

# Dating Suborder Polypodiineae (Eupolypods I) with Its Oldest Fossil

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## Abstract

Inferring divergence times between lineages is crucial for understanding biological evolutionary processes. The extraordinary species diversity of Eupolypods within the fern lineage has been interpreted as an ecological opportunistic response to the emergence of more complex, angiosperm-dominated ecosystems. This co-adaptation between Eupolypods and angiosperms has prompted ongoing investigations into the phylogenetic and diversification timelines of Eupolypods. In this study, we incorporate newly discovered fossils of Dryopteridaceae, including two species from both the stem and crown groups, to reanalyze the phylogenetic and diversification times of Eupolypods using total-evidence dating (TED or tip-dating) methods. Our analyses confirm that Eupolypods first diversified during the Jurassic and suggest that both subclades, Polypodiineae and Aspleniineae, underwent their earliest diversification during this period, challenging recent claims that Polypodiineae diversified no earlier than the Cretaceous. These results support a “long fuse” model, indicating that the initial emergence of Polypodiales preceded their diversification and extensive fossil record. Furthermore, our findings clarify the systematic position of Hypodematiaceae, indicating that it, along with Didymochlaenaceae, represents the basal lineages of Polypodiineae. This study highlights the critical role of fossil abundance and taxonomic composition in molecular dating analyses.

## Keywords

Eupolypods, Polypodiineae, Dryopteridaceae Fossils, Bayesian Tip-Dating

## 1. Introduction

Despite numerous phylogenetic and molecular dating studies that have con-

tinuously refined the time tree of ferns, there remain conflicting estimates regarding the diversification times of certain fern groups, particularly the Eupolypods (Table 1) [1]-[10]. Eupolypods, or eupolypod ferns, represent a highly diversified lineage and account for the majority of extant fern diversity, with nearly twice the number of species as all other non-eupolypod ferns combined (Figure 1) [2] [11]. Eupolypods are divided into two major clades: suborder Polypodiineae (eupolypods I) and suborder Aspleniineae (eupolypods II). The Polypodiineae, comprising 4665 species, is more species-rich than the Aspleniineae, which contains 3442 species (Figure 1) [2] [11]. Wang and Li [1] were the first to apply the TED method to estimate the divergence times within Eupolypods. However, their dating of Polypodiineae, the more diverse clade, has two significant limitations: 1) the absence of stem fossils, meaning only relatively recent fossils were used for Eupolypods I, and 2) an uneven fossil representation across lineages, particularly the lack of fossils for the highly diverse Dryopteridaceae.

Moreover, their study presents a controversial placement of Hypodematiaceae. While Wang and Li [1] proposed it as being located at the base of a clade within suborder Polypodiineae, recent studies suggest it forms a basal lineage within suborder Polypodiineae, representing an independent basal clade.

To address these issues, we incorporate four fossils morphologically attributed to Dryopteridaceae: the crown group fossils of *Elaphoglossum miocenicum* from the Miocene and *Polystichum pactovae* from the Oligocene, along with the stem group fossils *Cretacifilix fungiformis* and *Dryopterites beishanensis* from the Cretaceous (Table 2) [5] [12]-[26]. We also add sampling extant taxa of Hypodematiaceae, Didymochlaenaceae, and Davalliaceae (Table 3) [27]-[32]. In this study, we continue to apply the integrative tip-dating approach that combines molecular and morphological data to re-estimate divergence times for eupolypods, the most diverse of all major lineages of ferns, in the light of the stratigraphic records. By including newly added taxa fossil and extant taxa, we aim to determine when Eupolypods, especially suborder Polypodiineae, began diversifying, thus enhancing our understanding of their evolutionary history.

**Table 1.** Summary of fern phylogenetic analyses in previous studies and this study.

Study	Phylogenetic depth	Eupolypods/ Ferns sampled	Characters used	Dating methods	Eupolypod/Fern Fossils used	Ages of Total Aspleniineae	Ages of Crown Aspleniineae	Ages of Total Polypodiineae	Ages of Crown Polypodiineae
Wang and Li [1]	Eupolypods	214/218	Three plastid genes ( <i>rbcL</i> , <i>atpA</i> , and <i>atpB</i> ), 3841 bp	Bayesian Inference (MrBayes version 3.2.7a)	Tip calibrations 9/9	146.43 (122.8 - 170.25) Ma*	64.64 (49.16 - 91.16) Ma*	109.11 (80.78 - 141.85) Ma*	109.11 (80.78 - 141.85) Ma*
Nitta <i>et al.</i> [2]	Ferns	3311/5582	Plastid genes 12,716 bp	Penalized likelihood (treePL)	Node calibrations 16/51	~196 Ma**	163.0 Ma	~196 Ma**	161.1 Ma
Du <i>et al.</i> [3]	Polypodiales	162/214	Plastid 84 protein-coding genes and four rRNA genes, 76 448 bp	Penalized likelihood (treePL), Bayesian inference (BEAST), three root age constraints	Node calibrations 6/14	Between 144.22 and 200.3 Ma from six different dating schemes	Between 138.86 and 155.9 Ma from six different dating schemes	Between 144.22 and 200.3 Ma from six different dating schemes	Between 134.76 and 151.3 Ma from six different dating schemes
Qi <i>et al.</i> [4]	Vascular plants: ferns, lycophytes, seed plants	70/129	935, 501, 348, 267 and 146 nuclear gene sets from transcriptomes	Penalized likelihood (treePL)	Node calibrations 4/17	Cretaceous***	Cretaceous***	Cretaceous***	Cretaceous***

Continued

Regalado <i>et al.</i> [5]	Eupolypods	199/203	Three plastid genes ( <i>rbcl</i> , <i>atpA</i> , and <i>atpB</i> ), 3826 bp	Rating dating, <i>i.e.</i> , using the standard substitution rate for plastid DNA	No calibrations 0/0	165.02 (108.87 247.74) Ma	128.44 (85.16 192.02) Ma	165.02 (108.87 247.74) Ma	140.91 (93.96 211.16) Ma
Testo and Sundue [6]	Ferns, lycophytes	2468/3973	Six chloroplast markers ( <i>atpB</i> , <i>rbcl</i> , <i>rps4+rps4-trnS</i> IGS, <i>trnL+trnL-trnF</i> IGS), 8059 bp	Penalized likelihood (treePL)	Node calibrations 7/26	196.55 (194.82, 201.87) Ma	185.79 (183.78, 196.08) Ma	196.55 (194.82, 201.87) Ma	160.94 (158.62, 172.49) Ma
Rothfels <i>et al.</i> [7]	Ferns, seed plants	31/73	25 nuclear loci 35 877 bp from transcriptomes	Bayesian methods (MrBayes version 3.2.2)	Second node calibrations 2/12	112.42 (92.08, 133.13) Ma	94.96 (82.01, 109.91) Ma	112.42 (92.08, 133.13) Ma	96.09 (80.83, 109.82) Ma
Schuettpelz and Pryer [8]	Leptosporangiate ferns	242/400	Three plastid genes ( <i>rbcl</i> , <i>atpA</i> , and <i>atpB</i> ), >4000 bp	Penalized likelihood in r8s version 1.71	Node calibrations 5/24	116.7 (105.6, 144.9) Ma	103.1 (96.8, 126.5) Ma	116.7 (105.6, 144.9) Ma	98.9 (88.2, 127.9) Ma
Pryer <i>et al.</i> [9]	Vascular plants: ferns, lycophytes, seed plants	6/51	Four genes (plastid <i>rbcl</i> , <i>atpB</i> , <i>rps4</i> , and nuclear 18S rDNA), 4747 bp	Penalized likelihood in r8s version 1.60	Node calibrations 1/21	75.49 ± 7.66 Ma	/	75.49 ± 7.66 Ma	/
Schneider <i>et al.</i> [10]	Ferns, seed plants	19/42	Two plastid genes ( <i>rbcl</i> , <i>rps4</i> ), ~2500 bp	Penalized likelihood in r8s version 1.60	Node calibrations 2/14	104.69 Ma	94.52 Ma	104.69 Ma	93.61 Ma
This study	Eupolypods	228/232	Three plastid genes ( <i>rbcl</i> , <i>atpA</i> , and <i>atpB</i> ), 3841 bp	Bayesian Inference (MrBayes version 3.2.7a)	Tip calibrations 13/13	171.62 (149.28, 190.34)****	134.52 (108.80, 171.37)****	157.03 (142.63, 176.95)****	157.03 (142.63, 176.95)****

\*Ages are got from the dating scheme 3/72F (Table 4). \*\*Ages are estimated based on Figure 3 of Nitta *et al.* [10]. \*\*\*Ages are estimated based on suppl. Fig. S16 of Qi *et al.* [6]. “/” No ages were provided. \*\*\*\*Ages are got from the dating scheme 7/82F (Table 4).

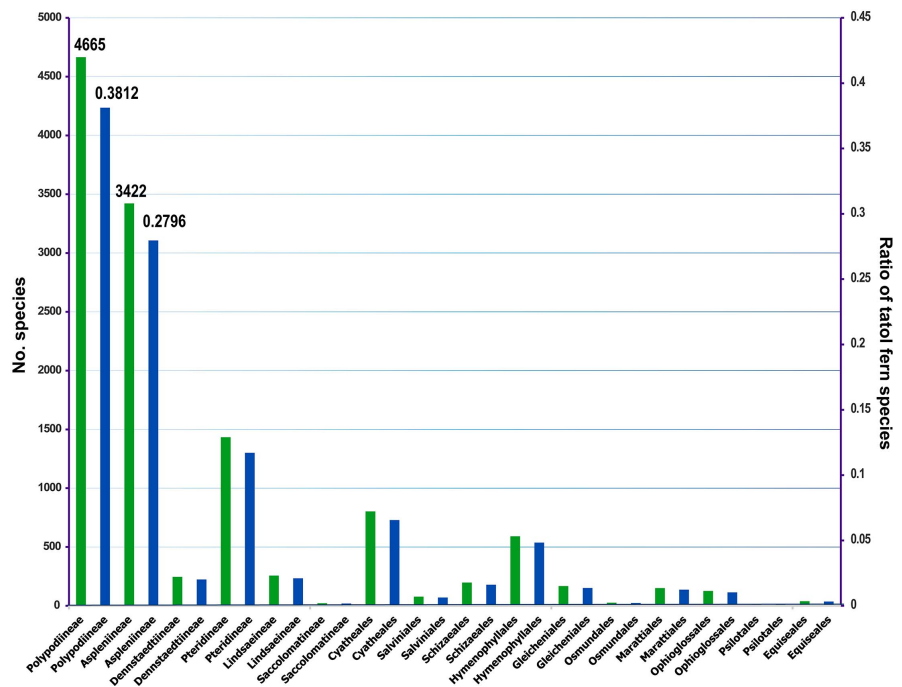


Figure 1. Diversity of Eupolypod Ferns (Order/Suborder). The 16 green bars represent species counts for 16 fern clades, while the blue bars indicate the relative proportions of each clade in relation to the total number of fern species. Numerical species counts and proportions are presented for two suborders of Eupolypods. Data and taxonomy are based on Nitta *et al.* [2] and the Pteridophyte Phylogeny Group I [11].

## 2. Analysis Methods

### 2.1. Sampling Taxa Set and Assembling Dataset

The data matrix of this study is assembled on the dataset from our previous work [1]. Four fossil taxa (Table 2) [5] [12]-[26] and ten extant taxa (Table 3) [27]-[32] are added to that combined dataset of Wang and Li [1]. The four newly added fossils, morphologically attributed to Dryopteridaceae, include crown group fossils *Elaphoglossum miocenicum* from the Miocene and *Polystichum pactovae* from the Oligocene, as well as stem group fossils *Cretacifilix fungiformis* and *Dryopterites beishanensis* from the Cretaceous. All fossils included in this study, along with their respective information, are presented in Table 2 [5] [12] [26]. The ten additional extant species, which were not included in the dataset of Wang and Li [1], belong to Davalliaceae (two taxa), Didymochlaenaceae (three taxa), and Hypodematiaceae (five taxa). Their DNA sequences were obtained from GenBank, with accession numbers and references provided in Table 3 [27]-[32]. The data matrix consists of 15 morphological characters, and the DNA sequence data, totaling 3826 bp from three plastid genes (*rbcL*, *atpA*, and *atpB*), remains identical to that used by Wang and Li [1].

**Table 2.** Fossils included in this study and their information.

Fossil taxa*	Selected references	Geological age and locality	Fossil ages (Ma), prior assignments, and affinities**
<i>Athyrium cretaceum</i> Chen and Meng	Chen <i>et al.</i> [12], Deng and Chen [13], Li <i>et al.</i> [14]	Neocomian (Hauterivian–Barremian), Lower Cretaceous, Liaoning, northeastern China	Uniform (100.00, 145.00), Aspleniineae
<i>Cretacifilix fungiformis</i> G. O. Poinar and R. Buckley	Regalado <i>et al.</i> [15], Poinar and Buckley [16]	Late Albian to earliest Cenomanian, Lower Cretaceous, Kachin State, northern Myanmar	Fixed (100.00), Polypodiineae
<i>Davallia walkeri</i> Conran, U. Kaulfuss, Bannister, Mildenhall and D. E. Lee	Conran <i>et al.</i> [17]	Early Miocene, Foulden Maar diatomite deposit, Otago, New Zealand	Uniform (20.44, 23.03), Polypodiineae
<i>Drynaria dimorpha</i> J. Y. Wu and B. N. Sun	Wu <i>et al.</i> [18]	Mangbang Formation, upper Pliocene, Yunnan Province, China	Uniform (2.58, 3.60), Polypodiineae
<i>Dryopterites beishanensis</i> Ren and Sun	Ren <i>et al.</i> [19]	Early Cretaceous (Hauterivian–Barremian), Zhongkouzi Basin, Beishan area, Northwest China	Uniform (129.40, 132.90), Polypodiineae
<i>Elaphoglossum miocenicum</i> Lóriga, A. R. Schmidt, R. C. Moran, K. Feldberg, H. Schneid and Heinrichs	Lóriga <i>et al.</i> [20]	Early Miocene (Burdigalian–Aquitainian), Dominican Republic, Santiago area	Uniform (15.97, 23.03), Polypodiineae

## Continued

<i>Holttumopteris burmensis</i> L. Regalado, H. Schneid., M. Krings and Heinrichs	Regalado <i>et al.</i> [5]	Late Albian to earliest Cenomanian, Lower Cretaceous, Kachin State, northern Myanmar	Fixed (100.00), Aspleniineae
<i>Onoclea sensibilis</i> L.	Pigg and Rothwell [21], Rothwell and Stockey [22]	Paleocene, Paskapoo Formation, central Alberta, Canada	Fixed (55.80), Aspleniineae
<i>Polystichum pactovae</i> Kvacek	Kvacek and Teodoridis [23]	Oligocene, Děčín Formation of the České středohoří Mts, Czech Republic	Uniform (23.03, 33.9), Polypodiineae
<i>Protodrynaria takhtajanii</i> Vikulin and Bobrov	Vikulin and Bobrov [24]	Paleogene flora of Tim in Russia	Fixed (33.90), Polypodiineae
<i>Thelypteris</i> sp. Aline M. Homes <i>et al.</i>	Homes <i>et al.</i> [25]	Late Eocene Pikopiko Fossil For- est, southern New Zealand	Fixed (34.40), Aspleniineae
<i>Woodwardia</i> <i>changchangensis</i> Naugolnykh and Song	Song <i>et al.</i> [26]	Middle Eocene of the Changchang Basin, Hainan Island, South China	Uniform (33.90, 56.00), Aspleniineae
<i>Woodwardia virginica</i> (L.) J. E. Smith	Pigg and Rothwell [21]	Middle Miocene Yakima Canyon flora of central Washington State, USA	Fixed (15.60), Aspleniineae

\*Fossils in pink are newly added this study. \*\*The fossils are ascribed to Aspleniineae or Polypodiineae based on originally described.

## 2.2. Set 12 Analytical Schemes for Bayesian Tip-Dating Analyses

Since the focus of this study is on the earliest divergence times of suborder Polypodiineae, or the total ages of Polypodiineae, we based our analysis on the dataset from Wang and Li [1]. The suborder Aspleniineae (eupolypods II) section of the dataset was kept unchanged, while we restructured the dataset by adding newly incorporated fossils belonging to suborder Polypodiineae. Each fossil or fossil group was combined with the constraint sets for family Dryopteridaceae or suborder Polypodiineae, resulting in 12 different analysis schemes (Table 4). Meanwhile, we used stepping-stone analysis [34] [35] to estimate marginal likelihoods for each model (Table 4). Our 12 tip dating analyses were performed in MrBayes 3.2.7a [35]-[37] following the manuals downloaded from <http://mrbayes.net>. The FBD model was used as the tree prior, the ages of fossil terminals were provided as uniform or fixed priors with bounds equal to the limits of the estimated ages of their deposits (Table 2). We used a “diversity” setting in the sampling strategy since we strived to include as many eupolypods terminals as possible, and set the sample probability prior to 0.0178; this was done because we included 214 terminals, while the diversity of the eupolypods lineage is currently of about 6000 species and we expected it contains about that same number of undescribed species. Our analyses were run for 20 million generations, sampling every 5000 generations. Bayesian posterior probabilities (PP) were calculated for the majority rule (>50%) consensus tree of all sampled trees after discarding the first 25% as burn-in. Visualized trees and all the nodes were checked in FigTree v.1.4 [38].

**Table 3.** GenBank accession numbers and references for extant taxa newly added in tip-dating analyses for this study.

Family	Representative species	GenBank accession numbers			Reference*
		<i>rbcl</i>	<i>atpA</i>	<i>atpB</i>	
Davalliaceae	<i>Davallia repens</i> (L.f.) Kuhn	MH392498	JF304018	MH392498	Ma <i>et al.</i> [27]; Kuo <i>et al.</i> [28] Ma <i>et al.</i> [27]
	<i>D. multidentata</i> Wall. ex Hook	MH392507	/	MH392507	Ma <i>et al.</i> [27]
Didymochlaenaceae	<i>Didymochlaena alpina</i> Li Bing Zhang & H. Shang	OP595169	OP595261	OP595231	Shang <i>et al.</i> [29]
	<i>D. amazonica</i> Li Bing Zhang & H. Shang	OP595168	OP595260	OP595230	Shang <i>et al.</i> [29]
	<i>D. solomonensis</i> Li Bing Zhang & H. Shang	MW323310	MW323335	MW323322	Shang <i>et al.</i> [30]
Hypodematiaceae	<i>Hypodematium glandulosum</i> Ching ex K. H. Shing	MZ957158	/	MZ957050	Fan <i>et al.</i> [31]
	<i>H. hirsutum</i> (Don) Ching	MZ957200	/	MZ957085	Fan <i>et al.</i> [31]
	<i>H. shingii</i> Li Bing Zhang, X. P. Fan & X. F. Gao	MZ957236	/	MZ957121	Fan <i>et al.</i> [31]
Hypodematiaceae	<i>Leucostegia amplissima</i> (Christ) C. W. Chen	MZ957135	/	MZ957032	Fan <i>et al.</i> [31]
	<i>L. immersa</i> Wall. ex C. Presl	AB232388	JF304009	MZ957036	Tsutsumi and Kato [32]; Kuo <i>et al.</i> [28] Fan <i>et al.</i> [31]

\*Three references are for three DNA accessions respectively. “/” No accessions were provided.

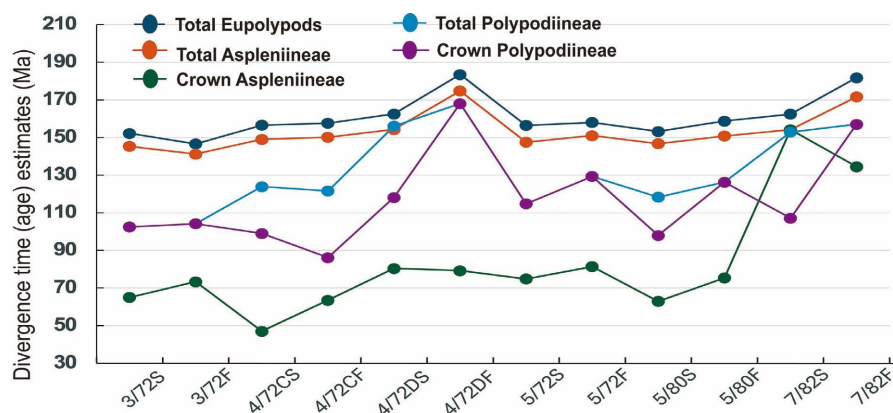
**Table 4.** Ages (in Ma) of total Eupolypods and its two subclades (median and 95% HPD) from tip dating under fossilized birth-death (FBD) priors\*.

Number of Fossil/extant taxa of Polypodiineae, fossil newly added**	Fossil Constraints*	Marginal likelihood**	Ages of Total Eupolypods	Ages of Total Aspleniineae	Ages of Crown Aspleniineae	Ages of Total Polypodiineae	Ages of Crown Polypodiineae
3/72, no newly added fossil	Suborder	-75046.58	152.15 (128.76, 173.80)	145.29 (121.05, 165.88)	65.04 (52.48, 86.28)	102.44 (71.96, 136.22)	102.44 (71.96, 136.22)
3/72, no newly added fossil	Family	-73065.95	152.95 (131.24, 174.44)	146.15 (135.31, 169.37)	78.74 (50.14, 113.44)	108.33 (81.63, 141.65)	108.33 (81.63, 141.65)
4/72, <i>Cretacifilix</i>	Suborder	-75046.58	156.57 (137.62, 179.14)	149.02 (127.03, 174.71)	47.01 (35.04, 91.13)	123.90 (105.05, 147.08)	99.01 (68.94, 116.70)
4/72, <i>Cretacifilix</i>	Family	-73754.00	157.61 (137.49, 178.41)	150.16 (126.87, 172.67)	63.51 (42.41, 111.12)	121.60 (101.88, 148.44)	86.19 (69.47, 106.80)
4/72, <i>Dryopterites</i>	Suborder	-75805.55	162.50 (147.15, 179.51)	154.26 (133.72, 173.91)	80.40 (57.86, 111.85)	<b>156.04</b> <b>(139.78, 171.47)</b>	118.12 (88.58, 148.50)
4/72, <i>Dryopterites</i>	Family	-73856.49	183.46 (171.22, 196.84)	174.77 (154.40, 192.85)	79.23 (55.51, 122.62)	<b>168.02</b> <b>(149.23, 188.79)</b>	168.02 (149.23, 188.79)
5/72, <i>Cretacifilix, Elaphoglossum</i>	Suborder	-75106.40	156.40 (134.84, 177.11)	147.55 (123.12, 170.25)	74.87 (55.37, 100.49)	114.81 (86.36, 145.38)	114.81 (86.36, 145.38)
5/72, <i>Cretacifilix, Elaphoglossum</i>	Family	-73525.47	158.01 (143.23, 175.36)	150.96 (130.57, 170.04)	81.38 (62.48, 109.26)	129.33 (115.28, 145.19)	129.33 (115.28, 145.19)
5/80, <i>Cretacifilix, Elaphoglossum</i>	Suborder	-77760.32	153.20 (133.22, 171.62)	146.74 (127.71, 166.00)	62.92 (46.05, 90.17)	118.34 (100.06, 147.65)	97.95 (75.71, 124.61)

## Continued

5/80, <i>Cretacifilix</i> , <i>Elaphoglossum</i>	Family	-75491.86	158.73 (142.94, 176.80)	150.80 (131.14, 169.85)	75.39 (56.58, 108.10)	126.24 (112.26, 142.02)	126.24 (112.26, 142.02)
7/82, <i>Cretacifilix</i> , <i>Dryopterites</i> , <i>Elaphoglossum</i> , <i>Polystichum</i>	Suborder	-79006.37	162.42 (143.92, 180.91)	154.15 (132.85, 176.14)	154.15 (132.85, 176.14)	<b>152.88</b> <b>(135.78, 173.45)</b>	107.1 (75.58, 134.99)
7/82, <i>Cretacifilix</i> , <i>Dryopterites</i> , <i>Elaphoglossum</i> , <i>Polystichum</i>	Family	-76503.86	181.78 (167.04, 195.63)	171.62 (149.28, 190.34)	134.52 (108.80, 171.37)	<b>157.03</b> <b>(142.63, 176.95)</b>	157.03 (142.63, 176.95)

\*All Bayesian analyses were conducted using relaxed clock model TK02 (autocorrelated lognormal; Thorne and Kishino [33]) with an offset exponential tree age prior, as implemented in MrBayes3.2.7a [35], ages of Total Suborder Polypodiineae in Jurassic were in bold. \*\*The four newly added fossils—*Cretacifilix fungiformis*, *Dryopterites beishanensis*, *Elaphoglossum miocenicum*, and *Polystichum pacltovae*—are listed by genus name only in the table. <sup>§</sup>Fossils were set constraint to family Dryopteridaceae or suborder Polypodiineae. <sup>§§</sup>Marginal likelihood (in natural log units, ln) were estimated using stepping-stone sampling (Xie *et al.* [34]).



**Figure 2.** Divergence times (median ages in Ma) of Eupolypods and its two subclades, estimated using a Bayesian tip-dating approach across 12 analytical schemes. The horizontal axis represents the 12 analytical schemes (see Table 3), where “S” and “F” respectively indicate whether fossils were constrained to the family Dryopteridaceae (indicated by F) or the suborder Polypodiineae (indicated by S).

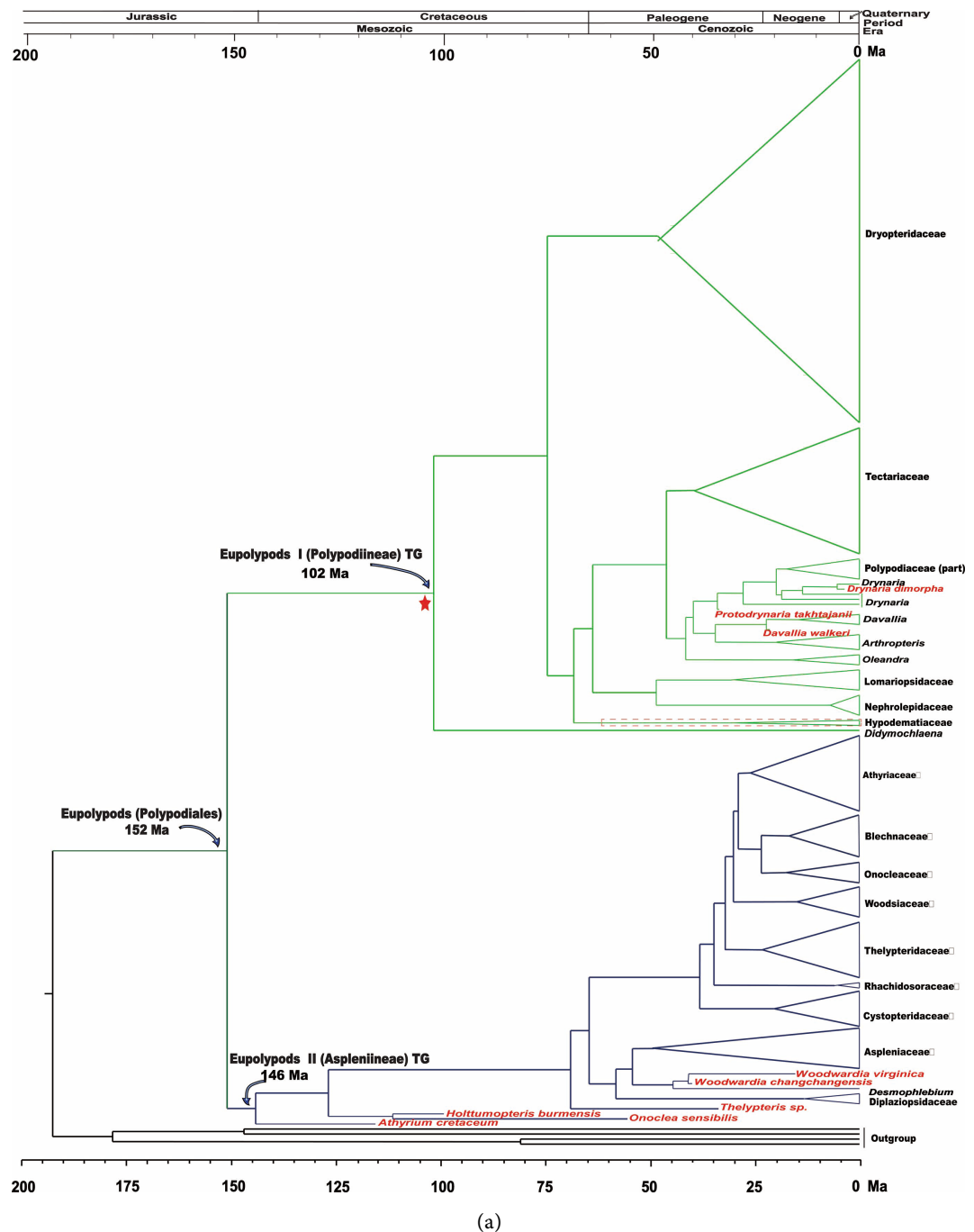
### 3. Results

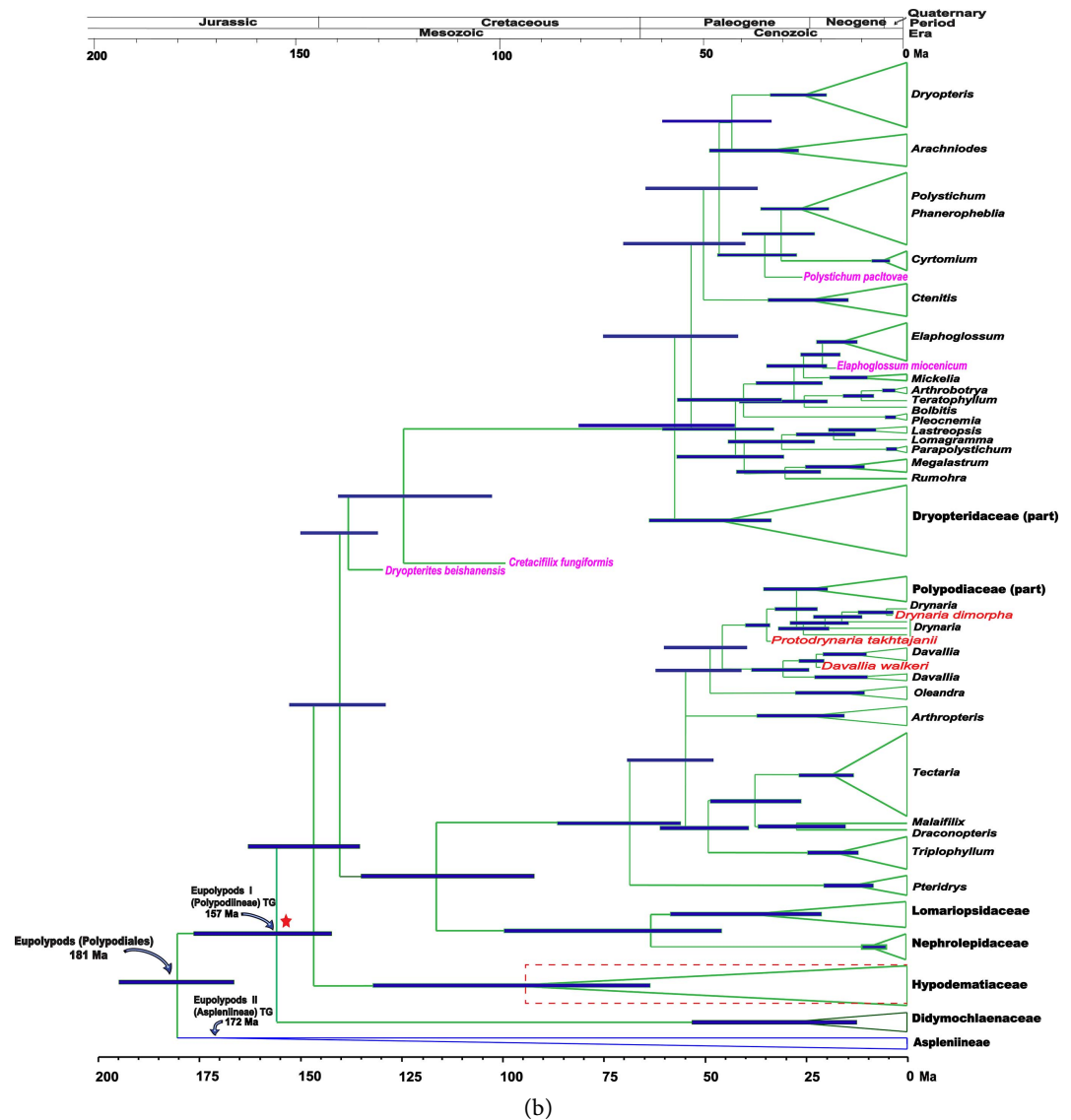
#### 3.1. Comparison among Different Tip-Dating Schemes

The divergence times (median ages in Ma) for Eupolypods and its two subclades obtained from the 12 analytical schemes (Table 4, Figure 2) show significant variation. However, all results indicate that the diversification of total Eupolypods occurred in the Jurassic, ranging from 152.15 - 183.46 Ma. The diversification of total eupolypod II (suborder Aspleniineae) also took place in the Jurassic, between 145.29 - 174.77 Ma, while eupolypod I diversified later. Among the 12 analytical schemes (Table 4), only four show that the earliest divergence time of suborder Polypodiineae occurred in the Jurassic, ranging from 158.88 - 168.02 Ma, and all four of these schemes include the earliest fossil of suborder Polypodiineae, *Dryopterites beishanensis* Ren et Sun from Early Cretaceous (Hauterivian-Barremian) of Northwest China. Our analysis suggests that the earliest divergence time of suborder Polypodiineae is determined by its earliest fossil record and the combinations that include this fossil, rather than being strongly influenced by whether the fossil or fossil combination is assigned to the constraint sets of family

Dryopteridaceae or suborder Polypodiineae. However, the estimated divergence times are slightly higher when the fossil or fossil combination is assigned to the family Dryopteridaceae compared to suborder Polypodiineae.

Based on our 12 analytical schemes, the marginal likelihoods (in natural log units, ln) estimated using stepping-stone sampling [34] consistently show higher values for the six analytical schemes where the constraint set is family, compared to the six schemes where the constraint set is suborder (Table 4). On the other hand, the values of marginal likelihoods decrease as the dataset size increases (Table 4).





**Figure 3.** Chronogram of eupolypod ferns constructed using a tip-dating approach and the Fossilized Birth-Death model within a Bayesian framework. Clades of the Suborder Polypodiinae (Eupolypods I) are shown in green, while those of the Suborder Aspleniinae (Eupolypods II) are shown in blue. The different systematic positions of Hypodematiaceae on chronogram (a) and (b) are highlighted with red dashed frames. (a) The chronogram of Eupolypods, modified from Wang and Li [1], includes only extant fern families and nine fossil taxa (in red), along with their relative extant genera. (b) The chronogram of Eupolypods I (Polypodiinae). Node bars represent 95% highest posterior density (HPD) intervals. Four newly added fossil taxa (in pink) are incorporated in this study. The three main lineages of eupolypods are indicated with their mean estimated ages, and the focal group of this study topic of this study, Suborder Polypodiinae, is highlighted with a red star.

### 3.2. Phylogenetic Positions of Dryopteridaceae Fossils

Our tip-dating tree (Figure 3(b)) shows that the phylogenetic placements of fossil taxa are mostly in accordance with their previous taxonomic attributions. Among the four newly added Dryopteridaceae fossils, the crown group fossils *Elaphoglossum miocenicum* [20] and *Polystichum pactovae* [23], which are expected to

belong to Dryopteridaceae, appear in the corresponding Dryopteridaceae lineage regardless of whether constraint sets are applied. Moreover, these two crown fossils have more concrete and accurate phylogenetic placements. For example, *Elaphoglossum miocenicum* [20] clusters with its extant *Elaphoglossum* relatives, and *Polystichum pacltovae* [23] clusters with its extant *Cyrtomium*-*Polystichum* relatives (**Figure 3(b)**). However, the situation is different for the stem group fossils *Cretacifilix fungiformis* [15] [16] and *Dryopterites beishanensis* [19] from the Cretaceous. The systematic positions of these stem group fossils in the tree depend on whether they are assigned to the constraint sets of family Dryopteridaceae or suborder Polypodiineae. If the two fossils are constrained to family Dryopteridaceae, they both occupy stem positions within Dryopteridaceae. If they are constrained to suborder Polypodiineae, they occupy stem positions within suborder Polypodiineae in our tip-dating tree (**Figure 3(b)**).

### 3.3. Phylogenetic Position of Hypodematiaceae

By incorporating ten additional extant taxa related to Hypodematiaceae (**Table 2**), our tip-dating analyses identify Didymochlaenaceae as the sister group to all other lineages within Polypodiineae, with Hypodematiaceae as the next earliest diverging lineage (**Figure 3(b)**). This suggests that Didymochlaenaceae represents the earliest divergence within Polypodiineae, followed by Hypodematiaceae. These findings contrast with those of Wang and Li [1], which placed Hypodematiaceae as the earliest diverging lineage within a clade of Polypodiineae, excluding Dryopteridaceae (**Figure 3(a)**).

## 4. Discussion

### 4.1. Diversification of Suborder Polypodiineae (Eupolypods I)

Polypodiineae (Eupolypods I) is the most species-rich lineage of ferns at the subordinal level (**Figure 1**), and analyses of its phylogenetic relationships and diversification times have been ongoing, resulting in considerable debate (**Table 1**). We will refrain from commenting on previous studies; instead, we focus on our findings in conjunction with our earlier analysis in Wang and Li [1] to explore the discrepancies in the diversification times of Polypodiineae. Among the current 12 analytical schemes (**Table 4**), only four indicate that the earliest divergence time of suborder Polypodiineae occurred in the Jurassic, ranging from 158.88 to 168.02 Ma (**Table 4**), and all four include the earliest fossil of suborder Polypodiineae, *Dryopterites beishanensis* [19]. Our study highlights the significant role of fossil abundance and taxonomic composition in molecular dating analyses. The results of our tip-dating analysis align with a few divergence time estimates derived from different molecular dating methods (node-dating [2] [3] [6] and rate-dating [5]). For the ages of crown Polypodiineae, all analytical schemes, except for 7/82S (**Table 4**, **Figure 2**), indicate that they are earlier than those of crown Aspleniineae, suggesting that crown Polypodiineae has a longer evolutionary history than crown Aspleniineae. This may explain its greater species richness compared to

Aspleniineae and its more ecological opportunistic response to the establishment of complex, angiosperm-dominated ecosystems.

#### 4.2. Ongoing Controversy on Phylogenetic Position of Hypodematiaceae

While our results in this study clarify the systematic position of Hypodematiaceae, indicating that it, along with Didymochlaenaceae, represents the basal lineages of Polypodiineae, this finding is only one of four analytical outcomes regarding its phylogenetic placement. Earlier studies have placed Hypodematiaceae nested with Didymochlaenaceae [8] [39]. Later, analyses of multiple chloroplast genes resolved Didymochlaenaceae as sister to the rest of Eupolypods I, Hypodematiaceae following [6] [28] [40], a result consistent with our findings (Figure 2(b)). However, recent plastid phylogenomic [2] [3] and nuclear phylotranscriptomic [4] analyses, based on more extensive sampling, have identified Hypodematiaceae as the most basal family within Polypodiineae. It is quite uncommon for analyses by Wang and Li [1] and Regalado *et al.* [5] to show that Hypodematiaceae is not positioned at the base of Polypodiineae but rather at the base of a clade within Polypodiineae (Figure 2(a)). The primary cause of these topological discrepancies appears to be differences in dataset sizes. Further investigation with expanded datasets is necessary to assess how these topological differences influence divergence age estimates compared to previous studies.

#### 5. Conclusion

This is our second attempt to combine morphological data from both extinct and extant taxa with DNA sequence data to estimate the diversification ages of eupolypods, the most species-rich fern lineage. The results further support previous hypotheses of Jurassic diversification across all eupolypods, indicating that both suborders, Polypodiineae and Aspleniineae, began diversifying during this period. However, estimates of Polypodiineae's diversification ages rely heavily on its earliest fossil records, underscoring the fossil record's critical role in calibrating clade origins. Tip-dating has once again proven to be an effective tool in a phylogenetic context. Ongoing research using tip-dating methods, along with new fossil discoveries, aims to shed more light on fern evolutionary history.

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#### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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