Cytogenetic features of sexual and asexual *Limonium* taxa (Plumbaginaceae)

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Abstract The genus *Limonium* (sea lavenders) is characterized by various taxonomically challenging complexes with great karyological diversity, and chromosomes ranging in size from diminutive to large. In this biosystematic study, our goal was to investigate cytogenetic features of twelve sexual and asexual taxa in a particular part of their distribution range. Genome size, chromosome number variation, and other cytological features were investigated in a representative set of sea lavenders with distinct leaf venation, present in the coastal areas of the Iberian Peninsula, Morocco, France, and the Channel Islands. Flow cytometric genome size determinations were made in plants from natural populations. Cell and nuclear areas of epidermal cells, as well as, chromosome numbers and length were measured in plants from ex situ collections. Our findings revealed positive and significant correlations between holoploid genome size and cell size, nuclei area and chromosome total length. Tetraploid taxa with pinnately veined leaves had significant lower holoploid and monoploid genome sizes along with lower cell and nuclei areas and chromosome sizes than tetraploid taxa with parallel-veined leaves. The results obtained are discussed in the context of breeding relationships known or suspected among these taxa. In conclusion, our results provide a basis for future evolutionary studies, and support that pollen-stigma dimorphisms and genome size allied with chromosome counts might be important taxonomic traits in *Limonium* in apomictic/sexual groups.

Keywords apomixis; chromosome number; flow cytometry; genome size; karyotyping; *Limonium*; Plumbaginaceae

Supplementary Material The Electronic Supplement (Tables S1 & S2) is available from https://doi.org/10.12705/676.10.S

INTRODUCTION

Limonium Mill. (sea lavenders; Caryophyllales: Plumbaginaceae) is a cosmopolitan species-rich genus of approximately 350 species of annuals, perennial herbs, shrubs and lianas, often adapted to extreme saline environments (Kubitzki, 1993). The plants have leafless and underground woody stems, with leaves usually in basal rosettes, and inflorescences usually of terminal panicles or corymbs, the ultimate branches consisting of spikelets of 1–5 flowers with 3 bracts aggregated into spikes, each flower giving rise to a small capsule with a single seed (Erben, 1993: fig. 1). Remarkable flower heteromorphisms such as heterostyly and pollen–stigma dimorphisms are found in *Limonium*. Heterostyly, although not typical in *Limonium*, is present in *L. vulgare* Mill. (Baker, 1948, 1966), whereas pollen–stigma dimorphism associated with a sporophytic self-incompatibility system is common in *Limonium* spp. (Baker, 1953a, b, 1966). In this system, A-type pollen grains (coarsely reticulate) germinate on papillose stigmas (papillate cells) and B-pollen type (finely reticulate) germinate on cob-like stigmas (polygonal cells), while the reverse combinations produce no successful fertilization (Baker, 1953a, b). Thus dimorphic pollen–stigma populations with plants featuring flowers with distinct pollen grain types and dissimilar stigma types appear to be outcrossing. In contrast, monomorphic self-incompatible populations showing only one pollen–stigma combination seem to produce seeds through apomixis (agamospermy; asexual reproduction through seeds) (Baker, 1953a, b, 1966). Therefore, the primary reproductive strategies of some *Limonium* species have been inferred upon analyses of such flower heteromorphism (Baker, 1953a, b; Erben, 1978, 1999; Ingrouille & Stace, 1986). Female gametophyte development studies reported tetrasporous (meiotic) embryo sacs of various types, including Adoxa, Fritillaria, Drusa, Penea and Gagea-ova types (Dahlgren, 1916; D'Amato, 1940, 1949; Hjelmqvist & Grazi, 1964; Róis & al., 2016). Facultative gametophytic apomicts like triploid *L. virgatum* (Willd.) Fourr. form tetrasporic (meiotic) embryo sacs of the Adoxa type, in parallel with apomictic diplosporic embryo sacs of the Erigeron type (studied as *Statice oleaefolia* var. *confusa* Godr.; D'Amato, 1949). Autonomous apomictic development seems also to occur in *L. multiflorum* Erben, which produces

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diplosporic (apomictic) embryo sacs of the Rudbeckia type (Róis & al., 2016). Nevertheless, for most putatively apomictic *Limonium* taxa, no information is available on the patterns of female gametophyte formation.

The effects of hybridization, polyploidy and apomixis appear to have all contributed to shape the radiation of *Limonium* spp. (Erben, 1978; Lledó & al., 2005). Various series of complex aggregates of sexual diploid species and putative asexual polyploid hybrids have been described (Erben, 1978, 1993). Molecular phylogenetic studies based on morphological traits, plastid DNA and nrITS sequences present an infrageneric classification of the genus, except for the species-rich Mediterranean clade that requires additional, dense sampling (Lledó & al., 2005; Malekmohammadi & al., 2017).

Karyological studies reported a high cytological variability in *Limonium*, with basic chromosome numbers varying from $x = 6$ to $x = 9$, being $x = 8$ and $x = 9$ the most frequent. Ploidy levels vary from diploid ($2x$) to hexaploid ($6x$), and chromosome numbers range from $2n = 12$ in *L. pectinatum* (Aitron) Kuntze (Larsen, 1958) to $2n = 72$ in *L. narbonense* Mill. (Georgakopoulou & al., 2006). It seems that triploids are by far the predominant cytotype in the Iberian Peninsula and in the Balearic Islands (Erben, 1978, 1979; Cowan & al., 1998; Castro & Rosselló, 2007). The most prevalent basic *Limonium* chromosome numbers in the Western Mediterranean are $x = 8$ and $x = 9$ found in multiple combinations to give a complete range of even and odd chromosome numbers (Erben, 1978, 1979; Castro & Rosselló, 2007).

In this biosystematic study our goal was to examine genome sizes and their relationships with chromosome numbers in 12 widespread sexual and asexual *Limonium* taxa with pinnate and parallel leaf venation, and a taxon with univeined leaves found on the coasts of Morocco, the Iberian Peninsula, France, and the Channel Islands (Erben, 1978, 1993; Lahondère & Biorét, 1996; Fennane & al., 2014). In these regions, the taxonomic group with pinnate venation is represented by *L. vulgare* and related taxa, which comprise sexual and putative apomict tetraploids (Erben, 1978, 1993; Cortinhas & al., 2015). The taxonomic group with parallel venation includes the *L. ovalifolium* complex of sexual diploids, the *L. binervosum* complex of facultatively apomictic tetraploids, and putatively apomictic triploids *L. algarvense* Erben and *L. normannicum* Ingrouille (Erben, 1978, 1993; Lahondère & Biorét, 1996; Caperta & al., 2017; Fennane & al., 2014). Finally, the sole species with univeined leaves is triploid *L. virgatum* (Erben, 1978, 1993). Most of the available cytogenetic data have been restricted to chromosome counts and, to a lesser extent, to reporting genome sizes ranging from $2n = 2x = 3.5$ pg/2C (*L. ovalifolium* (Poir.) Kuntze) to $2n = 4x = 7.7$ pg/2C (*L. multiflorum*) (Róis & al., 2012; Caperta & al., 2017). However, previous work suggested contrasting differences in chromosome size among taxa with pinnate or parallel venation (A. Caperta & A.S. Róis, unpub. data). In this study we addressed the relationships between genome size data, cell, and nucleotypic features in *Limonium* species representative of these regions, and their possible bearing on evolution in the genus. Specifically, we estimated genome size using flow cytometry, measured cell and nuclear areas and performed chromosome counts, and discussed the results obtained by correlating existing data on breeding system with the results described.

■ MATERIALS AND METHODS

Study species. — The *Limonium* taxa studied here are named following the treatments of Erben (1978, 1993, 1999), Stace (2010), and Cortinhas & al. (2015). Individual species can be grouped according to leaf venation type (Erben, 1998, 1993; Stace, 2010) and ploidy level (Erben, 1993; Ingrouille, 1985; Róis & al., 2012, 2018; Caperta & al., 2017) (Table 1). Their geographic distribution is given in Table 1 (based on Erben, 1993, 1999; Lahondère & Biorét, 1996; Stace, 2010; Fennane & al., 2014; Cortinhas & al., 2015; Brullo & Erben, 2016; Caperta & al., 2017).

Flow cytometric genome size estimations. — Genome size was assessed for 244 wild-collected individuals sampled randomly from 34 natural populations (three populations per species, except for *L. vulgare* where three populations were included, *L. virgatum* where only two populations were available, and one *L. normannicum* population; Electr. Suppl.: Table S1) using flow cytometry following the methodology described in Caperta & al. (2017). A second *L. normannicum* population (English Channel, Jersey, U.K., please see Electr. Suppl.: Table S1) was established ex situ using seeds provided by Kew's Millennium Seed Bank, U.K. (http://apps.kew.org). Briefly, nuclei were isolated from fresh leaf material following the procedure of Galbraith & al. (1983) in WPB buffer (Loureiro & al., 2007), using *Pisum sativum* 'Ctirad' (2C = 9.09 pg; Doležel & al., 1998) as internal reference standard. The nuclear suspension was filtered, stained with propidium iodide and analysed using a Partec CyFlow Space flow cytometer (532 nm green solid-state laser, operating at 30 mW; Partec, Görlitz, Germany). The following histograms/cytograms were obtained: fluorescence pulse integral in linear scale (FL); forward light scatter (FS) vs. side light scatter (SS), both in logarithmic (log) scale; FL vs. time; and FL vs. SS in log scale. For most samples, a polygonal region was defined in the latter graphic to include intact nuclei only, and this region was subsequently used to gate all the other graphics. At least 1300 particles per G_1 peak were analysed per sample (Suda & al., 2007). In most cases, the quality of the histograms was good, with CV values of both the sample (average CV of 4.17%) and internal reference standard (average CV of 2.82%) being, in most cases, below the 5% quality control threshold. When G_1 peaks presented higher CV values, samples were discarded, and a new sample was prepared until the defined quality standards were achieved. Usually two replicated independent runs were made per individual, being assured that a variation lower than 2% was obtained for each individual.

The value of genome size in mass units (2C in pg; sensu Greilhuber & al., 2005) was obtained for each individual analysed using the following equation: *Limonium* 2C nuclear DNA content [pg] = (*Limonium* G_1 peak mean / reference standard G_1 peak mean) * genome size of the reference standard. The

monoploid genome size (1Cx; sensu Greilhuber & al., 2005) was calculated by dividing the genome size with the ploidy level.

Cell and nuclear areas measurements. — Samples for measurements of 1029 cells and nuclei were obtained from cultivated plants (999 cells/nuclei per individual, except for *L. vulgare* with 80 cells/nuclei, *L. maritimum* with 60 cells/nuclei and *L. narbonense* with 49 cells/nuclei). These plants were obtained from seeds collected in the same natural populations used for flow cytometry. For cell and nuclear areas measurements, a modified procedure by Fuchs & al. (2015) was employed. Upper epidermal layers from healthy young leaves of two to three specimens from all studied species were collected under a stereomicroscope (Zeiss Stemi 2000, Jena, Germany) and were fixed in fresh ethanol : glacial acetic acid (3 : 1, v/v) solution at room temperature for 24 h. Afterwards, the tissues were placed in a microscope well-slide with 8 µl of 4′,6-diamidino-2-phenylindole (DAPI, 100 ng/ml in water) for 4 h. Tri-dimensional images of epidermis cells and their nuclei were acquired using a Zeiss epifluorescence microscope (Axioskop 2) and photographed with an AxioCam MRc5 digital camera (Zeiss). Cell and nuclear areas were measured using the ImageJ v.1.50f3 (https://imagej.nih.gov/ij/, Rasband, 2016) software.

Chromosome preparations and karyotyping. — Root-tips from three plants of each species obtained from the same seed parents collected in the natural populations for genome size estimations were used for chromosome preparations, following Caperta & al. (2017). Briefly, root tips were excised and cold treated for 36 h at 0°C. Root tips were then fixed in a fresh absolute ethanol : glacial acetic acid (3 : 1, v/v) solution overnight and stored in 70% ethanol at −20 °C. Afterwards, root tips were digested in a pectolytic enzyme mixture (2% cellulase [Sigma, St. Louis, Missouri, U.S.A.], 2% cellulase ‘‘Onozuka R-10’’ [Serva , Heidelberg, Germany], and 2% pectinase enzyme [Sigma] solution in 1× Enzyme Buffer [EB, 40 ml 0.1 M citric acid-1-hydrate and 60 ml of 0.1 M sodium citrate dihydrate; pH 4.8]) for 150 min at 37°C. Chromosome preparations were made in 60% acetic acid and stained with DAPI (1 mg/ml) in Vectashield (Vector Laboratories, Peterborough, U.K.). Chromosomes were observed with a ×63 or ×100 objective using a Zeiss Axioskop 2 fluorescence microscope and photographed with an AxioCam MRc5 digital camera (Zeiss). Well-spread chromosome complements were measured on micrographs recorded with a ×100 objective using the Axiovision 4.0 (Zeiss).

Statistical analyses. — Descriptive statistics were calculated for the holoploid genome size and monoploid genome size. Differences in holoploid and monoploid genome sizes were explored using generalized linear mixed models (GLMM), first, grouping species according to venation and ploidy level (fixed factor with the following categories: parallel venation – 2*x*, parallel venation – 3*x*, parallel venation – 4*x*, and pinnate venation – 4*x*, univeined – 3*x*) and including population nested within species as random factor; and second, considering all the species separately defining population as random factor.

An analysis of variance (one way-ANOVA) was used to assess differences in the cell and nuclei parameters between species (Khan & Rayner, 2003), with both variables square root transformed. A Tukey’s HSD test ($P < 0.05$) was used to identify the homogeneous groups when significant differences were found among species for a given parameter. Correlation between mean holoploid genome size and mean cell and nuclei areas were explored with Pearson Correlation. Correlation coefficients between nuclei and cell areas were also explored within groups of venation and for each species separately, with nuclei and cell areas square root transformed. Statistical analyses were performed in R v.3.1.1 (R Core Team, 2014).

■ RESULTS

The nuclear DNA content of the distinct ploidy groups differed significantly for both holoploid and monoploid genome sizes (Table 1; Fig. 1A, B). Holoploid genome size differed significantly between the three ploidy levels within the group with leaves with parallel venation ($P < 0.05$). Instead, the tetraploid species with leaves with pinnate venation (i.e., *L. maritimum*, *L. narbonense* and *L. vulgare*) presented significantly lower genome sizes than the tetraploid taxa with leaves with parallel venation (i.e., *L. binervosum*, *L. dodartii*, *L. multiflorum*), while being similar to the values obtained for the triploid species with parallel venation (Table 1; Fig. 1A, B). The taxon with univeined leaves, here represented by triploid *L. virgatum*, differed significantly from virtually all the other groups, including the triploid species with parallel venation ($P < 0.05$). These general patterns were also observed when the species were analysed individually (Table 1). The differences in holoploid genome size and ploidy levels resulted in significant differences also in monoploid genome size among taxa.

In general, measurements of cell and nuclei areas (Figs. 1C, D, 2, 3) in taxa with leaves with parallel venation revealed overall significant correlations between nuclear and cell areas ($r^2 = 0.477$, $P < 0.001$) that were non-significant within each species (except for *L. multiflorum*; Fig. 3B; Electr. Suppl.: Table S2); contrarily, taxa with leaves with pinnate venation and univeined leaves showed no correlations (overall: $r^2 = 0.038$, $P = 0.616$; within taxa: see Fig. 3C, D; Electr. Suppl.: Table S2). In species with parallel venation significant differences between cell area mean (F (7,729) = 163, $P < 0.001$) and nuclear area mean (F (7,729) = 16.29, $P < 0.001$) were found. By contrast, in species with pinnate venation, no differences among species in cell area mean (F (2,172) = 0.692, $P = 0.502$) and in nuclear area mean (F (2,172) = 12.85, $P < 0.001$) were detected. In taxa with leaves with parallel venation, genome size differences among ploidy groups (Table 1) were reflected by larger average areas of nuclei in tetraploid taxa (Fig. 3B; Electr. Suppl.: Table S2). Nuclei areas of tetraploid taxa with leaves with pinnate venation did not differ from tetraploid taxa with leaves with parallel venation (except for *L. multiflorum*). Overall correlations between genome size and cell, nuclei and chromosome size produced significant patterns: holoploid genome size was positively and significantly correlated with cell size ($r^2 = 0.856$, $P < 0.001$), nuclei area ($r^2 = 0.788$, $P = 0.002$) and chromosome total length ($r^2 = 0.899$, $P < 0.001$) (Fig. 3).

Calculations of total chromosome lengths in individuals with parallel or pinnately veined leaves, or univeined leaves

(24 individuals from ten taxa) with approximately the same degree of condensation revealed that metaphase cells with more chromosomes showed a larger chromosome complement length (Fig. 4; Table 2). Expectedly, tetraploid taxa presented in average higher total chromosome length than triploid and diploid taxa (Table 2). The same pattern was observed within taxa varying in the number of chromosomes.

For example, in *L. multiflorum* euploid and aneuploid individuals with parallel venation total chromosome length varied from 90.26 ± 14.20 µm in plants with $2n = 34$, to 103.81 µm in plants with $2n = 35$, and 187.30 ± 14.27 µm in plants with $2n = 36$ chromosomes. Nevertheless, tetraploid taxa with leaves with pinnate venation, which also presented $2n = 36$, showed shorter chromosomes lengths than tetraploid taxa with parallel venation in accordance with the differences observed in genome size estimates (Table 1; Electr. Suppl.: Table S2).

■ DISCUSSION

Plumbaginaceae with nearly 90% of coastal species, and in particular the genus *Limonium*, are among the best-represented taxa of coastal habitats (Kubitzki, 1993; Van der Maarel & Van der Maarel-Versluys, 1996). However, *Limonium* taxonomy is extremely complex given the intricate processes of polyploidization and hybridization, as well as unusual reproductive strategies (Erben, 1978; Lledó & al., 2005; Róis & al., 2016, 2018). On the coasts of the North Atlantic and Mediterranean, northwest Morocco and northwest France, and in the Channel Islands the genus *Limonium* is represented by taxa with leaves with parallel or pinnate venation or univeined leaves (Erben, 1993; Lahondère & Biorét, 1996; Fennane & al., 2014). In this study, chromosome counts and flow cytometry data from the same natural populations enabled us, for the first time, to interpret variation in nuclear DNA content in terms of variation in genome size and ploidy level.

The taxonomic relationships between the species studied here have been explored in previous studies. Morphometric and genetic data were used to define the relationships among species with parallel venation like diploid species of the *L. ovalifolium* complex (Erben, 1993, 1999; Róis & al., 2013), triploid *L. algarvense* (Malekmohammadi & al., 2017), and tetraploid agamospermous species of the *L. binervosum* complex (Ingrouille, 1984; Róis & al., 2013), and triploid *L. virgatum* with univeined leaves (Erben, 1993; Lledó & al., 2005; Malekmohammadi & al., 2017). A similar strategy has been applied to describe morphological and genetic differentiation patterns of the closely related *L. vulgare* species complex with

Table 1. Genome size estimates obtained in *Limonium* taxa with leaves with parallel or pinnate venation, or univeined leaves.

Species	Geographic distribution	Ploidy level	2C G.s. [pg]		1Cx G.s. [pg]		n
Parallel venation							
L. lanceolatum	IP, Mo	2*x*	3.60 ± 0.07[a]	3.60 ± 0.07[a]	1.79 ± 0.04[b]	1.80 ± 0.04[b]	25
L. nydeggeri	Mo, P	2*x*		3.59 ± 0.05[a]		1.80 ± 0.03[b]	19
L. ovalifolium	F, IP, Mo	2*x*		3.53 ± 0.07[a]		1.76 ± 0.03[b]	12
L. algarvense	IP, Mo	3*x*	5.72 ± 0.15[c]	5.76 ± 0.13[c]	1.91 ± 0.05[c]	1.92 ± 0.04[c]	24
L. normannicum	Ch	3*x*		5.64 ± 0.15[b,c]		1.88 ± 0.05[c]	10
L. binervosum	F, I, IP, UK	4*x*	7.51 ± 0.17[d]	7.41 ± 0.16[d]	1.88 ± 0.04[c]	1.85 ± 0.04[b,c]	18
L. dodartii	F, IP	4*x*		7.46 ± 0.05[d]		1.86 ± 0.01[b,c]	13
L. multiflorum	P	4*x*		7.63 ± 0.16[d]		1.91 ± 0.04[c]	21
Pinnate venation							
L. maritimum	P	4*x*	5.73 ± 0.12[c]	5.79 ± 0.06[c]	1.43 ± 0.03[a]	1.45 ± 0.02[a]	25
L. narbonense	F, P, Mo	4*x*		5.63 ± 0.11[b,c]		1.41 ± 0.03[a]	31
L. vulgare	F, IP, Mo; UK	4*x*		5.76 ± 0.10[c]		1.44 ± 0.02[a]	41
Univeined leaves							
L. virgatum	G, IP	3*x*	5.38 ± 0.12[b]	5.38 ± 0.12[b]	1.79 ± 0.04[b]	1.79 ± 0.04[b]	5
Statistical test			$\chi^2_4 = 3456.4$	$\chi^2_{11} = 8096.6$	$\chi^2_4 = 808.8$	$\chi^2_{11} = 1906.3$	
			$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	

Leaf venation types followed the descriptions in Erben (1978, 1993) and Stace (2010), and individual species geographic distribution followed that described in Erben (1993, 1999), Lahondère & Biorét (1996), Stace (2010), Fennane & al. (2014), Cortinhas & al. (2015), Brullo & Erben (2016), and Caperta & al. (2017): Ch, Channel Islands; F, France; G, Greece; I, Ireland; IP, Iberian Peninsula; Mo, Morocco; P, Portugal; UK, United Kingdom. Ploidy level, holoploid genome size (2C G.s.; in pg) and monoploid genome size (1Cx G.s.; in pg) are provided for each taxon and for each ploidy levels within major groups. Superscript letters denote groups of statistically similar observations; different letters denote groups that differ significantly at $P < 0.05$; the letters are ordered according to the mean values in ascending order. The number of individual plants analysed is also provided for each taxon (n). Descriptive statistics were calculated for the holoploid genome size and monoploid genome size. Differences in holoploid and monoploid genome sizes were explored using generalized linear mixed models.

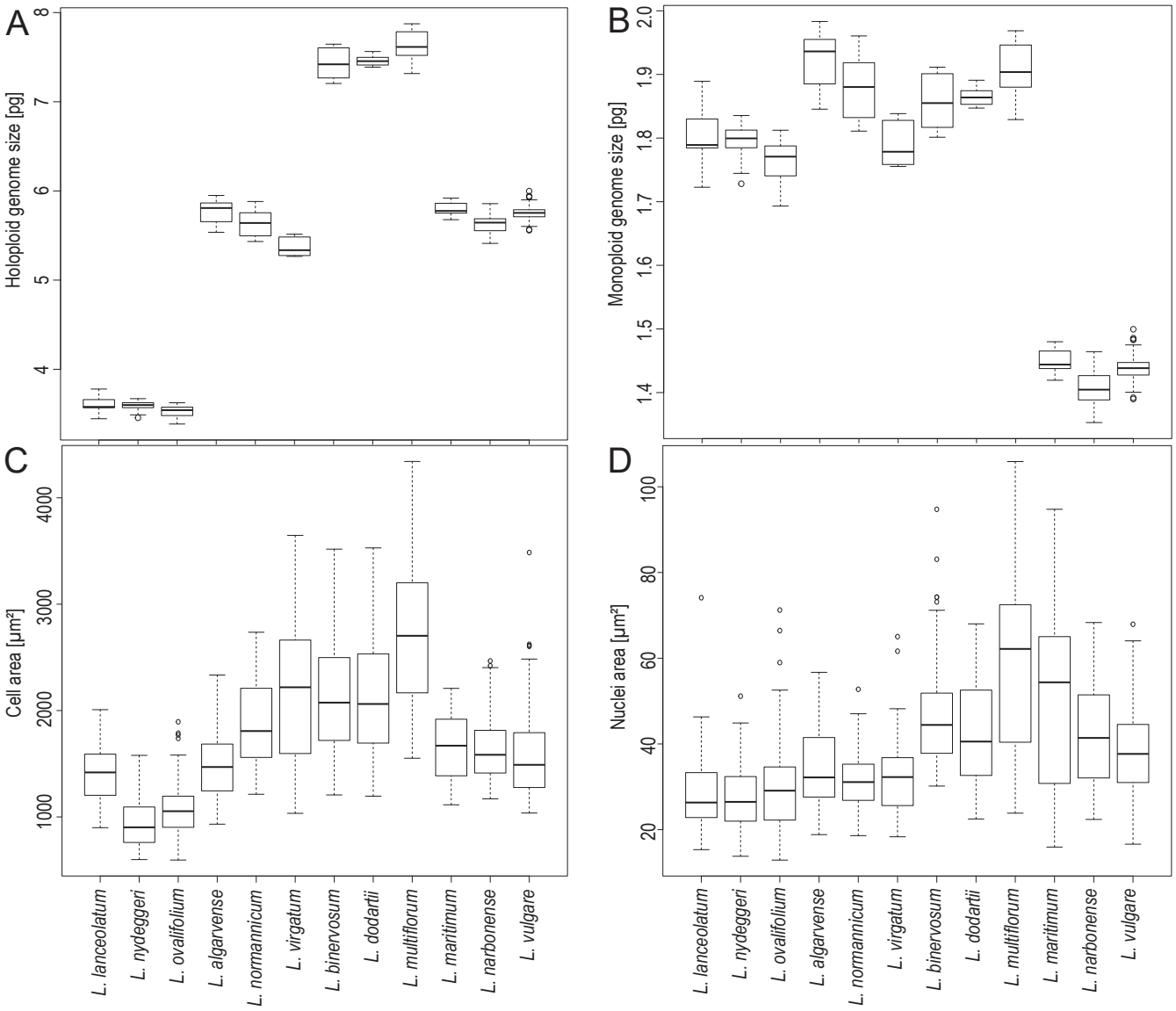

Fig. 1. Boxplots of holoploid **(A),** monoploid **(B),** and cell **(C)** and nuclei **(D)** areas in diploid and polyploid *Limonium* taxa with parallel or pinnately veined leaves, or univeined leaves.

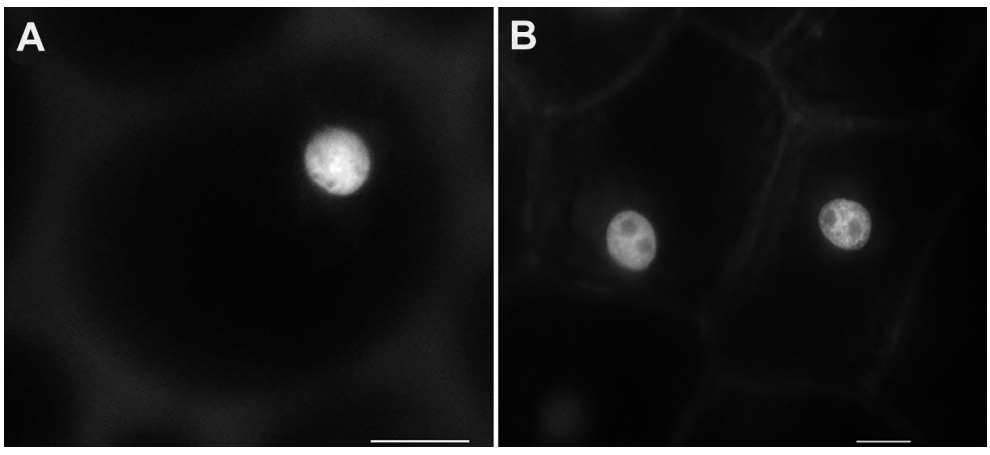

Fig. 2. Cell and nuclei of *Limonium* sp. leaf epidermal cells. Significant differences ($P < 0.01\%$) exist between cell and nuclear areas between diploid *Limonium nydeggeri* **(A)** and tetraploid *L. maritimum* **(B)**. — Scale bars: 10 µm.

pinnate venation (Erben, 1993; Lledó & al., 2005; Cortinhas & al., 2015; Malekmohammadi & al., 2017; Róis & al., 2018).

Monoploid genome sizes differed significantly between taxa with leaves with pinnate venation and taxa with parallel venation, with the latter being similar to the univeined taxon. Variation in nuclear DNA C-values in the studied species was more or less continuous within taxa. Despite tetraploid taxa have approximately the same chromosome numbers, species with leaves with pinnate venation have significantly smaller holoploid genome sizes than tetraploid species with parallel venation. Our results are further supported by other related cytological parameters measured in both groups, namely nuclear area and chromosome complement total length, which showed the same positive correlations. Similar observations were found in the carnivorous genus *Genlisea* A.St.-Hil., where large differences in genome size between species with the same chromosome number were detected (~18-fold). This is also in agreement with the 2.3× larger nuclear and 8× larger cellular volumes found in *Genlisea* species with large genomes (Fuchs & al., 2015). Furthermore our findings agree with results from

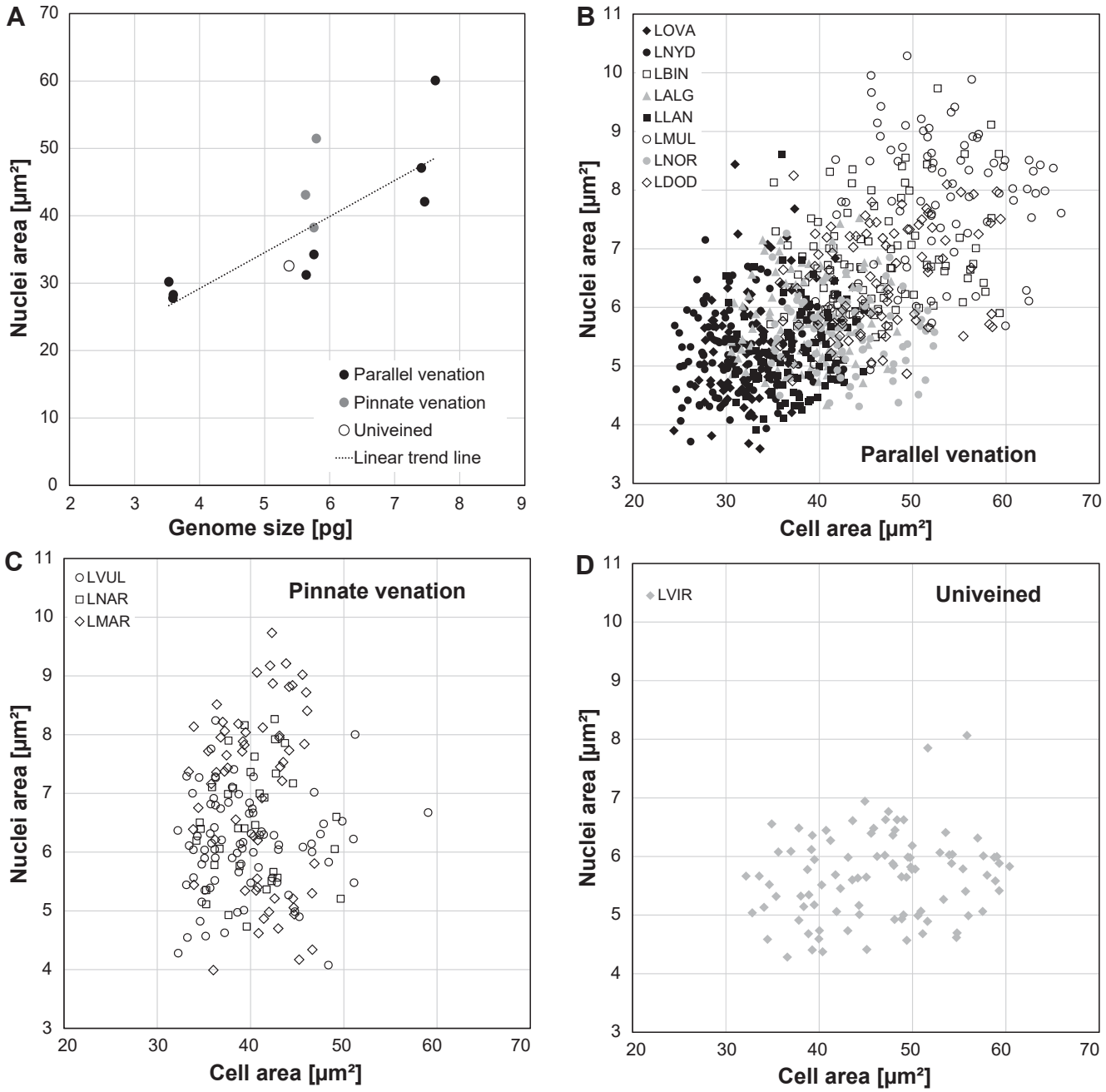

Fig. 3. Correlations among cytogenetic features observed in *Limonium* sp. in this study. **A,** Holoploid genome size was positively and significantly correlated with nuclei area; **B,** In taxa with parallel venation nuclear and cell areas are significantly correlated ($P < 0.001$); **C & D,** Contrastingly, no correlations are found in cell and in nuclear areas in taxa with pinnate venation **(C)** nor in species with univeined leaves ($P < 0.001$) **(D)**.

another work which revealed a positive correlation between nuclear DNA content and nuclear and cell volume in angiosperms (Jovtchev & al., 2006). Moreover, we found that taxa with a larger chromosome complement have a larger average total chromosome length. Among taxa with leaves with parallel venation or univeined leaves, monoploid genome size estimations were fairly similar among the taxa, and the ploidy levels revealed the additivity of chromosome sets expected in related polyploid taxa; i.e., tetraploids have approximately double the genome size of diploids, with the triploids having intermediate genome sizes. Tetraploid taxa with pinnately veined leaves present significantly lower homoploid genome sizes than diploid and polyploid taxa with leaves with parallel veined or univeined leaves.

Genome size variation in plants representative of each of the studied taxa with different leaf venation can be associated with a noticeable diversity in several other characters such as morphology, breeding systems, polymorphism in DNA methylation patterns, and plastid DNA haplotypes (Cortinhas & al., 2015; Róis & al., 2012, 2013, 2016). In taxa with parallel-veined leaves, genetic/epigenetic analyses showed a clear and pronounced discrimination between ploidy levels on the basis of epigenetic profiles, with tetraploids (*L. multiflorum*, *L. dodartii*) presenting lower levels of genetic variability, but higher levels of methylation than diploids (*L. ovalifolium*, *L. nydeggeri*) (Róis & al., 2013). Chloroplast DNA data for these taxa indicate species-specific haplotypes for both ploidy levels, with diploids presenting higher haplotype diversity than tetraploids (very little to no haplotype diversity; Róis & al., 2016). These observations could be most plausibly explained by the breeding systems of these taxa. While diploid *L. ovalifolium* reproduces sexually (Róis & al., 2012, 2016), the tetraploids *L. binervosum* and *L. multiflorum* are apomictic (Hjelmqvist & Grazi, 1964; Ingrouille & Stace, 1986; Róis & al., 2016). A pattern of "geographical parthenogenesis" was suggested for these taxa, with tetraploid apomicts tending to be distributed at higher latitudes than the diploid sexuals (Róis & al., 2016), while absent in the southern portions of diploid ranges (Caperta & al., 2017). Interestingly, triploid *L. algarvense*, which is closely related morphologically to the diploid *L. ovalifolium* (Ingrouille, 1985; Erben, 1993), has a genome size intermediate between the diploid and tetraploid taxa with parallel-veined leaves. *Limonium algarvense* occupies areas of sympatry in the southern limits of diploids (Caperta & al., 2017). It has been hypothesized that *L. algarvense* originated from crosses between *L. ovalifolium* and *L. binervosum* (Ingrouille, 1985) but this assumption remains to be tested. In both of the triploids *L. algarvense* and *L. normannicum* only the self-incompatible morph (monomorphic B-type pollen/papillate stigma) was found in individual plants (Ingrouille, 1985), which possibly indicates the presence of apomictic reproduction, as both taxa are able to produce viable seeds (Ingrouille, 1985; Caperta & al., 2017). Regarding the sole representative taxon with univeined leaves, the triploid *L. virgatum* has significantly different genome size compared

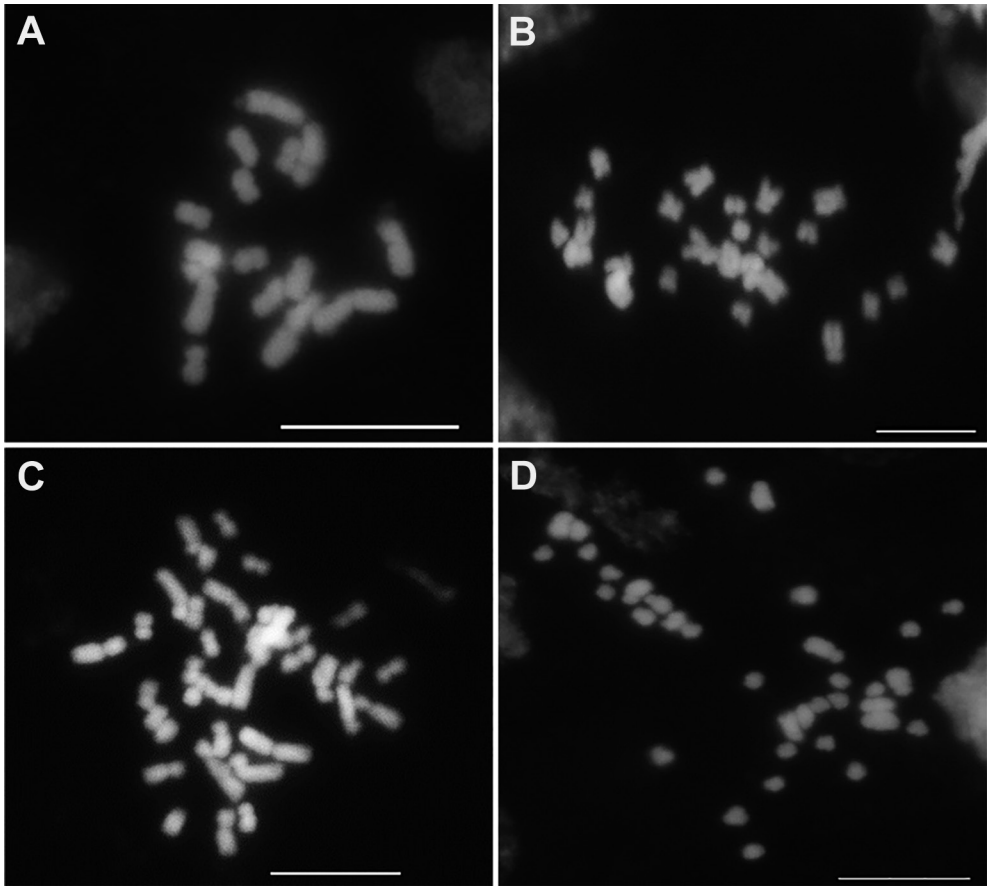

Fig. 4. Mitotic metaphase plates of DAPI-stained metaphase spreads from *Limonium* spp. with parallel and pinnate venation, or univeined leaves. **A,** *Limonium nydeggeri* (2*n* = 16 chromosomes); **B,** *Limonium virgatum* (2*n* = 27); **C,** *L. binervosum* (2*n* = 35); **D,** *L. narbonense* (2*n* = 34). — Scale bars: 10 µm.

Table 2. List of investigated *Limonium* taxa with parallel or pinnately veined leaves, or univeined leaves.

Taxa	Voucher	Chromosome counts		KT	Total chromosome length (µm)
		Observed (2*n*)	Literature (2*n*)		
Parallel venation					
L. nydeggeri	LnyCarI1 LnySRI1	16	15, 16, 17[a,c]	5	27.40 ± 9.17
L. ovalifolium	LovaCSI1 LovaFerI1	16	16[a,b]	6	39.57 ± 8.46
L. algarvense	LalgAI1	24, 26	251[d,e]	2 (24) 1 (26)	45.39 ± 14.16 (24) 58.34 (26)
L. binervosum	LbinSPMI1	34, 35	27, 35[a,b,c]	1 (34) 1 (35)	131.32 (34) 104.79 (35)
L. dodartii	LdodCSI2	36	35, 36[a,b]	1	156.19
L. multiflorum	LmulCRI1 LmulCRI2 LmulCRI7	34, 35, 36	32, 35, 36[a,b,c]	2 (34) 1 (35) 2 (36)	90.26 ± 14.20 (34) 103.81 (35) 187.30 ± 14.27 (36)
Pinnate venation					
L. maritimum	LmarArI1 LmarArI2	36	–	3	83.96 ± 8.85
L. narbonense	LnarVTSI1	34	36, 54, 72[b,f,g]	2	80.70 ± 2.65
L. vulgare	LvulBI2 LvulRAI1 LvulAI1	35, 36, 37	35, 36, 37[h,i]	3 (35) 1 (36) 1 (37)	116.34 ± 37.48 (35) 98.47 (36) 135.05 (37)
Univeined leaves					
L. virgatum	LvirCRI4	27	27[a,b]	9	65.82 ± 10.34

Voucher: referring to plants present in ex situ collections used for karyotype analysis; Chromosome counts observed in this study (Observed) and reported elsewhere in the literature are provided (Literature); KT: number of metaphases per individual analysed for karyotype.
a – Erben, 1978; b – Erben, 1993; c – Róis & al., 2012; d – Ingrouille, 1985; e – Caperta & al., 2017; f – Arrigoni & Diana, 1999; g – Georgakopoulou & al., 2006; h – Dawson & Ingrouille, 1995; i – Cortinhas & al., 2015.

to other triploids, being a facultative apomict as it forms tetrasporic (meiotic) embryo sacs of *Adoxa* type, in parallel with apomictic, diplosporic embryo sacs of *Erigeron* type (D'Amato, 1949). However, the breeding relationships between this taxon, and the abovementioned diploid and tetraploid taxa, or even with other triploid taxa are yet to be uncovered.

The closely related tetraploid taxa with leaves with pinnate venation *L. vulgare* and *L. narbonense* appear to be obligate outcrossers, according to the occurrence of heterostyly and pollen-stigma dimorphic populations (Erben, 1993; Dawson & Ingrouille, 1995; Cortinhas & al., 2015), and high male fertility (Erben, 1979). At least for *L. narbonense*, an autotetraploid origin is presumed, given the microsatellite amplification patterns in populations from eastern Spain (Palop-Esteban & al., 2011). By contrast, tetraploid *L. maritimum*, which present the same genome size and chromosome numbers as the above taxa, show homostylic flowers with a unique pollen–stigma morph (B), suggesting that it might reproduce through apomixis.

■ CONCLUSIONS

In conclusion, our findings revealed that genome size in combination with chromosome counts provide a valuable tool for studies of *Limonum* taxonomy. Taken altogether, our results support that morphologically related sexual diploids of the *L. ovalifolium* complex and *L. algarvense* putative apomictic triploids with leaves with parallel venation, present cytogenetic similarities among them. The same applies to facultative apomictic tetraploids of the *L. binervosum* complex with parallel-veined leaves, which display morphological similarities (other than leaf venation) with the triploid facultative apomict with univeined leaves, *L. virgatum*. All triploids showed an intermediate genome size, chromosome numbers and length in comparison with taxa of other ploidy groups. Interestingly, the cytogenetic features found in these triploids are similar to all studied tetraploid sexual/apomictic taxa with leaves with pinnate venation. Considering all this, the present study supports an independent evolution between taxa with parallel or pinnate venation.

■ AUTHOR CONTRIBUTIONS

ADC conceived and coordinated the study. SIRC and JL participated in study design. SIRC, ASR, SC and JL performed the comparative cytogenetic experiments and statistical analyses. ADC wrote the manuscript with the input from the other co-authors. All authors read and approved

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