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The third opinion on fern phylogenetics with novel insights into their mitogenome evolution

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Abstract

The current understanding of fern phylogeny is primarily based on plastid and nuclear sequences, but the third genome—the mitogenome—has remained practically unstudied. We inferred the first broad scale fern phylogeny based on mitogenomic data, obtained from the One Thousand Plant Transcriptomes Initiative project, and compared it with the plastid phylogeny. The trees were mostly congruent and corresponded to the current understanding of the fern phylogeny, but we observed different evolutionary patterns between the two genomes. Protein-coding markers located in the plastome had, on average, over two times higher substitution rate than the markers from the mitogenome. The similar rate variation pattern between the genomes in different fern lineages supports the idea that a common mechanism, like life history traits, drives the rates of molecular evolution. The few conflicting nodes we observed have also been difficult to resolve in other studies, suggesting that even genomic data may not suffice to resolve them.

Key words: mitochondrial marker, plastid marker, plastome, purifying selection, RNA editing

Introduction

Since the early days of molecular systematics, molecular phylogenetic studies in land plants have heavily relied on the plastid genome (plastome), due to its high copy number and more stable structure compared to the other two genomes (mitogenome and nuclear genome) (Zardoya 2020). Thanks to the high-throughput sequencing techniques, studies using the other two genomes have recently become more common, resulting in improved understanding of the evolution of plants and their genomes (e.g., Guo *et al.* 2016, Jackman *et al.* 2020, Kan *et al.* 2020, Sullivan *et al.* 2020, Zardoya 2020, Feng & Wicke 2023, Zumkeller *et al*. 2023). Whereas the plastome is structurally rather conserved, the plant mitogenome varies greatly in size, gene structure, mutation rate, and level of RNA editing, hampering its assembly and use in phylogenetic studies (Ogihara *et al.* 2005, Richardson *et al.* 2013, Bonavita & Rosaria 2016, Small *et al.* 2020, Zardoya 2020, Zumkeller *et al*. 2023). Despite the highly variable structure of plant mitogenomes, it has been broadly demonstrated that mitochondrial genes in flowering plants have lower substitution rates than plastid genes (Wolfe *et al.* 1987, Palmer & Herbon 1988, Cho *et al.* 2004, Parkinson *et al.* 2005, Richardson *et al.* 2013), and that some species seem to have practically frozen mitogenome (Richardson *et al.* 2013).

Mitogenomic sequencing has been predominantly done in angiosperms (Kan *et al.* 2020), mainly because of their economic value and importance in agronomy (Knoop 2013). More recently, mitogenomic studies of plants other than angiosperms have also been published. For example, it was found that the mitogenome of *Cycas taitungensis* C.F.Shen, K.D.Hill, C.H.Tsou & C.J.Chen in Shen (1994: 135) contains more than twice as many RNA-editing sites than the angiosperm mitogenomes (Chaw *et al.* 2008). Liu *et al*. (2019) inferred the phylogeny of mosses based on broad sampling of mitochondrial, chloroplast, and nuclear markers, and they concluded that multigenome approach can give confidence for resolving dubious clades. This last finding supports the statement of Gitzendanner *et al*. (2018), who mentioned that plastome data alone are likely not enough to resolve deep-level phylogeny.

In ferns, the rapid increment and availability of complete plastomes have facilitated large-scale plastome-based phylogenomics (e.g., Grewe *et al.* 2013, Labiak & Karol 2017, Gitzendanner *et al.* 2018, Kuo *et al.* 2018a, Lehtonen & Cárdenas 2019, Lehtonen *et al.* 2020, Liu *et al*. 2020, Du *et al.* 2021, Wei *et al.* 2021). Simultaneously, massive nuclear

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data sets obtained using transcriptomes or target probe techniques and high-throughput sequencing have been used for exploring fern phylogenetics (Rothfels *et al.* 2015, Qi *et al.* 2018, Shen *et al.* 2018, Wolf *et al.* 2018, One Thousand Plant Transcriptomes Initiative 2019, Breinholt *et al.* 2021, Fawcett *et al*. 2021, Pelosi *et al*. 2022). In contrast, the mitogenome has remained practically unused in fern systematics.

There are only few studies that investigated mitochondrial data in ferns. Wikström & Pryer (2005) used a single mitochondrial marker (*atp1*) along with plastid and nuclear sequences to investigate deep nodes in the fern phylogeny, and Rothfels & Schuettpelz (2014) investigated the molecular evolutionary rate of vittarioid ferns using two mitochondrial markers (*atp1*, *nad5*), together with chloroplast and nuclear markers. Likewise, Vangerow *et al*. (1999) investigated the structure and RNA editing of a single mitochondrial gene (*nad5*) across the fern phylogeny. Knie *et al*. (2016) further explored RNA editing in fern plastomes and mitogenomes and reported very low levels of editing in Marattiales and *Equisetum*. To our knowledge the only study that has so far applied broad mitogenomic data in fern systematics focused on Marattiales and used plastome data together with 39 mitochondrial markers to resolve congruent trees between the two genomes (Lehtonen *et al.* 2020). So far, only five complete fern mitogenomes have been reported (Guo *et al.* 2017, Song *et al.* 2021, Feng & Wicke 2022).

The molecular systematic studies over the past years have resulted in a generally accepted consensus view on fern systematics (PPG I 2016). Yet, despite the increasing availability of molecular data, some phylogenetic relationships among ferns remain controversial, such as the positions of *Equisetum*, Marattiales, and Gleicheniales (e.g., Rothfels *et al.* 2015, Kuo *et al.* 2018a, Shen *et al*. 2018). If fern mitochondrial genes are evolving in a different manner than plastid genes, we could expect the mitogenomic data to provide a complementing source of data that may help to elucidating those uncertain relationships. It should be noted that so far only maternal inheritance of plastomes and mitogenomes have been reported in ferns, but these genomes occasionally have more complex inheritance in plants (Kuo *et al*. 2018b).

In this study, we make use of the One Thousand Plant Transcriptomes Initiative (2019) transcriptome data 1) to generate the first mitogenomic phylogeny based on a taxonomically broad set of ferns, 2) to compare these results with phylogenomic hypothesis built from plastome data and the current understanding of fern phylogenetics, and 3) to explore how the two organellar genomes differ in their molecular evolution and phylogenetic signal.

Materials and methods

Origin of the data

We used raw sequence data obtained from the One Thousand Plant Transcriptomes Initiative (1KP) (2019) web page open data archives, which has made available transcriptome sequencing data for over 1 000 species of plants (http://www.onekp.com/samples/list.php). These valuable transcriptome data (Matasci *et al.* 2014, Carpenter *et al.* 2019) have been used in various studies, for instance, to compare mitochondrial and plastid phylogenies to investigate bryophyte molecular evolution (Bell *et al.* 2020). Here, we explore its utility to generate a baseline of the fern mitochondrial phylogeny.

The 1KP archives hold data from 58 fern samples. In order to produce orthologous contig sequences of known identity, we mapped the raw 1KP reads to mitochondrial and plastid reference marker sequences downloaded from GenBank, using Geneious Prime version 2021.1.1. The mitochondrial reference marker sequences were extracted from the published mitogenome of *Ophioglossum californicum* Prantl (1883: 351) (NC_030900; Guo *et al.* 2017), and the plastid reference marker sequences were extracted from the plastome of *Lindsaea linearis* Swartz (1800: 78) (MK705751; Lehtonen & Cárdenas 2019), an early diverging member of Polypodiales and therefore phylogenetically in a rather intermediate position relative to our samples.

Raw reads were mapped to the reference marker sequences using Geneious default parameters (Low Sensitivity and Trim paired read overhangs). For the analyses we used the markers for which we recovered sequence data for more than 35% of the species, and the species for which we recovered more than 30% of markers. This resulted in 33 mitochondrial and 31 plastid markers (Tables 1, 2, respectively), and 51 species belonging to 29 families for the final analyses, representing a broad sample of taxonomic lineages across the fern phylogenetic tree.

TABLE 1. Species and molecular markers included in mitogenome analyses. Colour intensity represents five different categories of data completeness for each marker (0%, 1–25%, 26–50%, 51–75%, and 76–100%). No data (0%) is depicted with no colour and over 75% alignment completeness is depicted with the most intense colour.

Species name	Sequence length (bp)	N° markers per spp	atp1	atp9	cob	cox1	cox2	cox3	matR	mttB	nad1 exon1	nad1 exon2	nad1 exon3	nad1 exon4	nad1 exon5	nad2 exon1	nad2 exon2	nad2 exon4	nad2 exon5	nad4L	nad4 exon3	nad5 exon2	nad6	nad7 exon1	nad7 exon2	nad9	rpl16	rpl2 exon2	rps10	rps11	rps12	rps2	rps7	sdh3	sdh4
Outgroup																																			
Selaginella apoda (Linné 1753: 1105) Fernald (1915: 68)	7607	19																																	
Austrobaileya scandens White (1993: 29)	14180	31																																	
Magnolia grandiflora Linné (1759: 1082)	13557	31																																	
Piper auritum Kunth (1815: 45)	13210	30																																	
Dioon edule Lindley (1843: 59)	13094	31																																	
Taxus cuspidata Siebold & Zuccarini (1846: 232)	10245	28																																	
Wollemia nobilis Jones et al. (1995: 173)	13230	30																																	
Ingroup																																			
Adiantum aleuticum (Ruprecht 1845: 49) Paris (1991: 112)	11969	26																																	
Adiantum raddianum Presl (1836: 158)	15673	33																																	
Anemia tomentosa (Savigny in Lamarck 1797: 652) Swartz (1806: 157)	8860	21																																	
Asplenium nidus Linné (1753: 1079)	6484	17																																	
Asplenium platyneuron (Linné 1753: 1069) Britton et al. (1888: 73)	13776	30																																	
Athyrium filix-femina (Linné 1753: 1090) Roth (1799: 65)	5615	15																																	
Blechnum spicant (Linné 1753: 1066) Roth (1794: 56)	14853	33																																	
Bolbitis repanda Schott (1834: 14)	8235	21																																	
Botrypus virginianus (Linné 1753: 1064) Michaux (1803: 274)	12346	31																																	
Ceratopteris thalictroides (Linné 1753: 1070) Brongniart (1821: 186)	14105	31																																	
Cryptogramma acrostichoides Brown in Franklin (1823: 767)	14475	31																																	
Culcita macrocarpa Presl (1836: 135)	11929	30																																	
Cystopteris fragilis (Linné 1753: 1091) Bernhardi (1805: 26)	13312	31																																	
Cystopteris protrusa (Weatherby 1935: 373) Blasdell (1963: 41)	11801	26																																	
Cystopteris reevesiana Lellinger (1981: 92)	13862	30																																	
Cystopteris utahensis Windham & Haufler (1991: 13)	12555	28																																	
Danaea nodosa (Linné 1753: 1070) Smith (1793: 420)	13676	29																																	
Dennstaedtia davallioides (Brown 1810: 158) Moore (1861: 305)	10211	23																																	
Deparia lobato-crenata (Tagawa) Kato (1977: 37)	9301	23																																	
Didymochlaena truncatula (Swartz 1801: 36) Smith (1841: 196)	5010	14																																	
Diplazium wichurae (Mettenius 1866: 237) Diels (1899: 226)	11357	27																																	
Dipteris conjugata Reinwardt (1828: 256)	13484	31																																	
Equisetum hyemale Linné (1753: 1062)	12559	27																																	
Gaga arizonica (Maxon 1918: 116) Fay W.Li & Windham in Li et al. (2012: 856)	12134	24																																	
Gymnocarpium dryopteris Newman (1851: XXIV)	11439	24																																	
Homalosorus pycnocarpos (Sprengel 1804: 112) Pichi Sermolli (1977: 246)	7951	18																																	
Hymenophyllum bivalve Smith (1841: 418)	5862	15																																	
Lindsaea linearis Swartz (1800: 78)	7393	13																																	
Lindsaea microphylla Swartz (1800: 79)	7286	20																																	
Lonchitis hirsuta Linné (1753: 1078)	11551	24																																	
Lygodium japonicum (Thunberg in Murray 1784: 926) Swartz (1800: 106)	8640	20																																	
Marattia attenuata LaBillardiere (1824: 9)	15470	32																																	
Myriopteris rufa Fée (1857: 77)	12318	24																																	
Nephrolepis exaltata (Linné 1759: 1326) Schott (1834: 3)	7700	15																																	
Onoclea sensibilis Linné (1753: 1062)	8963	20																																	
Ophioglossum vulgatum Linné (1753: 1062)	3458	12																																	
Osmunda javanica Blume (1828: 252)	7454	16																																	
Osmundastrum cinnamomeum (Linné 1753: 1066) Presl (1847: 326)	13596	30																																	
Pityrogramma trifoliata (Linné 1753: 1070) Tryon (1962: 68)	12661	26																																	
Plagiogyria japonica Nakai (1928: 206)	13699	31																																	
Pleopeltis polypodioides (Linné 1753: 1068) Andrews & Windham (1993:46)	15512	33																																	
Polystichum acrostichoides (Michx.) Schott (1834: 10)	9817	23																																	
Pteris ensiformis Burman (1768: 230)	14839	31																																	
Pteris vittata Linné (1753: 1074)	12045	26																																	
Thelypteris acuminata (Houttuyn 1783: 181) Morton (1959: 139)	6053	16																																	
Thyrsopteris elegans Kunze (1835: 507)	11688	28																																	
Tmesipteris parva Wakefield (1944: 143)	12104	29																																	
Vittaria appalachiana Farrar & Mickel (1991: 72)	12872	27																																	
Vittaria lineata (Linné 1753: 1073) Smith (1793: 421)	10766	24																																	
Woodsia ilvensis (Linné 1753: 1071) Brown (1810: 158)	13246	29																																	
Woodsia scopulina Eaton (1865: 91)	13550	28																																	
N° spp per marker in ingroup			51	46	50	44	47	47	43	17	26	32	24	20	24	16	37	40	34	31	35	45	45	42	45	32	46	48	50	46	50	40	44	39	30
Average read coverage in ingroup			173	68	54	70	34	30	25	12	15	12	17	15	20	9	15	17	18	15	34	20	13	22	37	20	55	37	60	108	35	68	40	27	20

We included in our analyses seven outgroup taxa selected from the 1KP dataset. These represented gymnosperms (three species: *Dioon edule* Lindley (1843: 59), *Taxus cuspidata* Siebold & Zuccarini (1846: 232), and *Wollemia nobilis* Jones *et al*. (1995: 173)), angiosperms (three species from the relatively early diverging lineages: *Austrobaileya scandens* White (1993: 29), *Magnolia grandiflora* Linné (1759: 1082), and *Piper auritum* Kunth (1815: 45)), and one lycophyte (*Selaginella apoda* (Linné 1753:1105) Fernald (1915: 68)). Sequence data were recovered and processed in the same way as for the ingroup species, but in order to improve the read match we used as additional reference sequences annotated genes from the available mitogenomes and plastomes of *Ginkgo biloba* Linné (1771: 313) (NC_027976, NC_016986) and *Selaginella moellendorffii* Hieron. in Engler & Prantl (1902: 680) (GL377739, HM173080; Banks *et al.* 2011).

Phylogeny reconstruction

We aligned the obtained marker sequences with MAFFT version 7.475 (Katoh & Standley 2013) using the default parameters. The single-marker alignments ranged from 57 to 1 452 bp in the mitochondrial dataset and from 246 to 2 253 bp in the plastid dataset. The sequence alignments were manually adjusted, when necessary, for example, by cutting the leading and trailing edges of highly varying completeness, in Mesquite version 3.70 (Maddison & Maddison 2021).

We first tested whether the markers of each organelle supported congruent phylogenetic signal by inferring trees for each mitochondrial and plastid marker in RAxML version 8.0.0 (Stamatakis 2014). We set the RAxML substitution

model to GTRGAMMA and ran rapid bootstrap analyses for each marker using 500 replicates followed by a full ML analysis. After verifying that individual marker trees did not show any significantly supported conflicting nodes (>70% bootstrap support) with iTOL version5 (Letunic & Bork 2021), we concatenated the markers into mitogenomic and plastome data sets for further analyses using SequenceMatrix version 1.9 (Vaidya *et al.* 2011). Final mitogenomic and plastome data matrices as well as their corresponding final trees are available in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S30584?x-access-code=b1821f4693fb19e6d713794111ab507&format=html).

TABLE 2. Species and molecular markers included in plastome analyses. Colour intensity represents five different categories of data completeness for each marker (0%, 1–25%, 26–50%, 51–75%, and 76–100%). No data (0%) is depicted with no colour and over 75% alignment completeness is depicted with the most intense colour.

Species name	Sequence length (bp)	N° markers per spp	accD	atpA	atpB	atpE	atpF	atpH	atpI	cemA	chlB	chlL	clpP	infA	ndhA	ndhB	ndhE	ndhI	ndhJ	ndhK	petA	petB	petD	psaA	psaB	psbA	psbB	psbC	psbD	psbE	rbcL	rpl16	rps7
Outgroup																																	
Selaginella apoda	20960	30																															
Austrobaileya scandens	15146	15																															
Magnolia grandiflora	12708	18																															
Piper auritum	15017	18																															
Dioon edule	16862	20																															
Taxus cuspidata	15959	18																															
Wollemia nobilis	17832	20																															
Ingroup																																	
Adiantum aleuticum	22463	28																															
Adiantum raddianum	24821	29																															
Anemia tomentosa	20564	24																															
Asplenium nidus	6931	11																															
Asplenium platyneuron	22267	26																															
Athyrium filix-femina	14663	17																															
Blechnum spicant	24568	28																															
Bolbitis repanda	17606	18																															
Botrypus virginianus	15792	20																															
Ceratopteris thalictroides	23120	28																															
Cryptogramma acrostichoides	25528	28																															
Culcita macrocarpa	26352	31																															
Cystopteris fragilis	25468	30																															
Cystopteris protrusa	24745	29																															
Cystopteris reevesiana	25742	31																															
Cystopteris utahensis	25422	30																															
Danaea nodosa	20630	23																															
Dennstaedtia davallioides	20889	26																															
Deparia lobato-crenata	17340	22																															
Didymochlaena truncatula	12552	19																															
Diplazium wichurae	24856	30																															
Dipteris conjugata	21033	26																															
Equisetum hyemale	15401	17																															
Gaga arizonica	25517	31																															
Gymnocarpium dryopteris	24294	28																															
Homalosorus pycnocarpos	18620	24																															
Hymenophyllum bivalve	13040	19																															
Lindsaea linearis	26369	31																															
Lindsaea microphylla	20349	26																															
Lonchitis hirsuta	22709	27																															
Lygodium japonicum	20701	25																															
Marattia attenuata	19684	24																															
Myriopteris rufa	23899	28																															
Nephrolepis exaltata	18267	23																															
Onoclea sensibilis	18974	22																															
Ophioglossum vulgatum	14014	15																															
Osmunda javanica	19772	24																															
Osmundastrum cinnamomeum	20816	25																															
Pityrogramma trifoliata	22334	28																															
Plagiogyria japonica	25726	29																															
Pleopeltis polypodioides	19625	23																															
Polystichum acrostichoides	23739	29																															
Pteris ensiformis	23909	29																															
Pteris vittata	23951	29																															
Thelypteris acuminata	11983	17																															
Thyrsopteris elegans	24922	30																															
Tmesipteris parva	19068	21																															
Vittaria appalachiana	22158	25																															
Vittaria lineata	18003	22																															
Woodsia ilvensis	25044	30																															
Woodsia scopulina	24772	30																															
N° of spp per marker in ingroup			39	49	42	38	25	38	45	23	43	46	43	30	35	37	30	32	30	36	42	47	47	49	49	51	50	51	49	47	50	42	50
Average read coverage in ingroup			42	110	250	187	46	269	92	46	68	316	178	50	57	103	83	123	232	110	117	165	168	917	877	811	308	874	988	264	344	322	208

Phylogeny calibration

We used the greedy algorithm in Partition Finder version 2.1.1 (Guindon *et al.* 2010, Lanfear *et al.* 2012, Lanfear *et al.* 2016) to select the best partition scheme and substitution models for the data under the Bayesian Information Criterion (BIC). We tested data partitioning by genes, by codons position, and by relative evolutionary rates using the approach described by Rota *et al*. (2018) with a division factor ranging from 2 to 16. The preferred scheme split both the mitogenome and plastome markers by codon positions into 12 partitions (Table 3).

We used BEAUti2 version 2.6.6 (Bouckaert *et al.* 2019) to set up the parameters for Bayesian inference in BEAST2 2.6.6 (Bouckaert *et al.* 2019). The site and clock models were unlinked, but tree model was linked between the partitions. We applied an exponential relaxed clock (Bouckaert *et al.* 2019) and birth-death tree model (Bouckaert *et al.* 2019). For the internal node calibration, we used 23 calibration points and applied uniform priors (Table 4). The minimum ages were set according to the estimated minimum ages of the selected fossils and the maximum age of 471 Ma was applied for all calibrated nodes based on the oldest known assemblages of land plants (Rubinstein *et al.* 2010). The same priors were applied in the mitogenome and plastome analyses. For both of these genomes, we performed five chains of 250 000 000 generations each, and sampled the chains every 10 000 generations. We also ran the analyses without data, i.e., sampling from the prior, to verify the effective priors and to compare them with the posterior. The prior was sampled like the posterior by performing five chains, but with ten times more generations in order to achieve convergence. The chains were combined with LogCombiner v2.6.6 (Rambaut & Drummond 2002–2021a) after removing the first 20 % of each chain as burn-in. TreeAnnotator (Rambaut & Drummond 2002–2021b) was used to obtain the maximum clade credibility trees. The resulting trees were visualised in FigTree v1.4.4. (Rambaut 2018). Convergence of the runs and effective sample sizes (ESS) of estimated parameters were checked in Tracer version 1.7.2 (Rambaut *et al.* 2018).

In order to compare the diversification patterns inferred from the two genomes we computed lineages-through-time (LTT) plots for 100 posterior trees randomly picked for both genomes using an R package ‘ape’ (Paradis & Schliep 2019).

Comparing molecular evolution between the organellar genomes

We used PhyKIT (Steenwyk *et al.* 2021) to compare evolutionary rates between the plastome and the mitogenome. These comparisons were based on individual marker trees inferred with RAxML, after removing the outgroups using the R package ‘ape’ (Paradis & Schliep 2019). In addition, we investigated evolutionary rates in some focal fern groups with strikingly short or long branches in ML trees by comparing the rates of these branches as estimated in the BEAST2 analyses. The average rates for the focal groups were calculated by multiplying the estimated mean rate of each branch belonging to the group by respective branch lengths (duration in time), summing up these multiplied rates and dividing the sum by the summed branch lengths (total time).

We furthermore calculated treeness divided by relative composition variability (RCV) index in PhyKIT. Treeness measures how equally the total tree length is divided among internal branches, whereas RCV indicates the degree to which site composition homogeneity is violated (Steenwyk *et al.* 2021). The combination of these metrics can be used to measure biases with negative impact on phylogenetic inference, with higher values of treeness/RCV index expected to produce trees of higher accuracy (Steenwyk *et al.* 2021).

We detected the presence of stop codons in some of the sequence reads, while other sequence reads lacked them. The stop codons were evident in specific sites in the alignment of various species, indicating that they represented a real pattern and were not just random sequencing errors. Given that RNA editing efficiency vary between tissues and developmental conditions so that partially edited sites can be present in the transcripts (Small *et al.* 2020), we assume these stop codons to represent sites of RNA editing. We counted the presence of these stop codons in each marker and reported the number in this study. However, these counts should be taken as minimum number estimates of the RNA editing sites, because our limited data most certainly prevented us from detecting all the sites with variable transcripts and we only counted premature stop codons, while in fact, other codons can as well be edited.

TABLE 3. Best partition schemes and substitution models for the mitogenome and plastome data obtained under Bayesian Information Criterion (BIC).

Organelles	Best Model	N° of sites	Markers in each partition
Mitochondria	GTR+I+G+X	741	rps12_pos1, atp1_pos1, rpl16_pos1
Mitochondria	GTR+I+G+X	768	atp1_pos2, cox1_pos2, atp9_pos2
Mitochondria	GTR+G+X	1529	atp1_pos3, nad1_exon1_pos3, rps10_pos3, rpl2_exon2_pos1, rps11_pos3, rps7_pos3, nad1_exon2_pos3, rpl16_pos3, rps12_pos3
Mitochondria	GTR+G+X	1539	nad7_exon2_pos1, atp9_pos1, nad1_exon4_pos1, nad5_exon2_pos1, cox1_pos1, nad1_exon3_pos1, nad7_exon1_pos1, nad4L_pos1, rps12_pos2, nad4_exon3_pos1, nad2_exon4_pos1
Mitochondria	GTR+G+X	1552	nad9_pos2, rps10_pos1, nad9_pos1, nad2_exon1_pos1, rps10_pos2, nad6_pos1, sdh4_pos1, rps11_pos2, rps2_pos2, rps7_pos2, atp9_pos3, rps2_pos1
Mitochondria	GTR+G+X	1614	nad1_exon1_pos1, cob_pos1, cox3_pos1, nad2_exon2_pos1, cox2_pos1, nad1_exon2_pos2, nad1_exon2_pos1, nad1_exon1_pos2, nad1_exon5_pos1, nad2_exon1_pos2, nad2_exon5_pos2, nad2_exon5_pos1
Mitochondria	GTR+G+X	1534	cob_pos2, nad5_exon2_pos2, nad1_exon3_pos2, nad7_exon2_pos2, cox2_pos2, cox3_pos2
Mitochondria	GTR+G+X	2385	rps2_pos3, nad9_pos3, mttB_pos3, sdh3_pos1, mttB_pos1, sdh3_pos3, nad2_exon1_pos3, sdh4_pos3, nad2_exon5_pos3, cox2_pos3, nad4L_pos3, cox3_pos3, cob_pos3, nad6_pos3
Mitochondria	GTR+G+X	1460	nad5_exon2_pos3, nad2_exon2_pos3, cox1_pos3, nad1_exon5_pos3, nad4_exon3_pos3, nad2_exon4_pos3, nad7_exon2_pos3, nad1_exon3_pos3, nad7_exon1_pos3, nad1_exon4_pos3
Mitochondria	GTR+G+X	1473	rpl16_pos2, matR_pos2, matR_pos3, rps7_pos1, rps11_pos1, matR_pos1, rpl2_exon2_pos3, rpl2_exon2_pos2
Mitochondria	GTR+G+X	627	sdh4_pos2, nad6_pos2, mttB_pos2, sdh3_pos2
Mitochondria	GTR+I+G+X	714	nad4L_pos2, nad1_exon4_pos2, nad4_exon3_pos2, nad7_exon1_pos2, nad2_exon2_pos2, nad2_exon4_pos2, nad1_exon5_pos2
Plastid	GTR+G+X	1271	ndhB_pos1, accD_pos1, ndhE_pos1, atpF_pos1, infA_pos1, cemA_pos1, infA_pos2, cemA_pos2
Plastid	GTR+G+X	760	accD_pos2, atpF_pos2, rps7_pos1, rps7_pos2
Plastid	GTR+I+G+X	4203	atpH_pos3, petB_pos3, atpI_pos3, clpP_pos3, atpB_pos3, petD_pos3, accD_pos3, atpE_pos3, cemA_pos3, petA_pos3, infA_pos3, atpF_pos3, chlB_pos3, atpA_pos3, rpl16_pos3, ndhI_pos3, ndhA_pos3, ndhE_pos3
Plastid	GTR+I+G+X	1823	rbcL_pos1, chlL_pos1, atpA_pos1, atpB_pos1, clpP_pos1
Plastid	GTR+I+G+X	4080	clpP_pos2, psbE_pos2, atpA_pos2, atpB_pos2, atpI_pos2, petD_pos2, psaB_pos2, chlL_pos2, atpH_pos2, psaA_pos2, petB_pos2, psbC_pos2
Plastid	GTR+I+G+X	2238	ndhA_pos1, rpl16_pos1, ndhK_pos1, ndhI_pos1, chlB_pos1, atpI_pos1, ndhJ_pos1, petA_pos1, atpE_pos1
Plastid	GTR+I+G+X	2342	ndhB_pos2, ndhA_pos2, ndhE_pos2, ndhJ_pos2, atpE_pos2, petA_pos2, chlB_pos2, ndhK_pos2, ndhI_pos2, rpl16_pos2
Plastid	GTR+I+G+X	2833	psaB_pos1, psaA_pos1, psbE_pos1, atpH_pos1, petD_pos1, petB_pos1, psbC_pos1, psbD_pos1
Plastid	GTR+I+G+X	247	chlL_pos3
Plastid	GTR+G+X	499	rps7_pos3, ndhB_pos3, psbE_pos3
Plastid	GTR+I+G+X	3139	ndhJ_pos3, ndhK_pos3, psbC_pos3, psbD_pos3, rbcL_pos3, psaB_pos3, psaA_pos3
Plastid	GTR+I+G+X	830	rbcL_pos2, psbD_pos2

TABLE 4. Prior calibration points and their references used to calibrate 23 points in this study with their posterior age estimates. All the ages indicated are stem ages except for the one marked with an asterisk, for which crown age is indicated to facilitate comparison with other studies.

Taxa	References for priors	Priors	Results of this publication		Testo & Sundue 2017	Rothfels *et al*. 2016	Lehtonen *et al*. 2018
			Age (mitogenome)	Age (plastome)	Age (cpDNA)	Age (nDNA)	Age (cpDNA)
Tracheophyta	Morris *et al*. 2018	420.7					
Euphyllophyta	Morris *et al*. 2018	385.57	469.18	466.77			
Spermatophyta	Morris *et al*. 2018	308.14	465.3	458.73			
Acrogymnospermaceae	Morris *et al*. 2018	308.14	323.94	337.39			
Agiospermae	Morris *et al*. 2018	125	323.94	337.39			
Magnoliids	Morris *et al*. 2018	110.87	133.08	134.38			
Ferns *	Stewart & Rothwell 1993	359	387.81	458.73	431.1	381.1	421.3
Equisetaceae	Stewart & Rothwell 1993	359	387.81	408.73	431.1	381.1	421.3
Leptosporangiates	Kenrick & Crane 1997	345	364.99	369.54	365.88	329.04	414.34
Marattiales	Gerrienne *et al*. 1999	323	348.99	369.54	365.88	329.04	414.34
Ophioglossales	Naugolnykh 2016	312	317.81	330.97	368.81	250.55	244.2
Gleichenaceae	He *et al*. 2021	298	247.91	260.96	278.13	196.09	289.44
Hymenophyllales	Axsmith *et al*. 2001	228	247.91	281.26	345.4	237.23	315.27
Osmundaceae	Serbet & Rothwell 1999	201.6	305.14	324.85			241.26
Lygodiaceae	Wikström *et al*. 2002	168	171.74	180.75	298.27	185.51	250.76
Cyatheales	Lantz *et al*. 1999	145	160.07	198.04	281.88	204.63	279
Thyrsopteridaceae	Li *et al*. 2020	98	122.94	115.52	206.54	71.69	140.81
Lindsaceae	Schneider & Kenrick 2001	99.6	128.92	147.21	247.07	184.18	182.43
Pteridaceae	Regalado *et al*. 2019	98	110.53	143.19	184.53	165.44	223.92
Eupolypods	Regalado *et al*. 2018	93.5	110.53	143.19	227.5	146.06	213.89
Thelypteridaceae	Berry 2019	66	68.35	70.32	117.03	71.07	119.5
Ceratopteris	Bonde & Kumaran 2002	65.5	74.92	85.23	184.53		144.2
Onoclea	Rothwell & Stockey 1991	55.8	72.58	57.8	85.93	28.81	66.46

Results

The total number of characters (bp) present in the final concatenated alignment was 15 936 and 26 854, in the mitochondrial and plastid datasets, respectively. The mitochondrial data matrix had 31% missing data and the plastid data matrix 26%. The average number of markers recovered per species was 25 from each organelle. The highest number of recovered markers per species included in the analysis was 33 for the mitogenome and 31 for the plastome, and the lowest number was 12 for the mitogenome and 11 for the plastome. The read coverage per marker varied between 9–173 in the mitochondrial data and 40–988 in the plastome data. Detailed information of the mitogenome and the plastome markers as well as their completeness is shown in Tables 1, 2, respectively.

In the plastid data set four markers were partially overlapping. The 5'—end of *ndhK* was overlapping with the 3'—end of *ndhC* and the 5'—end of *psbC* with the 3'—end of *psbD*. For the analysis, we entirely excluded *ndhC* because of the many ambiguities and incompleteness of the sequences. In the case of *psbD* and *psbC*, we included the complete *psbD*, but excluded the overlapping part from *psbC*.

Mitochondrial fern phylogeny reconstruction

Effective sample sizes (ESS) values, when sampling the "posterior", were generally high for both the mitogenome (for most parameters ESS well over 500) and the plastome (for most parameters ESS well over 300). However, the "prior"

did not converge as well and had low ESS values (99 and 69 for the mitogenome and the plastome, respectively). We assume that the poor convergence was caused by the high number of prior calibration points, forcing the prior to jump between a relatively limited number of possible conflicting states. Similarly, in the mitogenome data the "posterior" for the *Onoclea* Linné (1753: 1062) age estimate was apparently multimodal, probably caused by topological conflict, and resulted in a low ESS value (82). We do not believe these few poorly converged parameters compromise our results.

The inferred Bayesian tree topologies were mostly congruent between the genomes and similar to the general current understanding of the fern molecular phylogeny (Testo & Sundue 2016, Qi *et al.* 2018, Shen *et al.* 2018, Nitta *et al.* 2022). Our phylogenetic trees were generally highly supported, but the mitogenome tree had less fully supported nodes than the plastome tree (Fig. 1A, 2); while there were 17 nodes without full support (PP<1) in the mitogenome tree, the plastome tree had only five such nodes.

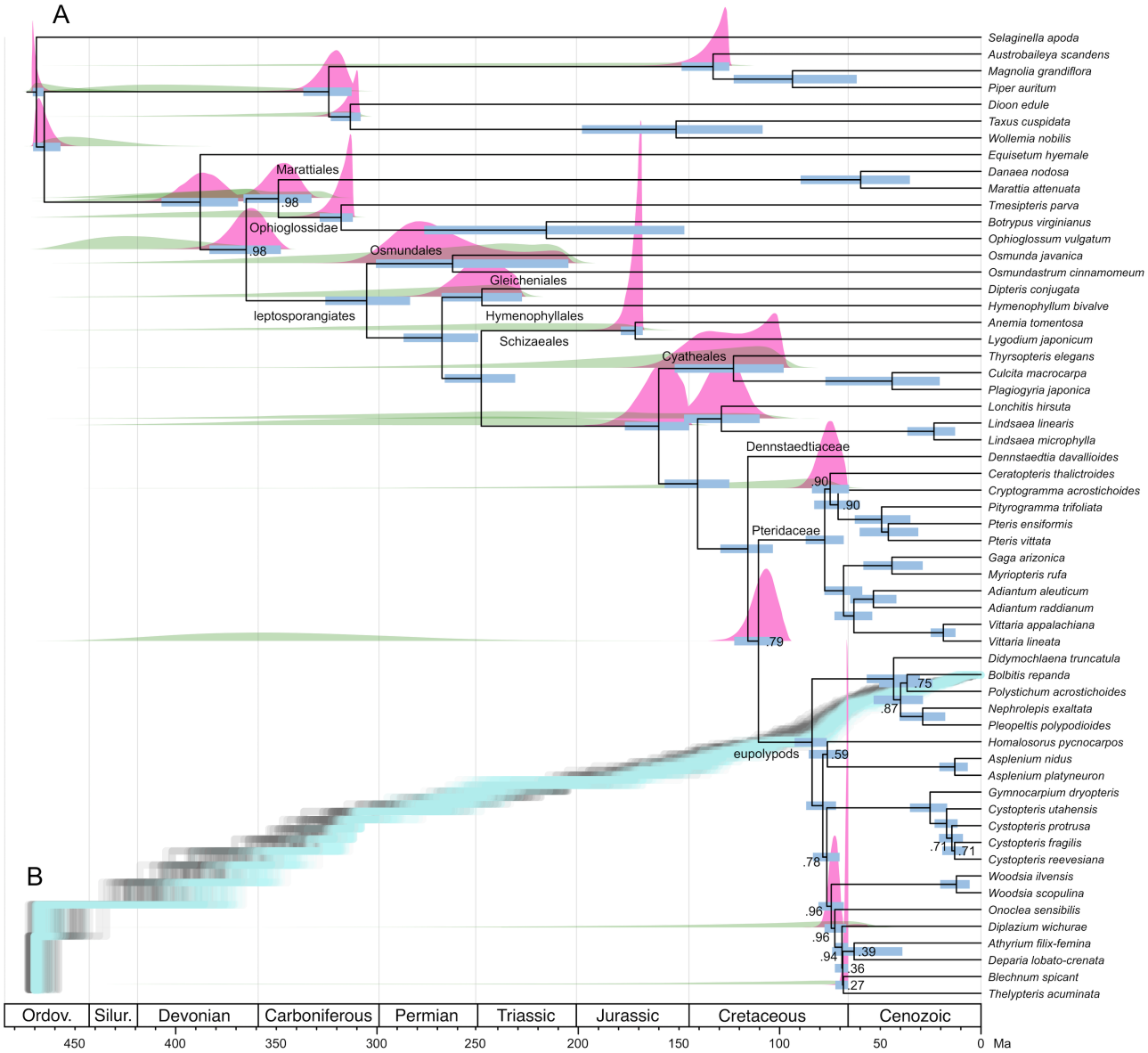

FIGURE 1. A. Time calibrated mitogenome tree based on 51 fern species and 33 markers. Blue horizontal bars show the 95% HPD ranges of estimated node ages. For the calibrated nodes both effective prior and posterior distributions are shown. Green curves represent the effective prior and magenta curves posterior distributions. Posterior Probability values are shown only for those nodes that were not fully supported. B. Lineages through time (LTT) plots for mitogenome shown in pale blue and plastome trees shown in grey. For LTT plots 100 trees were randomly picked from the posterior of both genomes.

Despite the general agreement between our mitogenome and plastome trees, some topological discordance was found between them. Marattiales (represented by *Danaea* Smith (1793: 420) + *Marattia* Swartz (1788: 128) was resolved as sister to Ophioglossidae (represented by *Tmesipteris* Bernhardi (1800: 131) + *Botrypus* Rich. ex Michx. in Michaux (1803: 274) + *Ophioglossum* Linné (1753: 1062) (with posterior probability pp = 0.98) in the mitogenome

tree (Fig. 1A), while in the plastome tree Marattiales was sister to the leptosporangiate ferns (pp = 1) (Fig. 2). Within leptosporangiates, *Dipteris* Reinw. in Hornschuch (1825: 3) and *Hymenophyllum* Smith (1793: 418) were resolved as a clade in the mitogenome tree (pp = 1), while in the plastome tree *Hymenophyllum* was resolved as sister to *Dipteris* and to the other non-osmundalean leptosporangiates (pp = 1). In the mitogenome tree, *Dennstaedtia* Bernhardi (1800: 124) was resolved as sister to the Pteridaceae + the eupolypods (pp = 0.79), but as sister to Pteridaceae in the plastome tree (pp = 0.95). The relationships between *Woodsia* Brown (1810: 158), *Onoclea*, *Thelypteris* Schott in Adanson (1763: 610), *Blechnum* Linné (1753: 1077), *Diplazium* Swartz (1801: 61), *Athyrium* Roth (1799: 31) and *Deparia* Hooker & Greville (1831 [1829]: 154) also varied between the trees (Figs. 1, 2). All the conflicting nodes were poorly supported in one or both of the trees (Fig. 1, 2).

The ML trees of the concatenated data were mostly congruent with the Bayesian trees, but Marattiales, Ophioglossidae, and *Equisetum* formed an unsupported clade in the mitogenome tree, and in the plastome tree *Dennstaedtia* was resolved as sister to Pteridaceae + eupolypods, however, without support (Fig. 3).

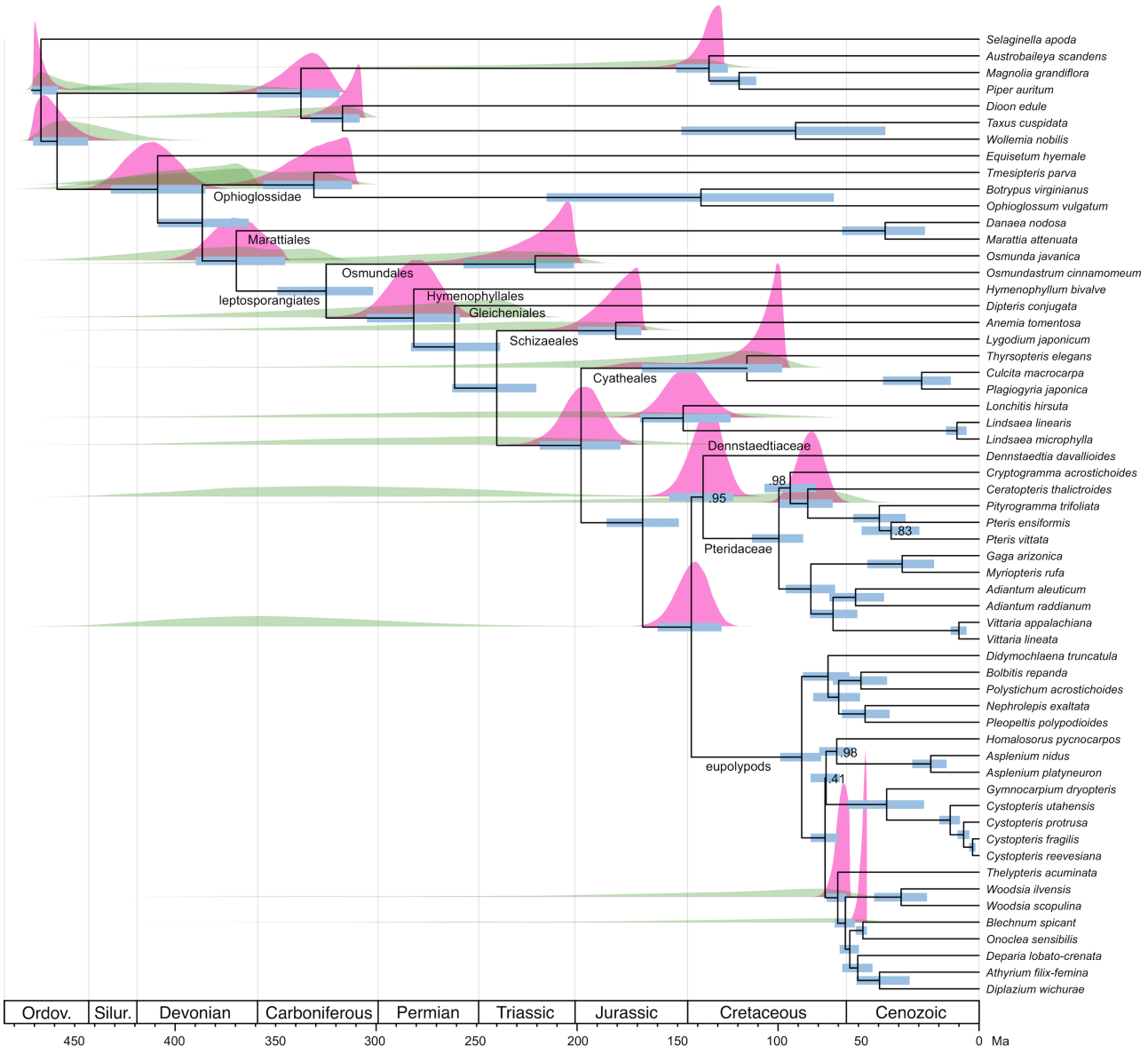

FIGURE 2. Time calibrated plastome tree based on 51 fern species and 31 markers. Blue horizontal bars show the 95% HPD ranges of estimated node ages. For the calibrated nodes both effective prior and posterior distributions are shown. Green curves represent the effective prior and magenta curves posterior distributions. Posterior Probability values are shown only for those nodes that were not fully supported.

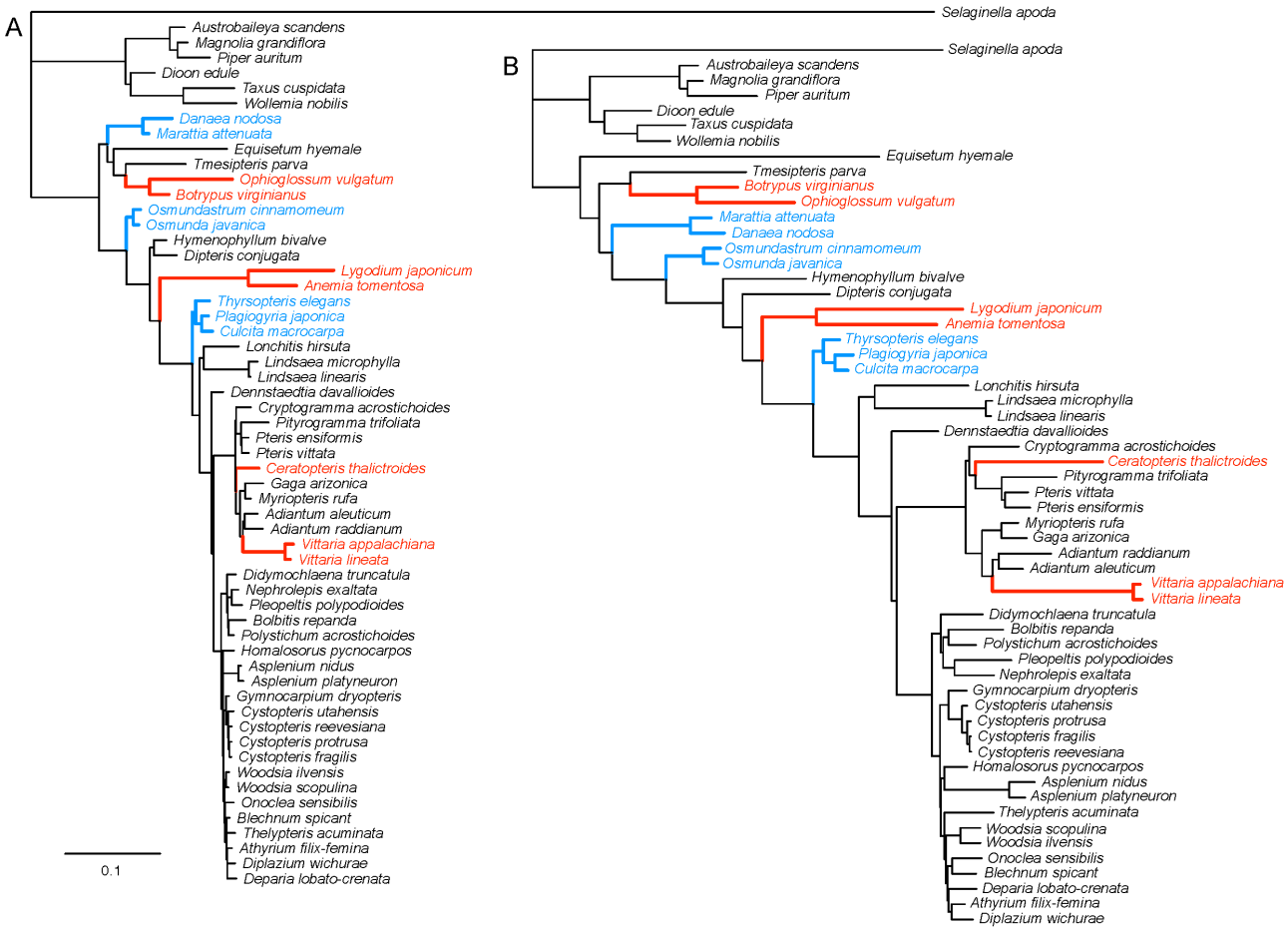

FIGURE 3. Lineages with conspicuous evolutionary rates discussed in the text (high rate indicated in red and low rate in blue). A. Maximum likelihood mitogenome phylogram. B. Maximum likelihood plastome phylogram.

Insights into evolutionary rates

A notable distinction between the genomes was the difference in estimated evolutionary rates. The evolutionary rate (measured for each marker as total tree length in PhyKIT) was on average more than two times higher in plastome markers (0.07 vs. 0.03) (Fig. 4). These inferences from the ML branch lengths were paralleled in the Bayesian time-calibrated analyses, whereby a higher mean rate was estimated for the plastome (Table 5). It should be noted that the rate estimates are affected by the RNA editing, as our data is based on transcriptomes with uncertain amount of edited sites.

TABLE 5. Mean evolutionary rates of mitochondrial and plastid markers in specific lineages, the rates relative to average rates of ferns, and the plastome to mitogenome ratio of rates.

Clade	Substitution rate		Relative rate		Ratio
	mitogenome	plastome	mitogenome	plastome	mitogenome / plastome
Ferns	1.94E-04	2.00E-04			1.03
Marattiales	9.449E-05	5.632E-05	0.49	0.28	0.60
Osmundales	4.06E-05	7.46E-05	0.21	0.37	1.84
Ophioglossidae	2.19E-04	1.24E-04	1.13	0.62	0.57
Cyatheales	1.96E-04	1.30E-04	1.01	0.65	0.66
Schizaeales	4.43E-04	2.67E-04	2.29	1.33	0.60
Ceratopteris	3.18E-04	5.37E-04	1.64	2.68	1.69
Vittaria	3.09E-04	5.39E-04	1.60	2.69	1.74
Asplenium	2.30E-04	4.72E-04	1.19	2.36	2.06

Several fern lineages showed elevated or decelerated rates. For example, the plastome markers in *Vittaria* Smith (1793: 431) evolved at a rate 2.7 faster than the fern average and the mitogenome markers 1.6 times faster (Table 5). Other fast evolving lineages were Schizaeales, *Ceratopteris* Brongniart (1821: 186), and *Asplenium* Linné (1753: 1078). Schizaeales had the mitogenome evolving at a rate 2.3 times higher than the average and plastome evolving at a rate 1.3 times faster than the average. Because of the very high rate in mitogenome, the plastome in Schizaeales actually had a lower rate than the mitogenome. This was also the case for Cyatheales and Marattiales, but these lineages generally had low evolutionary rates. Another slowly evolving lineage was Osmundales.

The average treeness/RCV index was higher for plastome than mitogenome markers, thus indicating that the mitogenome markers were on average more susceptible for biases that may negatively affect the phylogenetic inference (Fig. 4).

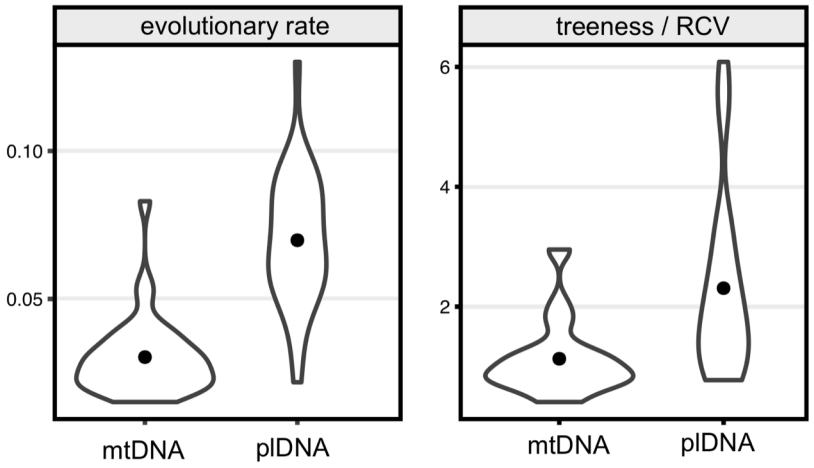

FIGURE 4. Molecular evolutionary patterns in the mitogenome and plastome markers. Mean values for each metric are represented by black points.

Insights about RNA-editing sites in the mitochondrial genome

We found presumed RNA-editing sites in 31 of the 33 mitogenome markers (94%) but only in eight of the 31 plastome markers (26%). The most recurrent presumed editing site detected was found in the codon UAA/CAA, but there were also editing sites in the codons UGA/CGA and UAG/CAG. No such editing sites were observed in *Equisetum* or in Marattiales. It should be noted that we only considered such editing sites that involved stop codons.

Divergence time

The mean divergence times estimated from the plastome data were generally older for the deeper nodes, but often younger for the shallow nodes, than divergence times estimated from the mitogenome data. For example, the fern crown group age was estimated as 387.81 Ma and 408.73 Ma in the mitogenome and plastome trees, respectively. The eupolypods stem age was 110.53 Ma and 143.19 Ma in the mitogenome and plastome trees, respectively (Table 4). In contrast, the split between the two *Lindsaea* Dryand. ex Smith (1793: 413) species was estimated older from the mitogenome (23.35 Ma) than plastome data (11.01 Ma), and the pattern was repeated in other very shallow nodes. This genomic pattern in age estimates resulted in a steadier increase of lineages when estimated from the plastome data (Fig. 1B).

Discussion

Our analysis represents the first taxonomically broadly sampled mitophylogenomic analysis of ferns. We recovered 33 mitogenome markers with a good taxonomic representation, which cover 40% of the protein coding genes found in complete fern mitogenomes (Guo *et al.* 2017). Even if the number of recovered markers remained relatively low, these data provided intriguing insights into the mitogenome evolution in ferns.

Despite the largely congruent mitogenome and plastome tree topologies they illustrated some of the most persisting uncertainties in the current understanding of the fern phylogeny. These include the relationships within eusporangiate ferns, which have remained notoriously incongruent even with large genomic data sets (Rothfels *et al.* 2015, Qi *et al.* 2018, Shen *et al.* 2018, One Thousand Plant Transcriptomes Initiative 2019, Breinholt *et al.* 2021). Both of our Bayesian trees support the now widely accepted topology with *Equisetum* as sister to all other ferns (Rai & Graham 2010, Knie *et al.* 2015, Rothfels *et al.* 2015, Labiak & Karol 2017, Liu *et al.* 2020). Our plastome tree also agrees with those studies by resolving Marattiales as sister to leptosporangiate ferns, but the mitogenome tree recovered Marattiales as sister to Ophioglossidae, albeit with low support. This same relation was recovered by Shen *et al*. (2018) and Pelosi *et al*. (2022) using transcriptome sequencing data.

The phylogenetic relationships of Hymenophyllales and Gleicheniales have also remained inconsistent (Kuo *et al.* 2018a). Depending on the type of data and the analytical method, Hymenophyllales has either been resolved as sister to Gleicheniales (e.g. Pryer *et al.* 2004, Schuettpelz & Pryer 2007, Lehtonen & Cárdenas 2019, Breinholt *et al.* 2021), as in our mitogenome tree, or sister to the remaining non-osmundalean leptosporangiate ferns (e.g. Lehtonen 2011, Kuo *et al.* 2018a, Du *et al.* 2022), as in our plastome tree.

Another persisting matter of contention is the relative phylogenetic positions of Pteridaceae and Dennstaedtiaceae (Hasebe *et al.* 1994, Schuettpelz & Pryer 2007, Wolf *et al.* 2015, Lehtonen & Cárdenas 2019, Du *et al*. 2021). More often Pteridaceae is resolved as sister to Dennstaedtiaceae + eupolypods (Lehtonen 2011, Rothfels *et al.* 2015, Qi *et al.* 2018, Shen *et al.* 2018, Lehtonen & Cárdenas 2019, Mu *et al.* 2020), but in our mitogenome tree Dennstaedtiaceae was sister to Pteridaceae + eupolypods, a topology that agrees with previous studies based on plastid data (Schuettpelz & Pryer 2007, Testo & Sundue 2016, Lehtonen *et al.* 2017). However, this topology contradicts the topology of our plastome tree, where Dennstaedtiaceae and Pteridaceae were resolved as sisters in the Bayesian analysis, a resolution that has previously been obtained by Du *et al*. (2021) based also on plastome data. Both hypotheses remained without significant support in our study. As well, individual marker trees had alternative resolutions irrespective of the genome for these families, but these topologies never received significant support.

Furthermore, other inconsistencies between our trees involved the genera *Woodsia*, *Onoclea*, *Thelypteris*, *Blechnum*, *Diplazium*, *Athyrium*, and *Deparia*. In our mitogenomic tree all these nodes lacked good support, however, the resolution in our plastome tree was fully supported and congruent with previous studies (e.g., Schuettpelz & Pryer 2007, Rothfels *et al*. 2012, Qi *et al.* 2018, Nitta *et al.* 2022). Inadequate taxon sampling may partly explain why our study could not resolve these conflicting relationships, but the persisting difficulties in resolving these clades may also indicate inherent complexity in their evolutionary origin, perhaps prohibiting their resolution.

The slower rate of evolution in plant mitochondrial genes compared with plastid genes is well documented (e.g. Palmer & Herbon 1988, Drouin *et al.* 2008, Richardson *et al.* 2013, Bell *et al.* 2020) and reinforced here. We inferred that mitogenome markers evolve at rate of 43% of the plastome markers when measured as total tree length, with a caveat that RNA editing may have biased our estimates to some degree.

We did not find evidence for RNA editing in the organellar genomes of Marattiales or *Equisetum*. Similarly, Lehtonen *et al*. (2020) reported absence or very low numbers of RNA editing sites in the plastome of Marattiales. In both studies the RNA editing was inferred from the presence of premature stop codons and therefore represent only the minimum level of RNA editing. Indeed, Knie *et al*. (2016) confirmed the presence of RNA editing in these groups, but with a very low frequency compared to most other ferns and possible absence of editing sites in the plastome of *Equisetum*. These results support the view that leptosporangiate ferns have much higher levels of RNA editing than eusporangiate ferns (Knie *et al*. 2016).

Lineage-specific rate heterogeneity is well documented in several fern groups (Schuettpelz & Pryer 2006). Reported examples include the slowly evolving Marattiales (Soltis *et al.* 2002, Lehtonen *et al.* 2020), Osmundales (Schneider *et al.* 2015), and Cyatheales (Korall *et al.* 2010), and the fast-evolving vittarioid ferns (Rothfels & Schuettpelz 2014, Grusz *et al.* 2016), *Ceratopteris* (Kinosian *et al.* 2020), Hymenophyllaceae (Schuettpelz & Pryer 2006) and Aspleniaceae (Rothfels *et al.* 2012). Our findings mostly support these reports and suggest that rate changes seem to be generally concordant between the fern mitogenome and plastome. The covarying rate heterogeneity has been associated in ferns with life history traits, such as generation time and gametophyte longevity (Grusz *et al.* 2016). Long living gametophyte phase may increase the probability of somatic mutation fixation and hence, the rate of molecular evolution (Klekowski 1984).

Our study reports the first age estimates for fern evolution based on mitogenomic data. These dates were largely congruent with the estimates from the plastome data, when using the same calibration points and priors, and with the previous studies. For example, the estimated age for the fern crown group was ca. 388 Ma for the mitogenome tree and ca. 409 Ma for the plastome tree. These estimates are within the range of previously published ages of ca. 430 Ma

(Testo & Sundue 2016), ca. 420 Ma (Lehtonen *et al*. 2017) and 381 Ma (Rothfels *et al.* 2015) (Table 4). Those studies applied exponential or lognormal prior age distributions in contrast to our uniform priors. Uniform priors are more conservative in the sense that they do not expect the fossils to represent the very earliest occurrences of their lineages, but also require hard maximum ages, which are difficult to justify. We applied the same very old maximum prior age for every calibrated node, thus allowing extremely wide prior distributions for most of the nodes. Despite this, we got age estimates falling within the published range of estimates. This indicates that the uniform priors actually might be preferable over the exponential priors, at least when a high number of calibration points are available across the whole depth of the tree.

The complex interplay of multiple priors may result in effective priors quite different from what was originally intended (Nascimento *et al.* 2017). We did not observe heavily distorted prior distributions despite having a high number of calibrated nodes. It is noteworthy, however, that for the deepest calibration points the posterior distributions were heavily left skewed, while the posterior distributions of the younger calibration points were mostly right skewed (Figs. 1, 2). This pattern was particularly clear for the mitogenome tree and resulted in less constant lineage accumulation in the LTT plots compared to the plastome tree. Overall, the skewed posterior distributions may indicate that the molecular clock was not fully capable of modelling rate heterogeneity, thus pushing the posterior towards the extremes allowed by the prior. Given the fossil record, we consider it unlikely that our priors would have been unrealistically narrow. The currently available molecular clocks are unable to model possible phylogeny-wide temporal rate variation caused by some common cause, such as elevated rates during the early diversification of the clades or after mass extinction events. Fern diversification patterns seem to be density-dependent (Lehtonen *et al.* 2017), and so may be the rate of molecular evolution.

On the other hand, the generally lower support and treeness/RCV ratio of the mitogenomic phylogeny may indicate less accurate phylogenetic estimate in comparison with the plastome data. In addition, the more common RNA editing in mitogenome may have introduced genomic biases in our rate estimates. Overall, our estimates of tree topology, diversification times and evolutionary rates are all based on RNA edited transcripts. This will, to some unknown extent, introduce non-random error in our results, as the rate of RNA editing varies between the genomes and taxa.

Conclusions

We reported the first broad fern phylogenetic tree based on mitogenomic data. This "third genome" generally supported the same topology as plastid and nuclear data, but showed different rates of molecular evolution. The evolutionary rate changes appear to covary between the plastome and mitogenome, and the covarying rate heterogeneity may be driven by life history traits. In the future, the intergenic regions of the mitogenome could provide very useful sequence data for lower-level phylogenetic studies, and the apparently common structural changes in the mitogenome may prove useful for resolving the deeper or currently unstable nodes, depending on their level of homoplasy. As such, this study provides a valuable starting point for future exploration of the fern mitogenome phylogeny and fern evolution in general.

Acknowledgments

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