

The Complete Chloroplast Genome of Tree Fern *Cyathea delgadii* and Its Comparison to other Cyatheales

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Abstract

The chloroplast genome of the tree fern *Cyathea delgadii* Pohl ex Sternb. was assembled and annotated to investigate its structure and evolution within the Cyatheaales order. The genome, assembled using Flye and Megamerger, has a total size of 165,248 bp, comprising a large single-copy (LSC) region of 94,738 bp, a small single-copy (SSC) region of 22,012 bp, and two inverted repeat (IR) regions of 24,251 bp each. It contains 89 protein-coding genes, eight rRNAs, and 33 tRNAs. Comparative phylogenomic analyses involving 19 species of Cyatheaales have revealed that the chloroplast genome of *C. delgadii* shares similarities in gene content with other ferns of the Cyatheaceae family; however, it demonstrates variations in both genome size and GC content. The overall guanine-cytosine (GC) content of *C. delgadii* was calculated to be 40.95%, with a significantly higher content of 44.03% observed in the intragenic regions. An analysis of codon usage indicated a preference for codons ending with adenine or thymine, which aligns with the genome's adenine-thymine (AT) richness. Variations in chloroplast genome size were observed across the Cyatheaales species, ranging from 154,046 bp in *Gymnosphaera denticulata* to 168,244 bp in *Dicksonia squarrosa*. Gene content analysis showed that most species have a conserved number of protein-coding genes, rRNAs, and tRNAs, suggesting structural stability. However, *Cibotium* has a reduced number of protein-coding genes (87), possibly due to gene loss or transfer to the nuclear genome. Phylogenetic analyses using both whole genome and SNP data showed comparable clustering among *Alsophila* and *Gymnosphaera* species, while *C. delgadii* occupied a basal to intermediate position. This study provides valuable genomic resources and insights into the evolution of Cyatheaales chloroplast genomes, emphasising both conserved features and specific adaptations within this group of ferns.

Introduction

The genus *Cyathea* represents one of the few remaining lineages of tree ferns on Earth. Alongside other genera such as *Dicksonia* and *Alsophila*, as well as the order Cyatheaales, it constitutes a unique and distinct flora. Ferns, in general, have undergone limited exploration within botanical research and have received less attention compared to angiosperms and gymnosperms (Sessa et al., 2015). This research gap is particularly apparent in studies related to agriculture and raw material extraction. Nonetheless, some evidence underscores the significance of the genus *Cyathea*, whether in terms of its shading properties that assist in maintaining humidity (Schneider and Schmitt, 2011), its role as a raw material to define specific properties, or in the extraction of compounds that possess hepatoprotective properties (Kiran et al., 2012). To date, there exists a paucity of genomic or transcriptomic databases accessible for ferns, with only a limited number of reference genomes available in public repositories (Goodstein et al., 2012; Wolf et al., 2015; IFERN, 2018; Li et al., 2018; Nitta et al., 2022). High rates of polyploidy are frequently regarded as a significant factor in the extensive hybridization and diversification of this group. Consequently, molecular methods are increasingly being integrated with classical taxonomy to differentiate and identify genera and species. (Whitham et al., 1991; Ekrt et al., 2010).

Chloroplasts are essential organelles in plant cells, playing a central role in sustaining life on Earth by converting solar energy into carbohydrates and releasing oxygen through photosynthesis (Sabater, 2018). Beyond their direct involvement in plant growth and development (Jansen et al., 2007; Cho et al., 2019; Chen et al., 2023), the chloroplast genome (cpDNA) is extensively documented in the literature as being predominantly maternally inherited (Whatley, 1982; Harris et al., 2009; Davis et al., 2010). In addition to its role in genetic transmission, this genome features a highly conserved structure among land plants, with a core set of introns established before the divergence of bryophytes and tracheophytes (Liu et al., 2020).

Comparative studies of cpDNA between charophytes and land plants indicate that numerous structural features of tracheophyte plastids are directly inherited from the ancestral algal population (Turmel et al., 2007). Furthermore, the linear arrangement of genes within plastid genomes has exhibited remarkable conservation among ferns, gymnosperms, and angiosperms across approximately 380 million years of independent evolution, suggesting that strong selective pressures have been at play to maintain this structure (Palmer and Stein, 1986). While the structure of the chloroplast genome is highly stable, the location of junctions between regions may vary due to the expansion and contraction of inverted repeat (IR) regions, reflecting significant evolutionary processes. In the case of ferns, both the organization and content of plastids remain exceptionally stable, with minimal loss or rearrangement throughout their evolutionary history (Liu et al., 2020).

The chloroplast genome of most photosynthetic organisms is divided into four regions: two inverted repeat (IR) regions, a small single-copy (SSC) region, and a large single-copy (LSC) region (He et al., 2020). The size and composition of these regions vary among taxa. Plant chloroplasts contain, on average, 110 to 130 genes, including those encoding proteins, rRNA, and tRNA (Sugiura, 1992; Chan and Bhattacharya, 2011). Furthermore, mutation rates are influenced by post-transcriptional modifications of cpDNA, such as adenosine-to-inosine conversion, a process that can introduce or eliminate splicing sites, affect base pairing, and alter RNA conformation or its interactions with other molecules (Wulff et al., 2011; Slotkin and Nishikura, 2013).

Given the limited number of studies on ferns and the extensive diversity of groups and taxa within this lineage, many questions regarding their phylogenetic position remain unresolved. High rates of interspecific hybridization and frequent polyploidy result in genomes that can exceed tens of gigabases, which makes genomic research challenging and expensive. In this context, the chloroplast genome emerges as a promising approach to address genetic and evolutionary questions, as it tends to be highly conserved due to its crucial role in energy production through photosynthesis. Any critical deletion, insertion, or substitution in its genes could compromise the organism's survival (Martín and Sabater, 2010).

In this study, we assembled and annotated the chloroplast genome of *Cyathea delgadii*, a tree fern widely distributed in South America and performed comparative phylogenomic analyses with the complete chloroplasts of 19 organisms from the order Cyatheaales to identify key differences and similarities among them.

Materials and Methods

Plant Cultivation and DNA Extraction

Fresh spores of *Cyathea delgadii* were collected from wild individuals in the Atlantic Forest, Brazil, and inoculated onto Petri dishes containing BCD medium (mineral medium supplemented with nutrients, pH 5.8). The plates were maintained in growth chambers under controlled conditions of photoperiod (16 hours of light and 8 hours of darkness) and a constant temperature of 20°C. After germination, the gametophytes were transferred to new Petri dishes containing BCDAT medium and cultivated under the same conditions until they reached sufficient size for DNA extraction.

Genomic DNA was extracted from 100 mg of fresh gametophyte tissue using the protocol described by Mayjonade et al. (2016) with modifications. Briefly, the tissue was grinded in liquid nitrogen, and the extraction was performed with SDS buffer (1.5% SDS, 100 mM Tris-HCl pH 8.0, 50 mM EDTA, 0.5 M NaCl) supplemented with 2% β -mercaptoethanol to minimize polyphenol oxidation. Following phase separation with CIA (chloroform/isoamyl alcohol, 24:1), subsequent precipitation with isopropanol and washes with 70% ethanol to, finally, resuspend the

DNA in ultrapure water. DNA quality was assessed by electrophoresis on a 0.8% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA), and initial quantification was performed visually based on molecular weight standards. For greater accuracy, the final concentration was determined by fluorometry using the Qubit 4.0 system (Thermo Fisher Scientific, Waltham, MA, USA) with the Qubit dsDNA HS Assay kit. All samples were diluted to a uniform concentration of 20 ng/μL for use in subsequent steps.

Chloroplast Genome Sequencing and Assembly

Genomic DNA sequencing was performed on the GRIDION platform (Oxford Nanopore Technologies, UK) to obtain long reads, conducted at the Bioinformatics Laboratory of the Antarctic Vegetation Studies Center of the Federal University of Pampa, São Gabriel campus, RS, Brazil. Prior to sequencing, DNA fragments smaller than 10 kb were removed using the Short Read Eliminator XS kit (Circulomics, PacBio Company, Baltimore, MD, USA), following the manufacturer's instructions. The genomic library was prepared with the Genomic DNA by Ligation kit (SQK-LSK109, Oxford Nanopore Technologies), and sequencing was performed on an R9.4.1 flow cell (Oxford Nanopore Technologies), generating raw reads of total DNA with an average length of 15 kb.

Basecalling of the reads was performed with Guppy software v5.0.17, integrated into the GRIDION device pipeline, using the high-accuracy mode. ONT adapters and artifacts were removed with Porechop program v0.2.4 (Wick, 2017, available at <https://github.com/rrwick/Porechop>). High-quality reads were filtered with NanoFilt v2.8.0 (De Coster et al., 2018), applying a quality threshold ($Q > 10$) and discarding reads shorter than 1000 base pairs. The filtered sequences were mapped against the chloroplast reference genome of *Gymnosphaera podophylla*. For improved assembly of the chloroplast subunits, the reference was divided into its four subunits, and each was used as a reference for Minimap2 v2.24 (Li, 2018) with parameters optimized for long reads (-ax asm5). Mapped reads were extracted with Samtools v1.15 (Danecek et al., 2021), and *de novo* genome assembly based on the chloroplast DNA subunits, using the size of each subunit as a genome size parameter, performed using Flye v2.9 (Kolmogorov et al., 2019). After assembly of each subunit, the regions of the contigs representing each subunit were trimmed, retaining approximately 2000 bases before and after the junction regions. The best contigs were then submitted to Megamerger (<https://emboss.bioinformatics.nl/cgi-bin/emboss/megamerger>) as reported in the literature by Redwan et al., (2015), where they obtained improved plastid genome assemblies following this pipeline.

Genome Annotation

The assembled plastid genome was annotated using GeSeq v2.03 (Tillich et al., 2017), configured to identify protein-coding genes, rRNAs, and tRNAs based on pteridophyte references available on the NCBI database. The circular structure of the genome was visualized with OGDRAW v1.3.1 (Greiner et al., 2019). Inverted repeat (IR) regions and their junction structures were analyzed and visualized with IRScope (Amiryousefi et al., 2018), configured to detect micro inversions and expansions/contractions in the LSC-IR and SSC-IR junction regions.

Phylogenomic and Single Nucleotide Polymorphism (SNP) Analysis

For phylogenomic analysis, complete chloroplast genome sequences of 18 species from the order Cyatheaales were obtained from the NCBI database (<https://www.ncbi.nlm.nih.gov/>), as listed in Table 1. The species *Ceratopteris thalictroides* (order Polypodiales) was included as an outgroup to root the phylogenetic trees. All sequences were annotated using the same pipeline applied to *C. delgadii* to ensure consistency. Global alignment of the genomes

was performed with BLASTn (Altschul et al., 1990), using the collinearity block-based approach implemented in ORPA applying the maximum likelihood method with 1000 bootstrap replicates (Bi et al., 2024), where the model chosen by the algorithm was GTR + F + R2. Phylogenetic trees were visualized on the iTOL v5 platform (Letunic and Bork, 2021).

Single nucleotide polymorphism (SNP) analysis was conducted with the REALPHY v1.12 pipeline (Amiryousefi et al., 2018), configured to identify highly conserved regions and minimize the impact of insertions and deletions. The complete genomes and SNP data were aligned individually with MAFFT v7.490 (Katoh, 2002) in automatic mode (--auto). SNP-based phylogenetic trees were generated with FASTTREE v2.1.11 (Price et al., 2009), using the General Time Reversible (GTR) model with bootstrap support (1000 replicates).

In conducting a syntenic analysis through a comparative analysis of plastid genomes, using the list of most common genes (Supplementary File 1) of ten fern species, the species included were: *C. delgadii*, *Sphaeropteris lepifera*, *Gymnosphaera khasyana*, *Sphaeropteris brunoniana*, *Alsophila costularis*, *G. podophylla*, *Alsophila austroyunnanensis*, *Plagiogyria subadnata*, *Dicksonia squarrosa* and *C. thalictroides*. These species encompass several clades within the order Cyatheaales and present representations of both tree ferns and associated lineages. Syntenic regions among the genomes were evaluated with SimpleSynteny v1.3 (Veltri et al., 2016), and dot plot analysis was performed with D-GENIES (Cabanettes and Klopp, 2018) to identify structural rearrangements in the genome comparisons.

Codon Usage and Relative Synonymous Codon Usage (RSCU) Analysis

Codon frequencies were calculated from the aligned chloroplast genomes using GCUA v1.2 (McInerney, 1998), and the results were visualized with CIMMiner (available at <https://discover.nci.nih.gov/cimminer/>). The relative synonymous codon usage (RSCU) in *C. delgadii* was determined using the Codon Usage Calculator ("Codon Usage Calculator - Bioinformatics Tools," n.d.), configured to analyze only protein-coding genes and exclude stop codons.

Table 1
Identification of the species used in this work with NCBI access number and the original reference

Taxon	Genbank Accession	References
<i>Alsophila austroyunnanensis</i>	OR575070.1	Zhu,M., Liang,Y., Su,Y. and Wang,T. Unpublished
<i>Alsophila gigantea</i>	MH603068.1	Wang et al., 2019
<i>Alsophila costularis</i>	MH684489	Wang et al., 2019
<i>Gymnosphaera denticulata</i>	MT726940.1	Zhu,M. Unpublished
<i>Plagiogyria subadnata</i>	MN623362.1	Liu et al., 2020
<i>Cibotium barometz</i>	MH105066.1	Liu,S., Wang,Z., Su,Y. and Wang,T Unpublished
<i>Sphaeropteris lepifera</i>	MN623357.1	Liu et al., 2020
<i>Gymnosphaera khasyana</i>	OR575071.1	Zhu,M., Liang,Y., Su,Y. and Wang,T. Unpublished
<i>Alsophila latebrosa</i>	MW620065.1	Wang,Z., Wang,R., Lin,L., Liu,R., Ma,S., Hong,Y., He,Z., Su,Y. and Wang,T. Unpublished
<i>Ceratopteris thalictroides</i>	OK524221.1	Zhou,X. Unpublished
<i>Sphaeropteris brunoniana</i>	MT543220.1	Zhu et al., 2020
<i>Cibotium cumingii</i>	OQ721088.1	Jiang,R.-H., Liang,S.-Q., Wu,F., Tang,L.-M., Qin,B., Chen,Y.-Y., Huang,Y.-H., Li,K.-X. and Zhang,X.-C. Unpublished
<i>Alsophila metteniana</i>	MN795320.1	Zhu,M. Unpublished
<i>Plagiogyria japonica</i>	PP861174.1	Wang,Y., Xie,P., Huang,K. and Li,H. Unpublished
<i>Dicksonia squarrosa</i>	KJ569698.1	Zhong et al., 2014
<i>Gymnosphaera podophylla</i>	MG262389.1	Liu et al., 2018
<i>Plagiogyria euphlebia</i>	MN027504.1	Yang et al., 2020
<i>Alsophila spinulosa</i>	PQ740943.1	Qin,B. Unpublished

Results and Discussion

Chloroplast genomes pose a significant challenge for the majority of assembly tools due to the presence of two identical yet inverted repeat regions (Turudić et al., 2022). Fern chloroplasts display the typical quadripartite structure seen in plants, along with variations in size and gene content that indicate evolutionary and ecological adaptations. The chloroplast genome of *C. delgadii* was assembled by mapping total DNA sequences to the plastid reference genome of *G. podophylla*. This assembly resulted in 163,172 mapped chloroplast sequences and an N50 value of 14 kb. To ensure assembly quality, reads shorter than 1 kb were discarded, achieving approximately 9200x coverage of the plastid genome. This high coverage facilitated accurate reconstruction of the typical quadripartite structure, consisting of a large single-copy (LSC) region of 94,738 bp, a small single-copy (SSC) region of 22,012 bp,

and two inverted repeat regions (IRa and IRb) each measuring 24,251 bp. The total estimated genome size was 165,248 bp, which aligns with findings in other species of the order Cyatheaales (Table 2).

The analysis of guanine-cytosine (GC) content revealed an overall value of 40.95% for the *C. delgadii* genome, characterised by an uneven distribution across various regions. The inverted repeat (IR) regions demonstrated a higher GC content of 44.03%, indicative of their functional stability, whereas the single-copy regions exhibited lower values, specifically 40.08% in the LSC and 37.51% in the SSC. This pattern of GC content variation bears similarities to that observed in other closely related ferns, such as *Alsophila gigantea* and *S. lepifera*, implying structural conservation within these regions.

The chloroplast genome size of *C. delgadii* (165,248 bp) is intermediate compared to the 18 species analysed (Table 2). The largest plastid genome within the dataset is attributed to *D. squarrosa* (168,244 bp), whereas the smallest genome was recorded in *Gymnosphaera denticulata* (154,046 bp). The organizational characteristics of the Large Single Copy (LSC) and Small Single Copy (SSC) regions exhibit substantial variation among species: *S. lepifera* possesses the largest SSC at 27,731 bp, while *Cibotium barometz* demonstrates the smallest LSC measuring 85,670 bp. Additionally, the Inverted Repeat (IR) regions display variability, with *C. delgadii* presenting IRs of 24,251 bp an intermediate measurement when comparing with species such as *Cibotium barometz* (29,177 bp) and *D. squarrosa* (30,201 bp), which possess longer IRs; conversely, the genus *Alsophila* is characterised by smaller IRs. Regarding gene content, *C. delgadii* encodes 89 protein-coding genes, eight rRNAs, and 33 tRNAs, totaling 130 genes. This pattern is consistent with other ferns of the Cyatheaaceae family, such as *Alsophila austroyunnanensis* (130 genes) and *P. euphlebia* (130 genes). This conservation suggests functional stability in photosynthetic and translational processes.

Table 2

Comparison of general features of fern chloroplast genomes. Para determinar o tamanho das subunidades utilizamos IRScope (Amiryousefi et al., 2018), os demais dados foram preenchidos manualmente com base nos arquivos genbank. o Conteúdo GC foi contabilizado utilizando

Species	Total genome size (bp)	LSC (bp)	SSC (bp)	IR (bp)	Protein-coding genes	rRNAs	tRNAs	GC content (%)	Total number of genes
<i>Cyathea delgadii</i>	164248	94738	22012	24251	89	8	33	40.95	130
<i>Alsophila austroyunnanensis</i>	161013	91787	21618	23804	89	8	33	41.23	130
<i>Alsophila gigantea</i>	161679	92307	21702	23835	89	8	33	41.35	130
<i>Alsophila costularis</i>	156675	86338	21625	24356	89	8	33	40.46	130
<i>Gymnosphaera denticulata</i>	154046	87975	21581	23245	89	8	33	40.58	130
<i>Plagiogyria subadnata</i>	159998	89960	21424	24307	89	8	32	42.94	129
<i>Cibotium barometz</i>	166087	85670	22063	29177	87	8	33	41.68	128
<i>Gymnosphaera khasyana</i>	155226	85855	21607	23882	88	8	32	40.64	128
<i>Alsophila latebrosa</i>	155724	85800	21620	24152	89	8	33	40.38	130
<i>Sphaeropteris lepifera</i>	162114	86327	27731	24028	89	8	32	40.8	129
<i>Sphaeropteris brunoniana</i>	156659	86196	22441	24011	89	8	33	40.26	130
<i>Cibotium cumingii</i>	167221	85641	22062	28759	87	8	33	41.69	128
<i>Alsophila metteniana</i>	161602	92292	21666	23822	89	8	31	41.33	128
<i>Plagiogyria japonica</i>	161371	91447	21438	24243	89	8	33	43.47	130
<i>Dicksonia squarrosa</i>	168254	85817	22035	30201	89	8	33	41.52	130
<i>Gymnosphaera podophylla</i>	166151	86762	21641	28874	91	8	33	41.88	132
<i>Plagiogyria euphlebia</i>	161046	90975	21441	24315	89	8	33	43.47	130
<i>Alsophila spinulosa</i>	156196	86313	21623	24130	92	8	33	40.44	133

Comparison of Plastid Genome Gene Content of *Cyathea delgadii* with Other Ferns

The same method was applied to all sequences to ensure the robustness and accuracy of comparisons between chloroplast genomic sequences. This approach minimizes methodological biases and allows a more reliable analysis of genomic differences and similarities. Moreover, consistency in processing is documented in the literature to diminish errors stemming from technical variations, thereby ensuring that any discrepancies observed between genomes genuinely reflect biological and evolutionary phenomena differences, rather than methodological artefacts (Phillips et al., 2004). Thus, standardization strengthens the integrity of the results, enabling more accurate and reproducible inferences in genome annotation.

The chloroplast genomes in this study exhibit a genetic composition that reflects both functional conservation and significant evolutionary variations, as previously evidenced (Table 2), which details the number of protein-coding genes, ribosomal RNAs (rRNAs), and transfer RNAs (tRNAs). Most of the analyzed species, including *C. delgadii*, some *Alsophila* species, and *Plagiogyria*, possess 89 protein-coding genes, 8 rRNAs, and between 31 and 33 tRNAs, suggesting a structural stability associated with essential photosynthetic functions. Upon analyzing *D. squarrosa*, it presents only 89 protein-coding genes, while *G. podophylla* and *A. spinulosa* reach 91 and 92 genes, respectively. Regarding tRNAs, variations from 31 (*Alsophila metteniana*) to 33 (*C. delgadii*, *A. austroyunnanensis*) are recorded, with intermediate values in *P. subadnata*, *G. khasyana*, and *S. lepifera* (32 tRNAs).

A comparative analysis with other ferns and mosses reveals similar patterns while highlighting distinct evolutionary differences. For example, the chloroplast of *Adiantum capillus-veneris* contains 88 protein-coding genes, eight rRNAs, and 37 tRNAs, as indicated by (Wolf et al., 2003), who investigated the evolution of fern plastomes. This increased tRNA count, which surpasses that of most species, implies a heightened translational diversity, potentially adapted to various environmental conditions. Conversely, *Pteridium aquilinum* exhibits genomic rearrangements that have led to a reduction of its plastome by up to 15%, as noted by Li et al. (2018), who examined ancestral fern genomes.

In mosses, such as *Physcomitrium patens*, the plastome contains 83 protein-coding genes, four rRNAs, and 31 tRNAs, according to Sugiura (1992). Compared to ferns, the number of protein-coding genes is slightly lower, while the number of rRNAs represents half of that found in the organisms of this study, which may reflect specific adaptations to life cycles. This difference might suggest that although ferns and mosses share common ancestors, their lineages diverged in genomic strategies, influenced by distinct ecological pressures. Given that the greater complexity of the environment during the transition from water to land and the subsequent conquest of terrestrial habitats led to the success of ferns, the presence of a larger number of rRNAs or even tRNAs could be a response to oxidative stress, adjusting the expression of antioxidant proteins and creating post-transcriptional modifications (Chan et al., 2010).

However, the significant reduction to 128 genes in *C. barometz*, for example, may result from gene loss or transfer to the nuclear genome. Evidence from various studies suggests that this type of transfer occurs continuously between organelles and the nucleus (Farrelly and Butow, 1983; Henze, 2001; Stegemann et al., 2003), contributing to the variation in the genetic content of organellar genomes (Yuan et al., 2002). This exchange mechanism may be associated with gene loss in response to environmental pressures, such as adaptation to shaded environments, where the reduction of redundant photosynthetic genes could prove advantageous (Valladares and Niinemets, 2008; Ruberti et al., 2012; Xu et al., 2018). Conversely, the increase to 91 genes in *G. podophylla* and 92 in *A. spinulosa* indicates duplications or the retention of genes with an adaptive role. The study conducted by Cullis et al. (2009) examines gene transfer between the nucleus and chloroplast; however, without specifying a particular cause for this transfer, it remains inconclusive to deduce a biotic or abiotic rationale for these occurrences. This leads to the

conclusion that such transfers are a natural and continuous process that has occurred throughout the evolution of plants.

The variations in the number of tRNAs (31 to 33) are equally intriguing. The reduction to 31 tRNAs in *A. metteniana* may indicate a translational optimization bias, which could be compensated by the import of tRNAs from the cytoplasm. Studies suggest that this conservation and the non-import of organellar ribosomal proteins is relatively conserved in some groups (Maier et al., 2013). The decrease in chloroplast tRNA genes may reflect that the organism lives in a specific ecological niche possibly adapted to variable environmental conditions. Tiller and Bock (2014) list a set of non-essential tRNAs; normally, 32 different types of tRNAs are needed to read all messages correctly, but there is variable base pairing (or superwobble) where some tRNAs can be flexible and fit into different positions, even if they are not exactly the same. Under this influence, the plant can continue to manufacture proteins without needing all 32 types of tRNAs (Zoschke and Bock, 2018).

Variation in GC Content

The variation in Guanine-Cytosine (GC) content within the plastid genome of ferns can reflect both structural factors and evolutionary and environmental pressures. GC content can affect the stability of the molecular structure of the plastid DNA double helix, potentially leading to different effects on its thermal resistance and the regulation of gene expression, as evidenced in the works of (Gao et al., 2009; Xinyu et al., 2019).

The plastid genome of *C. delgadii* has a GC content of 40.95%, a value similar to that of other species in the genera *Alsophila* and *Sphaeropteris*, such as *A. gigantea* and *S. brunoniana*, which exhibit 41.35% and 40.26% GC, respectively (Fan et al., 2021). However, species of the genus *Plagiogyria*, such as *P. japonica* and *P. euphlebia*, show significantly higher values (~ 43.5%), while *P. subadnata* presents 42.94% (Du et al., 2022), reflecting a conservation of this group regarding the thermostability of their DNA.

This increase in GC content may be associated with greater structural stability of the plastid DNA, making it more thermostable and less susceptible to UV radiation-induced damage, favoring its occurrence in more exposed and high-temperature environments (Gu et al., 2024). In contrast to species with high GC content, *A. costularis*, *A. latebrosa*, and *S. brunoniana* exhibit the lowest GC values among the analyzed species. This pattern is often associated with shaded or cold environments, although without freezing phases, which would represent another type of stress. In such conditions, rapid transcriptional regulation and an adjustment to maintain DNA with fewer hydrogen bonds may signal energy economy for polymerase function in situations requiring speed, ultimately being more advantageous than the greater structural stability of the DNA helix (Li et al., 2016).

Furthermore, the distribution of GC content among the different DNA regions, with a higher concentration in the inverted repeat (IR) regions due to the presence of rRNA and tRNA genes (Wolf et al., 2015) that are fundamental for the organelle's existence, reinforces the role of genomic organization in the compositional variation of cpDNA. Thus, the plastid GC composition of ferns demonstrates a balance between evolutionary constraints and facilitations and environmental pressures, where genomic structure and organization play fundamental roles, reflecting distinct strategies of adaptation to the environment (Novoa and Ribas De Pouplana, 2012; Kim et al., 2014).

Codon Usage in *C. delgadii* and Overall Amino Acid Frequency

Upon conducting an examination of the codon frequency in *C. delgadii* (Fig. 2), a distinct preference for specific synonymous codons was observed. The ATG codon, which encodes methionine (Met) and serves as a translation

initiator, displays a frequency of 100%, which is an anticipated pattern attributable to its conserved function across all organisms. Other codons, such as TAT (tyrosine, Tyr, 64.4%), GAT (aspartic acid, Asp, 69.2%), and GAA (glutamic acid, Glu, 63.0%), also emerge as significantly utilized, suggesting a favorable selection for these amino acids within chloroplast proteins. Conversely, codons such as CGC (arginine, Arg, 10.4%), GGC (glycine, Gly, 14.9%), TCG (serine, Ser, 12.4%), and CTG (leucine, Leu, 11.0%) exhibit lower representation, indicating that terminations in guanine or cytosine are less favored. Previous studies have indicated that in organisms with lower GC content, there exists a tendency to favor codons ending in adenine (A) or thymine (T), whereas GC-rich genomes prefer terminations in G or C (Ermolaeva, 2001; Chen et al., 2004). This pattern observed in *C. delgadii* implies that the chemical structure of the DNA may be associated with an adaptation for translational efficiency, as previously discussed, or to RNA stability (Parvathy et al., 2022).

The comparative analysis of codon usage among the species (Fig. 3) reveals consistency within genera or closely related taxonomic groups, despite the expected interspecific variations. Amino acids such as leucine (Leu), serine (Ser), and arginine (Arg), which are encoded by six codons each, exhibit high frequencies, reflecting a broader translational range offered by multiple synonymous options. Conversely, amino acids like methionine (Met) and tryptophan (Trp), which are restricted to a single translation codon, show lower frequencies a pattern correlated with the GC content of the chloroplasts (Iriarte et al., 2021).

As previously noted, plants belonging to the genus *Plagiogyria* are distinguished by their elevated GC content (~43%), which correlates with a preference for codons concluding with G or C. It is imperative to acknowledge that each individual exhibits a codon bias influenced by mutational pressures finely adjusted by the selective pressures essential for its survival (MacDonald, 2013). Moreover, these organisms predominantly inhabit mountainous regions of Southeast Asia and periodically encounter environmental pressures that may induce abiotic stress, with the high GC content serving as a beneficial factor in preserving DNA integrity against mutations (Moura et al., 2009). In contrast, genera such as *Alsophila* and *Gymnosphaera*, characterised by lower GC content, demonstrate a discernible inclination towards codons ending in A or T, akin to the pattern observed in *C. delgadii*.

The similarities in codon usage bias among closely related species, such as *Sphaeropteris* (with intermediate GC content) and *Cibotium* (with high GC content), reinforce the hypothesis that these patterns reflect phylogenetic relationships and specific adaptations. The genus *Cibotium*, for instance, may have developed a GC-rich chloroplast genome as a response to distinct ecological or functional pressures. Three-hydrogen-bond pairings have been observed as favored in bacteria, where factors such as translational selection, GC composition, and RNA stability influence synonymous codon usage (Ermolaeva, 2001). Considering that chloroplasts derive from prokaryotic ancestors, this connection reinforces the idea of evolutionary continuity between the bacterial and plant domains.

Evolutionary Considerations

The emergence of the Cyatheaceae family of the Cyatheales order occurred in the Upper Jurassic, possibly in Australasia or South America, with the probable distance between these groups being due to vicariance (Lantz et al., 1999). From a phylogenetic point of view, *C. delgadii* is closely related to *S. lepifera* and *S. brunoniana* which are found in Southeast and East Asia, both geographically distant from *C. delgadii*. What is most curious is the fact that the genus *Sphaeropteris* possibly arose in Australasia (Korall and Pryer, 2014), demonstrating conservation of species DNA. Furthermore, it is observed that the IR region of *C. delgadii* is approximately 1 kb larger than that of the closest species and approximately 3 kb smaller than that of *S. lepifera*, additionally, the size of the LSC region is

expanded by up to 7 kb, This type of variation has already been reported in Polypodiaceae where elongations or deletions tend to occur in the flank regions of the subunits (Liu et al., 2021).

The analyzed differences in the number of plastid genes and GC content among ferns may reflect distinct evolutionary pathways or adaptive pressures experienced by these organisms. Studies on plastid evolution indicate that, although conservation of the plastid genome is common in many lineages, specific adaptations, such as variations in GC content, play a significant role in genome stability and processes like GC-biased gene conversion, especially in plants (Singh et al., 2023). In *C. delgadii* and most of the analyzed species, the conservation of the total number of genes may indicate structural stability. In contrast, punctual differences in protein-coding genes and tRNAs suggest possible specific adaptations to environmental conditions, as seen in other plants and algae with distinct plastid genomes (Sibbald and Archibald, 2020).

As an outgroup for rooting the phylogenetic trees, we used *C. thalictroides*, which consistently appears as the outgroup in both approaches, corroborating its effectiveness for rooting the phylogeny. This molecular divergence may be related to different evolutionary dynamics and events that altered its genome, distancing it from the groups addressed in this work, as observed in studies of *Ceratopteris richardii* (Marchant et al., 2022), which evidenced a history of rapid nuclear genomic evolution in this lineage, including gene loss events and tandem duplications of nuclear genes. This genomic plasticity may significantly influence the inference of evolutionary distances, as reported for several organisms such as Zhen, birds, marine corals and plants as well (Passow et al., 2017; Baniaga et al., 2020; Rivera et al., 2021; Leung et al., 2022; She et al., 2023). This phenomenon was observed in depth by Tseng et al. (2024), corroborating this plasticity related to molecular evolution and highlighting hybridization as an important mechanism for the speciation of Thelypteridaceae ferns, in which genomic and transcriptomic patterns serve to elucidate gene evolution and adaptation, thereby providing a clearer understanding of the pathways for the organism's biological intricacies changes (Zeng-Qiang et al., 2024).

Moreover, we noted a comparable clustering among individuals of the genera *Alsophila* and *Gymnosphaera* in both phylogenetic trees (Figs. 4 and 5). It is pertinent to mention that in the polymorphism-based tree, *C. delgadii* is positioned medially, branching between the two aforementioned groups. Conversely, in the genomic tree, it occupies a basal position. Additionally, the consistent delineation of *Plagiogyria* and *Dicksonia* from the remaining taxa across both trees serves to reinforce the robustness of these phylogenetic relationships. The stability observed in these groupings can be elucidated by the low incidence of polyploidy within the Cyatheaceae, as evidenced in the genome of *A. spinulosa* (Huang et al., 2022).

Regarding *D. squarrosa* and its basal position in relation to alignments against *Cyathea* and *Alsophila*, it can be highlighted that this organism is not curated in the database and its sequence presents a gap starting at position 35.633 of the nucleotide sequence. Furthermore, we can highlight events in all analyzed organisms that may result from changes in gene regulation or a functional shift of these genes to the nucleus, something very common in plants (Lee, 2023). Indeed, experimental studies have already shown that the transfer of DNA from the chloroplast to the nucleus occurs frequently, allowing the integration of genome fragments and causing variations between species and organisms (Stegemann et al., 2003). Thus, the evolutionary complexity of the plastid genome differs in retention or loss patterns within the Cyatheaceae and related groups.

New approaches such as ORPA (Organelle Genomes for Phylogenetic Analysis), which incorporates information from the complete organelle genome, may contain greater accuracy, as it reduces distortions generated by specific evolutionary events that would be punctuated in polymorphism analysis. The ORPA method, as described by Bi et al., (2024), has proven to be highly efficient in the phylogenetic reconstruction of organellar genomes, in addition to

being faster and more accurate with the manipulation of entire genomes. ORPA's ability to handle phylogenomic conflicts reinforces its efficiency and capacity in detailed evolutionary studies. Thus, comparisons between methods are a caveat to the importance of integrating different inference methodologies to reach more solid conclusions about evolution. Meanwhile, polymorphism-based approaches can provide new insights into specific variations.

Given that the chloroplast typically possesses a quadripartite structure, it tends to exhibit some structural similarities at the junctions of these subunits. The comparison of junctions between the inverted repeat (IR) regions and the single-copy regions (LSC and SSC) of the chloroplast genome of species most phylogenetically related to *C. delgadii* highlights the structural differences among the plastid genomes of these ferns (Fig. 6), focusing especially on the variation in region size and the position of flanking genes such as *ndhB* and *ndhF*.

The *ndhB* and *ndhA* genes play an essential role in photosynthesis, forming the thylakoid complex in the chloroplast and are homologous to mitochondrial complex I (Sabater, 2021). They are also involved in the chloroplast NAD(P)H dehydrogenase (NDH) complex and participate in the reduction of plastoquinones in thylakoid membranes and in the cyclic electron flow of photosystem I and chlororespiration (Nixon, 2000; Peltier and Cournac, 2002). The *ndhB* gene is generally located partly within and flanking the IR regions, which confers greater stability and protection against mutations since these parts of the genome have a higher GC content, in addition to being in inverted regions where duplication of these genes provides a backup. Studies show that when the plant is subjected to stress such as high light and humidity or low temperature, this gene, as well as *ndhA*, tend to be overexpressed (Endo et al., 1999; Liu et al., 2021). The *ndhF* gene, on the other hand, is a gene from the same family that acts in electron transport, often located at the junction between the SSC region and the IRb. Its variable position at the IR/SSC junctions may suggest expansion or contraction events of the repeated regions, reflecting structural adjustments of the genome in response to the evolution of lineages.

It is worth pointing out that the anchoring of the *ndhF* gene is consistent across all organisms analyzed within *Cyatheales*, reflecting stability in the flanking position of the regions, despite variations in total size. The *ndhB* gene near the IRA/LSC junction follows a trend in its presentation in the totality of compared species, differing only in *C. delgadii* and *S. lepifera* where it is represented distant from the junction by up to 500 bases. In contrast, in the other species the gene overlaps the entire junction region in both directions. This non-standardization is possible due to the Irscope algorithm (Amiryousefi et al., 2018), developed for angiosperm chloroplast genomes, in the case of the *ndhB* gene, it has an intron (Freyer et al., 1995) which the mapping of this gene creates the impression of fragmentation.

Comparison between phylogenomically close genomes to *C. delgadii* reveals that some species present a moderate expansion of the IR region, while others present a shortening of this region. Expansion occurs when part of the SSC or LSC region is incorporated into IRs, which can result in gene duplication and potentially confer greater stability to the genome. In contrast, contraction occurs when part of the IR region is lost or transferred to the nucleus, reducing the size of that region and shifting the position of nearby genes. These changes are revealed from green algae to the angiosperm group as frequent in the evolutionary history of photosynthetic organisms (Wang et al., 2008; Zhu et al., 2016; Turmel et al., 2017).

The presence of genes such as *ndhB* within the IRs in some proven species and partially displaced to the LSC or SSC in others indicates different degrees of structural stability in these genomes. The *ndhF* gene is normally located in the IR/SSC region and its deallocation can be related to adaptation to different environments or selective pressures. Regions within the SSC can change 7 to 14 times more than repeated regions (Yi et al., 2012), in grasses when there is shortening of the SSC region this gene is usually at a disadvantage with the inverted region (Martín

and Sabater, 2010), whereas when there is elongation, a functional duplication of the gene may even occur (Seliverstov et al., 2009).

The synteny map (Fig. 7) elucidates several significant patterns of plastome organization. A highly conserved gene arrangement is discernible among *C. delgadii*, *S. lepifera*, *G. khasyana*, *A. costularis*, *G. podophylla*, and *A. austroyunnanensis*. These six species exhibit nearly identical gene blocks and orientations, indicating a strong conservation of plastid structure within the *Cyatheaceae* family. While *S. brunoniana* retains a substantial portion of the ancestral plastome structure, minor gene orientation and linkage shifts imply early divergence or lineage-specific rearrangements within the *Sphaeropteris* lineage. *Plagiogyria subadnata* demonstrates the most considerable rearrangements, featuring several gene inversions and disrupted synteny compared to the *Cyatheaceae* core group. These rearrangements could reflect deeper phylogenetic divergence or adaptive structural evolution of the plastome, as already reported for other land plants (Yu et al., 2023). *Dicksonia squarrosa* and *C. thalictroides* exhibit partial synteny with the *Alsophila* group and *Plagiogyria*, suggesting an intermediate plastome structure. Both species reveal inversions and transpositions involving tRNA clusters and genes such as *rpoC1*, *ndhF*, and *ycf1*, which are recognised hotspots for rearrangement. Specific genomic regions reveal frequent disruption across lineages particularly the region spanning *ycf1*–*ndhF*, which is often implicated in inversion events. The *trnL*–*CAA* junction and clusters of tRNAs also appear to be susceptible to rearrangement, as reported for algae and land plants (Sugiura, 1992; Letsch and Lewis, 2012; Lemieux et al., 2014).

The notable conservation observed among species of the *Cyatheaceae* family signifies a relatively stable plastome architecture within this group, which correlates with their phylogenetic proximity. The same was also highlighted by Huang et al. (2022) when comparing the genome of *A. spinulosa* with other fern species. This finding supports the use of plastome structure as a taxonomic marker at the genus level (Zuo et al., 2025). In contrast, the extensive rearrangements noted in *Plagiogyria* underscore the potential for rapid structural evolution within certain fern lineages (Du et al., 2022), which may be driven by recombination hotspots or variations in repeat content (Gao et al., 2011). The intermediate plastome patterns identified in *Dicksonia* and *Ceratopteris* suggest that plastome rearrangement is not exclusively clade-specific but may, rather, reflect a combination of lineage divergence, genome plasticity, and selective pressures (Robison et al., 2018; Wei et al., 2021; Du et al., 2022). From an evolutionary standpoint, these rearrangements could be linked to adaptations to environmental conditions (Lehtonen et al., 2017), reproductive strategies, or modifications in life cycles, although the functional correlations are yet to be thoroughly elucidated (Vera-Paz et al., 2022).

The comparative and structural analysis (Fig. 8) of the complete chloroplast genome of *C. delgadii* with other ferns reveals clear patterns of divergence and conservation, highlighting evolutionary relationships and genomic events. When compared to the outgroup *C. thalictroides*, *C. delgadii* exhibits extreme divergence, with 99.19% of the regions showing less than 25% identity. This expected event reflects the fact that *Ceratopteris* belongs to a distant lineage (Pteridaceae), confirming its appropriate use as an outgroup. In contrast, comparisons within the order Cyatheaales show varying degrees of similarity. Species from less related families, such as *Dicksonia* (Dicksoniaceae) and *Cibotium* (Cibotiaceae), exhibit moderate identity (50–75% in ~ 34–48% of the genome), while ferns from the *Cyatheaceae* family (*Alsophila*, *Sphaeropteris*, *Gymnosphaera*) show greater conservation, with ~ 28–30% of the genome displaying high similarity (> 75%). These results reinforce the phylogenetic proximity between *Cyathea* and the other members of *Cyatheaceae*, while highlighting the accumulation of divergence over time in the more distant lineages. It is also worth noting that the fern group is monophyletic and sister to the moss and spermatophyte clade. Gao et al. (2010), which justifies the high structural similarity between the organisms studied in this work given their proximity in the order Cyatheaales.

The absence of highly conserved regions (> 75% identity) in comparisons with *Dicksonia* and *Cibotium* may reflect both extensive genomic rearrangements and accelerated rates of evolution in these lineages, possibly associated with their distinct biogeographic history such as the early breakup of Africa during the fragmentation of Gondwana. In contrast, the greater similarity observed between Cyatheaceae genera (*Alsophila*, *Sphaeropteris* and *Gymnosphaera*) suggests a relatively stable genomic structure, consistent with their shared Gondwanan origin (Late Jurassic) (Schuettzel and Pryer, 2009) and vicariance patterns (Janssen et al., 2008; Korall and Pryer, 2014). However, even within the family, ~ 70% of the genome shows identity below 75%, indicating significant variation, probably influenced by limited transoceanic dispersal events such as the colonization of Africa by *Alsophila* in the Late Cretaceous (Mohr and Lazarus, 1994). The notable exception is *Gymnosphaera* podophylla, whose regions exhibit 100% identity below 75%, an atypical pattern that may be linked to its unique evolutionary history such as multiple recent dispersals and its colonization of Africa and subsequent expansion into America (Korall and Pryer, 2014).

The aggregated findings underscore the structural resemblance among the genomes of ferns categorised within the order Cyatheaales, concurrently disclosing variations in particular regions that may be associated with evolutionary adaptations or distinct regulatory strategies pertinent to this group. From a phylogenetic viewpoint, the disparities observed in the size and architecture of the chloroplast genome across these ferns may correlate with specific evolutionary modifications. The intermediate size of the *C. delgadii* genome implies that it sustains a balance between genetic efficiency and structural integrity stability.

The junction variations between the regions of the plastid genome has significant implications for both chloroplast evolution and the phylogenetic reconstruction of the family Cyatheaceae. Expansions and contractions of the inverted repeats (IRs) are frequently used in the inference of evolutionary relationships, as they can serve as indicators of divergences between lineages. Furthermore, the variation in the positioning of flanking genes offers valuable information for the development of genetic markers.

Declarations

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contribution

Field work, investigation, sampling, conceptualization and methodology by GFM, RPML and FCV; Images built by GFM and RPML; Writing- original draft preparation by GFM and F.C.V.; Data curation and validation by GFM, CBDM, TDVF, RDVF, RPML; Writing - review and editing by GFM, CBDM, RPML and FCV; Project administration, supervision and funding acquisition by FCV.

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Data Availability

All reads and assemblies are available in the NCBI repository. The cpDNA assembly has been submitted to Genbank/NCBI under ID PV686490 and the raw reads were submitted to the Sequence Read Archive (SRA/NCBI) in the BioProject ID: PRJNA1263463. Additional data is provided within the manuscript or supplementary information files

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Figures

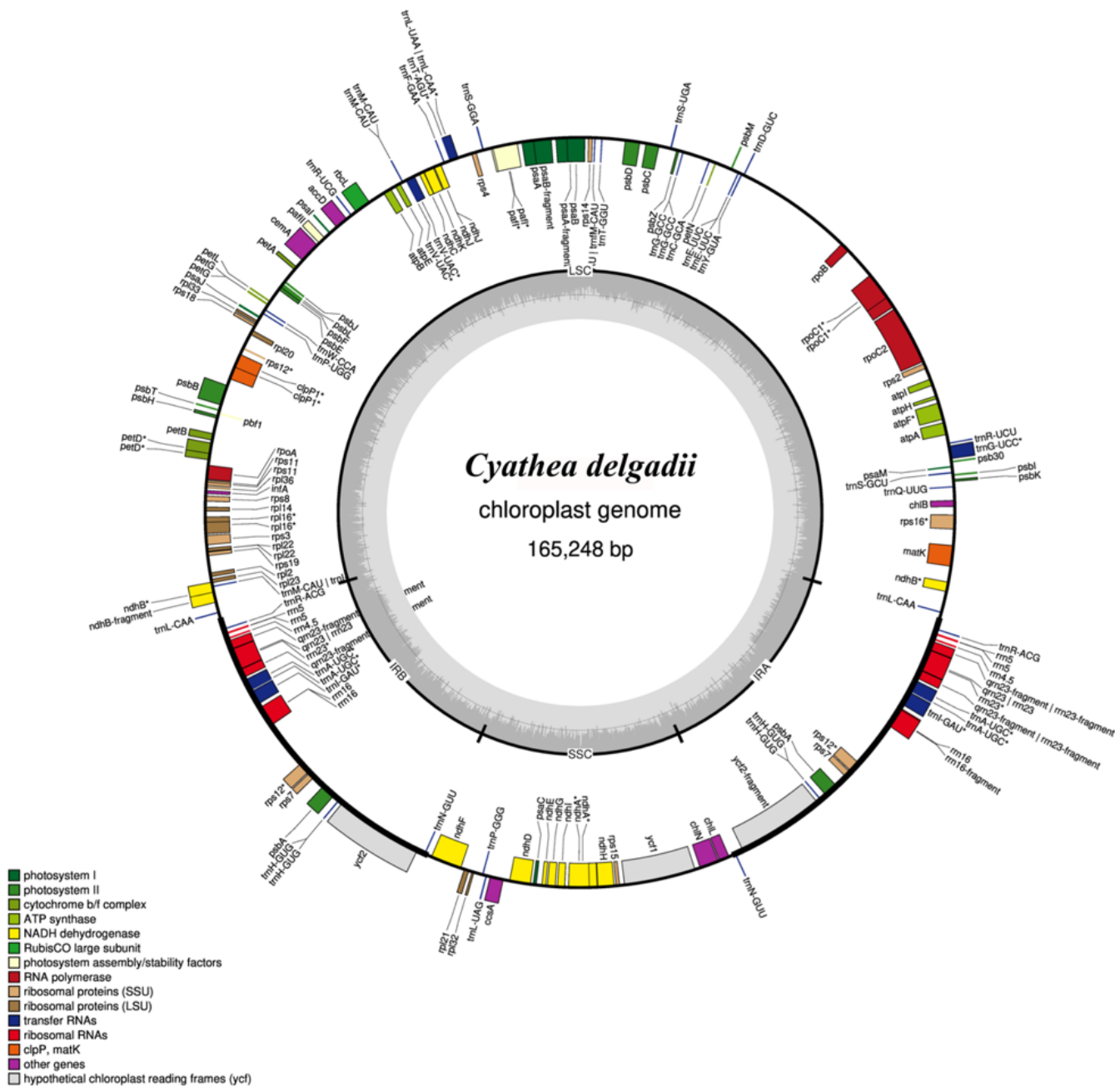


Figure 1

Chloroplast genome map of *C. delgadii*. Genes on the outer side of the circle are transcribed clockwise, while genes on the inner side are transcribed counterclockwise. Genes are color-coded according to their functional groups. The darker gray in the inner circle represents the GC content, while the lighter gray corresponds to the AT content. The

genome comprises a Large Single-Copy (LSC) region, a Small Single-Copy (SSC) region, and two Inverted Repeat regions (IRa and IRb), indicated by thick lines in the inner circle.

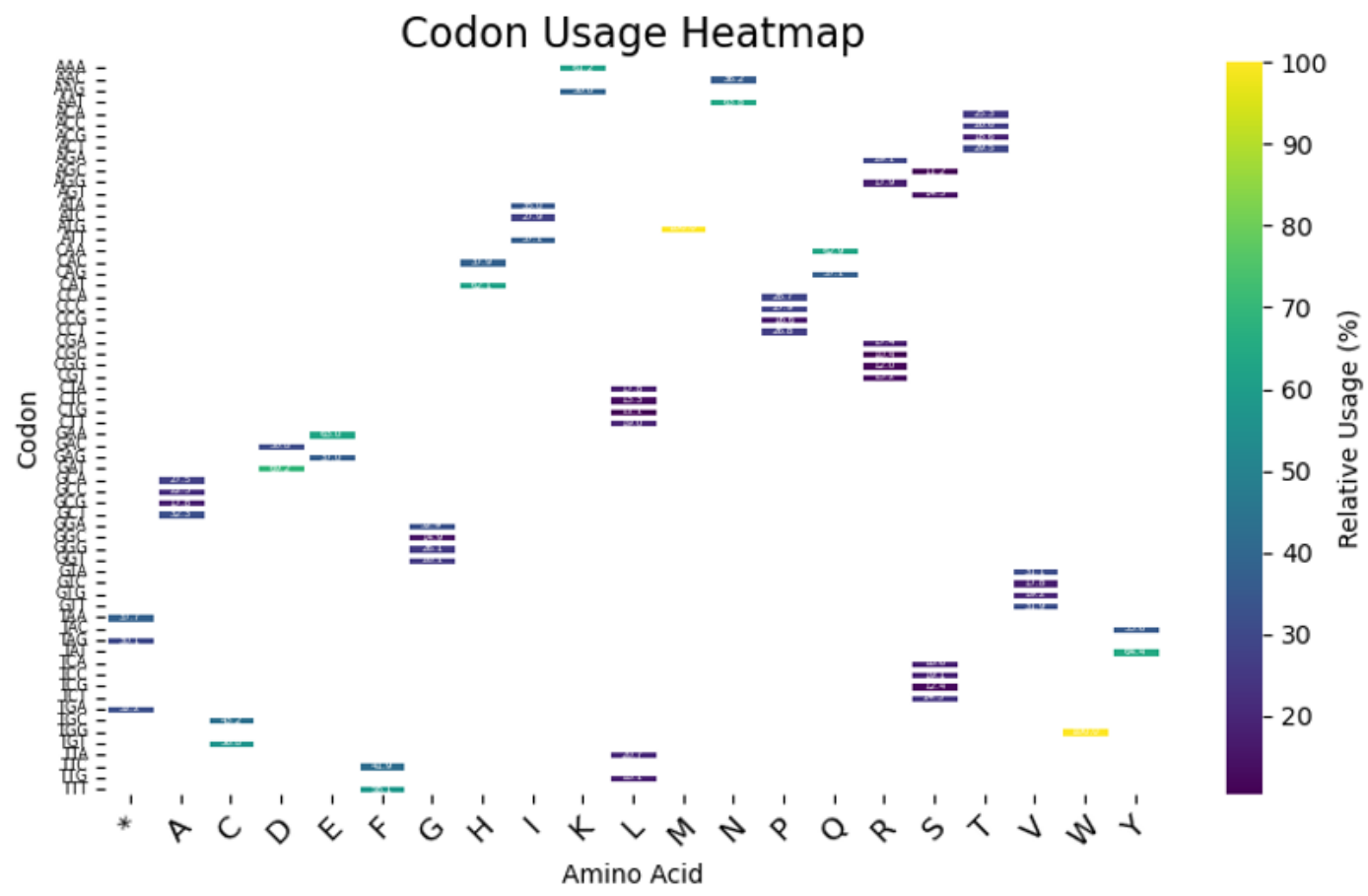


Figure 2

Heatmap displaying the relative synonymous codon usage (RSCU) percentages for each amino acid, based on a dataset from the Codon Usage Calculator (McGowan, 2023). Colors range from purple (low usage, ~20%) to yellow (high usage, ~100%). The x-axis represents amino acids, including an asterisk (*) denoting stop codons (TAA, TAG, TGA), while the y-axis lists the 64 possible codons. The numerical values within each cell represent the RSCU percentage of the codon relative to the total codons encoding the same amino acid. The ATG codon, encoding methionine (M), shows 100% usage, indicating it is the sole start codon in this context, while codons like TGG (encoding tryptophan, W) also exhibit 100% usage due to their assignment to a single codon.

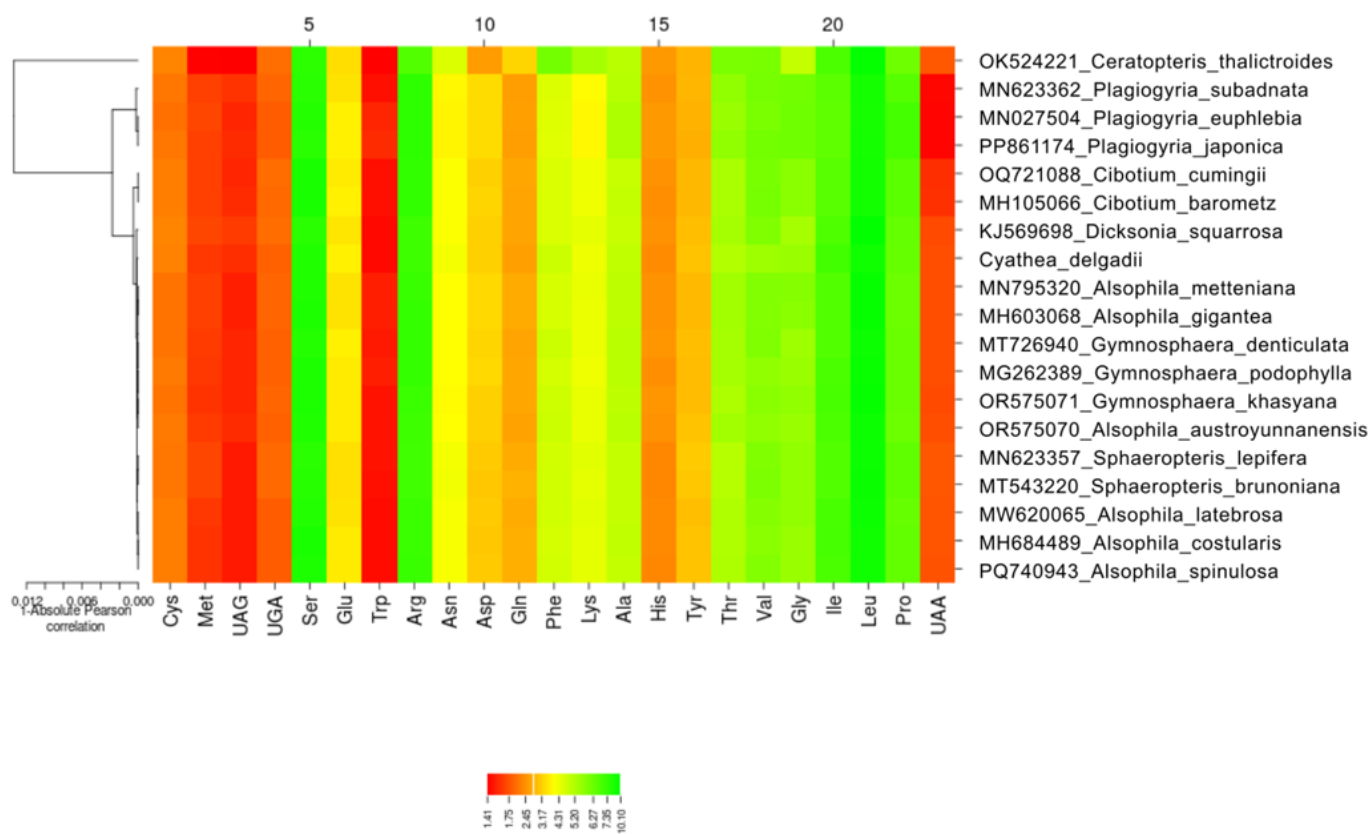


Figure 3

Heatmap of Synonymous Codon Usage Frequency in Cyatheales Species.

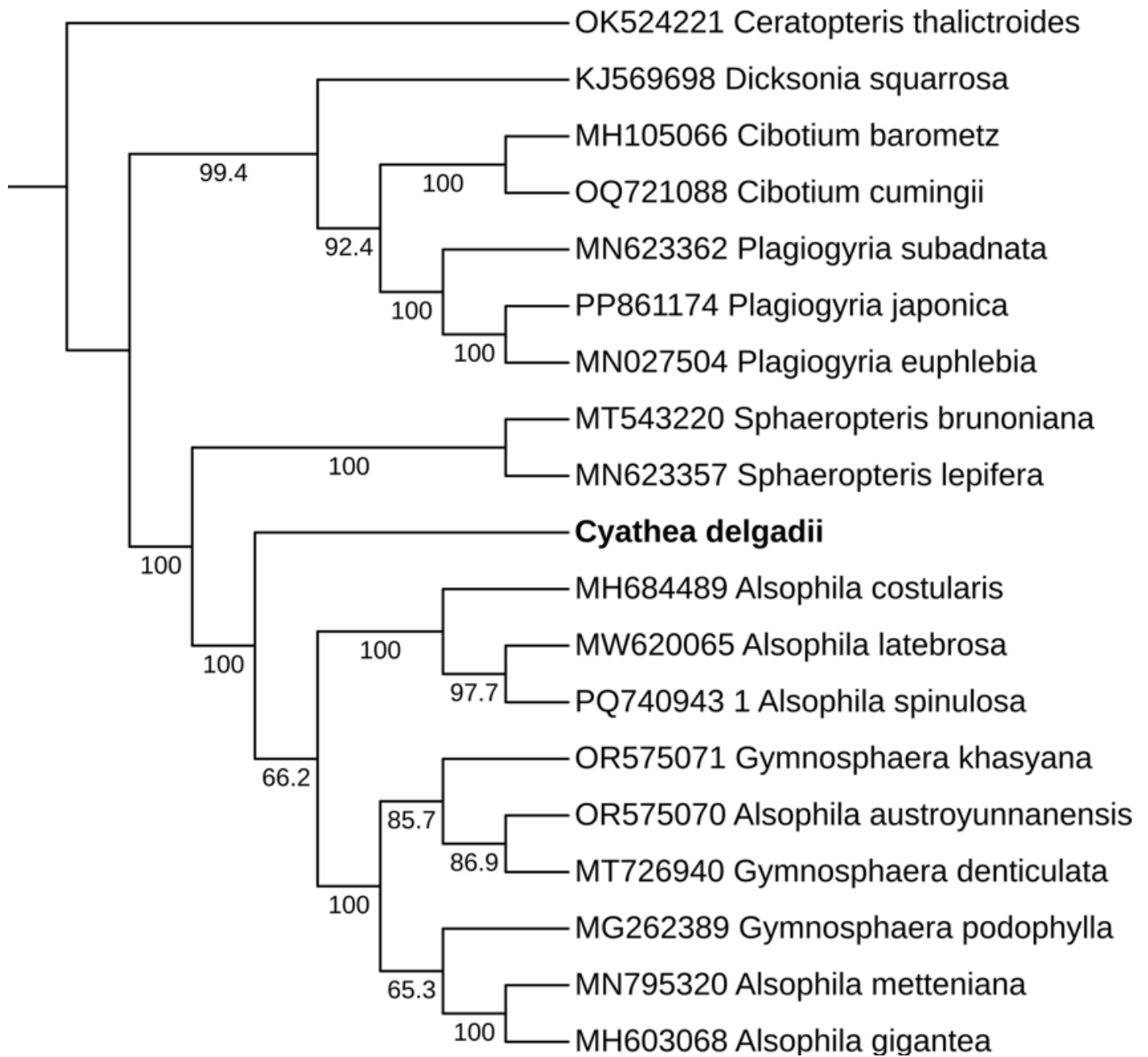


Figure 4

Phylogenomic tree of cpDNAs constructed with ORPA; the method used was GTR+F+R2.

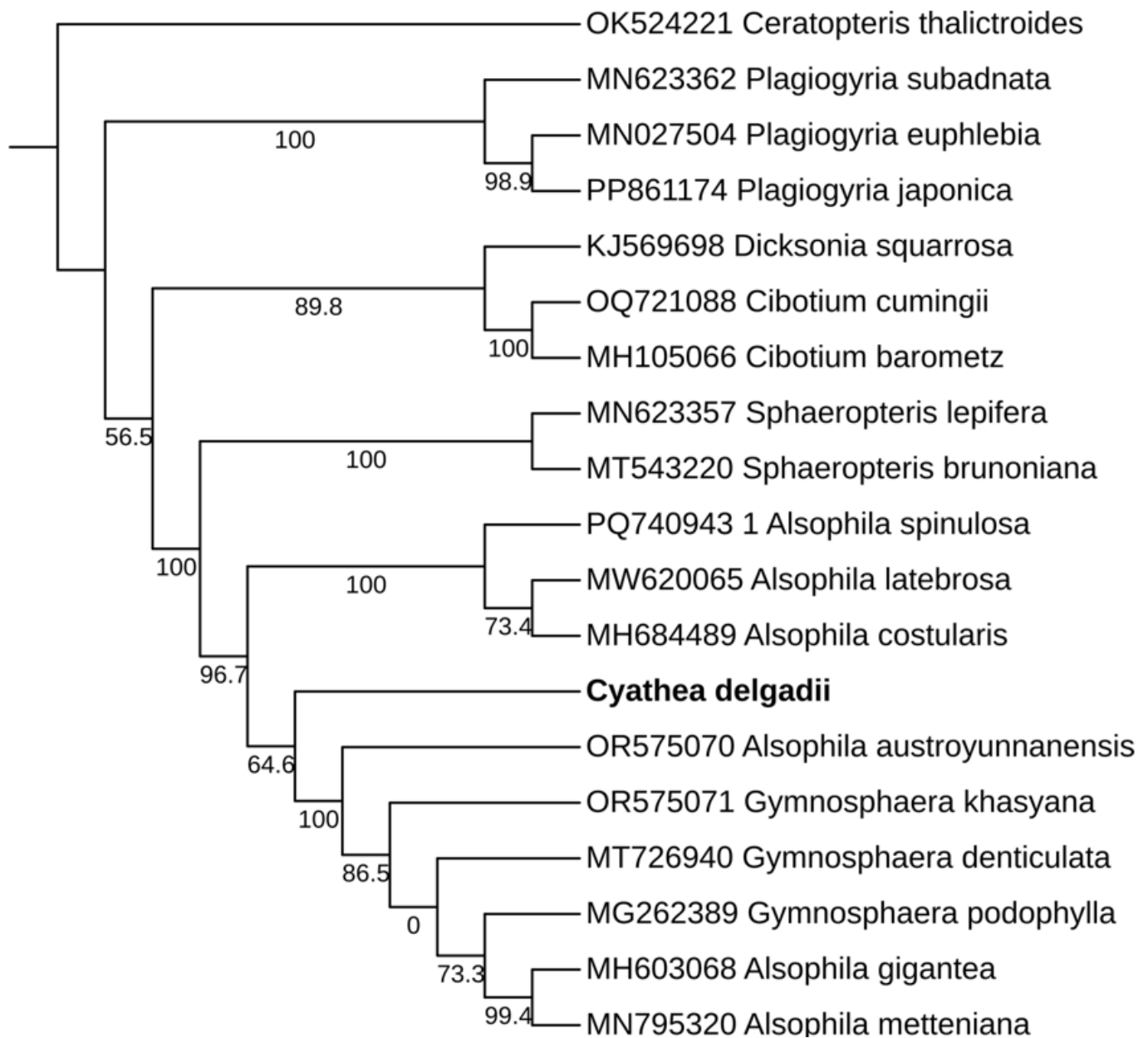


Figure 5

Polymorphism-based tree constructed using the method: (--auto) and GTR.

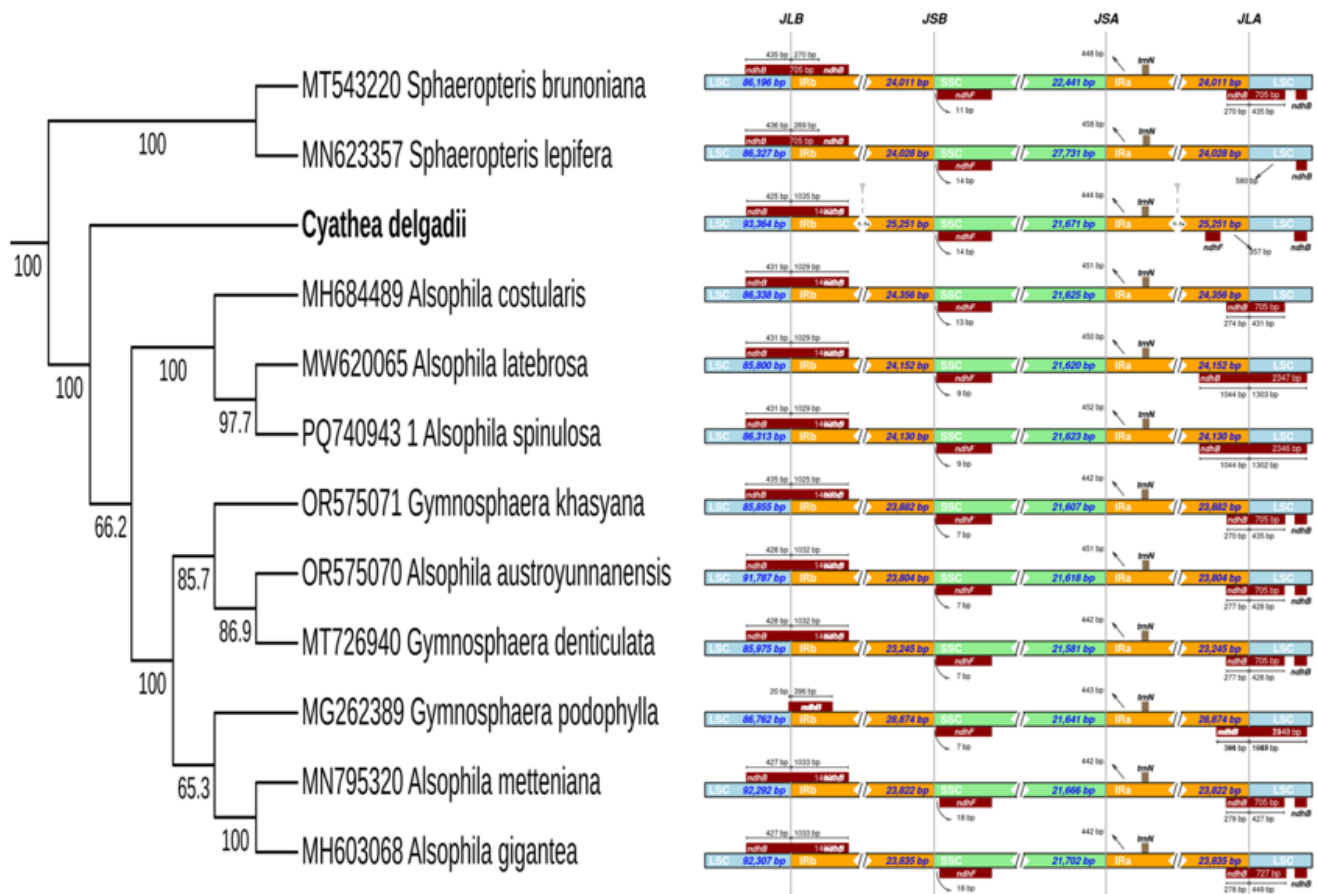


Figure 6

Comparison of chloroplast borders of the LSC, SSC, and IR regions among the species closest to *C. delgadii*.

Map of key genes highlighted in the literature as involved in rearrangements in the plastid genome of ferns.



Figure 8

Syntenic dotplot generated based on Minimap2 alignment between the cp genomes produced by D-GENIES. A: *Ceratopteris thalictroides*; B: *Dicksonia squarrosa*; C: *Cibotium barometz*; D: *Cibotium cumingii*; E: *Plagiogyria subadnata*; F: *Plagiogyria japonica*; G: *Plagiogyria euphlebia*; H: *Sphaeropteris brunoniana*; I: *Sphaeropteris lepifera*; J: *Alsophila costularis*; K: *Alsophila latebrosa*; L: *Alsophila spinulosa*; M: *Gymnosphaera khasyana*; N: *Alsophila austroyunnanensis*; O: *Gymnosphaera denticulata*; P: *Gymnosphaera podophylla*; Q: *Alsophila metteniana*; R: *Alsophila gigantea*. It is noteworthy that only *D. squarrosa* presents a truncated dot plot comparison in comparison to *C. delgadii*, which is the only organism analyzed that is not curated in the database and presents an incomplete region in the sequencing.

Supplementary Files

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