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# Phylogenomic evolutionary insights in the fern family Gleicheniaceae

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ABSTRACT

The pantropical fern family Gleicheniaceae comprises approximately 157 species. Seven genera are currently recognized in the family, although their monophyly is still uncertain due to low sampling in phylogenetic studies. We examined the monophyly of the genera through extended sampling, using the first phylogenomic inference of the family including data from both nuclear and plastid genomes. Seventy-six samples were sequenced (70 Gleicheniaceae species and six outgroups) using high throughput sequencing, including all seven currently recognized genera. Plastid and nuclear data were recovered and assembled; the nuclear data was phased to reduce paralogy as well as hybrid noise in the final recovered topology. Maximum likelihood trees were built for each locus, and a concatenated dataset was built for both datasets. A species tree based on a multispecies coalescent model was generated, and divergence time analyses performed. We here present the first genomic phylogenetic inferences concerning Gleicheniaceae, confirming the monophyly of most genera except *Sticherus*, which we recovered as paraphyletic. Although most of the extant genera of Gleicheniaceae originated during the Mesozoic, several genera show Neogene and even Quaternary diversifications, and our results suggest that reticulation and polyploidy may have played significant roles during this diversification. However, some genera, such as *Rouxopteris* and *Stromatopteris*, appear to represent evolutionary relicts.

## 1. Introduction

Gleicheniaceae C.Presl is a distinct leptosporangiate fern family that currently is considered to comprise approximately 157 species distributed into seven genera (Gonzales and Kessler, 2011; PPG, 2016; Liu et al., 2020). The plants have long-creeping rhizomes and pseudodichotomous branched fronds with indeterminate growth due to periodic apical dormancy (Holttum, 1957; Tryon and Stolze, 1989; Ostergaard Andersen and Ollgaard, 2001; Gonzales and Kessler, 2011; Lima and Salino, 2018). They are heliophytes, predominantly terrestrial, occasionally occurring on rocks, with low demands for mineral nutrients and often inhabiting disturbed areas such as roadsides (Penrod, 2000; Walker and Sharpe, 2010).

The numbers of recognized genera in the family have changed over time (Table 1). Smith (1793) initially considered the family to be monogeneric (*Gleichenia* Sm.). Diels (1900) later maintained all species in *Gleichenia*, separating the species into subgenera and sections. This infrageneric classification was initially followed by Christensen (1905), although that author later (Christensen 1938) recognized five genera (*Dicranopteris* Bernh., *Sticherus* C.Presl., *Gleichenia*, *Platyzoma* R.Br., and *Stromatopteris* Mett.). Copeland (1947) adopted the five genera of Christensen's classification but further recognized the genus *Hicriopteris* C.Presl. Ching (1940) segregated five genera from *Gleichenia* and proposed a new monotypic genus, *Gleichenella* Ching. Holttum (1947)

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initially considered the genera proposed by Christensen (1938), but later (Holttum, 1957, 1959) placed them in the subgenera *Gleichenia* and *Dicranopteris*. Nakai (1950) removed *Platyzoma* from Gleicheniaceae, positioning it in Platyzomataceae, which was later placed in Pteridaceae and subsumed in the genus *Pteris* L. (PPG, 2016).

Phylogenetic inferences based on chloroplast DNA sequences, including *atpA*, *atpB*, *rbcL*, and *rps4* (Pryer et al., 2004; Perrie et al., 2007; Schuettpelz and Pryer, 2007; Li et al., 2010), suggest that the family is composed of two clades. One clade comprises *Diplopterygium* (Diels) Nakai, with scaly rhizomes, as a sister of *Dicranopteris*, while *Gleichenella* has a rhizome covered exclusively by hairs, which has been considered a possible synapomorphy. The other clade comprises *Gleichenia*, *Sticherus*, and *Stromatopteris*, all with scaly rhizomes (Gonzales and Kessler, 2011). Liu et al. (2020) segregated *Gleichenia boryi* from the other *Gleichenia* species, placing it in *Rouxopteris* H.M. Liu due to its morphology and phylogenetic characteristics. In their phylogenetic topology, *Rouxopteris* is recovered as sister group to a clade formed by *Gleichenella* + *Dicranopteris* + *Diplopterygium*.

Despite these studies, based on phylogenetic reconstructions from a few plastid loci, and major advances in the application of molecular tools to the taxonomy of various fern and lycophyte groups (e.g., Gasper et al., 2017; Zhang and Zhang, 2017; Almeida et al., 2017; Testo et al., 2018; Lehtonen et al., 2020; Chen et al., 2022), the monophyly of the genera of Gleicheniaceae is still questionable in light of low sampling (PPG, 2016), as exemplified by the segregation of *Rouxopteris*, which was only discovered due to dense sampling in *Gleichenia* (Liu et al., 2020). Phylogenetic studies, associated with divergence time analyses, have provided valuable information for a better understanding of the evolutionary history of many groups, as they can contextualize the rise of their lineages (Testo et al., 2018; Testo and Sundue, 2016). Gleicheniaceae has previously been included in large-scale dated phylogenies (e.g., Pryer et al., 2004; Testo and Sundue, 2016), although these studies did not include all its genera. Recently, all Gleicheniaceae genera were included in a divergence-time analysis – but based on only a single plastid locus (Liu et al., 2020). Therefore, no multilocus dataset that tested all Gleicheniaceae genera has yet been utilized to estimate the divergence times of the extant lineages of the family.

In that context, we addressed the following questions: What are the phylogenetic relationships among the different Gleicheniaceae lineages? Are the genera of Gleicheniaceae, as currently circumscribed, monophyletic? Are the data recovered from the plastid and nuclear genomes congruent in terms of recounting the evolutionary history of the family? When did the main lineages of Gleicheniaceae arise?

## 2. Materials and methods

### 2.1. Sampling, sequencing, and read quality control

In the present study, 76 samples (70 Gleicheniaceae, representing about 44% of recognized species in the family, and six outgroups) from herbarium samples and tissue dried in silica gel were sequenced (Table 2). Samples of the seven genera of Gleicheniaceae sensu PPG I (2016) and Liu et al. (2020) were included (*Dicranopteris, Gleichenella, Gleichenia, Diplopterygium, Sticherus, Stromatopteris,* and *Rouxopteris*). Type species of all genera were sampled. DNA was extracted from silica-dried tissue using the DNeasy Mini Plant Kit (Qiagen); the CTAB protocol (cetyltrimethylammonium bromide) protocol was used for herbarium samples, following Doyle and Doyle (1990). The samples were sequenced using Rapid Genomics (Gainsville, USA) using target enrichment sequencing using GoFlag 451 probes (a set of 56,989 probes that covered 451 exons from 248 single or low-copy nuclear genes) (Breinholt et al., 2021). Samples were sequenced using the Illumina HiSeq 2500 platform, generating 150 bp paired-end reads. Raw data quality was verified using FastQC (Andrews, 2010, version 0.11.9); the filtering and trimming of low quality pair-end reads was performed using Trimmomatic (Bolger et al., 2014, version 0.36) (illuminaclip 2:30:10, leading 10, trailing 40). The trimmed raw data were assembled in two separate datasets: nuclear and chloroplast. Sequence reads were deposited in the NCBI Sequence Read Archive (Table 2) and recovered gene sequences and alignments are available on GitHub (https://github.com/lucaslima1618/phylo_gleich).

### 2.2. Chloroplast dataset

To build a partial plastome dataset, Geneious Prime 2021 (version 2021.1.1) (https://www.geneious.com) was used to assemble the trimmed raw data by reference. The annotated plastome of *Diplopterygium glaucum* [deposited at GenBank (NC_024158) (Kim et al. 2014)] was used as a reference. All assembled sequences were strictly aligned to the reference sequence using MAFFT (Katoh et al., 2009, version 7.48). We extracted 40 coding regions to build a partitioned dataset, then TrimAL (Capella-Gutierrez et al., 2009, version 1.2) was used in each partition to eliminate columns with>60% gaps. The result was a matrix with 73 terminals and approximately 15,000 bp.

### 2.3. Nuclear dataset

HybPiper (Johnson et al., 2016) was used to assemble the nuclear sequence reads derived from the GoFlag probe set for ferns. Considering

**Table 1**
Generic classification of Gleicheniaceae.

| Smith (1773) | Diels (1900) | Christensen (1905) | Copeland (1947) | Nakai (1950) | Holttum (1957) | Kramer (1990) | PPG, 2016 | Liu et al. (2020) |
|---|---|---|---|---|---|---|---|---|
| *Gleichenia* | *Gleichenia* | *Gleichenia* | ——//—— | ——//—— | ——//—— | ——//—— | ——//—— | ——//—— |
| ——//—— | Subg. *Eu-Gleichenia* | ——//—— | *Stromatopteris* | *Stromatopteris* | *Stromatopteris* | *Stromatopteris* | *Stromatopteris* | *Stromatopteris* |
| ——//—— | Subg. *Eu-Gleichenia* | Sect. *Eugleichenia* | *Gleichenia* | *Gleichenia* | *Gleichenia* | *Gleichenia* | *Gleichenia* | *Gleichenia* |
| ——//—— | ——//—— | Subsect. *Gleicheniastrum* | ——//—— | ——//—— | ——//—— | ——//—— | ——//—— | ——//—— |
| ——//—— | ——//—— | Subsect. *Calymella* | ——//—— | *Calymella* | ——//—— | ——//—— | ——//—— | ——//—— |
| ——//—— | Subg. *Mertensia* | Sect. *Mertensia* | ——//—— | ——//—— | ——//—— | ——//—— | ——//—— | ——//—— |
| ——//—— | ——//—— | ——//—— | ——//—— | ——//—— | ——//—— | ——//—— | ——//—— | *Rouxopteris* |
| ——//—— | Sect. *Diplopterygium* | Subsect. *Diplopterygium* | *Hicriopteris* | *Hicriopteris* | Subg. *Diplopterygium* | *Diplopterygium* | *Diplopterygium* | *Diplopterygium* |
| ——//—— | Sect. *Holopterigyum* | Subsect. *Holopterygium* | *Sticherus* | *Sticherus* | Subg. *Mertensia* | *Sticherus* | *Sticherus* | *Sticherus* |
| ——//—— | ——//—— | ——//—— | *Dicranopteris* | ——//—— | *Dicranopteris* | *Dicranopteris* | *Dicranopteris* | *Dicranopteris* |
| ——//—— | Sect. *Heteropterygium* | Subsect. *Heteropterygium* | ——//—— | *Dicranopteris* | Subg. *Dicranopteris* | ——//—— | ——//—— | ——//—— |
| ——//—— | ——//—— | ——//—— | ——//—— | *Gleichenella* | ——//—— | ——//—— | *Gleichenella* | *Gleichenella* |
| ——//—— | Sect. *Acropterygium* | Subsect. *Acropterygium* | ——//—— | *Acropterigyum* | Subg. *Acropterygium* | ——//—— | ——//—— | ——//—— |

**Table 2**
Voucher information for specimens used in this study and SRA accessions. Herbarium acronyms are according to Thiers (2020 onward: http://sweetgum.nybg.org/science/ih/).

| Taxon Name | Voucher Number | Voucher Location | Country of origin | Accession |
|---|---|---|---|---|
| *Dicranopteris dichotoma* | Takehara 2 | Herb Inst. Biologie Tohoku | Japan | SAMN33583381 |
| *Dicranopteris flexuosa* | Lima 220 | BHCB | Brazil | SAMN33583382 |
| *Dicranopteris linearis* | Kessler 13864 | GOET | New Guinea | SAMN33583383 |
| *Dicranopteris taiwanensis* | Wen-Liang 15282 | TAIF | India | SAMN33583384 |
| *Dicranopteris nervosa* | Lima 226 | BHCB | Brazil | SAMN33583385 |
| *Dicranopteris rufinervis* | Lima 213 | BHCB | Brazil | SAMN33583386 |
| *Dicranopteris seminuda* | Martinelli 17233 | RB | Brazil | SAMN33583387 |
| *Diranopteris spissa* | Salino 16256 | BHCB | Brazil | SAMN33583388 |
| *Dicranopteris subpectinata* | SWK 1717 | FU | Malaysia | SAMN33583389 |
| *Dicranopteris tetraphylla* | Tobagane 4641 | FU | Thailand | SAMN33583390 |
| *Diplopterygium bancroftii* | Øllgaard 35676 | AAU | Ecuador | SAMN33583391 |
| *Diplopterygium brevipinnulum* | Karger 1441 | GOET | Moluccas | SAMN33583392 |
| *Diplopterygium chinensis* | Tobagane 76 | FU | Taiwan | SAMN33583393 |
| *Diplopterygium conversum* | Jimenez 1434 | FU | Indonesia | SAMN33583394 |
| *Diplopterygium glaucum* | Karger 597 | GOET | Philippines | SAMN33583395 |
| *Diplopterygium longissimum* | Kessler 13546 | GOET | Malaysia | SAMN33583396 |
| *Diplopterygium norisii* | Karger 1099 | GOET | Malaysia | SAMN33583397 |
| *Diplopterygium volubilis* | Jimenez 1148 | FU | Indonesia | SAMN33583398 |
| *Diplopterygium* sp. 1 | T 3869 | FU | Thailand | SAMN33583399 |
| *Diplopterygium* sp. 2 | T 2084 | FU | Cambodia | SAMN33583400 |
| *Gleichenella pectinata* | Lima 225 | BHCB | Brazil | SAMN33583401 |
| *Gleichenia dicarpa* | Kessler 14281 | GOET | Australia | SAMN33583402 |
| *Gleichenia peltophora* | Karger 441 | GOET | Philippines | SAMN33583403 |
| *Gleichenia polypodioides* | Kessler 13836 | GOET | La Réunion | SAMN33583404 |
| *Rouxopteris boryi* | Hennequin 254 | P | La Réunion | SAMN33583405 |
| *Rouxopteris boryi* var. *madagascariensis* | Hennequin 276 | P | La Réunion | SAMN33583406 |
| *Sticherus aurantiacus* | Øllgaard 2504 | AAU | Ecuador | SAMN33583407 |
| *Sticherus bifidus* | Lima 210 | BHCB | Brazil | SAMN33583408 |
| *Sticherus blepharolepis* | Kessler 14840 | GOET | Colombia | SAMN33583409 |
| *Sticherus bolanicus* | Karger 2626 | GOET | New Guinea | SAMN33583410 |
| *Sticherus brackenridgei* | Lehnert 3615 | GOET | New Guinea | SAMN33583411 |
| *Sticherus brevitomentosus* | Solomon 17604 | MBM | Bolivia | SAMN33583412 |
| *Sticherus decurrens* | Lima 207 | BHCB | Brazil | SAMN33583413 |
| *Sticherus farinosus* | Kluge 9255 | GOET | Guadalupe | SAMN33583414 |
| *Sticherus ferrugineus* | Kessler 14699 | GOET | Colombia | SAMN33583415 |
| *Sticherus flabellatus* var. *compactus* | Kessler 14280 | GOET | Australia | SAMN33583416 |
| *Sticherus flagellaris* | Kessler 13821 | GOET | La Réunion | SAMN33583417 |
| *Sticherus fulvus* | Kluge 9259 | GOET | Guadalupe | SAMN33583418 |
| *Sticherus furcatus* | Testo 1518 | VT | Mexico | SAMN33583419 |
| *Sticherus gracilis* | Lima 212 | BHCB | Brazil | SAMN33583420 |
| *Sticherus habbemensis* | Kessler 14121 | GOET | New Guinea | SAMN33583421 |
| *Sticherus hirtus* | Kluge s.n. | GOET | Indonesia | SAMN33583422 |
| *Sticherus hypoleucus* | Kessler 14800 | GOET | Colombia | SAMN33583423 |
| *Sticherus jacha* | Jimenez 1615 | GOET | Bolivia | SAMN33583424 |
| *Sticherus lanosus* | Bach 1769 | GOET | Bolivia | SAMN33583425 |
| *Sticherus lanuginosus* | Lima 208 | BHCB | Brazil | SAMN33583426 |
| *Sticherus lechleri* | Jimenez 16362 | GOET | Bolivia | SAMN33583427 |
| *Sticherus loheri* | Karger 1001 | GOET | Indonesia | SAMN33583428 |
| *Sticherus maritimus* | Kessler 14839 | GOET | Colombia | SAMN33583429 |
| *Sticherus melanoblastus* | Kessler 14743 | GOET | Colombia | SAMN33583430 |
| *Sticherus milnei* | Kluge7003 | GOET | Indonesia | SAMN33583431 |
| *Sticherus montaguei* | Lehnert 3625 | GOET | New Guinea | SAMN33583432 |
| *Sticherus nervatus* | Kessler 14733 | GOET | Bolivia | SAMN33583433 |
| *Sticherus nigropaleaceus* | Lima236 | BHCB | Brazil | SAMN33583434 |
| *Sticherus nudus* | Lima 239 | BHCB | Colombia | SAMN33583435 |
| *Sticherus pallescens* | Lima 238 | BHCB | Colombia | SAMN33583436 |
| *Sticherus paulistanus* | Salino 8431 | BHCB | Brazil | SAMN33583437 |
| *Sticherus pruinosus* | Lima 325 | BHCB | Brazil | SAMN33583438 |
| *Sticherus remotus* | Jimenez 2709 | GOET | Bolivia | SAMN33583439 |
| *Sticherus revolutus* | Lima 240 | BHCB | Colombia | SAMN33583440 |
| *Sticherus rubiginosus* | Lima 237 | BHCB | Colombia | SAMN33583441 |
| *Sticherus salinoi* | Fernandes 771 | BHCB | Brazil | SAMN33583442 |
| *Sticherus simplex* | Asplund s.n. | R | Peru | SAMN33583443 |
| *Sticherus squamosus* | Lima 233 | BHCB | Brazil | SAMN33583444 |
| *Sticherus tomentosus* | Kessler 142728 | GOET | Colombia | SAMN33583445 |
| *Sticherus truncatus* | Karger 287 | GOET | Malaysia | SAMN33583446 |
| *Sticherus truncatus* | Jimenez1388 | FU | Indonesia | SAMN33583447 |
| *Sticherus vestitus* | Jimenez1151 | FU | Indonesia | SAMN33583448 |
| *Stromatopteris moniliformis* | Munzinger 1317 | P | New Caledonia | SAMN33583449 |
| *Hymenophyllum pulchellum* | Testo 909 | VT | Mexico | SAMN33583450 |
| *Trichomanes ankersii* | Testo 1242 | VT | Mexico | SAMN33583451 |
| *Dipteris conjugata* | Knapp 1438 | P | Taiwan | SAMN33583452 |
| *Danaea wendlandii* | Testo 784 | VT | Costa Rica | SAMN33583453 |
| *Danaea* sp. 1 | Testo 994 | VT | Panama | SAMN33583454 |
| *Danaea* sp. 2 | Testo 1440 | VT | Colombia | SAMN33583455 |

the HybPiper supercontig output (exons + introns), Hybphaser (Nauheimer et al., 2020, version 2.0) was used to access the quality of the sequences, exclude putative paralogs, access heterozygosity and haplotypic divergence, and to phase haplotypes, with the objective of reducing putative hybrid noise in the recovered topology. We followed Nauheimer et al. (2020) and Bloesch et al. (2022) and SNPs (single nucleotide polymorphism) were mapped and were only called when coverage was at least 10x, the allele frequency was at least 0.15, and the alternative allele occurred in at least 4 reads. All loci were removed that had an outlier value for the proportion of SNPs compared to other loci (datapoints of values that were above 1.5x the interquartile range above the third quartile were considered outliers).

### 2.4. Phylogenetic inferences

We used two phylogenetic approaches to recover evolutionary relationships within Gleicheniaceae. Maximum likelihood was employed to build two species trees with a partitioned matrix, one using plastid data (PST) and the other using nuclear data (NST). We also generated a coalescence-based species tree using the nuclear dataset (MSCT).

ModelTest-NG (Darriba et al., 2020) was used with both the nuclear and chloroplast datasets to select the best-fit model of evolution for each partition (recovered loci) based on Bayesian information criterion (BIC) (Supplementary Material 1). IQ-TREE2 was used to estimate the species tree from a concatenated partitioned matrix, defining each locus as a partition, with 1,000 ultrafast bootstrap replicates (Minh et al. 2020). IQ-TREE2 was also used to generate gene trees for nuclear loci and to estimate gene concordance factors (gCF) and site concordance factors (sCF). ASTRAL III (Zhang et al., 2018) was used with previously generated gene trees to infer the species tree based on a multi-species coalescent method. ASTRAL trees were scored to obtain an estimate of incomplete lineage sorting (ILS). The trees were rooted using *Dipteris conjugata* as an outgroup. We ran all analyses in the Sagarana HPC cluster (housed at Universidade Federal de Minas Gerais).

### 2.5. Divergence time analysis

TreePL (Smith and O'Meara, 2012) was used to estimate the divergence times of the main extant lineages of Gleicheniaceae. The TreePL run was optimized and conducted following the guide described by Maurin (2020). A smoothing value of $10^{-6}$ was chosen after comparing tree runs using $10^{-3}$, $10^{-6}$, and $10^{-9}$. Two fossil calibrations were used: *Gleichenia chaloneri* Herendeen & J. Skog, the most ancient known *Gleichenia* fossil (Herendeen and Skog, 1998), setting the crown age of *Gleichenia* within 99–112 million years ago (mya); and *Chansitheca wudaensis* Deng, Sun and Li, the most ancient Gleicheniaceae-like fossil (He et al., 2020; He et al. 2016), constraining the crown age of Gleicheniales to within approximately 297–298 mya. Since there is no conclusive evidence (such as a pseudo-dichotomic frond structure) that unambiguously aligns these Gleicheniaceae-like fossils with Gleicheniaceae, we chose to calibrate Gleicheniales crowns conservatively. Further discussion regarding the Gleicheniaceae fossils is available in Supplementary Material 2.

## 3. Results

The nuclear dataset recovered an average of 419 loci out of 451 (standard deviation = 40, median 434) (Supplementary Material 3), with 93% average loci coverage. We removed putative paralogs, samples with coverage below 50% in each locus, and loci with less than 50% of the sequences (Supplementary Material 3). Our final partitioned matrix had 294 loci and a length of approximately 150 thousand bp. Following the thresholds suggested by Nauheimer et al. (2020), we found high rates of heterozygosity (LH) and haplotype divergence (HD), with 18 ingroups showing loci heterozygosity above 80% with > 0% SNPs, and allele divergence above 1% [including 10 *Sticherus* (55%), seven *Dicranopteris* (38%), and one *Gleichenia* (5%)] (Table 3). The highest rates of haplotype divergence and heterozygosity were found in *Gleichenia peltophora* (87.88% with > 0% SNPs and 3.18% of allele divergence), and the lowest in *Gleichenella pectinata* (12.07% and 0.03%) (Table 3) (Fig. 1). Additionally, the results of the ASTRAL III-scored tree pointed out that 84% of quartet trees induced by the gene trees are in the species tree of our nuclear dataset.

The recovered topology of NST showed two main clades (Fig. 2), which were recovered in both PST and MSCT. The first clade (here named the Diplopteroid clade) is formed by *Diplopterygium*, as sister to a clade formed by *Gleichenella* + *Dicranopteris* (bootstrap = 100). Two clades within *Dicranopteris* were recovered, one with *Dicranopteris tetraphylla* + *Di. linearis* + *Di. taiwanensis* + *Di. dichotoma* (bs = 100) and another formed by *Di. speciosa* + *Di. subpectinata* plus the neotropical species (*Di. nervosa*, *Di. rufinervis*, *Di. seminuda*, *Di. spissa,* and *Di. flexuosa*) (Fig. 2) (bs = 100). This topology was recovered in all phylogenetic inferences, although there was some discordance regarding the relationships within the neotropical species of *Dicranopteris*.

Regarding *Diplopterygium*, *Dp. bancrofitii* (the only neotropical species of the genus) was sister to the remaining species in all topologies (bs = 100). In the NST, a clade formed by *Dp. norrisii* + *Diplopterygium* sp. 2 + *Dp. brevipinnulum* + *Dp. sordidum* was sister to a clade formed by *Dp. chinensis* + *Dp. volubilis* + *Diplopterygium* sp. 1 + *Dp. longissimum* + *Dp. conversum* + *Dp. glaucum* (Fig. 1) (bs = 100). This topology showed high concordance with the MSCT, except for *Dp. sordidum*, which came out as sister to *Dp. brevipinnulum* (Fig. 2, Supplementary Material 4). However, there was discordance between the recovered topologies of the nuclear dataset (NST and MSCT) and the plastid dataset regarding the affinities among Asian species of *Diplopterygium*. In the PST, *D. norrisii* emerged as a sister group of two clades, one formed by *Dp. volubilis* + *Diplopterygium* sp. 1 and the other formed by *Dp. sordidum* + *Dp. brevipinnulum* as a sister group of *Dp. chinensis* + *Diplopterygium* sp. 1 + *Dp. conversum* + *Dp. glaucum* + *Dp. longissimum* (Fig. 2, Supplementary Material 4).

In the second clade, named here as the Sticheroid clade, *Rouxopteris* was recovered as sister to all other lineages in all recovered topologies (Fig. 2) (bs = 100). However, with NST, *Rouxopteris* appeared as sister of a clade formed by *Sticherus milnei* + *S. truncatus*, which was, in turn, sister of *Gleichenia* + *Stromatopteris* plus the remaining *Sticherus* species (Fig. 2) (bs = 100). In the MSCT, *Rouxopteris* emerged as sister to *Stromatopteris* + a clade formed by *Sticherus milnei* + *S. truncatus*, which in turn is sister to *Gleichenia* + plus the remaining species of *Sticherus* (Supplementary Material 4). In the PST, *Rouxopteris* came out as sister to a clade formed by *Gleichenia* + *Stromatopteris*, which was, in turn, sister to a clade formed by *Sticherus milnei* + *S. truncatus* and the remaining species of *Sticherus* (Supplementary Material 4) (bs = 100). The phylogenetic placement of *Stromatopteris* in the MSCT was incongruent between the PST and NST topologies (Fig. 2, Supplementary Material 4). Within *Gleichenia*, *G. polypodioides* was recovered as sister to *G. dicarpa* + *G. peltophora* in the PST (Supplementary Material 4). Only plastid sequences were recovered for *G. dicarpa* (Fig. 2, Supplementary Material 4) (bs = 100).

The relationships within the clades formed by the remaining *Sticherus* species were not well resolved and incongruent among the recovered topologies, except for early diverging lineages and a few individual clades. In the NST, *Sticherus brackenrigdei* was recovered as sister to the other species, followed by *S. montaguei* as sister to two clades. One clade was formed by five species with distributions throughout Southern and Southeastern Asia and Oceania (*Sticherus flabellatus*, *S. bolanicus*, *S. loheri*, *S. hirtus*, and *S. vestitus*), including the neotropical *Sticherus nudus* as sister to *S. bolanicus* (all node with bs = 100). The other clade was formed by *Sticherus simplex* as sister to the remaining species (bs = 100), followed by *Sticherus flagellaris*, an African species, as sister to a clade formed by *Sticherus nervatus*, *S. pruinosus*, *S. lechleri*, and *S. revolutus* (bs = 99), which was, in turn, sister to the remaining species. Although the relationships among them are not well resolved, a few clades are well supported [e.g., the one formed by *S. squamosus*,

**Table 3**
Allele divergence and locus heterozygosity (showing the proportion of loci containing > 0% SNPs) rates for each sample.

| Sample | Allele Divergence | Locus Heterozygosity |
|---|---|---|
| *G. peltophora* | 3,18 | 87,88 |
| *S. montaguei* | 2,47 | 87,86 |
| *D. flexuosa* | 2,29 | 89,11 |
| *D. klotzschii* | 2,07 | 86,67 |
| *D. nervosa* | 2,05 | 90,62 |
| *D. spissa* | 1,93 | 86,41 |
| *D. seminuda* | 1,92 | 86,39 |
| *D. rufinervis* | 1,84 | 80,30 |
| *D. linearis* | 1,81 | 89,41 |
| *D. wendlandii* | 1,80 | 78,87 |
| *S. truncatus* | 1,61 | 66,42 |
| *S. lechleri* | 1,49 | 91,46 |
| *S. nudus* | 1,42 | 86,06 |
| *S. nervatus* | 1,40 | 91,50 |
| *S. revolutus* | 1,33 | 89,46 |
| *S. pruinosus* | 1,30 | 85,37 |
| *S. nigropaleaceus* | 1,15 | 86,70 |
| *S. bolanicus* | 1,10 | 81,95 |
| *S. salinoi* | 1,10 | 87,86 |
| *H. pulchellum* | 1,06 | 78,32 |
| *S. vestitus* | 0,94 | 78,01 |
| *D. norrisii* | 0,92 | 68,69 |
| *Danaea sp1* | 0,84 | 75,63 |
| *S. simplex* | 0,74 | 71,22 |
| *S. remotus* | 0,65 | 69,17 |
| *S. milnei* | 0,62 | 62,22 |
| *T. ankersii* | 0,57 | 26,85 |
| *Diplopterygium sp2* | 0,57 | 55,23 |
| *D. taiwanensis* | 0,56 | 28,92 |
| *S. squamosus* | 0,52 | 77,91 |
| *D. dichotoma* | 0,49 | 49,15 |
| *D. sordidum* | 0,47 | 62,77 |
| *S. aurantiacus* | 0,45 | 38,24 |
| *D. glaucum* | 0,44 | 53,66 |
| *S. ferruginosus* | 0,43 | 72,82 |
| *Danaea sp2* | 0,42 | 47,80 |
| *R. boryi* var. *madagascariensis* | 0,42 | 56,35 |
| *G. polypodioides* | 0,41 | 33,25 |
| *S. hirtus* | 0,41 | 45,63 |
| *D. subpectinata* | 0,40 | 53,83 |
| *S. moniliformis* | 0,39 | 29,97 |
| *D. longissimum* | 0,37 | 57,66 |
| *S. truncatus* | 0,35 | 29,22 |
| *D. tetraphylla* | 0,34 | 57,52 |
| *D. conjugata* | 0,32 | 50,01 |
| *S. loheri* | 0,31 | 45,48 |
| *R. boryi* | 0,31 | 19,50 |
| *S. flagellaris* | 0,29 | 47,80 |
| *D. conversum* | 0,28 | 38,54 |
| *D. chinensis* | 0,27 | 47,19 |
| *Diplopterygium sp1* | 0,26 | 40,88 |
| *S. jacha* | 0,26 | 44,99 |
| *D. brevipinnulum* | 0,25 | 47,93 |
| *G. dicarpa* | 0,25 | 25,13 |
| *S. blepharolepis* | 0,24 | 50,49 |
| *S. tomentosus* | 0,24 | 50,01 |
| *S. hypoleucus* | 0,23 | 50,98 |
| *S. rubiginosus* | 0,23 | 49,76 |
| *D. bancroftii* | 0,21 | 31,63 |
| *S. brackenridgei* | 0,21 | 45,63 |
| *S. pallescens* | 0,21 | 43,03 |
| *S. paulistanus* | 0,20 | 37,06 |
| *D. speciosa* | 0,19 | 43,50 |
| *S. bifidus* | 0,19 | 19,12 |
| *S. melanoblastus* | 0,19 | 45,12 |
| *S. lanosus* | 0,18 | 44,17 |
| *S. habbemensis* | 0,18 | 33,42 |
| *S. furcatus* | 0,16 | 37,84 |
| *D. volubilis* | 0,15 | 29,03 |
| *S. lanuginosus* | 0,15 | 31,28 |
| *S. flabellatus* | 0,11 | 21,27 |
| *S. brevitomentosus* | 0,11 | 20,72 |
| *S. farinosus* | 0,09 | 22,25 |
| *S. decurrens* | 0,08 | 21,62 |
| *S. fulvus* | 0,08 | 28,12 |
| *S. gracilis* | 0,07 | 23,02 |
| *S. maritimus* | 0,06 | 13,14 |
| *G. pectinata* | 0,03 | 12,07 |

*S. lanuginosus*, and *S. paulistanus* (bs = 100), and another formed by *S. maritimus*, *S. blepharolepis*, *S. ferruginosus*, *S. decurrens*, *S. bifidus*, and *S. fulvus*] (Fig. 2) (bs = 100). The clade formed by S. *nudus*, *S. bolanicus*, *S. flabelatus*, *S. loheri*, and *S. hirtus* was also recovered in the MSCT, although *S. nervatus*, *S. pruinosus*, *S. revolutus*, and *S. lechleri* did not form a clade (Supplementary Material 4).

The time-calibrated tree recovered an estimated origin of the Gleicheniaceae crown aprox. 121–125 mya (CI = [121.77–125.03], median = 123.4), of the Diplopteroid lineage crown at 105–115 mya (CI = [105.25–115.48], median = 110.36), and of the Sticheroid clade crown at 119–122 mya (CI = [119.32–122.63], median = 120.97) (Fig. 3). The split between *Gleichenella* and *Dicranopteris* was estimated to have occurred 49–62 mya (CI = [49.87–62.61], median = 56.24). Within the *Diplopterygium* lineage, the split of neotropical *D. bancroftii* from the other *Diplopterygium* species was estimated to have occurred 47–68 mya (CI = [47.29–68.03], median = 57.66). Within the Sticheroid clade, *Rouxopteris* diverged from the other lineages about 119–122 mya (CI = [119.32–122.63], median = 120.97), while *Sticherus s.s.* diverged from *Stromatopteris* + *Gleichenia* and the remaining *Sticherus* species about 114–117 mya (CI = [114.39–117], median = 115.85). The separation between *Stromatopteris* and *Gleichenia* occurred 107–109 mya (CI = [107.38–109.46], median = 108.42), while the remaining *Sticherus* diverged from *Stromatopteris* + *Gleichenia* clade about 111–113 mya (CI = [111.08–113.58], median = 112.33). (Fig. 3). Although the clade formed by the remaining species of *Sticherus* represents an old lineage, its species showed recent divergences approximately 3–0.5 mya (Fig. 3). In contrast, *Diplopterygium bancroftii* has an estimated separation from the other *Diplopterygium* species of about 47 to 68 mya. Finally, it is estimated that the diversification of *Dicranopteris* species took place between 14 and 3 mya (Fig. 3).

## 4. Discussion

### 4.1. Topology

Here we present the first family-level phylogenomic inference of Gleicheniaceae based on both nuclear and plastid data. Our analyses recovered two main clades, the Diplopteroid and Sticheroid clades, and clarified the phylogenetic relationships among all Gleicheniaceae genera. Our results support the current circumscription of the genera with the exception of *Sticherus*, which was recovered as paraphyletic, and highlight the phylogenetic and morphological uniqueness of the clade formed by *Sticherus milnei* + *S. truncatus*. However, the phylogenetic placement of the *S. milnei* + S. *truncatus* clade differs among the topologies recovered from the nuclear (NST and MSCT) and plastid datasets (PST). Therefore, the morphological characters of *Sticherus* will need to be reinterpreted and its circumscription revised. Importantly, *Sticherus laevigatus* (=*Sticherus truncatus*), the type species of *Sticherus*, appears as a clade independent of the majority of remaining *Sticherus* species. All recovered topologies in the Diplopteroid clade are congruent with respect to generic relationships and circumscriptions (Fig. 2, Fig. 4., Supplementary Material 4).

Our recovered topologies agree with Li et al. (2010) in terms of *Dicranopteris* + *Gleichenella* being sister to *Diplopterygium* and *Stromatopteris* + *Gleichenia* being sister to the remaining species of *Sticherus*, although these authors did not include *Rouxopteris boryi*. Liu et al. (2020) proposed the segregation of *Rouxopteris boryi* from *Gleichenia* due

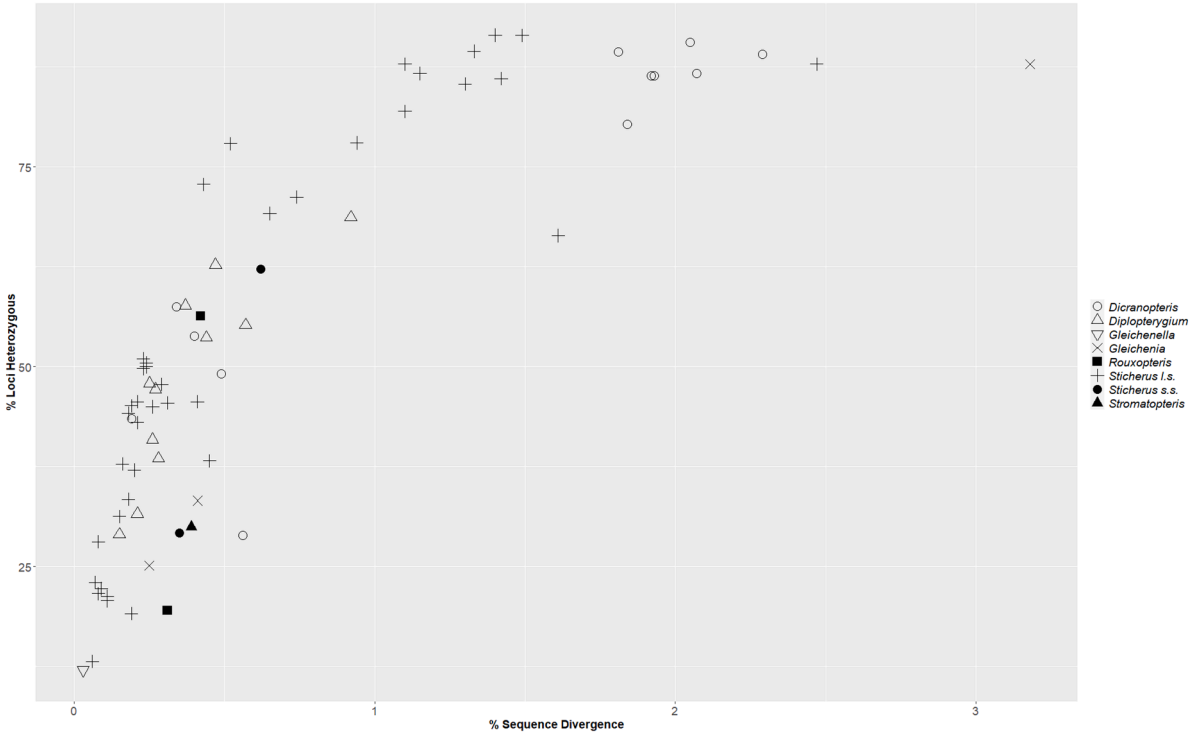


**Fig. 1.** Relationship between locus heterozygosity and allele divergence. Symbols correspond to representative of the following genera: white hexagon = *Dicranopteris*; white triangle = *Diplopterygium*; inverted white triangle = *Gleichenella*; multiplication sign = *Gleichenia*; black squares = *Rouxopteris*; cross = remaining species of *Sticherus*; black hexagon = *Sticherus* s.s.; black triangle = *Stromatopteris*.

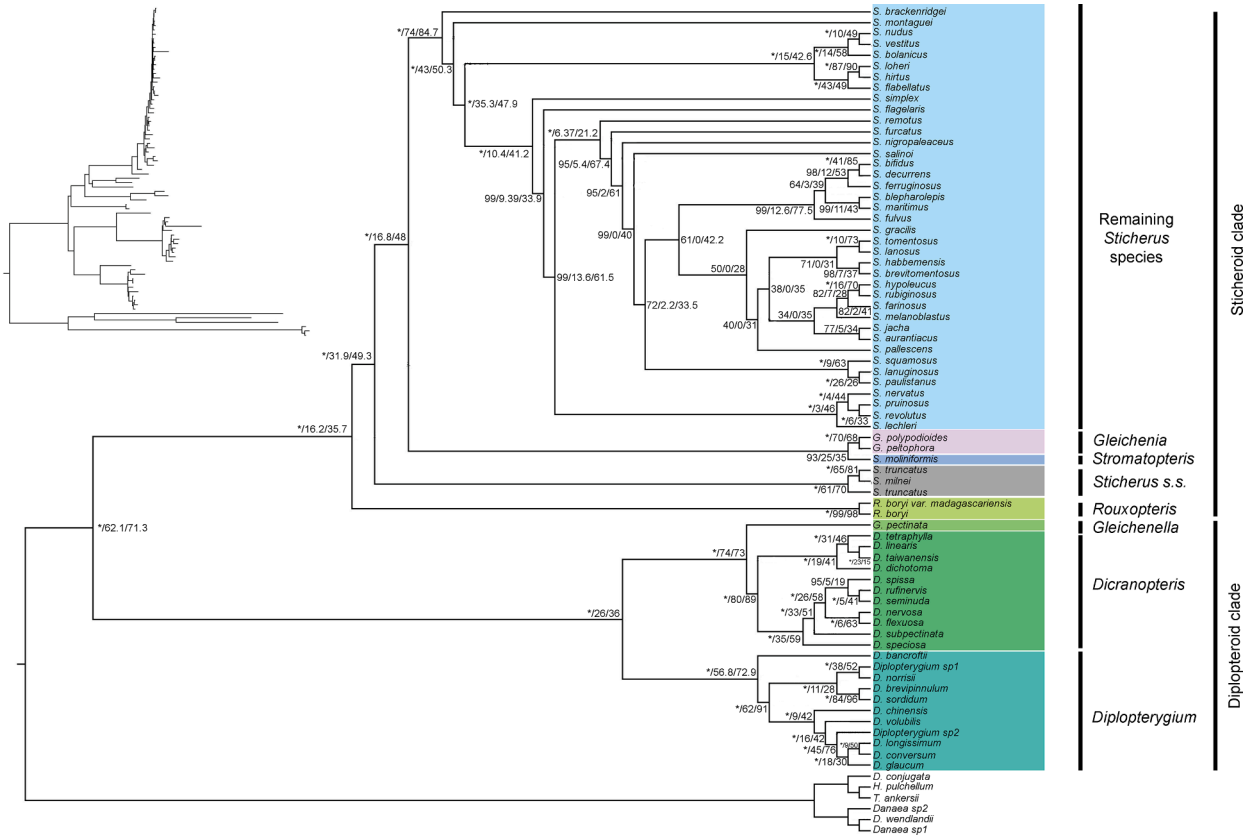


**Fig. 2.** Maximum Likelihood species tree generated from a partitioned nuclear matrix, with ultrafast bootstrap branch supports, gene concordance factor (gCF), and site concordance factor (sCF), respectively. * Indicates Ultrafast Bootstrap = 100.

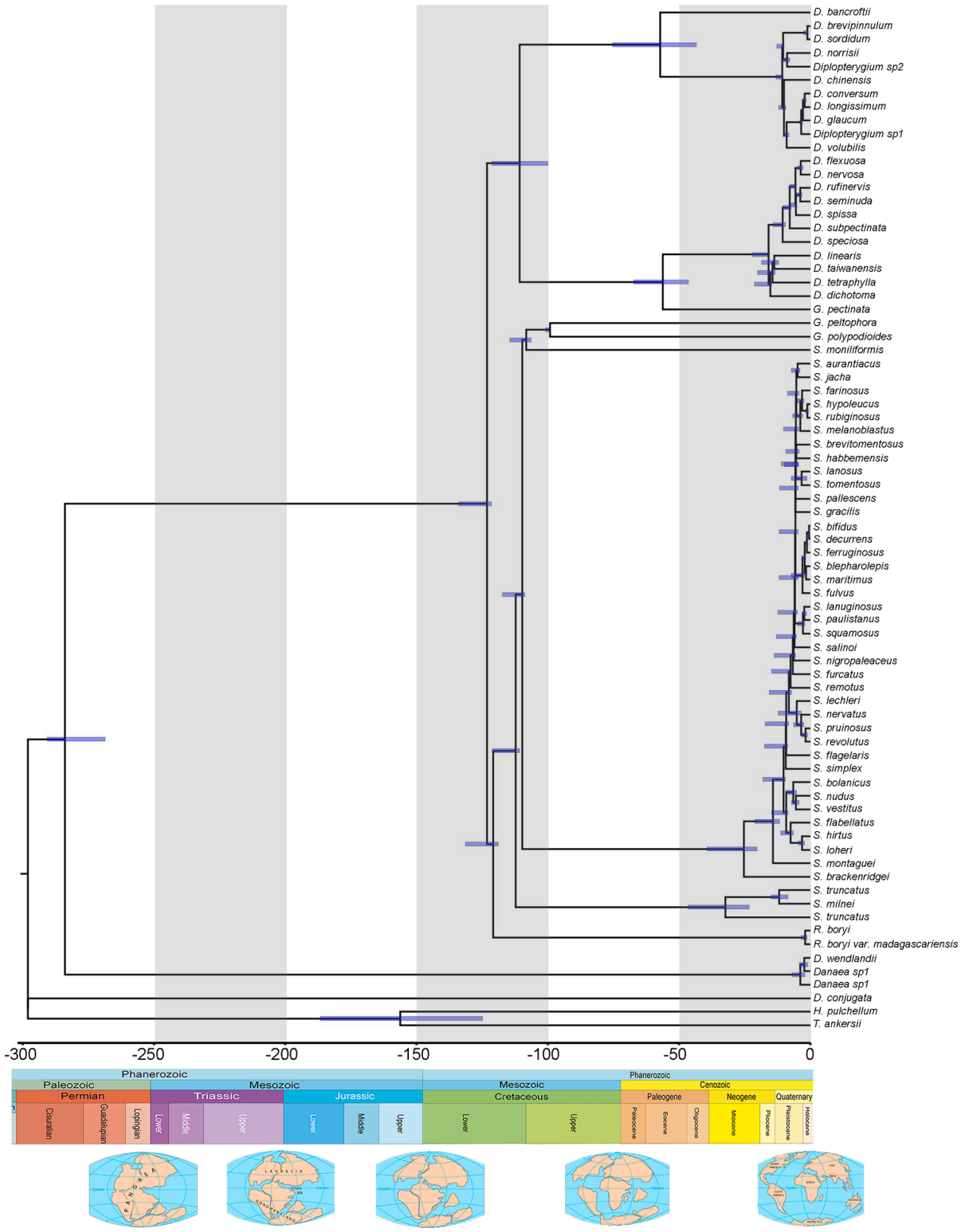


**Fig. 3.** Divergence time estimates for Gleicheniaceae based on the nuclear dataset. Blue lines at the nodes indicate 95% highest probability density intervals. Dates are given in million years (mya). Chronostratigrafic bar follows the International Chronostratigraphic Chart v2022/02 (www.stratigraphy.org). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to its morphological uniqueness and its placement in a phylogenetic hypothesis based on *rbcl* sequences. In their topology, *Rouxopteris* comes out with low support as sister to the Diplopteroid clade. In contrast, in our results, *Rouxopteris* is recovered with high support in all topologies as sister to the Sticheroid clade (Fig. 4.).

The taxonomy of Gleicheniaceae has traditionally been based on the rhizome and bud indument types (Holttum, 1957; Tryon and Stolze, 1989; Østergaard Andersen and Øllgaard 2001; Gonzales and Kessler, 2011). However, the first phylogenetic inferences focusing on the family demonstrated that these characters do not reflect the evolutionary

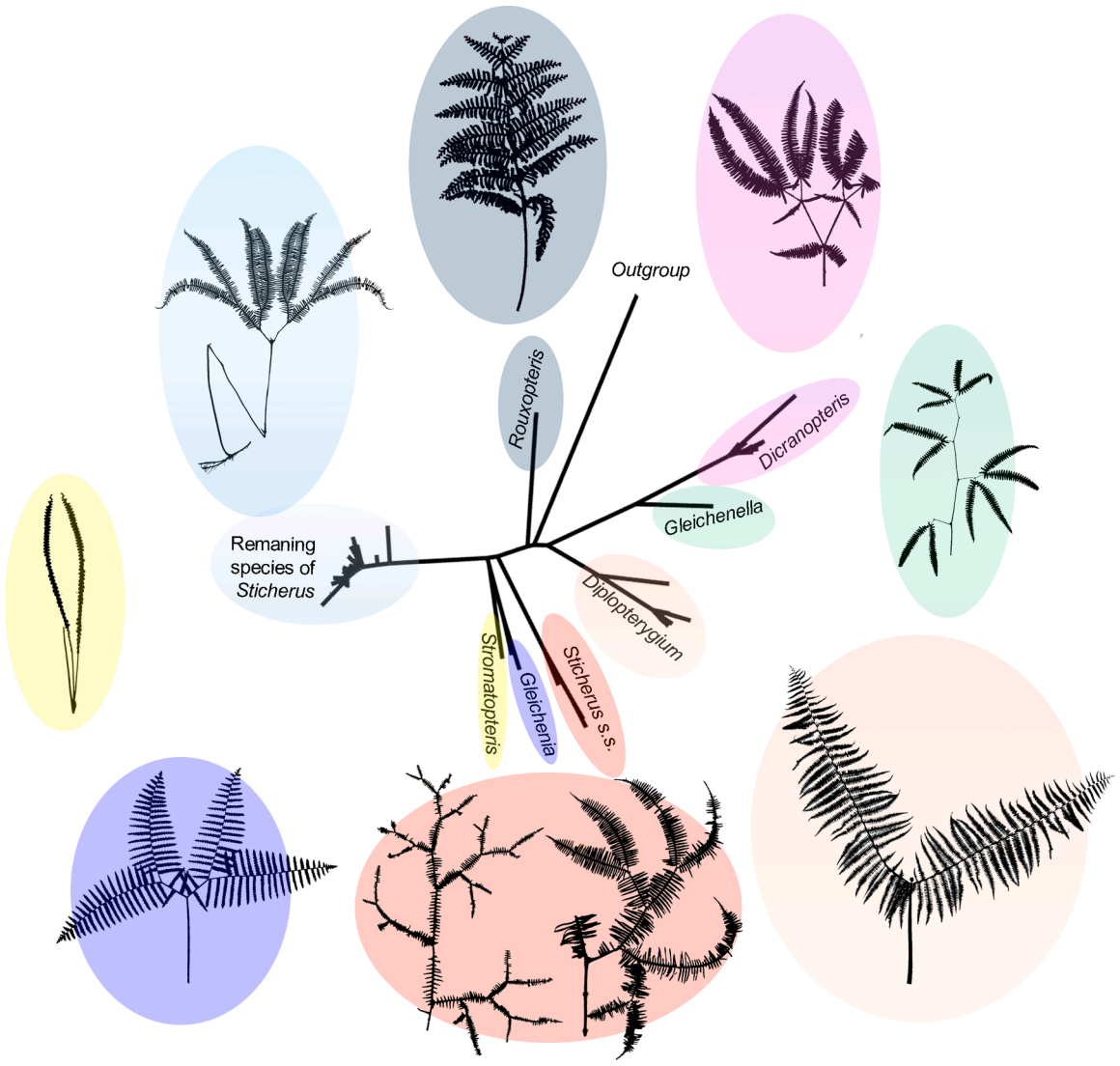


**Fig. 4.** Unrooted collapsed tree based on nuclear ML tree depicting generic relationships and illustrations of ramifications patterns observed in each genus of Gleicheniaceae.

affinities of the genera. For example, *Diplopterygium*, with scaly rhizomes and buds, is sister to *Dicranopteris* + *Gleichenella*, both with hairy rhizomes and buds (Liu et al., 2020; Li et al., 2010). The hairs found on some Gleicheniaceae, such as *Dicranopteris* and *Gleichenella*, can be interpreted evolutionarily, based on their morphology and the fossil record, as originating from reduced scales (Liu et al., 2020). Alternatively, they may have emerged independently several times in the family. Other Gleicheniaceae species, such as *Rouxopteris boryi* and *Gleichenia microphylla*, have scales (with ciliated margins) and hairs, as well as stiff stellate hairs on their rhizomes (Holttum, 1959; Liu et al., 2020). Several Asian species of *Diplopterygium* also have rhizomes and buds covered by scales, and bear stellate hairs on the abaxial surface of the rachis (Jin et al., 2013). Similarly, some species of *Sticherus* (e.g., *S. pruinosus*) have hairs on segments of the abaxial surfaces of secondary veins (Gonzales and Kessler, 2011; Lima and Salino, 2018). Gonzales (2003), however, did not recognize those structures as hairs but rather as reduced scales. Clearly, the evolution of scales and hairs, and their taxonomic significance, need to be explored in more detail in light of the new phylogenetic evidence.

### 4.2. Hybridization and polyploidization

In contrast to the well-resolved relationships between genera, in many cases in our study the relationships within genera are not well-resolved, especially in *Sticherus*. The lack of resolution and the recovered incongruences among the NST, PST, and MSC trees may be related, at different levels, to incomplete taxonomic sampling, incomplete lineage sorting, and introgression following hybridization (Rieseberg and Soltis, 1991; Dorado et al., 1992; Degnan and Salter, 2005; Drábková and Vlcek, 2010; Xu et al., 2012; Sigel, 2016; Bruun-Lund et al., 2017). Incomplete lineage sorting may also play a role in the incongruence between gene trees and species trees, especially in recently diverged species (Knowles and Carstens, 2007), such as those in *Sticherus* (most of them with divergence estimated to have occurred ca. 3–4 mya). The ASTRAL results (with about 84% of the quartet trees induced by the gene trees found in the species tree) indicate a significant amount of ILS in our dataset. The high rates of heterozygosity (LH) and haplotypic divergences (HD) found in many sampled species (Table 2) may be related to hybridization, introgression, and polyploidy events (Nauheimer et al., 2020). High LH and HD values may also be related to fixed heterozygosity in species of allopolyploid origin. This situation occurs when allopolyploid species retain sets of divergent gene copies inherited from

each parental species and may present phenotypes reflecting the additivity or synergy of the parental genomes in their respective ratios (Buggs et al., 2014; Soltis and Soltis, 2000; Sigel, 2016). Further, Ohlsen et al. (2022) recovered a topology of *Gleichenia dicarpa* as a polyphyletic taxon using chloroplast *rbcL* and *trnL-trnF* sequences, which may also be related to reticulation and hybrid formation. Thus, all these data suggest that hybridization and polyploidization may have been important evolutionary drivers in Gleicheniaceae.

It has long been proposed that some *Sticherus* species may have originated through hybridization events, based on evidence from both morphology (Gonzales and Kessler, 2011) and cytogenetic data (Jermy and Walker, 1985). Unfortunately, little progress has been made in testing these hypotheses of reticulate evolution in *Sticherus*. Although our analyses do not include described hybrids, our results support a hybrid origin for some species in this lineage. For example, *Sticherus nigropaleaceus*, one of the species that shows morphological intermediacy between two species groups in *Sticherus* (Prado and Lellinger, 1996; Gonzales and Kessler, 2011; Lima and Salino, 2018) showed high rates of LH and HD. Although no chromosome count has been performed so far for *S. nigropaleaceus*, its DNA c-value has been estimated at about 2–3 times that of other *Sticherus* species that have been investigated (Lima et al., 2021), supporting an allopolyploid origin. Similarly, all species in the taxonomically complex group of species of *Sticherus revolutus* (*S. nervatus*, *S. revolutus*, and *S. pruinosus*) showed high rates of LH and HD (Table 2). In the taxonomically equally complex genus *Dicranopteris*, most of the sampled species also showed high rates of LH and HD (above 80% and 1.0 % respectively) (Table 2), and these rates may be related to a series of autopolyploidy or allopolyploidy events (Lima et al., 2021). Traditionally, a broad taxonomic concept has been adopted for *Di. linearis* due to the lack of morphological and molecular studies (Chinnock and Bell, 1998; Perrie and Brownsey, 2015; Chen et al., 2017). However, the reported chromosome numbers of *Di. linearis* (n = 39, n = 40, 78, n = 80) (Lima et al., 2021) vary as much as their morphology and geographical distribution – and could be used to better understand the reticulation within the complex as well as to clarify species limits. The complex was recently the focus of molecular and morphological studies, and a few taxa were segregated from it (Wei et al., 2021), although further work using integrative tools is still needed. Furthermore, there has been discussion about the morphological distinctiveness of *Di. linearis* (with Asian and African distribution) and *Di. flexuosa* (with neotropical distribution) (Lima and Salino, 2018). All the investigated samples of *Di. flexuosa* have so far shown a base haploid number of 78, and 2C values of 9.16 pg (Lima et al., 2021), and therefore may represent polyploid populations. On the other hand, most *Di. linearis* chromosome counts have shown n = 39 as the haploid chromosome number, with 2C = 6.41 pg (despite some cases of polyploidy) (Clark et al., 2016). In the present study, these species had LH values above 89%, SNPs > 0%, and AD above 1.81%, which also suggests that their origins may be related to polyploidy events. In the genus *Gleichenia*, *G. peltophora* was the only species that showed high rates of LH and HD, and all samples of *Diplopterygium* showed low rates of LH and HD (Table 3). Therefore, it would appear that polyploidy and reticulation might not have played such significant roles in the diversification of these genera as proposed in *Sticherus* and *Dicranopteris* (Table 2) (Jermy and Walker, 1985; Lima et al., 2021).

In contrast, the monospecific genus *Gleichenella*, widespread in the Neotropics, showed the lowest rates of allele divergences and heterozygosity among all species sampled (Table 2). This might be related to its growth habit, as it usually occupies large areas along roadsides and on steep slopes. The extensive clonal expansion of its rhizome allows for great expansion, so that *Gleichenella* patches may be formed by a single individual. The reproductive isolation of *Gleichenella* may be related to effective pre- or postzygotic barriers, preventing hybrid formation (Roe et al., 2014), which, in turn, could be related to its early divergence time (estimated at around 49–62 mya). Additionally, chromosome counts point to the conservation of ploidy in the genus, with only putative dysploidy events being recorded (Lima et al., 2021). Similarly, *Rouxopteris* and *Stromatopteris*, two other monospecific genera with restricted distributions, showed low rates of LH and HD and may represent ancient relict lineages within the family with well-developed reproductive barriers to prevent hybrid formation.

It is well established that whole-genome duplication (WGD) events have played major roles in fern diversification (1KP, 2019; Huang et al., 2020), as have WGD and reticulation with high LH and HD rates (Sigel, 2016). Gleicheniaceae seem to have experienced a WGD before its radiation during the Mesozoic (Huang et al., 2020). Our results also support the hypothesis that polyploidy and reticulation played a major role in the diversification of *Sticherus* and *Dicranopteris* (Table 3). A gradient of LH and AD across the sampled species was also observed (Table 2), which may indicate the presence of unknown hybrids and putative introgression events (Nauheimer et al., 2020). Although no intergeneric hybrids have yet been recorded for the family, there is evidence that several putative hybrids and allopolyploids within the genera will require further investigation (Table 3).

### 4.3. Divergence time analysis

Gleicheniaceae is an ancient lineage of leptosporangiate ferns with an extensive fossil record spanning the Mesozoic (Gandolfo et al., 1997), although a recently described fossil from the Permian assignable to the family (*Chansitheca wudaensis*, 298 mya) indicates an even earlier origin (He et al. 2020). Our time divergence estimates corroborate previous estimates (Pryer et al., 2004; Schuettpelz and Pryer, 2007; Testo and Sundue, 2016; Liu et al., 2020), and support the hypothesis of the establishment and diversification of most Gleicheniaceae lineages during the Mesozoic (Schneider et al., 2004). It is hypothesized that the early divergence of the Gleicheniaceae crown occurred in connection with continental movements during the breakup of Laurasia and Gondwana during the late Mesozoic and early Cenozoic (Liu et al., 2020) (Fig. 3). Of the three geographically restricted genera in Gleicheniaceae, *Rouxopteris* (Madagascar and the Mascarenes - Liu et al., 2020) and *Stromatopteris* (New Caledonia - Kramer, 1990) are ancient lineages, and seem to be relicts from the early diversification of Gleicheniaceae lineages (Liu et al., 2020) (Fig. 3), whereas the neotropical *Gleichenella* (Ching, 1940; Mickel and Smith, 2004) diverged more recently from *Dicranopteris*.

Despite their early divergence and diversification during the Mesozoic, several early-diverged genera (such as *Dicranopteris*, *Diplopterygium* and *Sticherus*) underwent more recent diversification during the Miocene (Fig. 3). This pattern has also been observed in other lineages of seedless plants, such as *Phlegmariurus* (Testo et al., 2019) and *Isoëtes* (Pereira et al., 2017). This agrees with the angiosperm-driven diversification hypothesis, as presented for other fern lineages (Schneider et al., 2004; Du et al., 2021), in that much of the current species diversity is of Neogene or even Quaternary age. This diversification may have been associated with climate adaptation and niche diversification in montane regions and matches the pattern reported by Suissa et al. (2021) for general fern diversification.

## 5. Conclusions

Here we present the first genomic scale phylogenetic inferences concerning Gleicheniaceae, confirming the monophyly of *Dicranopteris*, *Diplopterygium*, *Gleichenella*, *Gleichenia*, *Rouxopteris*, and *Stromatopteris*, while recovering *Sticherus* as paraphyletic. We clarify the relationships of *Rouxopteris as belonging to the Sticheroid clade,* and corroborate the overall phylogenetic relationships recovered in previous works. Although Gleicheniaceae is an ancient lineage (with most of its extant genera diverging during the Mesozoic), several genera show more recent diversification, and our results suggest that reticulation and polyploidy have played significant roles during that process. However, some genera, such as *Rouxopteris* and *Stromatopteris*, appear to represent

evolutionary relicts. Future studies with expanded sampling should focus on a better understanding of species-level relationships. Additionally, integrative approaches should be applied to elucidate genera with reticulate evolutionary histories and to define species complexes.

## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

All authors made a substantial contribution to the concept and design of the study, to data collection, analysis and interpretation, writing and revising the manuscript, and adding intellectual content.

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## CRediT authorship contribution statement

**Lucas Vieira Lima:** Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Alexandre Salino:** Supervision, Funding acquisition, Writing – review & editing. **Michael Kessler:** Funding acquisition, Writing – review & editing. **Germinal Rouhan:** Writing – review & editing. **Weston L. Testo:** Data curation, Methodology, Writing – review & editing. **Caio Suzart Argolo:** Data curation, Methodology, Writing – review & editing. **GoFlag Consortium:** Funding acquisition, Methodology, Data curation. **Thaís Elias Almeida:** Supervision, Conceptualization, Methodology, Data curation, Formal analysis, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2023.107782.

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