SYSTEMATICS AND PHYLOGENY

Chromosome evolution in the cosmopolitan genus *Lycium* (Solanaceae)

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Abstract *Lycium* is the only member of tribe Lycieae (Solanoideae, Solanaceae), and it has a cosmopolitan distribution with its greatest diversity in southern South America, southern Africa, and southwestern North America. To date, there has been no attempt to synthesize and evaluate the significance of the available cytogenetical data from a phylogenetic perspective, which is the objective of this study. Firstly, new data on 27 taxa from all its range of distribution (FISH in all of them, banding in 23, Feulgen technique in 14) were provided to fill gaps in the information. The chromosome numbers of *L. australe*, *L. humile*, and *L. repens* were recorded for the first time. Species showed $x = 12$ with different ploidy levels (mostly diploid or tetraploid), small chromosomes, and symmetrical karyotypes. *Lycium fremontii* and *L. repens* were outstanding for having the highest numbers reported for the genus: $10x$ and $11x$, respectively. North American species showed comparatively longer chromosomes. Secondly, cytogenetical traits were mapped on a phylogenetic tree, using character mapping and ancestral states reconstruction, to understand the dynamics of the evolutionary changes. The main cytotaxonomical features were included: chromosome number, presence of polyploidy, total length of the haploid chromosome set, mean chromosome length, karyotype formula, A_1 and A_2 asymmetry indices, percentage of heterochromatin, number of $CMA^+/DAPI^-$ NORs bands, and number and position of 5S and 18S-5.8S-26S sites. The mean chromosome length, total haploid chromosome length, number of 18S-5.8S-26S loci, number of $CMA^+/DAPI^-$ NORs bands underwent comparatively few transitions compared with the ploidy level and number of 5S loci. The mapping of the characters on the phylogenetic tree showed that the most probable ancestral chromosome number was $2n = 24$ with several independent polyploidization events and one pair of each rDNA locus. The most likely ancestral condition for the genus would be: diploid, with small chromosomes, scarce heterochromatin, asynteny of rDNA loci, and one pair of both 18S-5.8S-26S and 5S loci.

Keywords ancestral state reconstruction; cytogenetics; heterochromatin; phylogeny; rDNA

Supporting Information may be found online in the Supporting Information section at the end of the article.

■ INTRODUCTION

The Solanaceae, or "Nightshades", is one of the most important angiosperm families serving mankind, ranking behind grasses, legumes, and brassicas. It includes about 2500 species in ca. 100 genera with its centre of diversification in South America and an estimated age of 17 million years (Olmstead & al., 2008; Särkinen & al., 2013). Several aspects deserve to be highlighted: (1) the enormous importance of some members as food plants, such as the potato (*Solanum tuberosum* L.), tomato (*S. lycopersicum* L.), peppers (several species of *Capsicum* L.), and eggplant (*S. melongena* L.); (2) many are sources of drugs used in medicine and pharmacology and as insecticides that can become powerful poisons in excess, as belladonna (*Atropa belladonna* L.) and mandrake (*Mandragora officinarum* L.); (3) several members are of ornamental interest, as species of *Petunia* Juss., *Schizanthus* Ruiz & Pav., and *Brugmansia* Pers.; (4) some taxa are fundamental as biological model systems in biotechnology, physiology and molecular biology, such as tobacco (*Nicotiana tabacum* L.), tomato, potato, and *Petunia*; and in addition, some are harmful weeds (Heiser, 1969; Hunziker, 2001). The family is widely distributed in tropical and temperate regions of the world, but there are only three genera with native cosmopolitan distributions: *Solanum* L., *Physalis* L., and *Lycium* L. (Hunziker, 2001).

Lycium is the only member of tribe Lycieae (subfam. Solanoideae), and it consists of about 90 woody species (Chiang-Cabrera, 1981; Bernardello, 1986; Bernardello & Hunziker, 1987; Venter, 2000; Levin & al., 2007, 2011); formerly, it included two small American genera (*Grabowskia* Schltdl., *Phrodus* Miers) that were recently synonymised with it (Levin & al., 2011). Knowledge of these plants has an ecological importance due to their adaptations to xerophytic and

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halophytic environments that allow them to grow in arid and semi-arid habitats. The genus has its greatest diversity in southern South America, southern Africa, and southwestern North America, with species distributed in the Mediterranean region and across Asia and Australia. It probably originated in South America 5 million years ago, with a single dispersal event from South America to Africa approximately 3.5 million years ago (Levin & al., 2007; Miller & al., 2011), although the recent finding of an Eocene *Physalis* fossil indicates that fossils are considerably older than corresponding molecular divergence dates (Wilf & al., 2017).

Chromosome studies on plants and animals have revealed significant variations in karyotypes, i.e., in chromosome number, size, shape and structure, gene localization, etc. (Stebbins, 1971; Sharma & Sen, 2002; Guerra, 2012). Thus, chromosomes are useful to infer phylogenetic relationships because they are hereditary elements and discrete units of mutation (Weiss-Schneeweiss & Schneeweiss, 2013). Karyotype features represent a significant aspect in plant speciation, since chromosomal differences establish immediate postzygotic crossing barriers (Rieseberg, 1997), and karyotype evolution is generally consistent with clade differentiation (Blöch & al., 2009). Cytogenetical data provide key characters for plant systematics and evolution (Stace, 2000), especially when combined with molecular phylogenies (Weiss-Schneeweiss & al., 2008; Baltisberger & Hörandl, 2016; Chiarini & al., 2018).

Lycium has $x = 12$ as the basic chromosome number, with a majority of diploid species with $2n = 24$ (Bernardello, 1982; Chiang, 1982; Stiefkens & Bernardello, 1996, 2000, 2002, 2006; Bernardello & al., 2008). To date, data are available on numbers and karyotypes of *Lycium* species from South and North America, southern Africa, and Asia, mostly derived using classical techniques (Stiefkens & Bernardello, 1996, 2000, 2002, 2006; Sheidai & al., 1999; Dongli & al., 2000; Bernardello & al., 2008; Stiefkens & al., 2009, 2010; Blanco & al., 2012; Chen & al., 2013).

Differential staining techniques can identify karyotype variation and species discrimination, even in the presence of morphologically similar karyotypes (De Souza Almeida & al., 2007; Moraes & al., 2007). A widely applied chromosome banding technique uses the sequential fluorescent dyes chromomycin A_3 (CMA) and 4′,6-diamidino-2-phenylindole (DAPI) (Schweizer, 1976). Fluorescent in situ hybridization (FISH) is another molecular cytogenetic tool that has been demonstrated to be effective in detecting chromosomal rearrangements involved in chromosome speciation (Srebniak & al., 2002; Melo & Guerra, 2003). Homologous chromosomes can be identified using FISH and related species can be compared and evolutionary trends can be distinguished (Chacón & al., 2012; Chiarini & al., 2014). The most common FISH markers are ribosomal DNA genes (5S and 18S-5.8S-26S rDNA), which are abundant and highly conserved in higher plants (Heslop-Harrison & Schwarzacher, 2011). Molecular techniques have scarcely been applied in *Lycium*: banding in some African and South American species (Stiefkens & al., 2010; Blanco & al., 2012) and FISH in a few South American species (Blanco & al., 2012).

To date, there has been no attempt to synthesize and evaluate the evolutionary significance of the available *Lycium* cytogenetical data, which is the objective of this study. Firstly, new data on 27 taxa (FISH in all of them, banding in 23, chromosome morphology in 14) are provided to fill gaps in the available information. Secondly, the existing cytogenetical data are reviewed, and chromosomal traits are mapped on a *Lycium* plastid phylogenetic tree (Levin & al., 2007), using character mapping and ancestral states reconstruction to understand the dynamics of the cytogenetical evolutionary changes; these traits were compared with the main exomorphological characteristics of the species.

The following chromosomal features were used: chromosome number, presence of polyploidy, total length of the haploid chromosome set, mean chromosome length, karyotype formula, A_1 and A_2 asymmetry indices, percentage of heterochromatin, number of $CMA^+/DAPI^-$ NORs bands, and number and position of 5S and 18S-5.8S-26S sites. We provide useful insights into the chromosome evolution of *Lycium* and its implication for species level in its systematics.

■ MATERIALS AND METHODS

Cytological analyses. — Table 1 includes collection data on the 33 accessions from 27 *Lycium* taxa studied here with the following techniques.

Conventional staining. – Mitotic chromosomes were analysed from root-tips obtained from primary roots of germinated seeds. Seeds were soaked in tap water for 24 hours and placed in Petri dishes lined with filter paper moistened with gibberellic acid (GA_3, 1000 ppm) and were watered regularly with this solution. Petri dishes were kept in the dark at 30°C. Root tips were cut when the primary roots were 2–10 mm long and were pretreated in a saturated solution of p-dichlorobenzene in water at room temperature for three hours. Root-tips were rinsed in distilled water and fixed in freshly made ethanol : glacial acetic acid (3 : 1) at room temperature for 24 hours. After fixation, root tips were hydrolyzed with 5N HCl at room temperature for 40 minutes and put into Feulgen solution (basic fuchsin) at room temperature in the dark for two hours (Jong, 1997). Root-tip meristem cells were isolated on a slide and squashed. Slides were made permanent in Euparal after removing the cover slips with liquid nitrogen to induce freezing. Cells selected for measurements were photographed with phase contrast optics on a Zeiss Axiophot microscope equipped with a Leica DFC300FX camera. Ten cells, each from a different individual, were photographed, and the lengths of the short arm (s), long arm (l), and total chromosome length (c) for each chromosome pair were taken. The arm ratios ($r = l / s$) were calculated and used to classify the chromosomes and determine homologues after Levan & al. (1964). Karyograms were constructed by organizing the chromosomes into groups according to their arm ratio

Table 1. Species studied by geographic origin with collection data and the technique applied to each one indicated with X.

Species	Voucher	Feulgen	CMA/ DAPI	FISH
Africa				
L. amoenum Dammer	SOUTH AFRICA. Northern Cape, lat. −29.985, long. 17.985, *Miller & al. 05-168* (MASS)	–	–	X
L. bosciifolium Schinz	NAMIBIA. Karas, lat. −27.62, long. 17.714, *Miller & al. 05-137* (MASS)	–	–	X
L. ferocissimum Miers	SOUTH AFRICA. Western Cape, lat. −34.198, long. 18.452, *Miller & al. 05-225* (MASS)	–	–	X
L. oxycarpum Dunal	SOUTH AFRICA. Western Cape, lat. −33.849, long. 20.856, *Miller & al. 05-206* (MASS)	–	–	X
L. tetrandrum L.f.	NAMIBIA. Karas, lat. −26.634, long. 15.151, *Miller & al. 05-151* (MASS)	–	X	X
Lycium sp.	SOUTH AFRICA. Western Cape, lat. −33.674, long. 19.55, *Miller & Levin 06-80* (MASS)	X	X	X
Australia				
L. australe F.Muell.	AUSTRALIA. South Australia, lat. −32.1901, long. 137.496, *Levin & Miller 14-205* (AD)	X	X	X
	AUSTRALIA. South Australia, lat. −32.0877, long. 132.985, *Levin & Miller 14-329* (AD)			
	AUSTRALIA. Western Australia, lat. −31.0169, long. 117.38, *Levin & Miller 14-574* (MASS)			
	AUSTRALIA. Western Australia, lat. −31.9119, long. 127.331, *Levin & Miller 14-370* (PERTH)			
Europe				
L. intricatum Boiss.	SPAIN. Alicante, 38°20′56″N, 00°28′41″W, *Chiarini 697* (CORD)	X	X	X
North America				
L. berlandieri Dunal	U.S.A. Arizona, Pima Co., *Levin & Miller s.n*. (MASS)	X	X	X
L. californicum Nutt. ($2n = 24$)	MEXICO. Sonora, Puerto Libertad, lat. 29.881, long. −112.642, *Miller & Levin 05-81* (MASS)	X	X	X
L. californicum ($2n = 48$)	U.S.A. Arizona, Pinal Co., lat. 32.78, long. −111.63, *Miller & Levin 04-12* (ARIZ)	X	X	X
L. exsertum A.Gray	U.S.A. Arizona, Pima Co., lat. 32.417, long. −110.938, *Miller & Levin 05-95* (ARIZ)	X	X	X
	U.S.A. Arizona, Pima Co., lat. 32.219, long. −111.004, *Miller & Levin 05-87* (ARIZ)			
	U.S.A. Arizona, Pinal Co., lat. 32.6, long. –111.25, *Miller 95-1* (ARIZ)			
L. fremontii A.Gray	U.S.A. Arizona, Pima Co., lat. 31.9376, long. −113.063, *Miller 01-4* (ARIZ)	X	X	X
	U.S.A. Arizona, Pinal Co., lat. 32.783, long. −111.633, *Miller 95-2* (ARIZ)			
L. pallidum Miers	U.S.A. Arizona, Pinal Co., lat. 32.542, long. −110.712, *Miller 97-20* (ARIZ)	X	X	X
L. parishii A.Gray	U.S.A. Arizona, Pima Co., lat. 31.9, long. −112.85, *Miller 97-22* (ARIZ)	X	X	X
South America				
L. ameghinoi Speg.	ARGENTINA. Santa Cruz, Río Chico, 47°20′09.1″S, 70°59′05.2″W, *Barboza & al. 3758* (CORD)	–	X	X
L. chilense var. *confertifolium* (Miers) F.A.Barkley	ARGENTINA. Chubut, Biedma, 42°47′07.5″S, 65°00′15.1″W, *Barboza & al. 2332* (CORD)	–	X	X
L. chilense var. *descolei* F.A.Barkley	ARGENTINA. Chubut, Río Senguer, 45°20′24.7″S, 69°55′47.8″W, *Barboza & al. 2376* (CORD)	X	X	X

(Continues)

Table 1. Continued.

Species	Voucher	Feulgen	CMA/DAPI	FISH
L. chilense var. *filifolium* (Gillies ex Miers) Bernardello	ARGENTINA. La Pampa, Toay, lat. −36.934, long. −64.2617, *Bernardello 111* (CORD)	X	X	X
L. ciliatum Schltdl.	ARGENTINA. La Rioja, Coronel Felipe Varela, 29°37′50.5″S, 68°03′05.6″W, *Urdampilleta 607* (CORD)	X	X	X
L. cuneatum Dammer	ARGENTINA. Formosa, Patiño, 24°42′38.5″S, 60°34′44.5″W, *Chiarini & al. 895* (CORD)	–	X	X
L. elongatum Miers	ARGENTINA. La Rioja, General San Martín, 31°34′30.3″S, 66°15′10.5″W, *Barboza & al. 2439* (CORD)	–	X	X
L. gillesianum Miers	ARGENTINA. Santa Cruz, Deseado, 47°49′43.7″S, 66°35′35.2″W, *Barboza & al. 3694* (CORD)	–	X	X
L. humile Phil.	ARGENTINA. Salta, Los Andes, 24°35′25.3″S, 67°08′12.5″W, *Barboza & al. 4347* (CORD)	X	X	X
L. morongii Britton	ARGENTINA. Chaco, 1° de Mayo, 27°11′26″S, 58°58′24″W, *Chiarini & al. 883* (CORD)	–	X	X
L. repens Speg.	ARGENTINA. Santa Cruz, Río Chico, 47°20′09.1″S, 70°59′05.2″W, *Barboza & al. 3759* (CORD)	X	X	X
L. tenuispinosum var. *friesii* (Dammer) C.H.Hitchc.	ARGENTINA. La Rioja, Chamical, 30°23′24.2″S, 66°18′59.8″W, *Urdampilleta 586* (CORD)	–	X	X

(from *m* to *sm*), ordering them by decreasing length within each category. Idiograms were based on the mean values obtained for measurements of all individuals for each species. Karyotype asymmetry was estimated using the indices of Romero Zarco (1986).

CMA/DAPI banding and FISH. – Mitotic chromosomes were examined in root tips obtained from germinating seeds as previously explained. Root tips were washed twice in distilled water (10 minutes each), digested with a 2% cellulase and 20% pectinase solution (from *Aspergillus niger*) at 37°C for 45 minutes, and squashed in a drop of 45% acetic acid (Schwarzacher & al., 1980). After coverslip removal in liquid nitrogen, the slides were stored at −20°C. Slides were stained with a drop of 0.5 $mg \cdot ml^{-1}$ CMA in McIlvaine buffer pH 7.0 and distilled water (1 : 1) containing 2.5 mM $MgCl_2$ for 90 minutes, and subsequently with 2 $\mu g \cdot ml^{-1}$ DAPI for 30 minutes and were mounted in McIlvaine's buffer-glycerol (1 : 1 v/v; Schweizer, 1976; Schweizer & Ambros, 1994). The amount of heterochromatin (CMA^+/$DAPI^-$ NORs bands, CG rich) was expressed as a percentage of the total length of the haploid karyotype. The position and number of rDNA sites were determined by FISH using two probes: pTa71 containing the 18S-5.8S-26S gene of wheat (Gerlach & Bedbrook, 1979), labelled with biotin-14-dATP, and a 5S rDNA fragment obtained by PCR from *Solanum stuckertii* Bitter (Chiarini & al., 2014), labelled with digoxigenin-11-dUTP. The FISH protocol followed Schwarzacher & Heslop-Harrison (2000) with minor modifications. The preparations were incubated in 100 $\mu g \cdot ml^{-1}$ RNAase, post-fixed in 4% (w/v) paraformaldehyde, dehydrated in a 70%–100% graded ethanol series and air dried. On each slide, 15 µl of hybridization mixture was added (4–6 $ng \cdot \mu l^{-1}$ probe, 50% formamide, 10% dextran sulphate, 20× saline sodium citrate [SSC] and 0.3% sodium dodecylsulphate [SDS]), previously denatured at 70°C for 10 minutes. Chromosome denaturation/hybridization was performed at 90°C for 10 minutes, 48°C for 10 minutes, and 38°C for 5 minutes using a thermal cycler (Mastercycler, Eppendorf). Slides were placed in a humid chamber at 37°C overnight. The 18S-5.8S-26S probe was detected with avidin-fluorescein isothiocyanate (FITC) conjugate. The 5S probe was detected with antidigoxigenin-rhodamine and then counterstained and mounted with 25 µl antifade Vectashield, containing 1.5 $\mu g \cdot ml^{-1}$ DAPI. Photomicrography was performed with an Olympus BX61 microscope equipped with a JAI CV-M4-CL monochromatic camera with Cytovision software (Leica Biosystems). At least ten metaphases of each accession from at least three different individuals were photographed with the microscope previously described, equipped with epifluorescence and a digital image capture system. The free software ImageJ v.1.5 (http://rsbweb.nih.gov/ij/) was used to merge the respective images obtained with the blue, red, and green filters.

Statistical methods. — Differences between species were calculated as Euclidean distances on the cytogenetic matrix of eight variables (suppl. Table S1) from 19 species. PERMANOVA (a permutation-based extension of multivariate analysis of variance to a matrix of pairwise distances) tested effects on multivariate patterns in accordance with five morphological variables (suppl. Table S1). Significance levels were calculated from 100,000 permutations of the residuals

under the reduced model. When a factor with more than two levels was identified as significant (at $\alpha = 0.05$), post-hoc pairwise tests were conducted. Logistic regression was used to study the relationship between relevant morphological traits and cytogenetic data. Data were modelled as a multinomial or binomial distribution according to the number of categories. All analyses were carried out in R (Maechler & al., 2018; R Core Team, 2018), using the "vegan" package (Oksanen & al., 2018).

Character mapping. — Chromosomal evolution was estimated on a maximum credibility tree of *Lycium*, using comparative methods. The tree was obtained by re-running the analyses of Miller & al. (2009, 2011) with sequences of four plastid DNA regions (*trnH-psbA*, *rpl32-trnL*, *ndhF-rpl32*, *trnD-trnT*; suppl. Table S2). Species in the resulting tree were pruned according to their availability for chromosomal data. Bayesian analysis was run with MrBayes v.3.1.2 (Huelsenbeck & Ronquist, 2001; Nylander & al., 2004) with a model of sequence evolution generated by MrModelTest v.2.2 (Nylander, 2004), which implements the hierarchical likelihood ratio test (hLRT) and the Akaike information criterion (AIC). Tracer v.1.5 (Rambaut & Drummond, 2007) was used to determine whether the MCMC parameter samples were drawn from a stationary distribution and adequate effective sample sizes for each parameter (ESS > 200) were reached. Sequences have an aligned length of 3666 bp. The ML analysis yielded two trees with −lnL = 6589.81093. Both ML trees show strong support for a monophyletic genus (bootstrap = 100). The tree was visualized with FigTree v.1.5.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Chromosomal features (see below) were mapped on 32 *Lycium* taxa from all its distribution area, with *Jaborosa squarrosa* (Miers) Hunz. & Barboza, *Nolana coelestis* Miers ex Dunal, and *Sclerophylax spinescens* Miers as outgroups (suppl. Table S1). The following traits were used to infer character history coded: discrete features = chromosome number, number of *sm* chromosomes, number of CMA^+/NOR bands, number of 5S and 18S-5.8S-26S rDNA sites; continuous characters = total length of the haploid complement (TLH), mean chromosome length (*C*), mean arm ratio (*r*), intrachromosomal (A_1) and interchromosomal (A_2) asymmetry indices, and percentage of heterochromatin (%Ht) (suppl. Table S1).

Table 2. Summary of the main chromosomal variables in the studied *Lycium* species.

Species	2*n*	Karyotype formula	TLH	*C*	*r*	*R*	A_1	A_2
Africa								
Lycium sp.	48	20 *m* + 4 *sm*	24.52	2.04	–	–	–	–
Australia								
L. australe	24	10 *m* + 2 *sm*	31.43	2.62	1.25	1.61	0.16	0.12
Europe								
L. intricatum	24	10 *m* + 2 *sm*	33.59	2.80	1.24	1.81	0.21	0.16
North America								
L. berlandieri	24	10 *m* + 2 *sm*	38.70	3.23	1.36	1.55	0.22	0.12
L. californicum	24	11 *m* + 1 *sm*	29.80	2.48	1.19	1.46	0.14	0.10
L. californicum	48	22 *m* + 2 *sm*	39.65	3.30	–	–	–	–
L. exsertum	48	20 *m* + 4 *sm*	40.26	3.35	–	–	–	–
L. fremontii	120	–	29.24	2.44	–	–	–	–
L. pallidum	24	10 *m* + 2 *sm*	29.98	2.50	1.37	1.49	0.25	0.11
L. parishii	24	11 *m* + 1 *sm*	30.67	2.55	1.15	1.36	0.12	0.08
South America								
L. chilense var. *confertifolium*	48	22 *m* + 2 *sm*	22.61	1.88	–	–	–	–
L. chilense var. *Descolei*	48	22 *m* + 2 *sm*	26.32	2.19	–	–	–	–
L. ciliatum	48	22 *m* + 2 *sm*	22.03	1.84	–	–	–	–
L. humile	24	11 *m* + 1 *sm*	21.45	1.79	1.34	1.71	0.16	0.11
L. repens	132	–	22.68	1.89	–	–	–	–

Data are diploid chromosome number (2*n*), karyotype formulae, total length of the haploid complement (TLH), mean chromosome length (*C*), mean arm ratio (*r*), ratio between the longest and shortest chromosomes of the complement (*R*), and intrachromosomal (A_1) and interchromosomal (A_2) asymmetry indices. Dashes indicate data not taken.

Ancestral character state reconstructions with maximum-likelihood criterion (Mk1 model, in which all changes are equally probable) were conducted in Mesquite v.3.2 (Maddison & Maddison, 2017) using the data matrix of the concatenated four plastid DNA regions in a pruned phylogenetic tree.

■ RESULTS

New cytological data. — All species show $x = 12$ with different ploidy levels, most of them are diploid or tetraploid (Table 2, Fig. 1). Chromosome numbers of *L. australe*, *L. humile*, and *L. repens* (Table 2) are recorded here for the first time. *Lycium fremontii* (10*x*, Fig. 1G) and *L. repens* (11*x*, Fig. 1J) are outstanding for having the highest numbers in the genus.

The karyotypes here reported for 10 species are new, showing symmetrical haploid formulae with 1–2 *sm* chromosomes (Table 2, Fig. 2). Chromosomes are small (Figs. 1, 2). The total length of the haploid complement ranges from 21.45 µm in *L. humile* to 40.26 µm in *L. exsertum*, with 2.35 µm being the overall mean chromosome length (Table 2). North American species show comparatively longer chromosomes (Fig. 3).

Two different heterochromatin types were detected with chromosome banding: (1) one pair of CMA^+ strong bands in

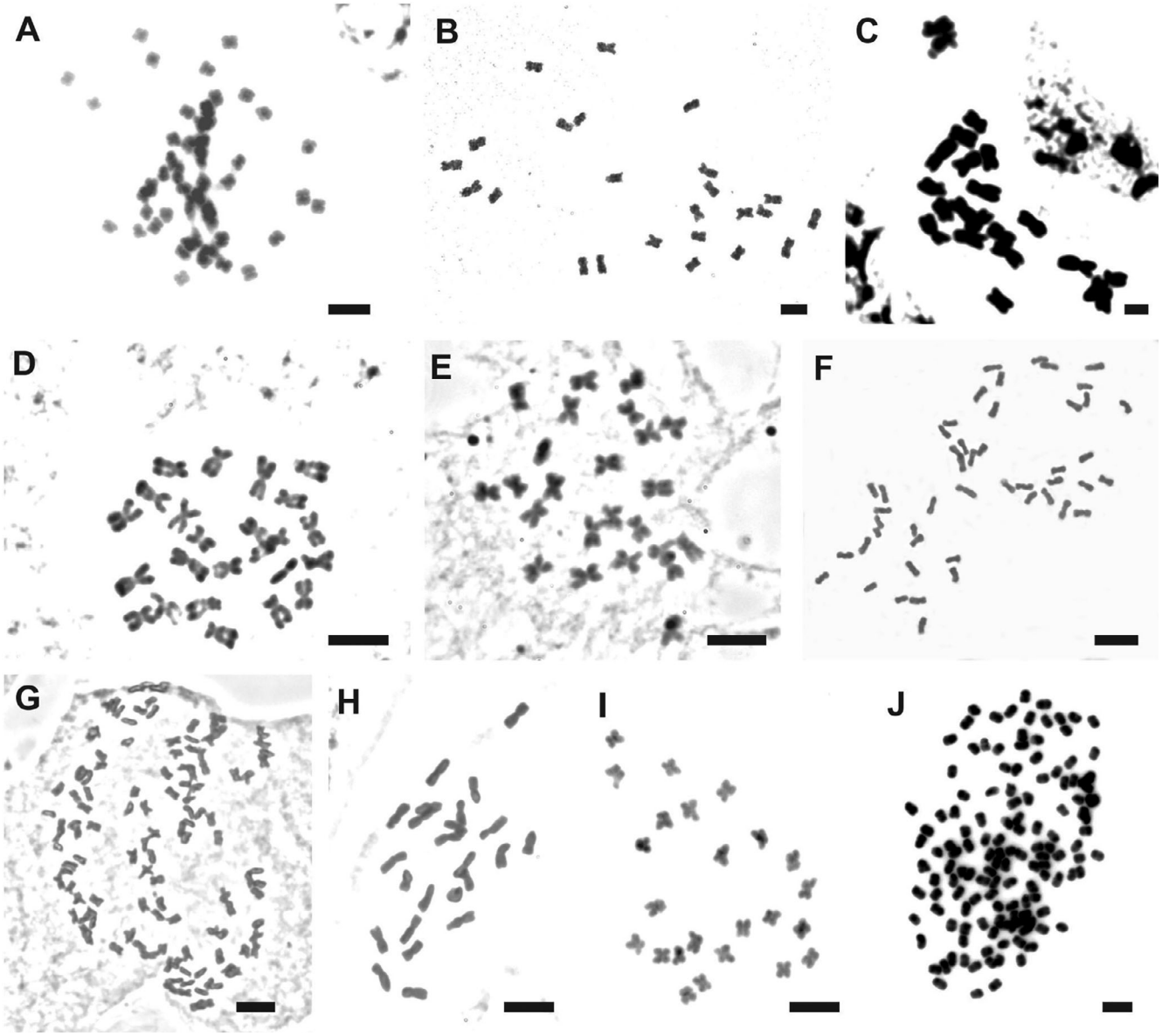

Fig. 1. Somatic metaphases of *Lycium* with Feulgen staining. **A,** *Lycium* sp. ($2n = 48$); **B,** *L. australe* ($2n = 24$); **C,** *L. intricatum* ($2n = 24$); **D,** *L. berlandieri* ($2n = 24$); **E,** *L. californicum* ($2n = 24$); **F,** *L. exsertum* ($2n = 48$); **G,** *L. fremontii* ($2n = 120$); **H,** *L. pallidum* ($2n = 24$); **I,** *L. humile* ($2n = 24$); **J,** *L. repens* ($2n = 132$). — Scale bars = 5 µm.

all species studied associated with the secondary constrictions (NORs) in a terminal position in pair #1, with the exception of *L. australe* in pair #4, and (2) additional $CMA^+/DAPI^-$ heterochromatin blocks not associated with NORs located in terminal or centromeric regions in three species: *L. berlandieri* and *L. exsertum* (terminal bands in all chromosomes), and *L. parishii* (four interstitial bands; Table 3, Figs. 2, 4). Only *L. australe* shows $CMA^-/DAPI^+$ bands in two chromosome pairs (Figs. 2, 4) and *L. elongatum* $CMA^+/DAPI^-$ heteromorphic bands associated with NORs (Fig. 4). The total amount of heterochromatin ranges from 0.63% (*L. intricatum*) to 3.55% (*L. parishii*) of the total karyotype length (Table 3).

Chromosomes with a secondary constriction are present in all the species studied and are labelled by FISH using the 18S-5.8S-26S probe. The number of genes corresponds to the ploidy level (from 2 in diploids to 11 in the undecaploid; Table 3, suppl. Figs. S1, S2). These signals coincide with the $CMA^+/DAPI^-$ blocks associated with NORs. The 18S-5.8S-26S loci are always located in *m* chromosomes; in the first pair in all taxa except in *L. australe* that are in the fourth pair (Fig. 2, suppl. Fig. S1). These loci are heteromorphic in *L. elongatum* and *L. tenuispinosum* var. *friesii* (suppl. Fig. S1).

In all the species studied, the position of the 5S rDNA probe is interstitial. From 2 to 10 hybridization signals were

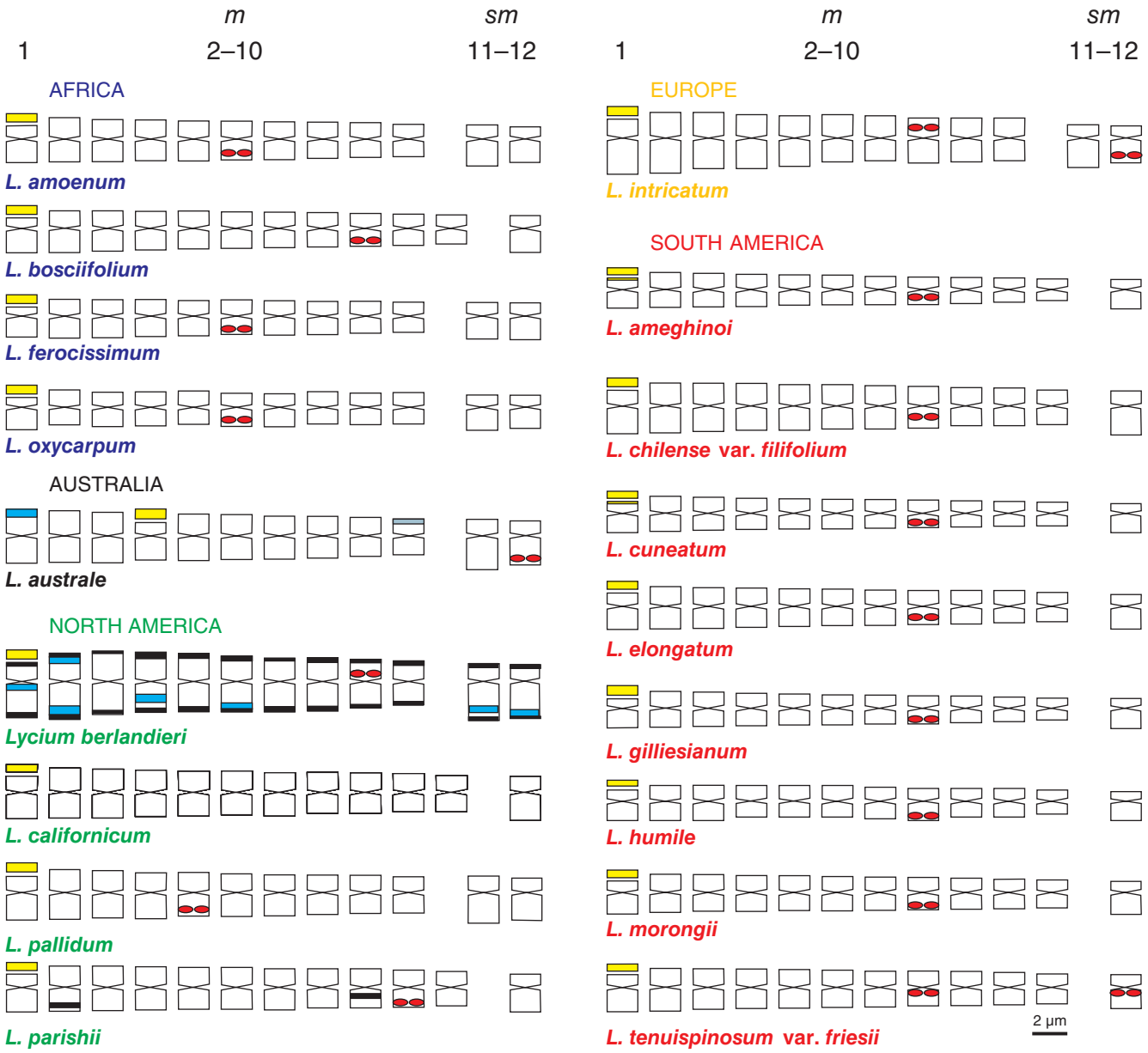

Fig. 2. Idiograms of *Lycium* species based on mean chromosome values; all chromosomes at the same scale, ordered by categories from *m* to *sm*. Black blocks = $CMA^+/DAPI^-$ bands; blue blocks = $DAPI^+$ after FISH bands; green blocks = $CMA^-/DAPI^+$ bands; red ellipses = 5S loci; yellow blocks = CMA^+/NOR and 18S-5.8S-26S loci.

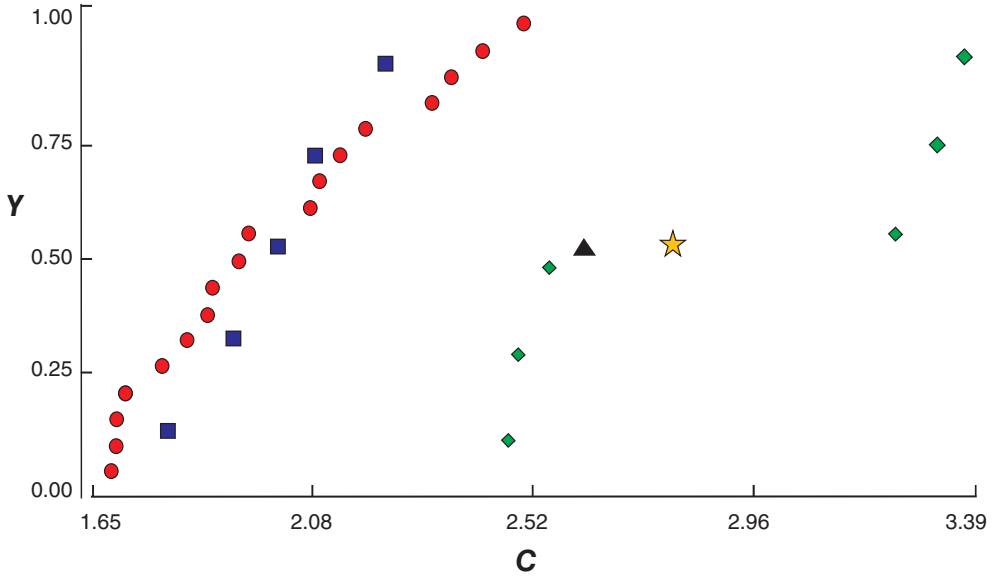

Fig. 3. Graph of the empirical distribution representing the mean chromosome length for *Lycium* species studied according to their geographical origin. Axes: y = cumulative probability and x = mean chromosome length (*C*). Symbols: Squares/blue = Africa, triangle/black = Australia, star/orange = Europe, diamonds/green = North America, circles/red = South America.

obtained with this probe. They are proportional to the ploidy level, except in *L. intricatum* and *L. tenuispinosum* var. *friesii*, which are diploid species but have four signals (Table 3, Fig. 2, suppl. Figs. S1, S2). The 5S sites are always separated from the 18S-5.8S-26S sites (Table 3, Fig. 2, suppl. Figs. S1, S2). Their position varies according to the species: all South American species show the 5S in pair #8, whereas in the species from other regions their positions were variable (Fig. 2, suppl. Figs. S1, S2).

$DAPI^+$ bands after FISH are rare in *Lycium*; they are not associated with NORs and do not coincide with $CMA^-/DAPI^+$ bands: present only in *L. berlandieri* (six pairs; Figs. 2, 4) and *L. exsertum* (in all chromosomes; Fig. 4).

Statistical analyses. — PERMANOVA analyses indicate significant differences in cytogenetical traits between species with different habits (erect versus prostrate shrubs, $P = 0.018$) and flower sex (hermaphroditic versus dioecious, pairwise post-hoc $P < 0.001$), whereas the other morphological traits examined show no significant differences (Table 4). Logistic regression results only show an association between flower sex and total chromosome length ($P < 0.023$).

Character mapping and ancestral state reconstruction. — Some characters undergo few transitions (mean chromosome length, total haploid chromosome length, mean arm ratio, asymmetry indices, percentage of heterochromatin, number of 18S-5.8S-26S loci, number of $CMA^+/DAPI^-$ NORs bands) compared with others that are more variable (chromosome number, number of *sm* chromosomes, number of 5S loci).

The mapping of the cytogenetical characters on the plastid phylogenetic tree shows that the most probable ancestral chromosome number is $2n = 24$ with several independent polyploidization events (Fig. 5). Concerning the number of 18S-5.8S-26S (Fig. 6) and 5S (Fig. 7) loci, the ancestral condition is probably one pair of repetitive rDNA units. The remaining cytological variables, i.e., the number of *sm* chromosomes (Fig. 8), percentage of heterochromatin, A_1 and A_2 indexes, do not show a clear pattern in the phylogeny. From these results, the most probable ancestral condition for the genus would be: diploid, with small chromosomes, scarce heterochromatin, asynteny of rDNA loci, and one pair of both 18S-5.8S-26S and 5S loci.

■ DISCUSSION

Cytogenetic traits. — In *Lycium*, change in chromosome number is not associated with speciation events. Most species examined so far (ca. 65% of the genus) show $x = 12$, mainly at the diploid level. The base number $x = 12$ is the most common for Solanaceae, which has an informally named "$x = 12$ clade" that includes ca. 2300 species (Olmstead & al., 1999, 2008; Särkinen & al., 2013); it has been suggested that this base number is apomorphic (Olmstead & al., 2008).

Polyploidy is the most frequent cause of number variation in angiosperms. In Solanaceae, polyploidy is not widespread, but it arose in 22 genera, including the large genera *Solanum*, *Nicotiana*, and *Lycium* (cf. Chiarini & al., 2018). *Lycium* shows a polyploid series: $2x$, $3x$, $4x$, $6x$, $8x$, $10x$, and $11x$, with the highest levels here reported. Polyploidy has been correlated with habit, life form, mating system, geographic range, and invasion of new habitats, due to the suggested advantages of genome duplication (Stebbins, 1985; Wendel, 2000; Soltis & al., 2003, 2014; Sonnleitner & al., 2015). In *Lycium*, there is

Table 3. Summary of the main molecular chromosomal variables in the studied *Lycium* species.

Species	%Ht	$CMA^+/DAPI^-$/NOR	$CMA^+/DAPI^-$	45S	5S
Africa					
L. amoenum	–	2	0	2	2
L. bosciifolium	2.92	2	0	2	2
L. ferocissimum	2.83	2	0	2	2
L. oxycarpum	1.79	2	0	2	2
L. tetrandrum	–	–	–	6	6
Lycium sp.	–	–	–	4	4
Australia					
L. australe	2.57	2	2	2	2
Europe					
L. intricatum	0.63	2	0	2	4
North America					
L. berlandieri	2.69	2	24	2	2
L. californicum ($2n = 24$)	–	2	0	2	2
L. californicum ($2n = 48$)	–	4	0	4	4
L. exsertum	3.32	4	48	4	4
L. fremontii	3.27	10	0	10	10
L. pallidum	–	2	0	2	2
L. parishii	3.55	2	4	2	2
South America					
L. ameghinoi	1.54	2	0	2	2
L. chilense var. *confertifolium*	2.70	4	0	4	4
L. chilense var. *descolei*	–	4	0	4	4
L. chilense var. *filifolium*	–	2	0	2	2
L. ciliatum	2.75	4	0	4	4
L. cuneatum	1.56	2	0	2	2
L. elongatum	0.87	2	0	2	2
L. gillesianum	1.37	2	0	2	2
L. humile	1.26	2	0	2	2
L. morongii	0.72	2	0	2	2
L. repens	2.77	11	0	11	11
L. tenuispinosum var. *friesii*	1.35	2	0	2	4

Data are percentage of heterochromatin (%Ht), number of $CMA^+/DAPI^-$ bands associated to NORs ($CMA^+/DAPI^-$/NOR), number of chromosomes with $CMA^+/DAPI^-$ bands ($CMA^+/DAPI^-$), number of 18S-5.8S-26S signals (45S), and number of 5S signals (5S). Dashes indicate data not taken. Data on CMA^+/NOR^- and $CMA^+/DAPI^-$ for African species from Stiefkens & al. (2010).

an association of polyploidy and gender dimorphism (Venter, 2000; Miller, 2002; Yeung & al., 2005): dimorphic North American species are 4*x* or 8*x* and dimorphic southern African species are 6*x*, whereas cosexual species with cytologically known numbers are 2*x* (suppl. Table S1). Miller & Venable (2000) suggested that in *Lycium* polyploidy and gender dimorphism have evolved in concert (see also Ashman & al., 2013). However, recently, in *L. carolinianum* (Blank & al., 2014) dimorphism was not perfectly correlated with a higher ploidy level. As karyotypes of polyploids are difficult to make because homologous pairs are quite similar, there are no data to compare. In addition, percentage of heterochromatin, number of $CMA^+/DAPI^-$ NORs bands, and the number and position of 5S and 18S-5.8S-26S sites are unknown for these species (suppl. Table S1).

Lycium shows small chromosomes of less than 3.30 µm (mean 2.12 ± 0.33, $n = 45$; suppl. Table S1) and symmetrical karyotypes (average values: arm ratio = 1.27, $A_1 = 0.16$, $A_2 = 0.12$) (Stiefkens & Bernardello, 1996, 2000, 2002, 2006; Sheidai & al., 1999; Dongli & al., 2000; Bernardello & al., 2008; Stiefkens & al., 2009, 2010; Blanco & al., 2012; Chen & al., 2013); both features are common in the family (cf. Chiarini & al., 2018). Karyotypes have *m* and *sm* chromosomes of rather similar size; only *L. bridgesii* has one *st* pair (Bernardello & al., 2008). South American species are quite homogeneous in their karyotype formula with one *sm* pair (except *L. bridgesii*), whereas species from other regions are more variable (with 2–4 *sm* pairs).

The number of satellited pairs is constant in diploid *Lycium* (one pair on the short arm of the longest *m* pair), the number of satellites being correlated with the ploidy level. Nevertheless, in three Asian diploid species two pairs with satellites were detected (Sheidai & al., 1999; Dongli & al., 2000). The sites of the satellites observed by Feulgen staining were confirmed with FISH (Stiefkens & Bernardello, 1996, 2000, 2002, 2006; Bernardello & al., 2008; Stiefkens & al., 2009, 2010), as found in other Solanaceae as well (Lim & al., 2000; Fregonezi & al., 2006; Moscone & al., 2007; Acosta & Moscone, 2010; Chiarini & al., 2014, 2018).

According to Bennett & Leitch (2012), the majority of angiosperm genera do not display any variation in the amount of DNA higher than 2-fold. As DNA amounts in *Lycium* are poorly known (data are only available for 10 species; 1C-value average = 1.90 ± 0.49 pg; suppl. Table S1), more data are needed to suggest the direction of the evolutionary changes.

Lycium has a low rate of cytogenetical changes with numerous small rearrangements not detected at the cytological level, as proposed for Solanaceae (Stebbins, 1971; Bernardello & Anderson, 1990; Bernardello & al., 1994; Wu & Tanksley, 2010). A karyotype orthoselection may have occurred in *Lycium*, conserving similar complements throughout a higher taxon as they are more stable (Bernardello & al., 2008). This hypothesis was proposed for the maintenance of complements with *m* and *sm* chromosomes of approximately the same length (Brandham & Doherty, 1998; Moscone & al., 2003; Stiefkens & al., 2009, 2010), although it is not

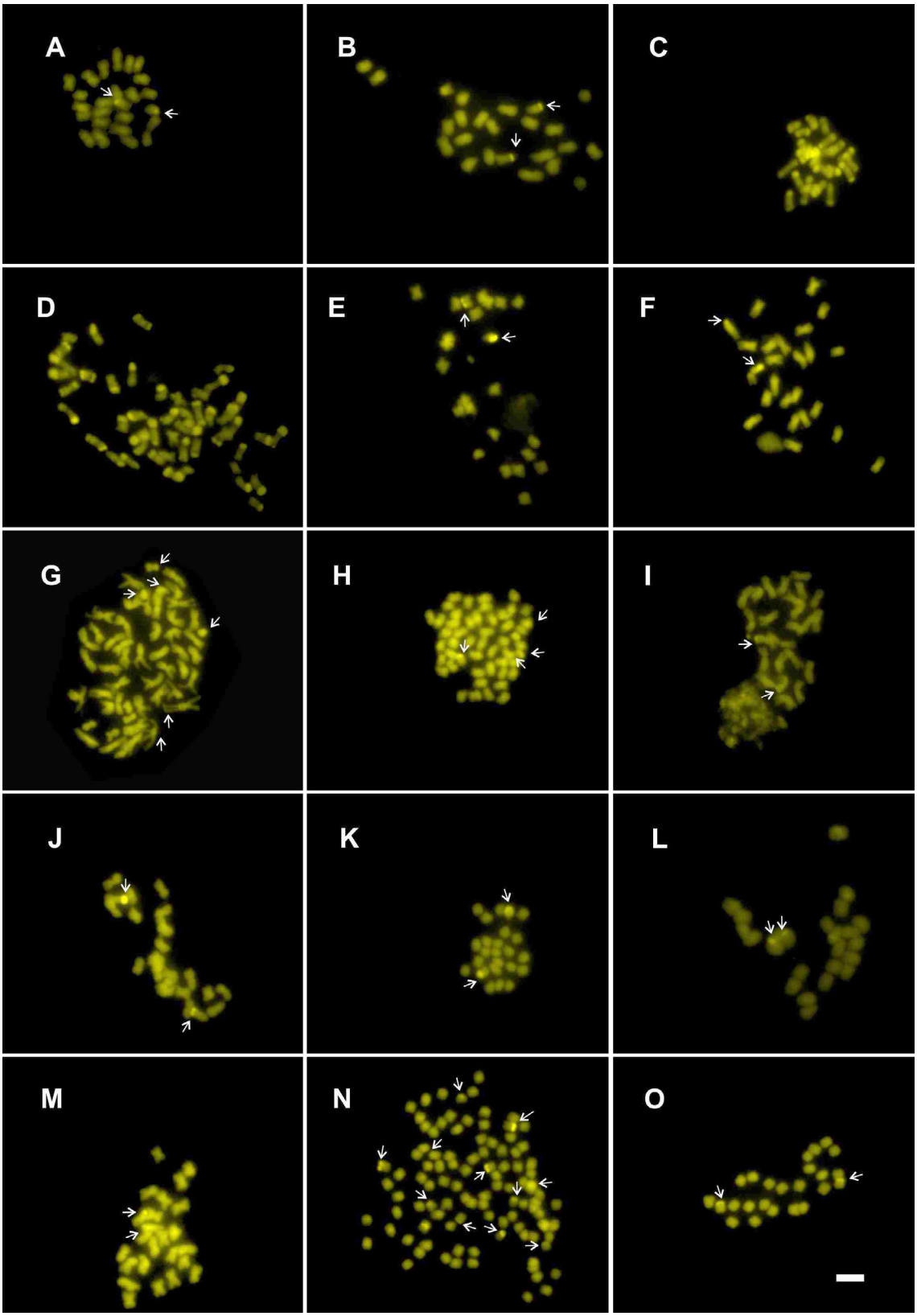

Fig. 4. Fluorochrome chromosome banding (CMA) in *Lycium* species illustrating the heterochromatin patterns found. **A,** *L. australe*; **B,** *L. intricatum*; **C,** *L. berlandieri*; **D,** *L. exsertum*; **E,** *L. parishii*; **F,** *L. ameghinoi*; **G,** *L. chilense* var. *confertifolium*; **H,** *L. ciliatum*; **I,** *L. cuneatum*; **J,** *L. elongatum*; **K,** *L. gillesianum*; **L,** *L. humile*; **M,** *L. morongii*; **N,** *L. repens*; **O,** *L. tenuispinosum* var. *friesii*. — Arrows indicate $CMA^{+}/DAPI^{-}$ NORs bands. All at the same scale. Scale bar = 5 μm.

Table 4. Results of PERMANOVA analyses.

	MS	F	Pr(>F)
Calyx shape	6.2	0.01	0.977
Corolla shape	287.8	0.55	0.642
Flower sex	5110.4	9.73	**0.002**
Fruit type	13.7	0.03	0.992
Habit	4321.1	8.22	**0.018**
Residual	525.5		
Total	684.0		

Bold values are statistically significant. Abbreviation: MS = Mean squares.

easy to determine the direction of this evolution because reversals might occur (Stace, 2000).

The rate of 0.19 rearrangements per chromosome per million years was estimated for Solanaceae, which represents a moderate rate of chromosome evolution where paracentric inversions of conserved syntenic segments would be the primary mechanism involved (Doganlar & al., 2002, 2014; Wu & al., 2009). In addition, Wu & Tanksley (2010) estimated this rate of chromosomal evolution, calculating 0.1 ~ 1 inversions and 0.2 ~ 0.4 translocations per million years across different species. As the chromosome number in Solanaceae is mostly constant, Wu & Tanksley (2010) proposed that the family has a modest rate of chromosomal evolution.

In plants, heterochromatin is commonly situated in similar chromosome regions, regardless of the distance from the

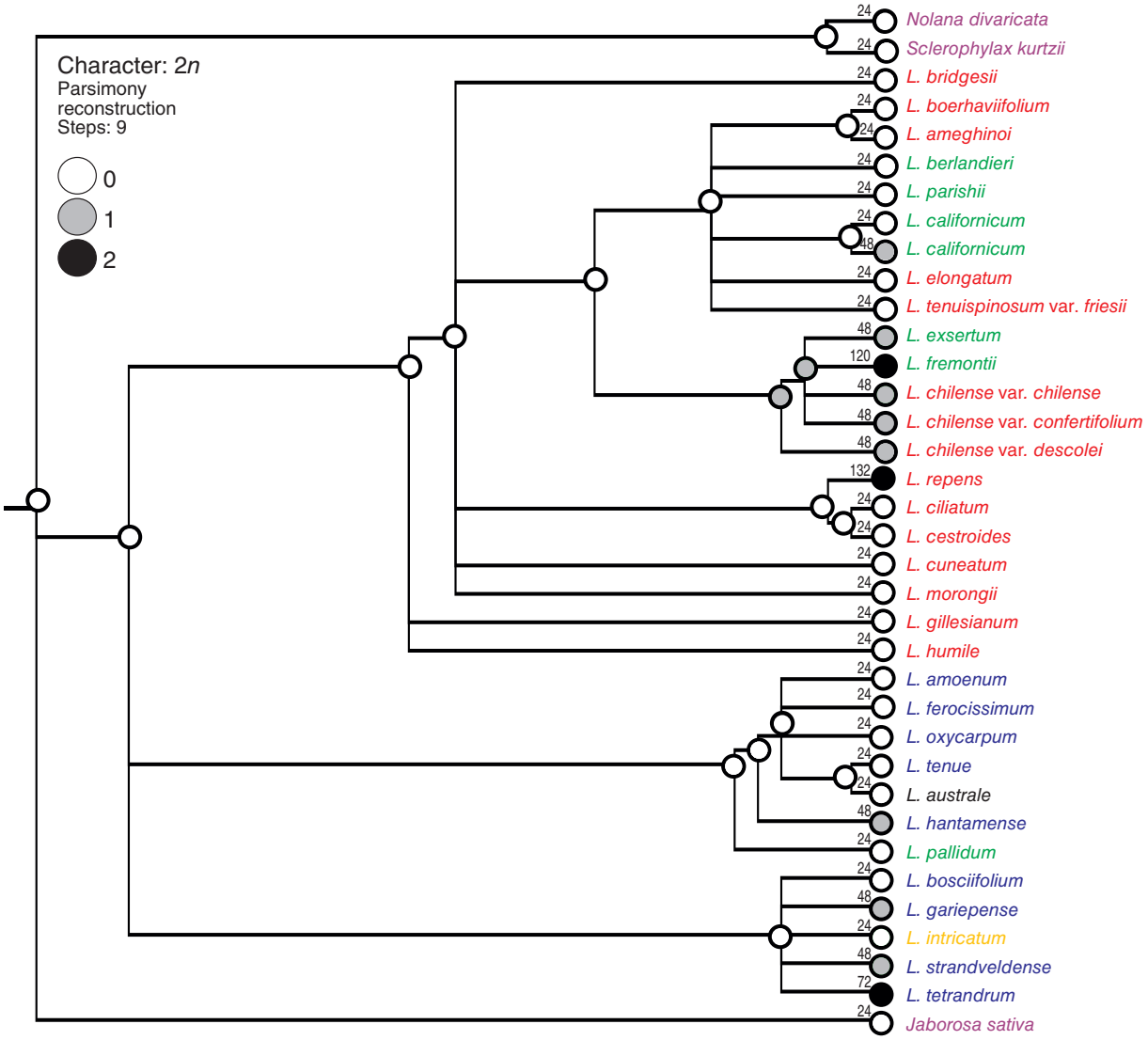

Fig. 5. Ancestral reconstruction of chromosome number in *Lycium* using parsimony and Bayesian approaches. The pie diagrams at nodes show the relative probability of the possible states (white, 0 = diploid; grey, 1 = tetraploid; black, 2 = polyploid greater than 48). Chromosome number is at branch end. Geographic origin of the species is indicated in colour letters: Red = South America, green = North America, blue = Africa, black = Australia, orange = Europe, violet = outgroups.

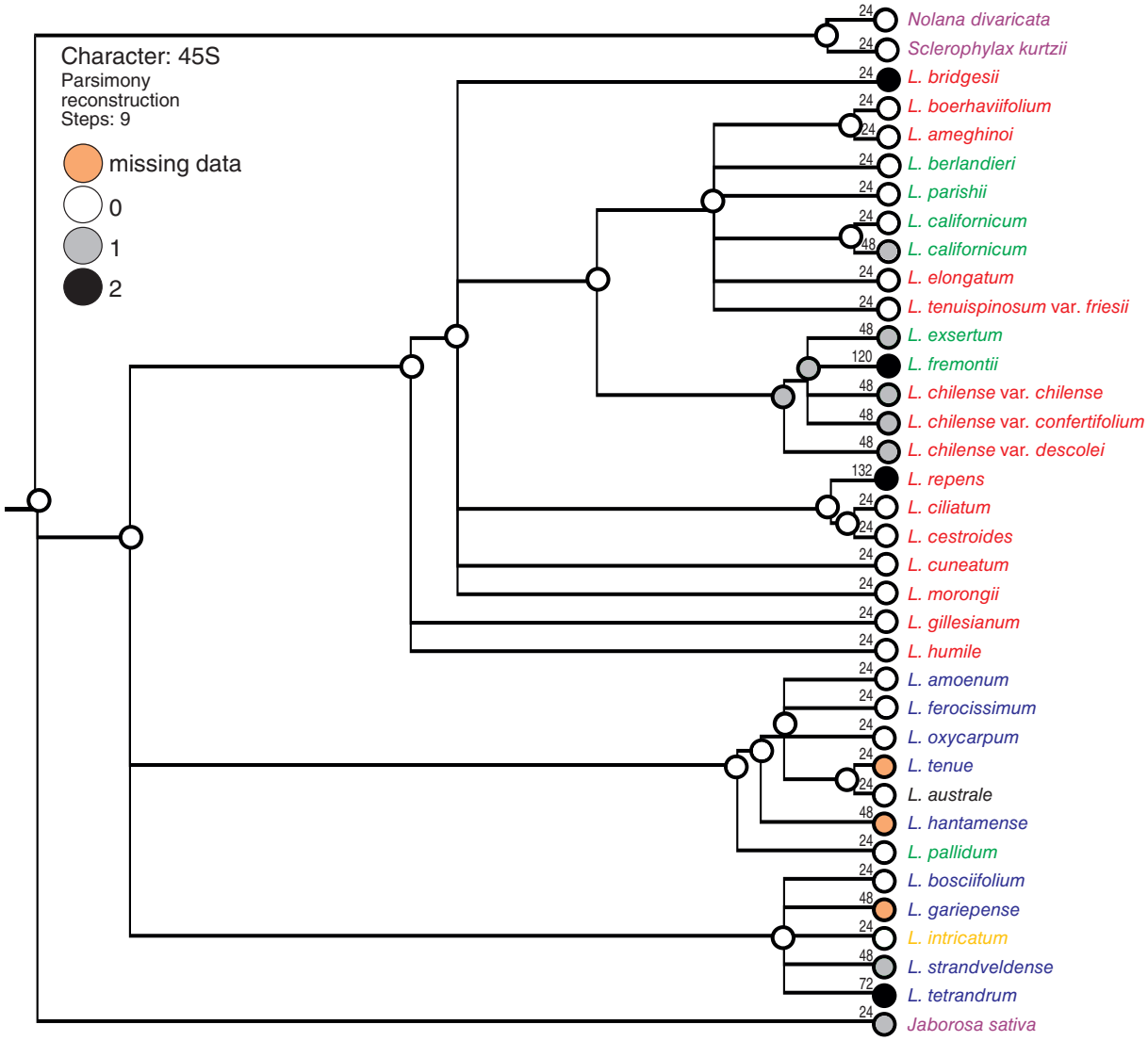

Fig. 6. Ancestral reconstruction of number of 18S-5.8S-26S loci in *Lycium* using parsimony and Bayesian approaches. The pie diagrams at nodes show the relative probability of the possible states (white, 0 = two 18S-5.8S-26S loci; grey, 1 = four 18S-5.8S-26S loci; black, 2 = more than four 18S-5.8S-26S loci). Chromosome number is at branch end. Geographic origin of the species is indicated in colour letters: Red = South America, green = North America, blue = Africa, black = Australia, orange = Europe, violet = outgroups.

centromere, resulting in generalized bands with similar distribution in most chromosomes and which can be either associated with NOR or be telomeric, interstitial, or proximal (Guerra, 2000). Solanaceous genera with symmetrical karyotypes, such as *Lycium*, present simpler heterochromatin patterns, whereas species with many heterochromatin bands have more asymmetrical karyotypes (Stiefkens & al., 2009; Blanco & al., 2012; Chiarini & al., 2016, 2018).

$CMA^{+}/DAPI^{-}$ bands associated with NOR are the most common in angiosperms (Kenton & al., 1993; Guerra, 2000; Lim & al., 2000; Fregonezi & al., 2006; Moscone & al., 2007; Chiarini & al., 2018) and in the 27 *Lycium* species examined (Stiefkens & al., 2010; Blanco & al., 2012; our data), regardless of their geographical origin. Although more banding studies are needed in the genus, the available data suggest that these bands are restricted to pair #1 in terminal position. Considering that fluorochrome banding is useful in the identification of homeologous pairs between species (Moscone & al., 1996), this NOR-bearing chromosome pair is likely to be homeologous. Heterochromatic $CMA^{+}/DAPI^{-}$ bands not associated with NOR were only detected in six species from several continents (Stiefkens & al., 2010; Blanco & al., 2012; Table 3).

Both rDNA sites have been used to estimate karyotype similarity and to understand cytogenetical evolution (Garcia & al., 2012; Weiss-Schneeweiss & Schneeweiss, 2013). The 5S and 18S-5.8S-26S rDNA loci are generally localized on different chromosomes in angiosperms (Garcia & al., 2009; Roa & Guerra, 2012), as found in this study; however, in some Solanaceae, synteny has been reported (cf. Chiarini & al.,

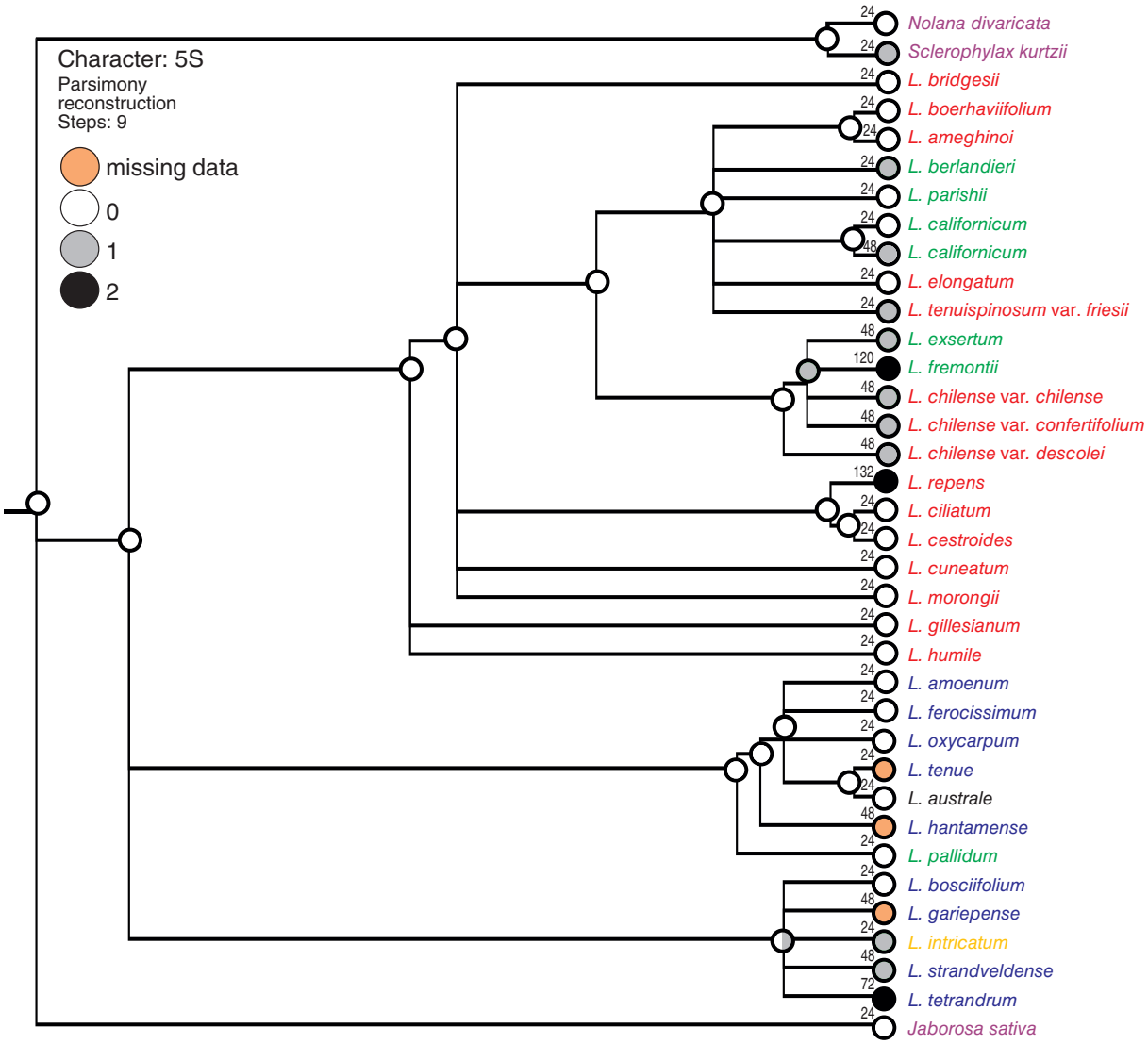

Fig. 7. Ancestral reconstruction of number of 5S loci in *Lycium* using parsimony and Bayesian approaches. The pie diagrams at nodes show the relative probability of the possible states (white, 0 = two 5S loci; grey, 1 = four 5S loci; black, 2 = more than four 5S loci). Chromosome number is at branch end. Geographic origin of the species is indicated in colour letters: Red = South America, green = North America, blue = Africa, black = Australia, orange = Europe, violet = outgroups.

2018). Two and four 18S-5.8S-26S sites per diploid karyotype are the most frequent in plants (Roa & Guerra, 2012). In *Lycium*, there are always two signals in diploids, whereas polyploids have a proportionally higher number (Blanco & al., 2012; Table 3, Fig. 6); thus, this gain is associated with polyploidy events (Hasterok & al., 2006; Pellicer & al., 2013).

There is differential evolution of the rDNA genes (Roa & Guerra, 2012). The 18S-5.8S-26S (Figs. 2, 6) sites in *Lycium* are more stable than the 5S (Figs. 2, 7), which are variable in position and number. There are two 5S sites per diploid genome, and polyploids proportionally increase the number of these sites (suppl. Figs. S1, S2), as detected in angiosperms and other Solanaceae (Roa & Guerra, 2012; Chiarini & al., 2018); exceptionally the diploids *L. intricatum* and *L. tenuispinosum* var. *friesii* present two pairs of signals (Fig. 7, suppl. Fig. S1), probably due to locus duplication.

Character mapping. — Considerable work has been published concerning phylogenetic relationships in *Lycium*, including several studies that explore the utility of nuclear markers in addition to plastid data. Miller (2002), Levin & Miller (2005), Levin & al. (2007, 2009a,b) and Miller & al. (2009, 2011) compared the phylogenetic utility of sequence data from nuclear (nitrate reductase, granule-bound starch synthase I, nuclear ribosomal internal transcribed spacer, and conserved ortholog set II) and chloroplastic DNA (spacers *trnT-trnF* and *trnD-trnT*) in tribe Lycieae. Although trees were generally concordant, there were some notable areas of incongruence. Nuclear data have their own set of challenges, especially in young groups like *Lycium*,

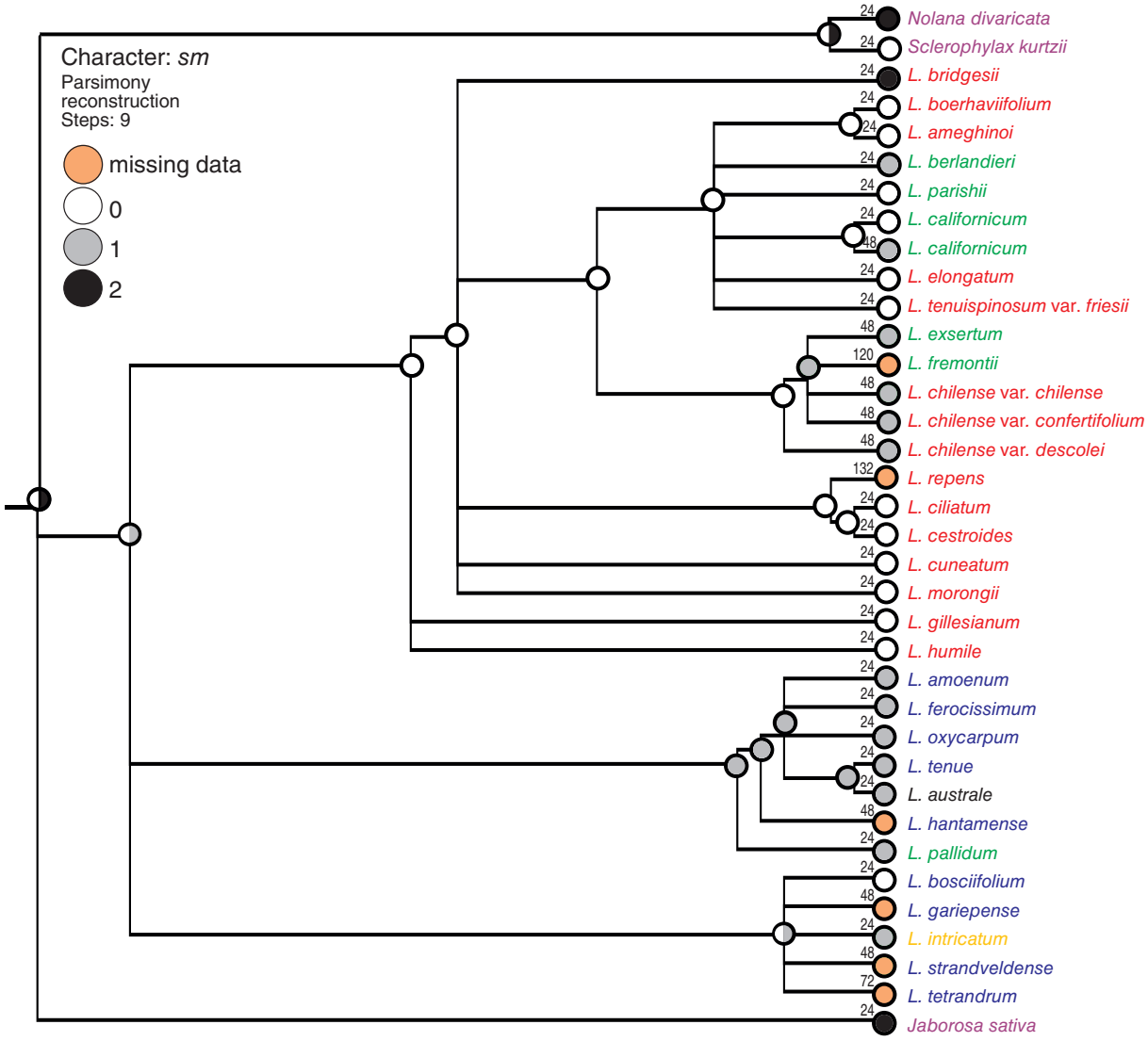

Fig. 8. Ancestral reconstruction of number of *sm* pairs in *Lycium* using parsimony and Bayesian approaches. The pie diagrams at nodes show the relative probability of the possible states (white, 0 = one *sm* pair; grey, 1 = two *sm* pairs; black, 2 = more than two *sm* pairs). Chromosome number is at branch end. Geographic origin of the species is indicated in colour letters: Red = South America, green = North America, blue = Africa, black = Australia, orange = Europe, violet = outgroups.

including incomplete concerted evolution for ITS (Miller, 2002), or precise determination of paralogy/orthology for nuclear markers (e.g., see Levin & al., 2009a,b). Importantly and relevant to this study, such challenges are exacerbated in polyploid taxa. Additionally, many of the polyploid *Lycium* species appear to be of hybrid origin, making phylogenetic reconstruction using nuclear data unreliable for those taxa. However, certainly it should be noted that a plastid-only analysis reflects solely maternal relationships. Larger comparative phylogenetic analyses with many markers and next-generation sequence data are beyond the scope of the present study, but are currently in preparation (Miller & Levin, in prep.). Given these issues and that the focus of the present study was character mapping and not phylogeny building, we chose to include plastid data only, which in our opinion is a conservative choice.

According to the mapping of the *Lycium* cytogenetical data, $2n = 24$ is ancestral in the genus and is a stable character for the "$x = 12$ clade" (Särkinen & al., 2013). Polyploidy arose independently in several African, South American, and North American species (Fig. 5, suppl. Fig. S3). In each of these three regions one species thrives with extremely high numbers: *L. tetrandrum*, *L. repens*, and *L. fremontii*, respectively; however, they have no particular morphological or ecological features to explain this phenomenon.

Regarding the total haploid chromosome length, in the ancestral *Lycium* condition it is between 20 and 25 µm, and the mean chromosome length is 1.69–2.15 µm. The outgroups have comparatively longer chromosomes, which suggests that the evolution of the genus started with a genomic reduction that was conserved during its diversification.

Stebbins (1971) suggested that the increase in karyotype asymmetry is associated with derived taxa. However, *Lycium* shows the opposite trend, as is also found in other Solanaceae (cf. Chiarini & al., 2018) and angiosperms (Mercado-Ruaro & Delgado-Salinas, 1998). Within *Lycium*, there is a tendency to a decrease in asymmetry as well as in chromosome length (Fig. 6, suppl. Fig. S3); *L. bridgesii*, which branches early in the tree, has the most asymmetrical karyotype in the genus (Bernardello & al., 2008), and the outgroups are also more asymmetrical (Chiarini & al., 2016; Lujea & Chiarini, 2017).

The ancestral heterochromatin percentage is 0.48%–0.78%. As a whole, this amount is conserved in the diversification of the genus, but in a mainly North American clade with species with long chromosomes, there is a notorious heterochromatin gain. Angiosperms with longer chromosomes tend to have more heterochromatic bands than those with smaller chromosomes (Guerra, 2000; Moscone & al., 2006; Chiarini & al., 2014), which applies to *Lycium*.

The most parsimonious hypothesis for the rDNA loci evolution in *Lycium* would be the presence of one locus as plesiomorphic. In several polyploid species, duplications of these rDNA loci have occurred independently after species divergence (Figs. 6, 7). This gain was associated with polyploidy events and/or increase of the genome length in species from other angiosperms (Hasterok & al., 2006; Pellicer & al., 2013), as may have occurred in *Lycium*. Only the basal diploid *L. bridgesii* shows all chromosome pairs with 18S-5.8S-26S loci (Blanco & al., 2012). This phenomenon was also observed in some species of *Nicotiana* and *Solanum*, where pericentromeric regions with sequences highly homologous to intergenic rDNA spacers were identified (Stupard & al., 2002; Lim & al., 2004). Unequal recombination, transposition and conversion/homogenization of repeats between loci have been proposed as mechanisms responsible for this mobility (Volkov & al., 2004; Raskina & al., 2008).

Our *Lycium* data can be compared with a recent cytogenetical revision in a phylogenetic framework on *Solanum*, the main genus of the family, with nuclear and chloroplastic sequences (Chiarini & al., 2018). The ancestors of both genera share these features: diploid with $2n = 24$, one locus of 18S-5.8S-26S and 5S genes, both asyntenic, but they differ in that the *Solanum* ancestor has a karyotype with *st* and/or *t* chromosomes. In *Solanum*, chromosomal variables are homoplastic with reversions in all branches, whereas in *Lycium* there are no reversions.

As a whole, *Lycium* species grow in arid and semi-arid environments and have their typical vegetative features: low shrubs, often with thorns, and small narrow leaves. Floral morphology is relatively uniform across the species, but specific floral features (or combinations of them) may be diagnostic. Most species have multi-seeded fleshy berries, some with various degrees of sclerification, or drupaceous fruits with two one-seeded pyrenes (Chiang-Cabrera, 1981; Bernardello, 1983, 1986; Levin & al., 2007). Pairwise analyses for the interaction of cytogenetical and morphological traits of the PERMANOVA detected significant differences in species with erect and prostrate habit and hermaphroditic and dioecious sex. The prostrate species are South American, diploid, hermaphroditic, and live in extreme environments. On the other hand, the dioecious taxa are North American or southern African and are polyploids. There is a reported connection between polyploidy and sexual system in some angiosperm clades (Glick & al., 2016). A biological explanation for this fact is the hypothesized effect of polyploidy on the transition from hermaphroditism to gynodioecy.

Our study points out the value of exploring cytogenetical data in a phylogenetic context, as they are significant traits that are not frequently examined in plant evolution. It has provided insights into the chromosome changes in different species during the morphological diversification in *Lycium*.

■ CONCLUSIONS

a) According to the ancestral state reconstruction, the common ancestor of *Lycium* species is diploid ($2n = 24$, $x = 12$), with a comparatively asymmetrical karyotype with *sm* and *st* chromosomes, an average chromosome length of $C = 1.69–2.15$ µm, and two asyntenic 18S-5.8S-26S and 5S loci.

b) The presence of polyploidy and the number of 5S loci are homoplastic, arising independently several times at different places in the phylogeny.

c) There are no significative evolutionary derivations from the cytogenetical features of the ancestor.

d) The examined characters can be ordered from more to less conservative, as follows: Asynteny of rDNA loci – chromosome number – number of 18S-5.8S-26S loci – mean chromosome length – number of 5S loci –karyotype formula.

■ AUTHOR CONTRIBUTIONS

Design of the research: LS, MLLP, GB; performance of the research: LS, MLLP, RAL, JSM, GB; data analysis, collection, or interpretation: LS, MLLP, RAL, JSM, GB; plant collection: LS, MLLP, RAL, JSM, GB; writing the manuscript: LS, MLLP, RAL, JSM, GB. — MLLP, https://orcid.org/0000-0003-4244-7807; GB, https://orcid.org/0000-0002-1915-9949

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