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CELLULAR AND MOLECULAR BIOLOGY

State of the art in cytogenetics, insights into chromosome number evolution, and new C-value reports for the fern family Gleicheniaceae

LUCAS VIEIRA LIMA, SAULO MARÇAL DE SOUSA, THAÍS ELIAS ALMEIDA & ALEXANDRE SALINO

Abstract: Studies concerning the cytogenetics of Gleicheniaceae have been scarce, especially those employing evolutionary approaches. Two chromosome number evolutionary models have been hypothesized for Gleicheniaceae. One proposes that ancestral haploid numbers were small and that the chromosome numbers of extant species evolved through polyploidy. The other model proposes that, at the genus level, fern chromosome evolution occurred from ancestors with essentially the same high chromosome numbers seen in living lineages. Neither of those hypotheses has been tested based on phylogenetic frameworks. We sought to (i) present the state of the art of Gleicheniaceae chromosome numbers; (ii) test the two evolutionary models of chromosome numbers within a phylogenetic framework; (iii) test correlations between DNA contents and chromosome numbers in the family. We report here DNA C-values for five species, which increases the number of investigated taxa nearly twofold and report two new genera records. Ancestral state chromosome reconstruction corroborates the hypothesis that ancestral chromosome numbers in Gleicheniaceae were as high as those of extant lineages. Our results demonstrate the possible role of dysploidy in the evolutionary chromosome history of Gleicheniaceae at the genus level and suggest that the relationship between chromosome number and DNA content does not appear to be linear.

Key words: Dysploidy, ferns, flow cytometry, Gleicheniales, polyploidy.

INTRODUCTION

Ferns and lycophytes stand out among vascular plants for their distinct genomic evolutionary histories, with the conservation of high chromosome numbers in taxa with diploid gene expressions (Haufler 1987, 2002, 2014). Evidence shows that those plants underwent multiple cycles of polyploidy (whole-genome duplications - WGDs) (1KP 2019, Huang et al. 2020), with subsequent diploidization involving gene silencing, but without apparent chromosome losses, so that high chromosome numbers were retained (Haufler 2002, 2014). Some putative gene loss is possible, however, as Liu et al. (2019) demonstrated that the range of genome sizes in ferns arose not only from repeated cycles of polyploidy but also through clade-specific constraints governing DNA accumulation and/or loss.

Whole Genome Duplication played a major role in fern and lycophyte speciation (Wood et al. 2009) and influenced not only chromosome numbers but also genome sizes (*e.g.,* Klekowski & Baker 1966, Leitch & Leitch 2012, 2013, Barker 2013, Henry et al. 2015). The DNA content showed high variability in ferns (Obermayer et al. 2002), ranging from 1C = 0.25 pg in *Salvinia cucullata*

Roxb. ex Bory (Li et al. 2018) to 1C = 150.61 pg in *Tmesipteris obliqua* Chinnock (Hidalgo et al. 2017). Although a few fern lineages show exceptionally large (or very small) genomes, ferns are typically characterized by medium-sized genomes. They are distinctive as compared to other land plants, however, as the only group with a correlation between genome size and chromosome numbers (Nakazato et al. 2008, Clark et al. 2016).

Even with significant advances in molecular studies of ferns and lycophytes, our knowledge concerning DNA C-values and the genome sizes of those plants remains incipient when compared to angiosperms. The Plant DNA C-values Database (Leitch et al. 2019), for example, contains DNA C-value data for 12,273 species but cites only 246 ferns (about 0.2% of all fern species) and 57 lycophytes (about 4% of all lycophyte species).

Although recent studies have shed light on the evolution of fern genome sizes (Clark et al. 2016, Liu et al. 2019), sampling data is still scarce, and it will be important to expand fern genome size information to better understand their genomic evolution (Bennett & Leitch 1995, Bennett et al. 2000).

The first interpretation of the evolutionary significance of C-values in ferns was made by Obermayer et al. (2002), based on a well-supported phylogenetic hypothesis of vascular plants (Pryer et al. 2001). Clark et al. (2016) subsequently significantly increased the number of fern species with documented C-values, providing evolutionary significance to a well-supported phylogeny of the group. A positive correlation between the genome sizes of ferns and lycophytes and their chromosome numbers was observed (Nakazato et al. 2008, Obermayer et al. 2002). The fern family Gleicheniaceae was included in previous works, but it was represented only by five species distributed in two genera (Kuo & Li 2019, Clark et al. 2016).

In that context, genome size studies together with the analysis of chromosome numbers represent important steps for genetic variation studies, phylogenetics, taxonomy, and evolution, and for understanding genome structure and diversity (*e.g.,* ploidy levels and expression, and nuclear architecture). Studies dealing with the cytology of Gleicheniaceae have been scarce (*e.g.,* Walker 1966, 1973, 1990, Mickel et al. 1966, Löve 1976, Tindale & Roy 2002), especially those employing evolutionary approaches. Sorsa (1968) proposed an evolutionary model for chromosome numbers in Gleicheniaceae and hypothesized two ancestor haploid numbers in the family (n=17 and n=11) from which all extant species evolved by polyploidy. A different point of view about chromosome number evolution in ferns, however, was proposed by Duncan & Smith (1978). Those authors hypothesized that, at the generic level, fern evolution occurred from ancestors that had essentially the same high chromosome numbers observed in living ferns. Neither of those hypotheses has been tested within a phylogenetic framework, and more studies are therefore needed to increase our knowledge of fern cytogenetics and test possible evolutionary patterns within a phylogenetic framework.

We, therefore, sought to (i) review what is already known about chromosome numbers in Gleicheniaceae; (ii) test the hypotheses of Sorsa (1968) and Duncan & Smith (1978) regarding ancestral chromosome numbers at the genus level in Gleicheniaceae; and (iii) report new DNA C-values for the family, increase taxa sampling, and evaluate the relationships between DNA content and chromosome numbers.

MATERIALS AND METHODS

Chromosome numbers and ancestral state reconstructions

Chromosome numbers for Gleicheniaceae were obtained from the EyeChrom online database (Rivero et al. 2019) and through an extensive literature review. The phylogenetic hypothesis used to infer chromosome ancestral state reconstructions was generated based on a data matrix of three plastid genome regions (*atpA, atpB,* and *rbcL*) available at GenBank (Table I). The data was assembled and aligned using MUSCLE, implemented in MEGA X (Kumar et al. 2018), and the best-fitting model of molecular evolution was determined using jMODELTEST v.2.1.4 (Darriba et al. 2012) based on Bayesian information criterion (Schwarz 1978). Bayesian inference was used to estimate a tree using MRBAYES v.3.2 (Ronquist et al. 2012), treating each region as a separate partition. The analysis consisted of two independent runs, with four simultaneous Markov chains running three million generations, with a random starting tree, at a temperature of 0.2, and sampling one tree every 100 generations. Convergence was verified by examining ESS (effective sample size) and PSRF (potential scale reduction factor) using TRACER v.1.6 (Rambaut et al. 2014), with a 10% burn-in. The remaining trees were used to assess topology in a strict consensus.

The basal chromosome number was inferred using ancestral state reconstruction in CHROMEVOL v.2.0 (Glick & Mayrose 2014). Character states were optimized using a model assuming a constant rate of chromosome gain, loss, and duplication, along with an estimated rate of no duplication (as that model was selected based on the output of the initial analyses with 10 models of chromosome evolution and chosen using Akaike information criteria).

Table I. GenBank accession for phylogenetic framework.

Taxa	rbcl	atpA	atpB
Matonia pectinata R. Br.	EU352307	EF463789	EF588716
Dicranopteris linearis (Burm. f.) Underw.	KU936634	DQ390557	AY612694
Diplopterygium bancroftii (Hook.) A.R. Sm.	EF463224	DQ390558	EF588713
Gleichenella pectinata (Wild.) Ching	EF588693	EF588671	AY612697
Gleichenia dicarpa R. Br.	AF313584	EF463736	AF313550
Sticherus bifidus (Wild.) Ching	EF463226	EF463737	EF463447
Stromatopteris moniliformis Mett.	AY612685	DQ390578	EF463448
Rouxopteris boryi (Kunze) H.M. Liu	KF992488	-	-

DNA C-values

Details and vouchers of the five species studied in the present work are presented in Table II. We used flow cytometry to estimate the DNA C-values of the species. Approximately 20 to 30 mg of young and fresh leaves of each species studied and the same amount of young leaf tissue from the internal reference standard (*Pisum sativum*, 9.09 pg) was chopped into ice containing 1 mL WPB buffer solution (0.2 M Tris. HCl, 4 mM $MgCl_2.6H_2O$, 2 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM sodium metabisulfite, 1 % PVP-10, 1 % (v/v) Triton X-100, pH 7·5) (Galbraith et al. 1983, Dolezel et al. 1998, Loureiro et al. 2007). The suspension was filtered through a 50-µm mesh and stained with 25 µL propidium iodide (10 mg L–1) (Sigma-Aldrich, USA) supplemented with 2.5 µL RNAse (20 mg L–1). For each run, at least 10,000 nuclei were analyzed per sample on a CytoFLEX cytometer (Beckman Coulter, USA). Histograms and statistical analyses were obtained using CytExpert Software version 2.0.1. DNA content was estimated using the G1 peak position of the internal standard as a reference following Dolezel & Bartos (2005). A Pearson correlation test between chromosomal numbers and DNA contents was performed using RStudio (2020).

Table II. Gleicheniales species with C-values reports. Family names follow PPG I (2016).

Families	Taxa	2C	1C	Voucher	Reference
Dipteridaceae	*Dipteris chinensis* Christ	4.85	2.43	HM Liu s.n.	Clark et al. (2016)
Dipteridaceae	*Dipteris conjugata* Reinw.	4.90	2.45	Schuettpelz 770	Clark et al. (2016)
Gleicheniaceae	*Diplopterygium blotianum* (C.Chr.) Nakai	3.90	1.95	Kuo 4408	Kuo & Li (2019)
Gleicheniaceae	*Diplopterygium glaucum* (Thunb. Ex Houtt.) Nakai	4.50	2.25	Kuo 4408	Kuo & Li (2019)
Gleicheniaceae	*Diplopterygium chinensis* (Rosesnt.) DeVol	3.90	1.95	Kuo 4410	Kuo & Li (2019)
Gleicheniaceae	*Diplopterygium bancroftii* (Hook.) A.R. Sm.	6.51	3.26	HM Liu s.n.	Clark et al. (2016)
Gleicheniaceae	*Dicranopteris linearis* (Burm. f.) Underw.	6.41	3.21	M. Christenhusz 7200	Clark et al. (2016)
Gleicheniaceae	*Dicranopteris flexuosa* (Schrad.) Underw.	9.16	4.58	LV Lima 66	Perez et al. (2021)
Gleicheniaceae	*Gleichenella pectinata* (Willd.) Ching	4.49	2.24	LV Lima 62	Present study
Gleicheniaceae	*Sticherus bifidus* (Wild.) Ching	10.90	5.45	LV Lima 90	Present study
Gleicheniaceae	*Sticherus gracilis* (Mart.) Copel.	6.48	3.24	LV Lima 212	Present study
Gleicheniaceae	*Sticherus lanuginosus* (Fée) Nakai	10.77	5.38	LV Lima 64	Present study
Gleicheniaceae	*Sticherus nigropaleaceus* (Sturm) J. Prado & Lellinger	18.32	9.16	LV Lima 94	Present study

RESULTS

Gleicheniaceae comprises approximately 120 species (PPG 2016), although chromosome numbers have been counted for only 37 species (23%) (Table III). Of the seven genera currently accepted for the family, six have at least one species with a known chromosome count (except *Rouxopteris* H.M. Liu). The lowest haploid number found in the family was n=20 (in some *Gleichenia* species), and the highest was n= 80 (*Dicranopteris linearis*) (Table III). *Sticherus*, a genus comprising approximately 94 species, has chromosome data available for only nine species (9.5%), although representing 42% of all chromosome counts recorded for the family (Figures 1 and 2). Data is available for five species of *Gleichenia* (40% of the recognized species in the genus), four species of *Diplopterygium* (16%), and three species of *Dicranopteris* (15%), as well as for the monotypic genera *Gleichenella* and *Stromatopteris* (Table III). The haploid number for most of the studied species of *Sticherus* is n=34; some species (*e.g., S. tenera, S. urceolatus*, *S. interjectus*, *S. jamaicensis*, and *S. revolutus*) showed n=68. *Gleichenia* showed two different haploid counts among the species investigated. The most common was n=20 (17 specimens), followed by n=22 (3 specimens). *Gleichenia microphylla* showed both chromosome counts (n=22 and n=20) in different populations (Figure 1, Table III). Only three species of *Diplopterygium* have had their chromosome numbers investigated: *D. bancroftii*, *D. farinosum*, and *D. glaucum* all showed n=56, while *D. longissimum* showed n=20. *Dicranopteris* showed different haploid counts, including n=78 (45% of the counts), n=39 (34%), n=68 (7%), n=80 (7%), and n=40 (3%) (Table III, Figure 1). *Gleichenella pectinata* showed two different haploid counts in the specimens investigated (n=43 and n= 44). *Stromatopteris* (a lineage represented by a single species confined to New Caledonia) showed n=39, although by only a single chromosome count (Table III, Figure 1).

The tree resulting from phylogenetic inference agrees with the topology recovered by Liu et al. (2020) and PPG I (2016) (Figure 2). Two different clades were recovered, one with *Rouxopteris* as the sister group of a clade formed by *Diplopterygium* as the sister group of *Dicranopteris+Gleichenella*. The other clade is composed of *Sticherus* as sister to the clade including *Stromatopteris+Gleichenia*. The basal node of Gleicheniaceae had its ancestral chromosome number recovered as n=46, while the clade including *Rouxopteris*, *Diplopterygium*, *Dicranopteris*, and *Gleichenella* was recovered with n=48. In the clade including *Diplopterygium*, *Dicranopteris*, and *Gleichenella* the ancestral number recovered was n=51, while the clade *Dicranopteris+Gleichenella* showed n=45 (Figure 2). In the clade including *Sticherus*, *Stromatopteris*, and *Gleichenia* the ancestral number recovered was n=42, while in *Stromatopteris+Gleichenia* the number recovered was n=40.

Regarding DNA contents in Gleicheniaceae, we increased here the sampled species in the family by eight, reporting five new c-values: *Gleichenella pectinata* (2C=4.49), *Sticherus bifidus* (2C=10.90), *Sticherus gracilis* (2C=6.48), *Sticherus lanuginosus* (2C=10.77), and *Sticherus nigropaleaceus* (2C=18.32) (Table II). We, therefore, report the C-values for two genera for the first time: *Sticherus* and *Gleichenella* (Table II). Despite the low sampling of C-values in Gleicheniaceae, the correlation coefficient between chromosome numbers and DNA content was 0.47 (Supplementary Material - Figure S1).

Table III. Chromosome numbers in Gleicheniaceae. CN= Chromosome number.

Species	CN(n)	Reference
Dicranopteris linearis (Burm. f.) Underw.	39,78, 80	Mehra & Singh (1956), Roy & Singh (1975), de Lange et al. (2004)
Dicranopteris flexuosa (Schrad.) Underw.	18, 68, 78	Löve (1976), Walker (1973), Sorsa (1968)
Dicranopteris pedata (Houtt.) Nakaike	78	Nakato (1988)
Diplopterygium bancroftii (Hook.) A.R.Sm	56	Mickel et al. (1966)
Diplopterygium farinosum (Kaulf.) Nakai	56	Mickel et al. (1966)
Diplopterygium glaucum (Thunb. ex Houtt.) Nakai	56	Mehra & Singh (1956)
Diplopterygium longissimum (Blume) Nakai	20	Fabbri (1963)
Gleichenia alpina R.Br.	20	Tindale & Roy (2002)
Gleichenella pectinata (Willd.) Ching	43, 44	Walker & Ortega (1992), Jermy & Walker (1985), Sorsa (1968)
Gleichenia circinata (Sw.) C.Chr.	20	Brownlie (1958)
Gleichenia dicarpa R.Br.	22	Brownlie in Fabbri (1965)
*Gleichenia microphyll*a (R.Br.) C.Chr.	20, 22	Brownlie in Fabbri (1963) Brownlie (1961)
Gleichenia rupestris R.Br.	20	Tindale & Roy (2002)
Sticherus bifidus (Willd.) Ching	34	Walker & Ortega (1992)
Sticherus brittonii (Maxon) Nakai	34	Walker & Ortega (1992)
Sticherus brackenridgii (E.Fourn.) H.S.John	34	Brownlie (1965)
Sticherus cunninghami (Hew ex Hook.) Ching	34	Brownlie (1958)
Sticherus flabellatus (R.Br.) H.St.John	34	Brownlie (1961)
Sticherus furcatus (L.) Ching	34	Walker (1966, 1990)
Sticherus hypoleucus (Sodiro) Copeland	34	Walker (1990)
Sticherus interjectus (Jermy & T.G.Walker) J.Gonzales	68	Jermy & Walker (1985)
Sticherus intermedius (Baker) Chrysler	34	Walker (1990)
Sticherus jamaicensis (Underw.) Nakai	68	Walker (1966, 1990)
Sticherus lobatus N.A.Wakef.	34	Tindale & Roy (2002)
Sticherus nudus (Moritz) Nakai	34	Walker & Ortega (1992)
Sticherus pallescens (Mett.) Vareschi	34	Walker & Ortega (1992)
Sticherus remotus (Kaulf.) Chrysler	34	Jermy & Walker (1985)
Sticherus retroflexus (J.Bommer ex Christ) Copeland	34	Walker (1990)
Sticherus revolutus (Kunth) Ching	68	Walker (1990), Walker & Ortega (1992)
Sticherus rubiginosus (Mett.) Nakai	34	Walker & Ortega (1992)
Sticherus strictissimus (Christ) Copeland	34	Walker (1990)
Sticherus tenera (R. Br.) Ching	34, 68	Tindale & Roy (2002), Thrower (1963)
Sticherus urceolatus M. Garrett, Kantvilas & Laws	68	Tindale & Roy (2002)
Sticherus milnei (Baker) Ching	34	Holttum & Roy (1965)
Sticherus × pseudobifidus (Jermy & T.G.Walker) J.Gonzales	51	Jermy & Walker (1985)
Sticherus × subremotus (Jermy & T.G.Walker) J.Gonzales	51	Jermy & Walker (1985)
Stromatopteris moniliformis Mett.	39	Bierhorst (1968)

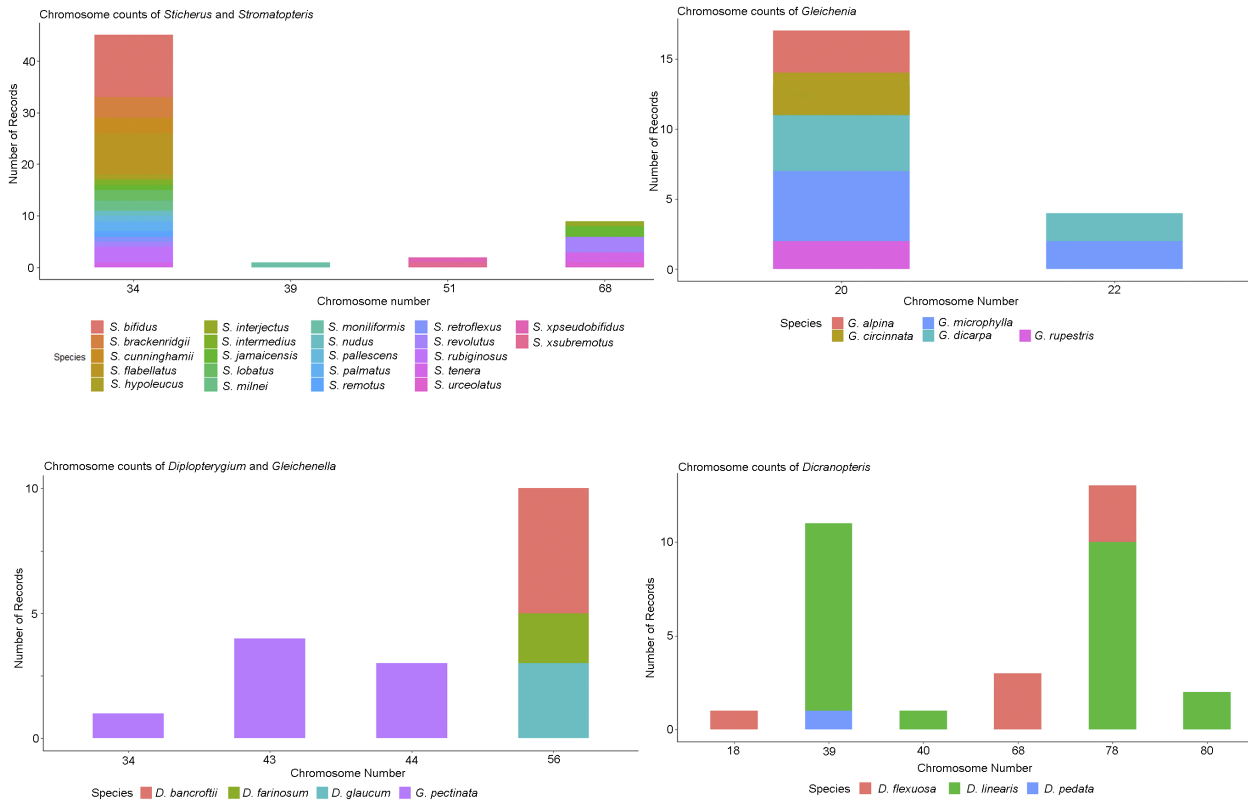

Figure 1. Distribution of chromosome numbers in the sampled species.

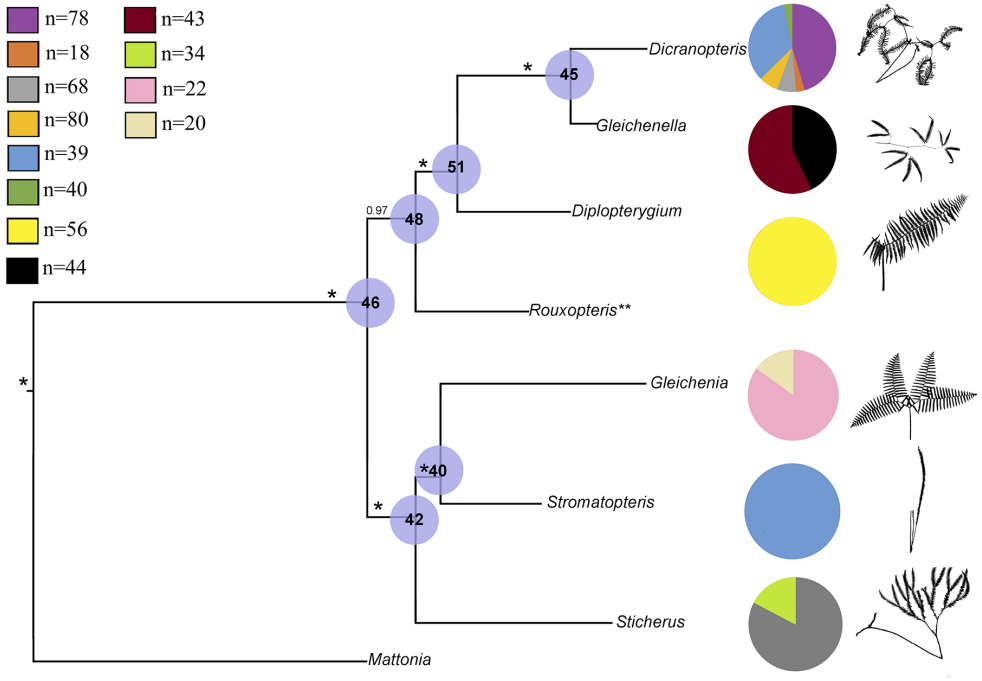

Figure 2. Phylogenetic inference with ancestral chromosome number reconstructions. Bayesian strict consensus tree, inferred from three plastid markers (*atpA*, *atpB*, and *rbcL*) (* indicates posterior probability equals 1.0; ** *Rouxopteris* is recently segregated genus from *Gleichenia* and it has no chromosome counts to the date). Pie charts show the frequency of chromosome numbers in each genus.

DISCUSSION

Chromosome counts

Polyploidy events are common in ferns and have likewise been observed in Gleicheniaceae, as was hypothesized by Sorsa (1968), Duncan & Smith (1978), and later by Haufler (2002, 2014). *Sticherus* has straightforward examples of polyploidy. That family showed only two haploid numbers among the species studied (n=34 and n=64) (*e.g.,* Walker & Ortega 1992, Walker 1966, 1990, Brownlie 1958, 1961, Brownlie in Fabbri 1965, Tindale & Roy 2002). *Sticherus tenera* showed different haploid numbers in different populations (34 and 68) (Tindale & Roy 2002, Thrower 1963), and it may represent a species with different diploid and polyploid cytotypes.

In addition to polyploidy, other events can induce chromosome number variations (either increasing or decreasing them), including aneuploidy and dysploidy, which may have played important roles in Gleicheniaceae chromosomal evolution. When one or more chromosomes are lost or gained by aneuploidy, there will presumably be deletions or duplications of many genes – resulting in unbalanced, lethal, or sub-vital constitutions, so that those types of chromosome number variations have no apparent evolutionary meaning (Guerra 2008). Dysploidy, on the other hand, can induce increases or decreases in haploid chromosome numbers without resulting in unbalanced or lethal constitutions (Friebe et al. 2005).

Polyploidy seems to be rather common in *Dicranopteris*, as it showed counts of n = 39 and n = 78 (Mehra & Singh 1956, Roy & Singh 1975, de Lange et al. 2004, Löve 1976, Walker 1973). Other haploid counts, however, have been found in the genus, such as n=40 (Manton & Sledge 1954). Both dysploidy and polyploidy events may have played roles in evolutionary changes in the chromosome numbers of that genus of Gleicheniaceae. One population of *Dicranopteris linearis* investigated showed n=40, and two others showed n=80. As n=39 is one the most frequent haploid number found in the genus, ascending dysploidy followed by polyploidization could explain those haploid numbers. *Dicranopteris flexuosa* also showed a possible case of dysploidy, with a chromosome decrease, with two specimens from different populations showing n=68 (Araujo in Löve 1976). We hypothesize that from n=39, a dysploidy event occurred, resulting in a chromosome decrease and a count of n=34, followed by a polyploidy event resulting in individuals with n=68.

Another possible dysploidy series was observed in *Gleichenia,* with n=20 and n=22 (*e.g.*, Walker & Ortega 1992, Brownlie 1958, Tindale & Roy 2002), especially in *G. microphylla*, which shows populations with both haploid counts (Brownlie 1961, Brownlie in Fabbri 1965). Similarly, *Gleichenella pectinata*, a widespread neotropical species, may also present cases of dysploidy series. Sorsa (1968) observed 44 chromosomes in four specimens from three different localities in Porto Rico, but also found populations with n=43. Those different chromosome counts were similarly reported in specimens from different populations in Jamaica (Walker 1966), Trinidad (Jermy & Walker 1985), and Mexico (Smith & Mickel 1977).

In addition to dysploidy events, another possible explanation for the variations seen in *Gleichenella* and *Gleichenia* would be the presence of B chromosomes – which are supernumerary, usually with preferential heritage, deviating from the usual Mendelian segregation (Houben 2017). There is no evidence to date, however, which could confirm the existence of B chromosomes in Gleicheniaceae, and more cytogenetics studies will be needed to test that possibility.

Additional cases of haploid numbers in Gleicheniaceae remain unexplained, such as in *Diplopterygium*. That genus has had only four species investigated, with three showing n=56, and *D. longissimum* showing n=20 (Mickel et al. 1966, Mehra & Singh 1956), which could be explained by dysploidy and polyploidy events (or may represent chromosome miscounts). Further attention should therefore be paid to *D. longissimum,* as its chromosome count is quite discrepant when compared to the other species analyzed.

Ancestral state reconstruction

The ancestral state reconstruction (Figure 2) recovered by the best-fitting model corroborates the hypothesis of Duncan & Smith (1978) that the ancestral chromosome numbers in Gleicheniaceae were as high as those of extant lineages. The ancestral chromosome number recovered in the first clade was 51 (Figure 2) in the node of *Diplopterygium* and *Dicranopteris*+*Gleichenella*. In that case, we hypothesize that ascendant dysploidy events resulted in a lineage with a basic chromosome number n=56, represented by the genus *Diplopterygium*. Despite low sampling in that genus, the chromosome counts were constant (n=56) among the investigated species, which may represent stability through the chromosomal evolutionary history of the genus.

The ancestral chromosome number recovered in other genera in that clade (*Dicranopteris* and *Gleichenella*) was n=45. *Dicranopteris* showed five different chromosome number counts (n=39, n=40, n=68, n=78, and n=80). Subsequent chromosome decreases would have to be assumed in a scenario with an ancestral number of n=45. In both cases, populations with n=80 and n=78 may have arisen through polyploidy. The second and less frequent count was n=68 (Table III), which could have resulted from an autopolyploidization event in a population having n=34. No population of *Dicranopteris* has yet been found with n=34, but that does not exclude the possibility of additional chromosome losses followed by subsequent autopolyploidization. More species and populations need to be sampled to construct a better panorama of the evolutionary history of chromosome numbers in *Dicranopteris*. As mentioned above, *Gleichenella* showed two different chromosome counts (n=44 and n=43). The ancestral chromosome number recovered (n=45) suggests a trend of chromosome loss in the lineage.

Regarding the second clade, ancestral character reconstruction showed ancestral numbers as high as those of extant lineages. A significant reduction in chromosome numbers (from n=42 to n=34) was observed in *Sticherus* as compared to the ancestral number recovered; additionally, no evidence of dysploidy was observed in the genus, only cases of autopolyploidy.

The clade formed by *Stromatopteris* +*Gleichenia* has a hypothetical ancestral chromosome number n=40, which implies a reduction of one chromosome in the former genus. Two haploid numbers have been reported in *Gleichenia* (n=20 more frequently, and n=22 less frequently). We hypothesize that there was a reduction by half of the total number of ancestral chromosomes, in this case, resulting in n=20; the chromosome count of n=22 might be the consequence of a subsequent event of ascending dysploidy, as has been documented in other fern genera (*e.g.,* by Wang et al. 2010 in *Lepisorus* [Polypodiaceae], and by Bellefroid et al. 2010 in *Asplenium* [Aspleniaceae]).

The same chromosome number may have independently appeared twice in different genera. Although the haploid number of *Stromatopteris* is the same as one of the haploid

numbers of *Dicranopteris* (n=39) (Bierhorst 1968), it may not represent a homologous condition, as *Stromatopteris* is placed in a different clade with *Gleichenia* (n=20 and n=22) and *Sticherus* (n=34 and n=64). Thus, additional studies will be required focusing on fern cytology, and evolutionary patterns will need to be examined in the light of phylogenetic studies. Further attention must also be paid to *Gleichenia*, as its monophyly is still questionable due to low sampling in phylogenetic analyses.

C-values

Chromosome numbers alone are not sufficient to fully understand the evolutionary cytogenetics of ferns. Liu et al. (2019) demonstrated, using *Asplenium* (Aspleniaceae) as a model, that the evolution of fern genome sizes is not shaped solely by chromosome number changes arising from polyploidy, but also by constraints on the average quantity of DNA per chromosome. The differences in DNA contents observed in different lineages may be related to chromosome size, and not necessarily to ploidy levels. We, therefore, examined the DNA contents of five Gleicheniaceae species and present here, for the first time, C-values for two Gleicheniaceae genera, *Sticherus* and *Gleichenella*, and likewise increased the number of sampled species in the family to eight by reporting new c-values for five species (Table II, Figure 3).

The differences in DNA contents observed among *Sticherus* species could be related to chromosome size, and not just ploidy levels. We

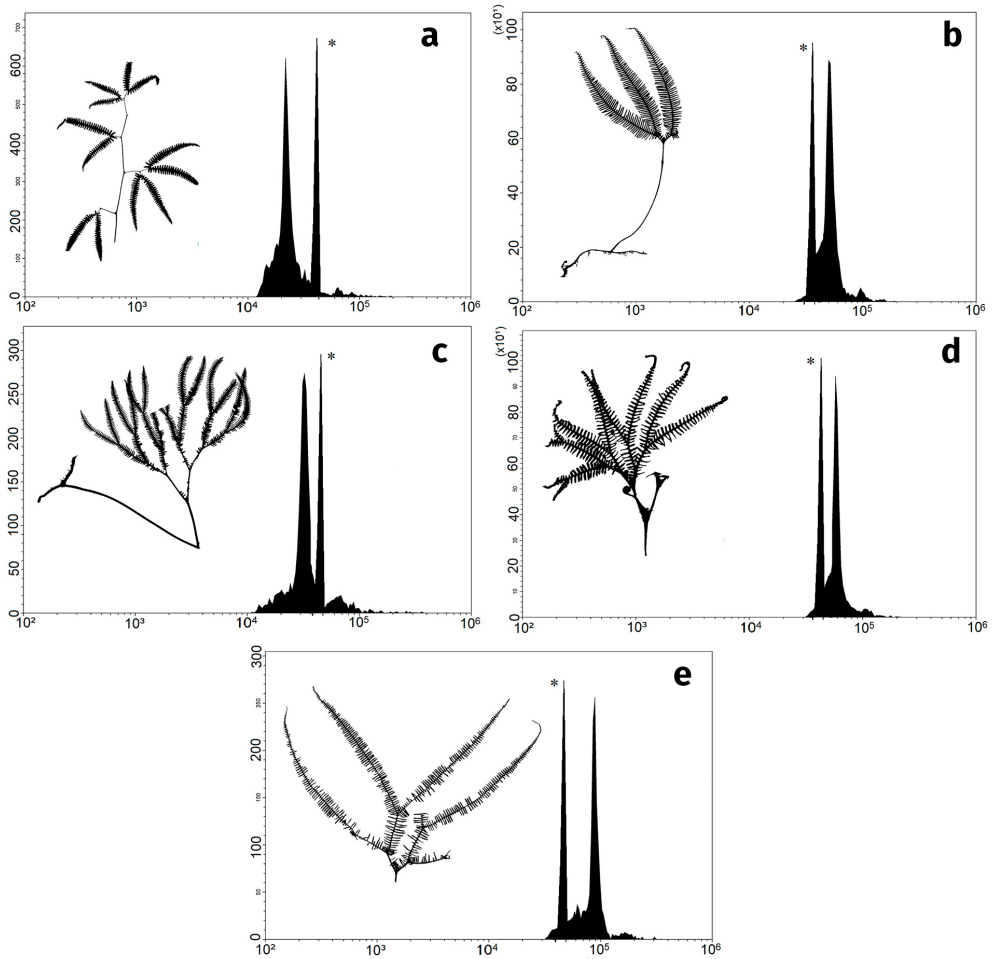

Figure 3. Cytometry Histograms. a. *G. pectinata* (CV=4.47, Sd= 0.3). b. *S. bifidus* (CV= 5.01, Sd= 0.3). c. *S. gracilis* (CV= 4.7%, Sd= 0.4). d. *S. lanuginosus* (CV= 4.3, Sd= 0.4). e. *S. nigropaleaceus* (CV= 4.7, Sd= 0.2) *Internal control (*Pisum sativum*). Sd= Standard deviation.

observed 3-fold variations in the DNA contents of the four *Sticherus* species examined, which ranged from 6.48 pg in *Sticherus gracilis* to 18 pg in *Sticherus nigropaleaceus*. The DNA contents of *Sticherus lanuginosus* (10.77 pg) and *S. bifidus* (10.9 pg) were similar and may be good examples of the chromosome number stability observed in the genus. The difference in the DNA content of *S. nigropaleaceus*, as compared with the other species of the genus so far investigated, may represent a case of polyploidy. Although no chromosome counts have so far been made for *S. gracilis*, its DNA content may be related to chromosome size, as the chromosome numbers in *Sticherus* usually are stable (Table III), with few cases of polyploidy (18%).

Despite the low sampling of C-values in Gleicheniaceae, our results indicate that chromosome numbers and DNA contents in Gleicheniaceae may be uncorrelated. *Gleichenella* showed the lowest DNA content in the family (4.49 pg) and has n=44, while *Sticherus*, which usually shows n=34, had the highest DNA content values, ranging from 6.48 pg to 18.32 pg. The DNA contents of *Diplopterygium bancroftii* (n=56) and *Dicranopteris linearis* (n=39) are similar (6.51 pg), which may be related to the lack of correlation between DNA content and chromosome numbers in the family; further attention must be given to *Stromatopteris*, *Rouxopteris*, and *Gleichenia*. Despite low sampling in the family, our results are close to the projections made by Clark et al. (2016), who estimated the mean of DNA content of Gleicheniales to be 10 pg.

CONCLUSIONS

Our chromosome ancestral state reconstructions corroborate the hypothesis that the ancestral chromosome numbers in Gleicheniaceae were as high as those now seen in extant lineages. The duplication of whole chromosome numbers (polyploidy), as well as the dysploidy series, appear to have played important roles in Gleicheniaceae chromosome evolution. We emphasize here the importance of cytogenetic studies as well as the need for more chromosome counts and DNA content data for the Gleicheniaceae (together with better-resolved phylogenetic inferences) to elucidate chromosome evolution in the group. The analysis of DNA C-values suggests that chromosome numbers and DNA contents may not be correlated in Gleicheniaceae, but an expanded sampling of DNA C-values and chromosome counts will be needed to verify that hypothesis.

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SUPPLEMENTARY MATERIAL

Figure S1.

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LUCAS VIEIRA LIMA[1]
https://orcid.org/0000-0003-1517-7100

SAULO MARÇAL DE SOUSA[2]
https://orcid.org/0000-0001-8229-9330

THAÍS ELIAS ALMEIDA[3]
https://orcid.org/0000-0002-1611-1333

ALEXANDRE SALINO[1]
https://orcid.org/0000-0003-0104-7524

[1]Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Departamento de Botânica, Laboratório de Sistemática Vegetal, Av. Antônio Carlos, 6627, 31270-901, Belo Horizonte, MG, Brazil

[2]UniversidadeFederal de Juiz de Fora, Instituto de Ciências Biológicas, Departamento de Biologia, Laboratório de Genética e Biotecnologia, Rua José Lourenço Kelmer, s/n, 36036-900 Juiz de Fora, MG, Brazil

[3]Universidade Federal do Oeste do Pará, Herbário HSTM, Avenida Marechal Rondon, s/n, 68040-070 Santarém, PA, Brazil

Correspondence to: **Lucas Vieira Lima**
E-mail: lucaslima1618@gmail.com

Author contributions

L.V. Lima: Contributions to the conception and design of the study, data collection, data analysis and interpretation, preparation of the manuscript draft, contribution to the critical revision, and the addition of intellectual content. S. M. Sousa: Contributions to data collection, analysis and interpretation, manuscript preparation, critical revision, and the addition of intellectual content. T. E. Almeida and A. Salino: Contributions to manuscript preparation, critical revision, and the addition of intellectual content.