheses are numbers of ovules/embryo sacs examined.
[e]Frequency of embryos = number of embryos/number of ovules analysed × 100. Numbers in parentheses are numbers of ovules/embryo sacs examined.
[f]Frequency of ovules = number of ovules with sexual or apomictic origin/ number of ovules analysed × 100.

nuclei did not divide further. Thus, the mature embryo sacs were six-nucleate (Fig. 4G). Other tetrasporic-origin embryo sacs were also produced, namely of the *Adoxa* (Fig. 4H, I) and *Drusa* types (Fig. 4J). Occasionally, unreduced, diplosporic (apomictic) embryo sacs of *Rudbeckia* type were formed (Table 3).

By contrast, *L. multiflorum* only produced diplosporous, meiotically unreduced eggs of the *Rudbeckia* type (Table 3). During meiosis I, prophase cells showed a single nucleolus (Fig. 5A) and in metaphase plates bivalent and univalent chromosomes scattered over the spindle were observed (Fig. 5B).

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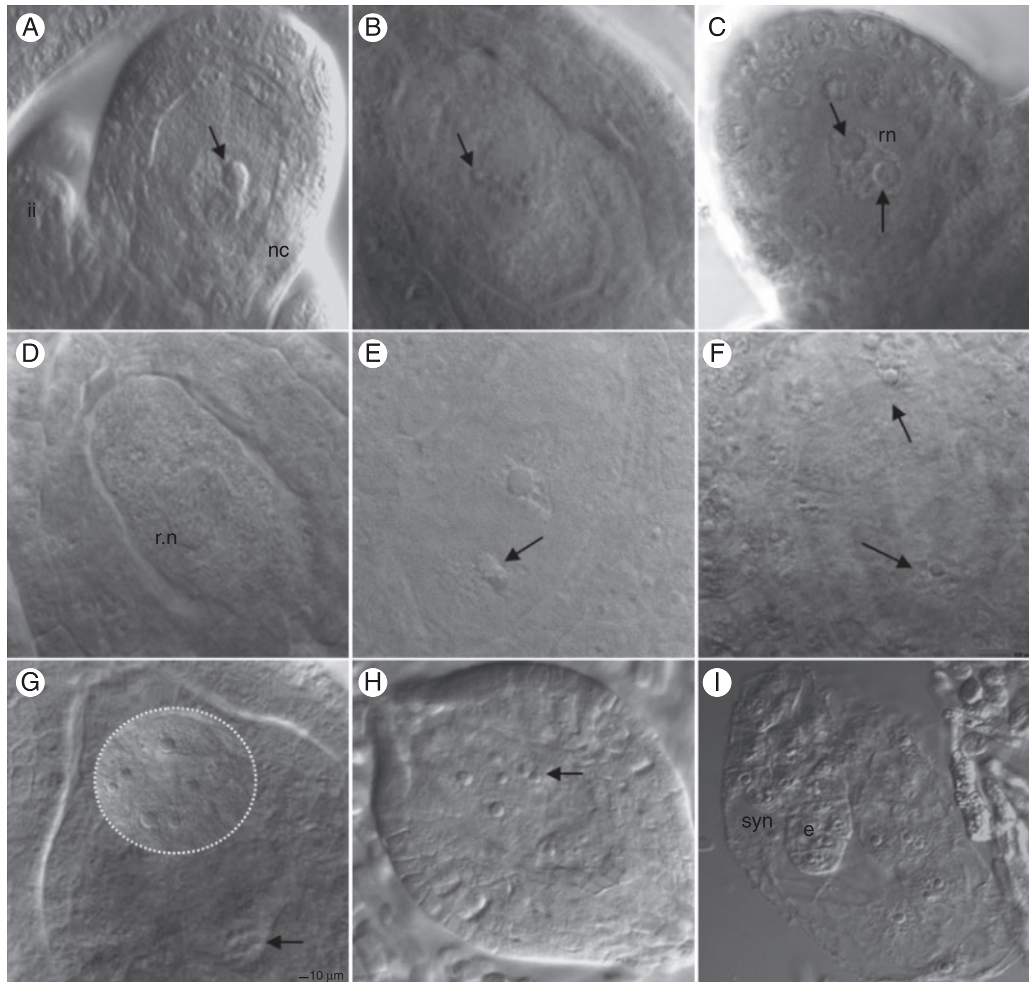

Fig. 5. Megaspore and embryo sac formation in *Limonium multiflorum* (differential interference contrast). Each image is oriented so that the micropyle is towards the top of the page and the chalaza is towards the bottom. (A–D) Plant with accession number 2009I4VF (Vale dos Frades, Lourinhã). (E, F) Plant 2009I9CR (Cabo Raso, Cascais) (G) Plant 2009I4VF (Vale dos Frades, Lourinhã). (H) Plant 2010I5FL (Foz do Lizandro, Mafra). (I) Plant 2009I4VF (Vale dos Frades, Lourinhã). (A) Megasporocyte within ovule showing one nucleolus. (B) First metaphase exhibiting laggard chromosome (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 (rn). (E) Two-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 (arrowed). (F) Embryo sac at four-nucleate stage resulting from mitotic-like division of megasporocyte; arrows indicate spindles. (G) Detail of egg cell apparatus (in dotted oval) in a mature embryo sac; antipodal cell is arrowed. In this composite image the area within the dotted oval, taken at a different light exposure, was copied into the background of the figure. (H) Mature embryo sac of *Rudbeckia* type. (I) Parthenogenetic embryo formation within a mature sac. Abbreviations: e, embryo; ii, integuments; rn, restitution nucleus; nc, nucellus; syn, synergid.

After anaphase I, a reconstitution nucleus was formed, irregular in shape, slightly elongated and somewhat constricted in the middle (Fig. 5C). This was followed by progression of the restitution nucleus through a mitotic anaphase, which in most cases formed a tripolar spindle (Fig. 5D). Embryo sacs at the two-nucleate stage showed one nucleus with one large nucleolus positioned at the micropylar end and another two-nucleolated nucleus at the most chalazal end (Fig. 5E). After a karyokinetic nuclear division at the two-nucleated stage, a four-nucleate gametophyte was produced (Fig. 5F). 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 (Fig. 5G). The central part of the embryo sac contained more than one protoendospermatic cell (four in the case illustrated; Fig. 5G), and at the chalazal-most region a variable number of antipodal cells with two nucleoli each were usually present (Fig. 5H). The ultimate development of the egg cell appeared to occur autonomously, as no pollen tubes were observed at the micropyle or at the extension of the gametophyte, and the first developmental stages of the embryo proceeded with perfectly intact synergids and polar nuclei (Fig. 5I).

DISCUSSION

Phylogeographical patterns

In order to compare cpDNA diversity in diploid and tetraploid plant species growing in sympatry as well as allopatry on the continental Portuguese coast, we analysed cp haplotypes in 18 populations of six *Limonium* species. Our results reveal that haplotype and nucleotide diversity are higher in diploids than in tetraploids, where moderate to no diversity at all is found.

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Remarkably, these data are concordant with previous MSAP studies on populations of these tetraploid species, in which little to no genetic and epigenetic variation is detected (Róis *et al.*, 2013). The frequency of endemic, unique haplotypes is $\geq$50 % for both species complexes (Table 2), which could be interpreted as lineage differentiation within and between complexes.

Among diploid species, *L. nydeggeri* revealed the highest haplotype and nucleotide diversity, and in the tetraploid species complex *L. multiflorum* presented the highest values for these parameters. Noticeably, both species represent two Lusitania endemics, the only ones for which the entire distribution range was sampled. It is interesting to note that *L. multiflorum*, which has a restricted distribution on the west coast of the Iberian Peninsula, has lower haplotype diversity than *L. nydeggeri*, which is more widespread and is distributed in both the west and south-west coasts of Portugal (Erben, 1978, 1993, 1999). For the remaining species, haplotype diversity is most probably underestimated as they also grow on the Atlantic coasts of the British Isles and France, and on south-west Atlantic and Mediterranean coasts (Erben, 1993; EUNIS, 2014). Considering the centrally located cp haplotypes for both *L. nydeggeri* (H2, H11, H13 and H19) and *L. multiflorum* (H2), our data suggest that the Lusitania region represents the centre of diversity for these species (Figs 1 and 2).

Our network analyses demonstrated differentiation between diploid and tetraploid lineages, some species-specific lineages and suprahaplotypes that can be shared by up to five taxa. Suprahaplotype H11 is shared by the three diploid taxa, and *L. lanceolatum* haplotypes appear to be derived from diploid lineages. In contrast, all tetraploid species share the suprahaplotype H2, from which derived, species-specific haplotypes were found, including haplotypes H3 and H4 in *L. dodartii* and haplotype H1 in *L. binervosum* (Fig. 2). The suprahaplotype H2 is the most frequent and seems to be a central haplotype that is shared by both diploid and tetraploid species. Nevertheless, its derived haplotypes are present in distinct diploid or tetraploid lineages, with haplotype H2 as the likely common ancestral haplotype (Fig. 2). In apomictic complexes, common ancestry followed by sequence divergence has been demonstrated in e.g. *Boechera* (Sharbel and Mitchell-Olds, 2001; Dobeš *et al.*, 2004; Kiefer *et al.*, 2009) and *Ranunculus* (Paun *et al.*, 2005; Hörandl *et al.*, 2009). Moreover, our data suggest that tetraploids were generated independently four times: haplotypes H2 and connected haplotypes (see haplotypes coloured yellow in Fig. 2A), H7 and H9.

Reproductive findings in diploid and tetraploids

Our results on floral heteromorphies, female gametophyte development and pollination experiments further demonstrate distinct reproductive modes in diploid and tetraploid *Limonium* species. Although both *L. ovalifolium* and *L. multiflorum* plants exhibit the 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 abnormal pollen grains without the typical exine patterns, and are therefore male-sterile (Róis *et al.*, 2012). As reported in other biological systems, poor quality of pollen due to mutation accumulation (Muller's ratchet) relaxes selection for a male function and will result in many of the progeny sired by asexuals being weak or inviable (Mogie *et al.*, 2007).

Here we show for the first time that *L. ovalifolium* mostly forms meiotically reduced tetrasporic embryo sacs (Table 3) of the *G. ova* type (Romanov, 1957), although tetrasporous female gametophytes of *Adoxa* and *Drusa* types are also found, as earlier reported for other *Limonium* spp. (Dahlgren, 1916; D'Amato, 1949; Hjelmqvist and Grazi, 1964). Moreover, a very low frequency of diplosporic embryo sacs of the *Rudbeckia* type (Battaglia, 1946) were also produced in this species (Table 3). Also, pollen exclusion tests in bagged self-incompatible *L. ovalifolium* plants result in the production of a very small number of seeds in the absence of self-compatible pollen, suggesting apomixis as a hypothetical reproductive event occurring at the diploid level. As rarely reported in literature, 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). At present our data are insufficient to provide solid evidence of apomixis at diploid level and need further conclusive support from genetic analyses, which are ongoing with SSRs (simple sequence repeats).

In contrast, in *L. multiflorum* meiotically unreduced, diplosporous embryo sacs of the *Rudbeckia* type (Battaglia, 1951) are produced and autonomous apomixis was observed. In other polyploid apomicts (e.g. *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, polyploid *Limonium transwallianum* forms reduced tetrasporous *Fritillaria* and *Drusa* types and unreduced diplosporous embryo sacs (Hjelmqvist and Grazi, 1964). However, tetraploid *L. multiflorum* only originates apomictic embryo sacs. Moreover, pollen exclusion tests reveal that this species produces a moderate to high number of viable seeds per scape, even in the presence of pollen grains with various morphological abnormalities. Therefore, we hypothesize that endosperm formation is pollen-independent, as has been found in other apomicts (Aliyu *et al.*, 2010). Cytohistological and scanning electron microscopy studies of mature seeds of *L. ovalifolium* and *L. multiflorum* showed that the embryo was enveloped by an endosperm tissue that was formed exclusively of polygonal starch grains with a well-defined hilum in the centre, often with radiating clefts (Róis *et al.*, 2012).

Interestingly, a common origin of apomixis and tetraspory has been hypothesized based on the identification of similar cytological characteristics during ovule development (Carman *et al.*, 1991; Carman, 1997; Peel *et al.*, 1997*a*, *b*). 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 in the surviving megaspore (Crane and Carman, 1987), as we also detected here.

Conclusions: linking cpDNA distribution patterns with reproductive modes – evidence of geographical parthenogenesis

Phylogeographical analysis of diploid and tetraploid *Limonium* spp. shows that diploids are more widespread along the Portuguese coast than tetraploids (Figs 1 and 2).

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Interestingly, tetraploids of agamospermous *L. binervosum* have a broad distribution in higher latitudes, extending to the coasts of north-west France and the British Isles (Ingrouille and Stace, 1985, 1986; Lahondère and Biorét, 1995, 1996). Even if northern populations of *L. binervosum* species complex (British Isles) are all tetraploid, diploid *L. ovalifolium* extends north to the coast of France and overlaps there only with tetraploid *L. dodartii*. Hence, it seems that the observed geographical pattern is not a strictly exclusive pattern of ploidy levels. Moreover, for the *Limonium* coastal species studied it appears that latitudinal ice sheet limits, but no altitudinal ones, during the quaternary glaciations (Hewitt, 2011) are involved in the observed geographical pattern by permitting the northern expansion of southern populations/species to suitable but non-optimal habitats, with the subsequent differentiation/adaptation of these front-edge populations.

Indirect estimates of *Limonium* breeding systems based on pollen–stigma dimorphisms (Baker, 1953*c*; Erben, 1978), in addition to our data, show that *L. ovalifolium* are outcrossers with hypothetically very rare apomictic events, whereas male-sterile *L. multiflorum* plants (Róis *et al*., 2012) reproduce through autonomous apomixis. In general, the majority of species of the *L. binervosum* group are characterized by self-incompatible monomorphic pollen–stigma combinations, and pollen is either not produced or is of very low stainability (0–38 %), although well-stained large pollen grains are also observed (Ingrouille and Stace, 1985). Even though it has been suggested species in this group are agamosperms (Ingrouille and Stace, 1985), tetraploid *L. transwallianum* (belonging to the *L. binervosum* complex) is a facultative apomict that produces both reduced tetrasporous and unreduced diplosporous embryo sacs (Hjelmqvist and Grazi, 1964). Thus, it appears that in this species complex both obligate and facultative apomixis may occur. Unfortunately, flow cytometric seed screening studies in diploid and tetraploid *Limonium* species did not enable the determination of their modes of reproduction since each mature seed profile was only characterized by a single embryo peak (Róis *et al*., 2012). Despite our use of high-throughput methods to measure seed formation, we were unable to identify sexual versus apomixis seed production, in addition to autonomous apomixis.

The occurrence of autonomous apomixis, where the need for pollen is completely eliminated, is taxonomically infrequent and occurs in species characterized by 'geographical parthenogenesis', as in many Asteraceae (Noyes, 2007; Hörandl *et al*., 2008). Autonomous apomixis appears to have a role in colonization scenarios (Baker, 1955, 1967; Baker and Stebbins, 1965) and genetic bottlenecks (Hörandl, 2010). Here, the phylogeographical patterns of some tetraploid *Limonium* species suggest the formation of clones restricted to single sites, e.g. *L. binervosum* (populations CM, SJ), *L. dodartii* (populations PCo, PV) or *L. multiflorum* (population FL). In these populations it is possible that uniparental reproduction might alleviate the reduced fecundity resulting from pollen limitation and compensate for the lack of suitable pollinators in the colonized region (Rambuda and Johnson, 2004) after long-distance dispersal of seeds by birds, wind and water along the coast. In conclusion, our phylogeographical and reproductive biology findings suggest a pattern of geographical parthenogenesis in diploid and tetraploid coastal *Limonium* species.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: the intron *trnL* and intergenic spacer *trnL–trnLF* sequence haplotypes of *L. ovalifolium* and *L. binervosum* species complexes. Table S2: pollen exclusion tests and crossing experiments in *L. ovalifolium* and *L. multiflorum*. Figure S1: median-joining network of cpDNA sequence regions in populations of *L. ovalifolium* and *L. binervosum* species complexes. Figure S2: Bayesian phylogenetic analysis of haplotypes. Figure S3: scanning electron microscopy photographs of floral and ovule development in *L. multiflorum* and *L. ovalifolium*.

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