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Title Page

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Running title

Re-terrestrialization in epiphytic ferns

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**Re-terrestrialization in the phylogeny of epiphytic plant lineages: Microsoroid ferns as a case study**

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**ABSTRACT:**

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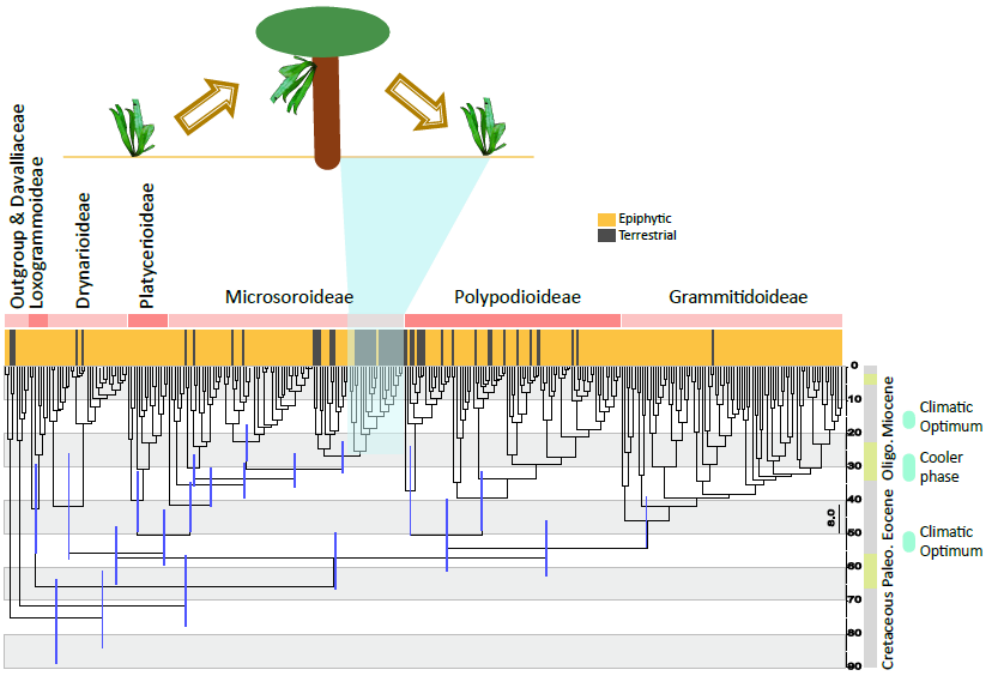
The fern family Polypodiaceae, with over 1,600 species, is not only one of the most species-rich families of ferns, but also a major contributor to the vascular epiphytic diversity throughout the tropics. Although the vast majority of species belonging to this family prefer to grow as epiphytes, several species colonize successfully rheophytic, lithophytic, and even terrestrial habitats. Here, we explore the hypothesis that non epiphytic habitat preferences, including terrestrial growth, evolved secondarily with epiphytes being the plesiomorphic habitat preference. The results of phylogenetic analyses, based on dense taxon sampling and four chloroplast DNA regions, were integrated with divergence time estimates and ancestral character state reconstructions to test these predictions. Both fossils and secondary calibration data were incorporated to obtain divergence time estimations. The results support the prediction of multiple transitions from epiphytic/lithophytic to terrestrial/rheophytic habitats occurring mainly in the Microsoreae lineage. The change in niche preferences coincides with niche colonization opportunities created by climatic fluctuations and geographical changes during the Oligocene and Miocene periods.

Graphical Abstract

This image shows the phylogenetic relationship of Oleandraceae, Davalliaceae, and Polypodiaceae with six subfamilies, the Oleandraceae selected as outgroup. With the integration of the growth form dataset, display the distribution of terrestrial species within Polypodiaceae. A clade marked with blue color indicates *Leptochilus*-MG5, in which the terrestrial species is dominant. This change in niche preferences is associated with competition in upper forest canopy habitats, influenced by the geographical change or climate fluctuations during the Oligocene-Miocene periods.

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**KEYWORDS:**

Ancestral state reconstruction, divergence time, fern, epiphyte, Microsoreae, re-terrestrialization

## 1 Introduction

Based on current consensus of the phylogenetic hypotheses of leptosporangiate fern diversification, the extant lineages, especially within the polypod clade, diversified simultaneously with the spread of angiosperm-dominated forests during the Late Cretaceous and Early Cenozoic (e.g., Schneider et al., 2004a; Schuettpelz & Pryer, 2009; Watkins & Cardelús, 2012; Testo & Sundue, 2016; Benton et al., 2021; Du et al., 2021). As a consequence of the transformation of terrestrial ecosystems with the rise of angiosperms, new epiphytic niches in the canopies of modern tropical rain forests became available that were subsequently successfully occupied by angiosperms and also ferns, lycophytes and even bryophytes (Zotz, 2013; Feldberg et al., 2014).

The widespread angiosperm-dominated forests arguably provided amble opportunities for ferns, but the colonization of these habitats required specific adaptations, especially enhanced

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water stress responses and mechanisms to deal with the reduced availability of crucial nutrients such as phosphorus and nitrogen (Schneider et al., 2004a; Tsutsumi & Kato, 2006; Schuettpelz & Pryer, 2009; Schneider et al., 2010; Lagomarsino et al., 2012; Watkins & Cardelús, 2012; Kato & Tsutsumi, 2013; Testo & Sundue, 2014; Nitta et al., 2020). Thus, a wide range of morphological and physiological adaptions are expected to correspond to an onset of radiation in the epiphytic habitat space (e.g., Tsutsumi & Kato, 2006; Schneider et al., 2010; Lagomarsino et al., 2012; Watkins & Cardelús, 2012; Kato & Tsutsumi, 2013; Testo & Sundue, 2014; Nitta et al., 2020). Until now, two syndromes have been found. The first responds to the paucity of phosphate and nitrogen resources by either revolving litter-collecting leaves (Schneider et al., 2010) with nest leaves evolving in the genera *Drynaria* (Bory) J.Sm. (Janssen & Schneider, 2005) and *Platycerium* Desv. (Kreier & Schneider, 2006) or by establishing mutualistic ant-fern associations as in the old-world genus *Lecanopteris* Reinw. and the new-world genus *Microgramma* subgroup *Solanopteris* Copel. (Gay, 1993; Schneider et al., 2010). The second syndrome addresses the challenge of water stress mainly by enhancing desiccation tolerance, as illustrated by the resurrection fern *Pleopeltis polypodioides* (L.) E.G. Andrews & Windham (Pessin, 1925), facultative Crassulacean acid metabolism (CAM) photosynthesis, as reported in *Microsorum* Link (Holttum & Winter, 1999), *Platycerium* (Ong et al., 1995), and *Pyrrosia* Mirb. (Wong & Hew, 1976; Chiang et al., 2013), and a range of morphological adaptations such as water storage cells in some species of Platyceroideae and induments, such as stellate hairs, enforcing protection and/or water uptake (Müller et al., 1981; John & Hasenstein, 2017).

Polypodiaceae are arguably the most suitable for studying the evolution of epiphytes in ferns because this family is not only one of the most epiphyte-rich fern families, with approximately 89% epiphytes and hemiephytes (Zotz et al., 2021c), but also includes non-epiphytic lineages. Subfamilies of Polypodiaceae have a high percentage of species classified as epiphytes because of their exclusive or dominant habitat preference (Zotz et al.,

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2021c) ranging from approximately 95% in Drynarioideae, 94% in Platyceroideae, 90% in Polypodioideae, 89% in Grammitioideae, 84% in Loxogrammoideae, and 83% in Microsoroideae. Among these, genera with a low frequency of epiphytes are mainly found in the subfamily Microsoroideae (Zotz et al., 2021c). In particular, *Leptochilus* Kaulf. contains only a few epiphytes with most species being either lithophytic, rheophytic or even terrestrial. This pattern led to the hypotheses that some species of Microsoroideae have evolved from ancestors with epiphytic habitat preferences to exploit aquatic (rheophytes), lithophytic, or even terrestrial habitats (Kreier et al., 2008; Testo & Sundue, 2014).

Previous studies have considered two main trends in the ecological shifts of lineages including epiphytic and terrestrial plants. Firstly, the origin of epiphytic plants from terrestrial growing ancestors by gradually adapting to the epiphytic habitats via transitional states evolving either climbing on other plants, or growing on rocks (Tsutsumi & Kato, 2006; Kato & Tsutsumi, 2013). Secondly, epiphytic plant lineages have been considered to have 'returned' to grow in soil, or on rocks, showing a process of re-terrestrialization or secondary terrestrialization (Wikström et al., 1999; Kato & Tsutsumi, 2013). Hemiepiphytes are considered intermediates with possible transitions in both directions (Testo & Sundue, 2014). Until now, the factors shaping the evolutionary directions of these processes have not been fully uncovered, but the dynamics of the availability of empty or low competition niches have been considered in studies focusing on orchids (Sosa et al., 2016) and the lycopod *Phlegmariurus* Holub (Wikström et al., 1999). In both cases, the evolution of epiphytism and re-terrestrialization were most likely influenced by various environmental changes. For example, the development of epiphytism was enabled by the rise of angiosperm forests and the ecological changes that followed (Wikström et al., 1999), while factors affecting terrestrialization most likely include climatic fluctuations (Sosa et al., 2016) or geographical changes, such as Andean orogeny and the development of open, alpine habitats (Wikström et al., 1999). For Polypodiaceae, several studies have also provided valuable insight into the

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evolution of substrate preferences (Schneider et al., 2010; Testo & Sundue, 2014; Sundue et al., 2015), and factors such as colonization of novel habitats and climatic fluctuations are considered to have had important impacts on the diversification of this family (Schneider et al., 2010; Sundue et al., 2015).

To explore the argument of global climate fluctuations or that geographic opportunities caused by geological changes have enabled secondary terrestrialization in Polypodiaceae, especially in the microsoroid lineages, a dated phylogenetic framework is needed, taking into account previous attempts to estimate the divergence times for major notes of these lineages (e.g., Kreier et al., 2006; Wang et al., 2012; Sigel et al., 2014; Sundue et al., 2014, 2015; Wei et al., 2017) within the context of the limited fossil record of these ferns (van Uffelen, 1991). Fortunately, several new fossils of Polypodiaceae have been discovered recently (Su et al., 2011; Jacques et al., 2013; Schneider et al., 2015; Huang et al., 2016; Sundue & Poinar, 2016; Xie et al., 2016; Xu et al., 2017; Kaulfuss et al., 2019), providing important data points for evolutionary studies of this family.

In this study, we conducted extended phylogenetic analyses incorporating divergence time estimations combined with reconstructions of substrate preferences with the aim to reconstruct the evolution of habitat preferences in Polypodiaceae, with special attention on the secondary terrestrialization of Microsoroideae. The main aims of the study were: 1) to obtain an overall view of the epiphytic and terrestrial species of Polypodiaceae, 2) to estimate the divergence time of terrestrial clades within the family, and 3) to elucidate environmental changes as the key factor driving secondary terrestrialization in epiphytic fern lineages.

## 2 Material and Methods

### 2.1 Taxon sampling and chloroplast DNA sequencing

Three fern families were sampled, including the secondary hemiepiphyte Oleandraceae and the two mainly epiphytic families Davalliaceae and Polypodiaceae. Previous studies have shown that Oleandraceae and Davalliaceae are the most closely related families of

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Polypodiaceae (e.g., Schuettpelz & Pryer, 2009). Of these, Oleandraceae was selected as an outgroup. For Polypodiaceae, the taxa were sampled from all six subfamilies following the Pteridophyte Phylogeny Group (PPG I, 2016), with all genera or groups included whenever possible (Table S1). The genera and groups used in this study mainly follow the PPG I (2016), but are also based on the latest studies, especially for Microsoroideae (Testo et al. 2019a; Chen et al., 2020). We attempted to sample comparable proportions of species in each family. In total, four and five species of Oleandraceae and Davalliaceae, respectively, were sampled, while 305 species of six subfamilies of Polypodiaceae were included. This represented approximately 18% of all species in the family, with seven species of Loxogrammoideae (22% of the species diversity sampled), 15 species of Platycerioideae (21%), 29 species of Drynarioideae (20%), 83 species of Polypodioideae (27%), 85 species of Grammitidoideae (9%), and 86 species of Microsoroideae (47%). A summary of the dataset is presented in the Table S1.

Four chloroplast markers (*rbcL*, *rps4+rps4-trnS*, *trnL+trnL-trnF*, *atpB*) were used for the analyses, with the first three obtained for most of the species. The *rbcL* sequences were available for all 314 species, *rps4+rps4-trnS* and *trnL+ trnL-trnF* for 312 and 313 species, respectively, and the *atpB* sequences for 168 species. All the sequences were downloaded from GenBank, with most sequences of Microsoroideae generated in our previous studies (Chen et al., 2020, 2021). Voucher information and GenBank accession numbers are provided in Table S1.

2.2 Data on substrate preference

Data on substrate preferences were compiled from regional flora treatments and other relevant studies (e.g., Brownlie, 1969; Stolze, 1981; Parris, 1986; Tryon & Stolze, 1993; Orchard, 1998; Baksh-Comeau, 2000; Lindsay & Middleton, 2012 onwards; Wu et al., 2013; Chen et al., 2017; Parris, 2018), as well as monographs and other taxonomic publications (e.g., Evans, 1968; Lellinger, 1972; Hovenkamp, 1986; Hennipman & Verduyn, 1987;

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Rödl-Linder, 1990; Bosman, 1991; Smith et al., 1991; Bishop & Smith, 1992; León, 1992; Nooteboom, 1994; Nooteboom, 1997; Hovenkamp, 1998; Kessler & Smith, 2005; Smith et al., 2006; Schuettpelz & Pryer, 2009; Hirai et al., 2011; Labiak, 2011; Hovenkamp & Ho, 2012; Labiak, 2013; Lehnert, 2013; Testo & Sundue, 2014; Moran & Labiak, 2017; Testo et al., 2019a). Final scores were checked by examining herbarium vouchers when necessary. We scored the substrate preference for all taxa, either as "epiphytic & lithophytic" or "terrestrial & rheophytic" (Fig. 1), with the former indicating whether the roots were on another plant, or on rocks, while the latter indicated that the roots were in soil or water. Hemiepiphytic was not used as a character state due to the lack of unambiguous ecological information for most species. Recent studies argue that its records require further study because of ambiguity in the applied concept (Zotz et al., 2021a, 2021b). Substrate preference plasticity of individual species may result in ecological diversity (Zotz, 2013; Zotz et al., 2021c). We used the most common description if there was conflict between published studies, with an aim to avoid scoring taxa as polymorphic.

2.3 Phylogenetic analyses

The DNA sequences were aligned using MUltiple Sequence Comparison by Log-Expectation (MUSCLE) (Edgar, 2004), as implemented in Mesquite 3.61 (Maddison & Maddison, 2019). The alignments obtained were visually checked for ambiguously aligned regions that were subsequently excluded. The most suitable partition and model were determined with PartitionFinder v2.1.1 (Lanfear et al., 2017) using the Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway (Miller et al., 2010). Phylogenetic trees were inferred based on Maximum Likelihood (ML), parsimony and Bayesian Inference (BI), as implemented in the programs IQ-TREE 2.0.5 (Minh et al., 2020), PAUP* 4.0a (Build 166) (Swofford, 2002), and both Bayesian Evolutionary Analysis Sampling Trees (BEAST) 1.10.4 (Suchard et al., 2018) and MrBayes 3.2.7a (Ronquist et al., 2012). Most of the analyses were conducted using the CIPRES portal (phylo.org).

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For ML analysis, the dataset was partitioned by marker and the analyses were performed using the GTR+I+G model and Bayesian Information Criterion (BIC), as determined by PartitionFinder. We evaluated the node support by 1000 ultrafast bootstrap replicates (UFBoot; Minh et al., 2013), a Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT; Guindon et al., 2010), and the Bayesian-like transformation of aLRT (aBayes; Anisimova et al., 2011). The single tree with the highest likelihood score was then employed as a starting tree in BEAST dating analysis (see section 2.4). For parsimony, the heuristic search was performed using Tree Bisection Reconnection (TBR) and bootstrap support was evaluated with 1000 replicates. All character states were treated as unordered and equally weighted, and gaps were treated as missing data.

For BI analyses, Markov chain Monte Carlo (MCMC) simulations ran independently twice in both BEAST and MrBayes, with a total number of runs of 200 million and 250 million generations, respectively. In the BEAST analyses, phylogeny and divergence time estimation are performed simultaneously (see section 2.4). In the MrBayes analyses, a GTR+I+G model was used, with one cold and three hot chains for each run, and these were stopped when the standard deviations of the split frequencies were below 0.05. The posterior distributions of all parameters and their associated statistics were examined using Tracer v. 1.6 (Rambaut et al., 2014) to ensure convergence of the chains and sufficient sampling of the generations. The consensus trees of both BI analyses (BEAST and MrBayes) were calculated based on all trees sampled and excluded the first 25% of the sampled trees, which were discarded in the burn-in phase. The posterior probabilities (PP) from both BI analyses were calculated. All tree topologies of the various analyses were visualized using the program FigTree, v1.4.2 (Rambaut, 2014).

2.4 Divergence time estimation

Divergence times were estimated with BEAST 1.10.4 using the CIPRES portal. For parameter setting in Bayesian Evolutionary Analysis Utility (BEAUti) 1.10.4, the clock

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models and topologies of the data partitions were linked, whereas the models of nucleotide substitution were unlinked across partitions, which were determined using PartitionFinder. An uncorrelated lognormal relaxed clock, and birth-death model for tree prior (Gernhard, 2008) were applied, with the ML tree being used as a starting tree.

We calibrated the chronogram using six fossils of Polypodiaceae (Van Uffelen, 1991; Kvaček, 2001; Su et al., 2011; Jacques et al., 2013; Schneider et al., 2015; Xie et al., 2016; Xu et al., 2017; Kaulfuss et al., 2019), also applied the node ages of previous analysis to calibrate two basal nodes (Schuettpelz & Pryer, 2009). In total, eight calibration points were used (Table 2; Table S2). In addition, the fossil *Protodrynaria takhtajanii* Vikulin & A. Bobr. (Vikulin & Bobrov, 1987) has been used to calibrate the divergent time for Polypodiaceae in previous studies (Schuettpelz & Pryer, 2009; Du et al., 2021), but the assignment of this fossil is ambiguous due to poor preservation of critical characters such sorus and venation (Schneider, pers. comm.). To access the influence of *P. takhtajanii* on the divergence time estimation, we carried out divergence time analysis with and without this fossil included (Table 2). Two of the secondary node calibration points were modeled using exponential distribution priors, whereas the lognormal distribution model was applied to all other calibration points (Table 2). For each of the calibration schemes using several calibrations, two runs of 200 million generations each were performed with tree and parameter sampling every 20,000 generations. We used LogCombiner v1.10.4 (part of the BEAST package) to combine results from the two runs and TreeAnnotator v1.10.4 (also part of the BEAST package) to compute the maximum clade credibility tree. FigTree 1.4.2 was used to visualize the maximum clade credibility tree and the associated chronogram.

2.5 Ancestral state reconstruction of substrate preference

The phylogenetic trees obtained from the BEAST analysis were used to estimate the probabilities of the ancestral states in BayesTraits v3.0 (Pagel & Meade, 2006). We used R to randomly choose 100 post burn-in trees to incorporate phylogenetic uncertainty. Ancestral

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states were reconstructed for 70 chosen nodes (n1-n70 in Fig. 1), representing important internodes and clades. The Multistate model was selected for the MCMC analysis, with Bayesian reversible-jump hyperprior (RJHP) selected to specify an exponential prior. We compared six RJHP exponential priors ranging from 0-0.01 to 0-100 and an optimal value of 0-0.1 was chosen. All analyses used 10 million iterations, in which chains were sampled every 1000 iterations with a burn-in of five million iterations. With the 100 trees and a score of substrate preference, the analysis generated the proportion of the likelihood associated with each of the possible states at each node. We then visualized the proportion of ancestral states using R and mapping to the BEAST consensus tree (Fig. 1).

## 3 Results

### 3.1 Phylogenetic analysis

In all topologies, Loxogrammoideae was the first subfamily within Polypodiaceae to diverge. The topologies from parsimony and both BI analyses were generally congruent with Drynarioideae as a sister clade of Platycerioideae and Microsoroideae (Figs. 2B, 2C, 2D), while Polypodioideae and Grammitidoideae were closely related, including the latter nested in the former (Figs. 2B, 2D), or as sister clades (Fig. 2C). In ML analysis, however, Drynarioideae was a sister of the clade comprising of the other four subfamilies (Fig. 2A), with low branch-support values (aLRT = 57% / aBayes = 0.67 / UFBoot = 65%). In addition, Polypodioideae was not monophyletic in the ML, BI of MrBayes and parsimony topologies (Figs. 2A, 2B, 2D), while in the topology from BEAST analysis, it was (PP = 0.78, Fig. 2C). The BEAST topology was used in the final presentation of the results (Fig. 1).

### 3.2 Ancestral state of substrate preference

The scored substrate preferences and the results of the Bayesian reconstruction are displayed as graphs in Fig. 1, while detailed information can be found in Table 1. The results show that epiphytic & lithophytic was the main substrate preference in Polypodiaceae, as well as in most of the internal nodes (Fig. 1, Table 1). However, seven nodes showed fairly equal

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probability values for different character states, including nodes n15 (with a relative probability of terrestrial/rheophytic 0.43 and epiphytic/lithophytic 0.57), n18 (0.43 and 0.57), n23 (0.56 and 0.44), n27 (0.55 and 0.45), n29 (0.54 and 0.46), n42 (0.57 and 0.43), and n53 (0.40 and 0.60), respectively (Table 1). Of these, node n15 was a point where Microsoreae separated from its sister clade (Fig. 1), and nodes n18 and n23 were within Microsoreae (Fig. 1). Nodes n27 and n29 belonged to the *Campyloneurum* clade, with the former representing the crown divergence of *Campyloneurum* (Fig. 1).

Five nodes showed a clearly higher probability of terrestrial & rheophytic substrate preference, including node n17, with relative probabilities of 0.91 (Table 1), n19 (0.94), n22 (0.97), n24 (0.996), and n30 (0.994), respectively (Table 1). Of these, n30 was within *Campyloneurum*, while the other four nodes were within the Microsoreae clade (Fig. 1), with node n17 representing the crown divergence of Microsoreae, n19 representing MG4, n22 representing crown divergence of MG5 plus *Leptochilus*, and n24 representing *Leptochilus* clade (Fig. 1). For Microsoreae, only the clade of core *Microsorum* (n20) showed epiphytic & lithophytic substrate preference (Fig. 1, Table 1). In the *Campyloneurum* clade, the terrestrial & rheophytic clade (n30) was a sister to the epiphytic & lithophytic clade (Fig. 1).

3.3 Divergence time estimates

In the divergence time analyses without *Protodrynaria takhtajanii* as a calibration point, the estimated time for Davalliaceae and Polypodiaceae to separate from their common ancestor was ca. 61-84 Ma BP (n2 in Fig. 1, Table 1), whereas diversification of the crown group Polypodiaceae was estimated to have started ca. 56–78 Ma (n3) BP. The divergence dates (crown ages) obtained for the six subfamilies were 40–61 Ma for Polypodioideae (n26), 26–58 Ma for Drynarioideae (n7), 39–54 Ma for Grammitidoideae (n32), 29–56 Ma for Loxogrammoideae (n4), 31–49 Ma for Platycerioideae (n9), and 35–49 Ma for Microsoroideae (n10) (Fig. 1, Table 1). The diversification of the terrestrial & rheophytic clade Microsoreae (n17) was estimated to have taken place ca. 22–32 Ma BP, during the early

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Oligocene to Miocene. The currently accepted genera showed divergence dates ranging between ca. 3 and 37 Ma BP, with the earliest diverged genera including *Campyloneurum* C. Presl of Polypodioideae (24–50 Ma), *Pyrrosia* of Platycerioideae (23–40 Ma), and *Loxogramme* (Blume) C. Presl of Loxogrammoideae (18–37 Ma). Most of the remaining genera have diverged (crown ages) in the mid to late Miocene (Fig. 1), including the terrestrial subclade within *Campyloneurum* (n30, ca. 7–15 Ma). In the analyses with *P. takhtajanii* as the calibration point, the divergence times were estimated to be shorter (Table 1).

## 4 Discussion

### 4.1 Phylogenetic relationships

Our results show Loxogrammoideae as the basal branch of Polypodiaceae, and the close relationship between Platycerioideae and Microsoroideae and between Polypodioideae and Grammitidoideae were discovered in all the topologies (Figs. 1, 2). The placement of Drynarioideae differs in ML topology compared with topologies obtained in BEAST, MrBayes, and parsimony analyses, but the support values related to Drynarioideae are weak in all four topologies (Fig. 2). Previous studies have shown uncertain placements of Drynarioideae and genus *Synammia* (Schneider et al., 2004b; Schneider et al., 2006; Schuettpelz & Pryer, 2007; Testo & Sundue, 2016; Qi et al., 2018; Du et al., 2021), and the most recent studies (Wei et al., 2021; Wei & Zhang, 2022) have revealed a sister relationship between these two. Insufficient taxonomic sampling, paucity of phylogenetic signal, or long-branch attraction have been proposed as reasons for ambiguous topologies (Wei et al., 2021; Wei & Zhang, 2022). In our analyses, paraphyletic Polypodioideae, relative to Grammitidoideae, is found in topologies obtained in the ML, MrBayes, and parsimony analyses (Figs. 2, 3). Polypodioideae has recently been further divided into four subfamilies (Wei & Zhang, 2022). However, monophyletic Polypodioideae was found in our BEAST analysis (Figs. 1, 2C), although with a relatively low support value (PP = 0.76).

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4.2 Divergence time estimates and the fossil record

Several fossils of Polypodiaceae have been reported in recent years. These belong to different subfamilies, including Microsoroideae (Jacques et al., 2013; Xie et al., 2016; Xu et al., 2017; Kaulfuss et al., 2019), Polypodioideae (Schneider et al., 2015), Drynarioideae (Su et al., 2011; Huang et al., 2016), and Grammitidoideae (Sundue & Poinar, 2016). These reports enabled time calibration for Polypodiaceae. However, fossils need to be chosen carefully for calibration to avoid the influence of the insufficient information of some records, or uncertain hypotheses about their relationship. For example, fossils of *Microsorum* sensu lato may represent different lineages (Kreier et al., 2008; Chen et al., 2020). Recently found fossils of Microsoroideae have been assigned to *Goniophlebium* (Blume) C. Presl (Xu et al., 2017), *Neolepisorus* Ching (Xie et al., 2016), and *Palaeosorum* F.M.B. Jacques & Z.K. Zhou (Jacques et al., 2013; Kaulfuss et al., 2019). Of these, *Palaeosorum* includes two species: *P. ellipticum* F.M.B. Jacques & Z.K. Zhou from Yunnan of China and *P. waipiata* U. Kaulf., Conran, Bannister, Mildenh. & D.E. Lee from New Zealand. The former has morphological characters such as an elliptical leaf shape and lax pinnate venation that resemble *Microsorum reticulatum* Ching ex L. Shi (= *Neolepisorus ensatus* (Thunb.) Ching), and affinity with *Neolepisorus* (Jacques et al., 2013; Wu et al., 2013). The latter, *P. waipiata*, was assigned to the tribe Lecanoptereae of *Microsorum* s.l. based on frond morphology, arrangement of the sori, and spore ornamentation (Kaulfuss et al., 2019; Chen et al., 2020, 2021). The verrucate spores are morphologically similar to spores found in species of *Zealandia* Testo & A.R. Field (Chen et al., 2021). The *Goniophlebium* fossil, *G. macrosorum* Xu & Zhou, also has in situ spores with verrucate surface (Xu et al., 2017), but its regular verrucae differ from those found in *P. waipiata* (Chen et al., 2021). For groups with ambiguous phylogeny like the microsoroid ferns, spore ornamentation may be useful to identify their phylogenetic placement (Chen et al., 2021).

To date, the oldest fossil of Polypodiaceae seems to be *Protodrynaria takhtajanii* from Russia,

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which is dated from Upper Eocene to Lower Oligocene (Van Uffelen, 1991; Jacques et al., 2013) and has been used to calibrate the time when Polypodiaceae separated from Davalliaceae (Schuettpelz & Pryer, 2009), or the stem of Drynarioideae (Du et al., 2021). However, the taxonomic assignment of this species is still controversial. Although the close relationship of *P. takhtajanii* with the drynarioids have been considered by some authors (Vikulin & Bobrov, 1987; Jacques et al., 2013; Du et al., 2021), the fossils do not preserve enough of the characters required to defend this assignment (Van Uffelen, 1991; Su et al., 2011; Testo & Sundue, 2016). For comparison, we conducted the divergence time estimation with and without *P. takhtajanii*, and the analysis with *P. takhtajanii* resulted in younger estimates than analysis without it (Table 1).

The fossils that most likely belong to Davalliaceae and Polypodiaceae are listed in Table S2. Six of them are used in this study, including three from Microsoroideae, two from Polypodioideae, and one from Drynarioideae (Table 2; Table S2). In addition, two basal divergent points (n1 and n2) based on previous studies were used (Schuettpelz & Pryer, 2009; Testo & Sundue, 2016). Our results show that the crown divergence of Polypodiaceae is slightly older (ca. 66 Ma BP, highest posterior density (HPD): 56–78 Ma, Table 1) than the 56 Ma estimated by Schuettpelz & Pryer (2009), although the difference is not significant. This is clearly earlier (ca. 25 Ma) than presented by Testo & Sundue (2016) and closer to 60 Ma (HPD: 45–92 Ma) as obtained by Rothfels et al. (2015) based on the use of nuclear genes, and ca. 71 Ma as estimated by Du et al. (2021) using plastomes.

4.3 Evolution of substrate preference

Davalliaceae and Polypodiaceae are important epiphytic lineages of ferns, and the evolution of substrate preferences has been studied in these groups (Tsutsumi & Kato, 2006; Kato & Tsutsumi, 2013). Our results agree with these previous studies. Epiphytism is the most plausible ancestral state in both families, with the divergent points of Davalliaceae and Polypodiaceae (n2) and crown of Polypodiaceae (n3) showing the relative probabilities of

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epiphytic & lithophytic as approximately 0.9 (Fig. 1; Table 1). Even though epiphytic & lithophytic is the dominant substrate preference, the terrestrial & rheophytic substrate preference is the ancestral state in some of the clades within Polypodiaceae, especially in Microsoreae of Microsoroideae (n17 in Fig. 1). Most of the genera and groups within Microsoreae have terrestrial & rheophytic substrate preference in the ancestral state, including *Leptochilus* (n24, relative probability 0.99), MG4 (n19, 0.93), and MG5 (n23, 0.55) (Table 1), showing that terrestrialization developed from epiphytic ancestors. In addition, the discovery of the primary hemiepiphyte *Dendroconche ampla* (F. Muell. ex Benth.) Testo, Sundue, & A.R. Field (= *Colysis ampla*) in the lineage close to Microsoreae has been interpreted as further evidence of this evolutionary process of returning to the ground from epiphytic habitats (Testo & Sundue, 2014). Compared to development of epiphytism, converse evolution from epiphytism to hemiepiphytism has been hypothesized to be more common in ferns due to preadaptation (Testo & Sundue, 2014).

The development of epiphytic Polypodiaceae shows a successful occupation of novel ecological niches that emerged in angiosperm-dominated tropical forests during the late Cretaceous to early Cenozoic (Schneider et al., 2004a; Schuettpelz & Pryer, 2009; Watkins & Cardelús, 2012; Qi et al., 2018), with secondary hemiepiphytes such as *Oleandra* Cav. representing a transition in this process (Tsutsumi & Kato, 2006; Kato & Tsutsumi, 2013). Our results show the divergence of Polypodiaceae, including epiphytism and secondary terrestrialization, to coincide with major changes in climate (Fig. 1). The overall climate trends in the Cenozoic mostly show development from warm towards cooler temperatures, with several fluctuations in between. The major phases include the warmer Cretaceous/Tertiary (K/T) boundary, Paleocene/Eocene thermal maximum (PETM), and early Eocene climatic optimum (EECO), followed by the cold phase of the Early Oligocene, and the middle Miocene climatic optimum (MMCO) (Zachos et al., 2001). Our time estimation shows that the diversification of Polypodiaceae mostly likely took place in the K/T boundary

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until the PETM periods (Fig. 1; Table 1), which is consistent with the results of previous studies (Schuettpelz & Pryer, 2009; Testo & Sundue, 2016). The warmer temperatures of this period most likely resulted in a rapid expansion of rainforests and a sudden increase in available canopy niches, as well as the overall higher atmospheric humidity, which are all beneficial to the development of epiphytes (Watkins & Cardelús, 2012; Benton et al., 2021). Suitable warm temperatures also facilitated the divergence of various subfamilies. Divergence time estimation shows that most subfamilies diversified during PETM and EECO periods, including Drynarioideae (n6 in Fig. 1), Platycerioideae and Microsoroideae (n8), as well as Grammitidoideae and Polypodioideae (n25).

All six subfamilies of Polypodiaceae mainly utilize the upper layers of forests, including Microsoroideae, with an estimated relative probability for an epiphytic ancestral state of approximately 0.8 (n10 in Table 1). Within Microsoroideae, the terrestrial lineage Microsoreae diverged in the early to middle Oligocene, when it separated from its common ancestor with Lepisoreae ca. 31 (26–36) Ma BP (Table 1), with the relative probability between epiphytic and terrestrial being almost equal (n15). After this point, the terrestrial state became dominant in the Microsoreae lineage (Table 1, Fig. 1). According to paleoclimatological studies, a cooler climate and middle-latitude desertification during this period decreased the area of broad-leafed forests and available habitats accordingly (Zachos et al., 2001; Potter & Szatmari, 2009; Quan et al., 2012). This most likely increased competition in upper canopy environments. Although diversification rate between terrestrial and epiphytic species obtained by Schuettpeltz & Pryer (2009) have been challenged by Testo & Sundue (2016), the rapid increase in terrestrial fern diversification is synchronous with the early Oligocene, which is shown as a turning point of the diversification curve in Figure 2 of Schuettpelz & Pryer (2009). This Oligocene turning point for terrestrial ferns coincides with the cooler climate period, indicating that climate changes may affect the evolution of substrate preference by altering the availability of different habitats. A similar evolutionary

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trend has been reported in orchids during the mid-Eocene to Oligocene, or more recently such as Pliocene to Quaternary, revealing that terrestrialization allowed plants to occupy a wider range of habitats, for example in semi-arid areas and in open savanna vegetation (Sosa et al., 2016; Martins et al., 2018).

In addition to climate, geographical changes such as widespread orogenic events and continental uplift during the Miocene also changed the topography, which created various new habitats to host novel plant diversity (Potter & Szatmari, 2009). Unlike the terrestrialization of Microsoreae, most of the genera in its sister clade Lepisoreae remained epiphytic/lithophytic (Fig. 1). The rise of the Himalayas and the Qinghai-Tibetan region greatly affected the diversification in *Lepisorus* (Wang et al., 2012; Sundue et al., 2015), with higher diversification rates indicating dispersal to novel habitats (Sundue et al., 2015; Testo & Sundue, 2016). In addition to the Himalayas, the uplift of the Andean range was also a key tectonic event during the Cenozoic, and an important factor greatly affecting climate of South America (Zachos et al., 2001; Sepulchre et al., 2010). For species distributed in South America, such as *Campyloneurum*, the formation of the Andean range and its effect on climate may also have affected the evolution of substrate preference, and this has been proposed as the reason for the diversification in *Phlegmariurus* of Lycopodiaceae (Wikström et al., 1999; Testo et al., 2019b).

## 5 Conclusions

Based on the fossils from three subfamilies, including Microsoroideae, Polypodioideae, and Drynarioideae, the divergence of Polypodiaceae can be estimated to have taken place mainly during the K/T boundary and PETM period (Fig. 1; Table 1). Polypodiaceae seem to show substrate preference evolution in both directions, including the development of epiphytism in the early history of the family, and secondary terrestrialization both in Microsoreae of subfamily Microsoroideae and *Campyloneurum* (Fig. 1). Both evolutionary trends seem to coincide with major climatic and geographical changes during the Cenozoic. Secondary

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terrestrialization of Microsoreae may have been driven by the cooler climatic conditions causing a decline of available canopy habitats or opening new ecological opportunities in non epiphytic habitats. The proposed causality between climate and diversification mediated by changes in substrate, may be tested in future studies by applying derived statistical models exploring alternative scenarios. As arguably expected, the Andean orogeny may have played an important role in the evolution of *Campyloneurum*. To examine this in further detail, related biogeographical information, including species distribution, climatic fluctuations, and tectonic plate motion data have to be integrated carefully (e.g., Antonelli et al., 2009).

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Table 1 Estimates for ancestral habitats and divergence dates at nodes n1 – n70 of Fig. 1. Ma = Megaannum (1 million years). Numbers in bold indicate the highest possibilities of habitat preferences.

| Node | Bayesian reconstruction | | Node age (Ma) without *P. takhtajanii* for calibration | Node age (Ma) with *P. takhtajanii* for calibration |
|---|---|---|---|---|
| | terrestrial & rheophytic | epiphytic & lithophytic | | |
| n1 | 0.1384 | **0.8616** | 75.28 (63.62–88.93) | 71.09 (63.6–82.87) |
| n2 | 0.1071 | **0.8929** | 71.73 (61.3–84.16) | 67.55 (60.41–77.9) |
| n3 | 0.0935 | **0.9065** | 65.72 (56.41–77.55) | 61.6 (53.66–71.64) |
| n4 | 0.1079 | **0.8921** | 42.39 (29.23–55.82) | 40.62 (29.1–52.19) |
| n5 | 0.0766 | **0.9234** | 57.32 (49.59–66.53) | 53.29 (46.26–61.26) |
| n6 | 0.1218 | **0.8782** | 55.72 (47.76–65.03) | 51.25 (43.67–59.82) |
| n7 | 0.1016 | **0.8984** | 41.75 (25.77–57.49) | 38.07 (23.51–52.72) |
| n8 | 0.1156 | **0.8844** | 50.5 (42.66–59.46) | 46.74 (39.5–54.88) |
| n9 | 0.0733 | **0.9267** | 40.14 (31.36–49.38) | 37.25 (28.99–45.41) |
| n10 | 0.1626 | **0.8374** | 41.39 (34.53–49.43) | 38.92 (32.67–45.84) |
| n11 | 0.1708 | **0.8292** | 35.51 (30.04–41.74) | 33.68 (28.99–39.2) |
| n12 | 0.0232 | **0.9768** | 16.2 (15.28–18.54) | 16.14 (15.27–18.31) |
| n13 | 0.2143 | **0.7857** | 33.5 (28.69–39.24) | 31.85 (27.49–36.77) |
| n14 | 0.1305 | **0.8695** | 30.25 (26.1–35.62) | 29.09 (25.52–33.57) |
| n15 | 0.4319 | **0.5681** | 30.6 (25.82–35.99) | 29.01 (24.69–33.9) |
| n16 | 0.0339 | **0.9661** | 22.49 (17.35–28.01) | 21.45 (16.73–26.44) |
| n17 | **0.9097** | 0.0903 | 26.64 (22.35–31.7) | 25.16 (20.81–29.71) |
| n18 | 0.4270 | **0.5730** | 24.47 (19.76–29.32) | 23.07 (18.83–27.88) |
| n19 | **0.9353** | 0.0647 | 20.23 (14.81–25.97) | 19 (13.34–24.37) |
| n20 | 0.1045 | **0.8955** | 20.22 (15.38-25.3) | 19.01 (14.41-23.8) |
| n21 | 0.0175 | **0.9825** | 13.52 (9.29–18.07) | 12.65 (8.68–17.02) |
| n22 | **0.9662** | 0.0338 | 25.24 (20.92–30.14) | 23.84 (19.62–28.42) |
| n23 | **0.5571** | 0.4429 | 16.7 (9.69–23.92) | 15.58 (9.02–22.48) |
| n24 | **0.9959** | 0.0041 | 20.32 (16.2–24.85) | 19.22 (15.35–23.45) |
| n25 | 0.0840 | **0.9160** | 54.18 (46.32–62.77) | 50.68 (43.8–58.23) |
| n26 | 0.1861 | **0.8139** | 50.48 (39.83–61.02) | 47.45 (37.97–56.22) |

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| | | | | |
|---|---|---|---|---|
| n27 | **0.5541** | 0.4459 | 36.99 (23.91–50.05) | 34.21 (22.14–46.58) |
| n28 | 0.2539 | **0.7461** | 16.93 (11.84–22.96) | 15.99 (11.1–21.66) |
| n29 | **0.5420** | 0.4580 | 15.18 (10.73–20.32) | 14.29 (10.3–19.33) |
| n30 | **0.9937** | 0.0063 | 10.82 (7.11–15.41) | 10.21 (6.72–14.55) |
| n31 | 0.0773 | **0.9227** | 39.17 (31.48–48.82) | 37.7 (31.16–46.7) |
| n32 | 0.0390 | **0.9610** | 45.89 (39.13–53.95) | 42.64 (36.43–49.56) |
| n33 | 0.0523 | **0.9477** | 22.11 (13.15–33.28) | 20.65 (12.4–30.82) |
| n34 | 0.0076 | **0.9924** | 3.11 (0.95–6.62) | 2.93 (0.88–6.35) |
| n35 | 0.0560 | **0.9440** | 26.32 (17.62–36.54) | 25.06 (16.48–34.35) |
| n36 | 0.0241 | **0.9759** | 16.33 (10.63–23.76) | 15.66 (10.63–22.21) |
| n37 | 0.0346 | **0.9654** | 17.21 (11.99–23.49) | 16 (11.16–21.84) |
| n38 | 0.1673 | **0.8327** | 2.86 (1.58–4.64) | 2.72 (1.4–4.38) |
| n39 | 0.0238 | **0.9762** | 16.39 (11.45–22.28) | 15.25 (10.55–20.68) |
| n40 | 0.0655 | **0.9345** | 21.72 (13.32–31.51) | 20.37 (12.34–28.9) |
| n41 | 0.0607 | **0.9393** | 30.72 (22.99–39.46) | 28.49 (21.24–36.52) |
| n42 | **0.5679** | 0.4321 | 6.16 (1.91–13.6) | 5.92 (1.76–13.22) |
| n43 | 0.0546 | **0.9454** | 23.92 (23.11–25.99) | 23.82 (23.08–25.5) |
| n44 | 0.0449 | **0.9551** | 22.48 (19.09–24.94) | 22.36 (19.1–24.75) |
| n45 | 0.1960 | **0.8040** | 12.91 (7.24–19.26) | 12.47 (6.6–19.33) |
| n46 | 0.0316 | **0.9684** | 13.78 (8.48–19.36) | 13.54 (8.22–19.21) |
| n47 | 0.0123 | **0.9877** | 11.15 (7.27–15.74) | 10.73 (7.04–15.27) |
| n48 | 0.0867 | **0.9133** | 17.01 (12.6–22) | 16.28 (11.92–20.74) |
| n49 | 0.0047 | **0.9953** | 5.09 (2.28–8.97) | 4.8 (2.14–8.41) |
| n50 | 0.1454 | **0.8546** | 13.17 (9.59–17.47) | 12.6 (9.26–16.52) |
| n51 | 0.3526 | **0.6474** | 6.6 (5.44–8.94) | 6.47 (5.4–8.52) |
| n52 | 0.1275 | **0.8725** | 10.69 (7.23–14.73) | 10.21 (6.87–13.86) |
| n53 | 0.4009 | **0.5991** | 8.68 (5.32–12.38) | 8.32 (5.2–11.83) |
| n54 | 0.0094 | **0.9906** | 4.59 (2.29–7.47) | 4.39 (2.25–7.22) |
| n55 | 0.0332 | **0.9668** | 20.31 (15.54–25.57) | 19.32 (14.73–24.19) |
| n56 | 0.0121 | **0.9879** | 17.98 (13.61–22.79) | 17.06 (13–21.8) |
| n57 | 0.1757 | **0.8243** | 34.66 (24.84–45.87) | 33.28 (23.67–43.96) |
| n58 | 0.0680 | **0.9320** | 33.58 (28.14–41.26) | 32.62 (28.07–39.54) |
| n59 | 0.1147 | **0.8853** | 12.33 (8.17–17.7) | 11.78 (7.9–16.7) |
| n60 | 0.0389 | **0.9611** | 29.31 (26.08–34.9) | 28.74 (25.92–33.33) |
| n61 | 0.0945 | **0.9055** | 26.22 (21.3–32.38) | 25.65 (20.57–30.99) |
| n62 | 0.0423 | **0.9577** | 27.1 (25.72–30.91) | 26.89 (25.7–30.04) |
| n63 | 0.1028 | **0.8972** | 16.68 (12.26–21.6) | 15.98 (11.64–20.67) |
| n64 | 0.0256 | **0.9744** | 16.04 (9.9–23.3) | 15.31 (9.37–22.53) |
| n65 | 0.0364 | **0.9636** | 20.8 (18.3–23.61) | 20.58 (18.2–23.22) |

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| | | | | |
|---|---|---|---|---|
| n66 | 0.0449 | **0.9551** | 18.78 (17.07–21.13) | 18.63 (16.91–20.84) |
| n67 | 0.0836 | **0.9164** | 35.5 (26.6–44.38) | 32.86 (24.6–41.04) |
| n68 | 0.0281 | **0.9719** | 41.85 (35.56–49.22) | 38.92 (33.3–45.38) |
| n69 | 0.0202 | **0.9798** | 39.27 (33.21–45.97) | 36.58 (31.18–42.24) |
| n70 | 0.0230 | **0.9770** | 38.09 (32.35–44.72) | 35.47 (30.29–41.12) |

Table 2 Age constraints used to calibrate and estimate divergence dates in Bayesian Evolutionary Analysis Sampling Trees (BEAST), with time in millions of years (Ma). A dash indicates lack of data. The symbol (†) indicates the fossil was used only for comparison purposes.

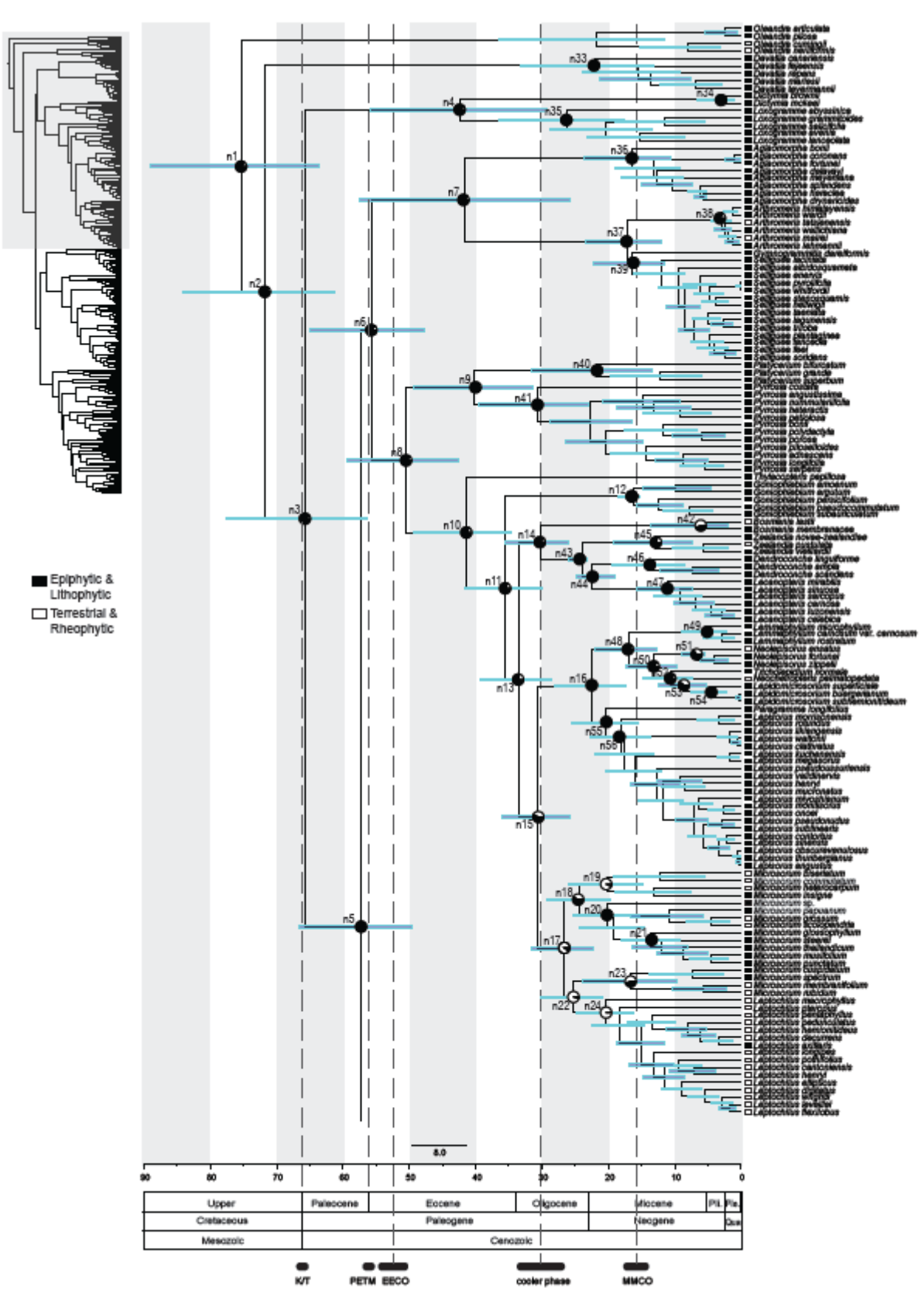
| Min Age | fossil | Clade | Stem/ Crown, Node | Priors |
|---|---|---|---|---|
| 5.3 | *Aglaomorpha heraclea* | *A. Heraclea*; Drynarioideae | Stem | lognormal |
| 15.2 | *Goniophlebium macrosorum* | *Goniophlebium*; Microsoroideae | Crown (n12) | lognormal |
| 5.3 | *Neolepisorus chingii* | *Neolepisorus*; Microsoroideae | Crown (n51) | lognormal |
| 23 | *Palaeosorum waipiata* | *Zealandia*; Microsoroideae | Stem (n43) | lognormal |
| 15.8 | *Pleopeltis dominicensis* | *Pleopeltis* subclade including *P. disjuncta*; Polypodioideae | Crown | lognormal |
| 25.6 | *Polypodium radonii* | *Polypodium*; Polypodioideae | Crown (n64) | lognormal |
| 33.9 | *Protodrynaria takhtajanii* † | Drynarioideae | Stem (n6) | lognormal |
| 63.6 | secondary node calibrations | Oleandraceae, Davalliaceae, and Polypodiaceae | Crown (n1) | exponential |
| 60.4 | secondary node calibrations | Davalliaceae, and Polypodiaceae | Crown (n2) | exponential |

Figure Captions

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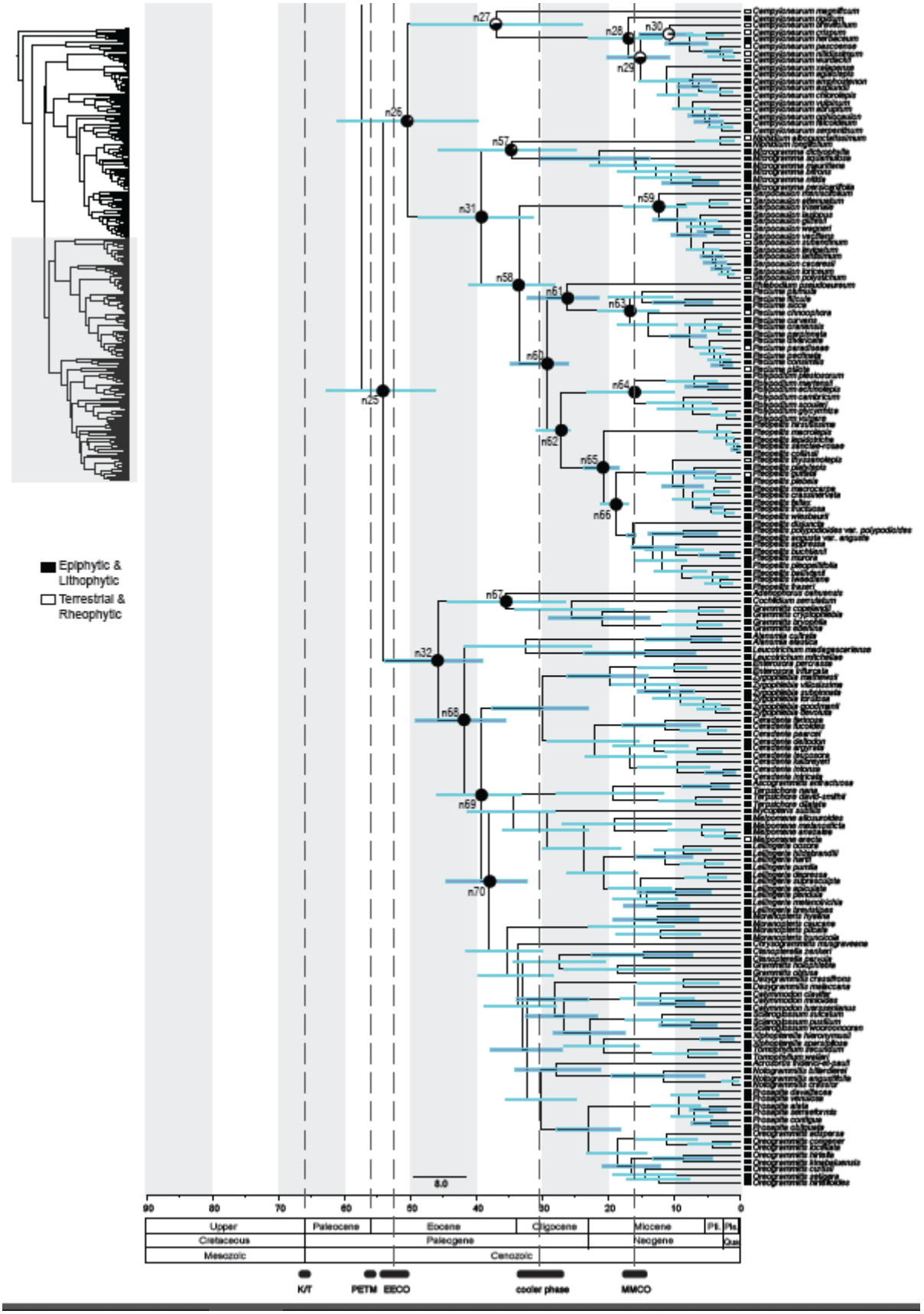



**Fig. 1.** Molecular dating estimates and ancestral area reconstructions inferred by Bayesian Evolutionary Analysis Sampling Trees (BEAST) and BayesTraits, respectively. The blue bars correspond to 95% highest posterior density (HPD) intervals of the median divergence dates. The pie chart at each node indicates the ancestral states. Detailed data of n1-n70 can be found

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in Table 1. The climate period marks are based on Zachos et al. (2001). Abbreviations: K/T, Cretaceous/Tertiary boundary; PETM, Paleocene/Eocene thermal maximum; EECO, early Eocene climatic optimum; MMCO, middle Miocene climatic optimum.

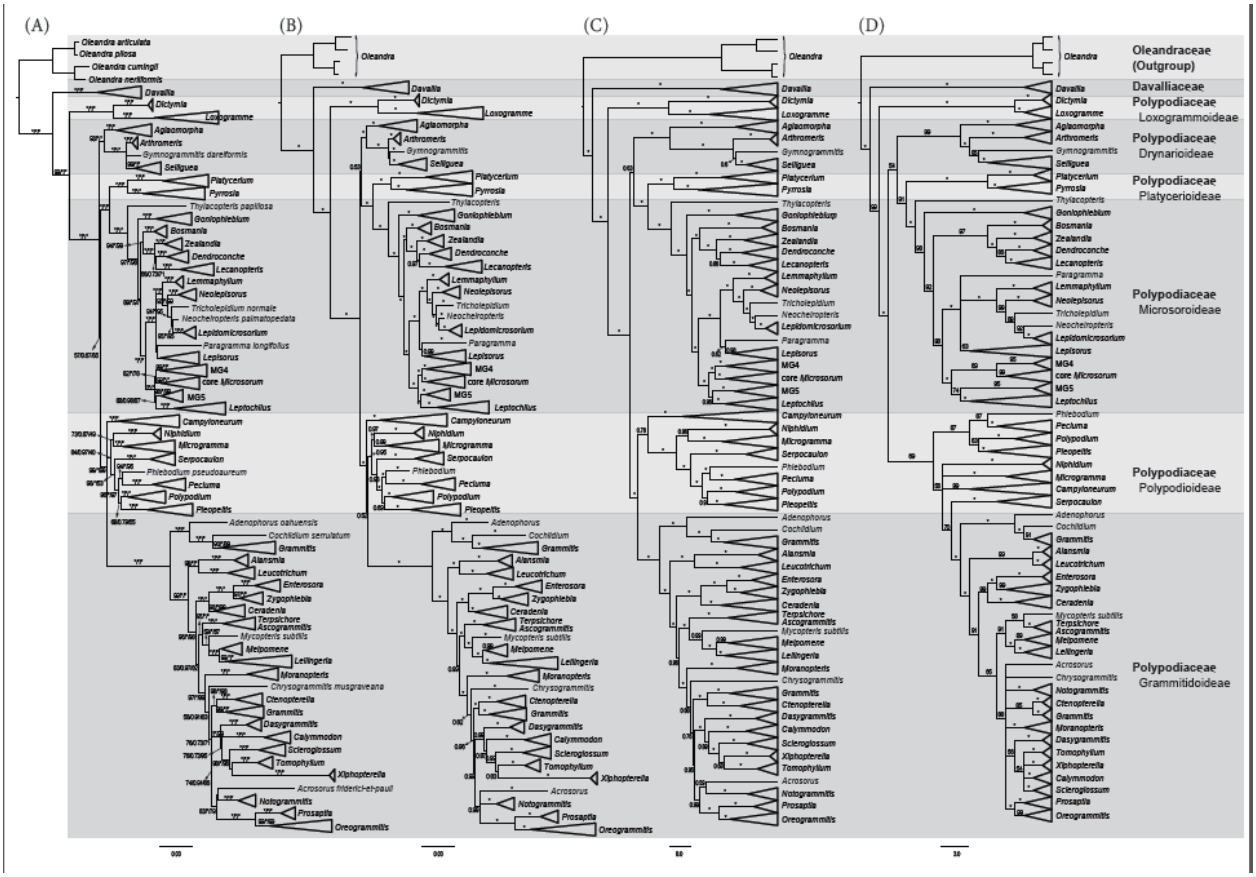



**Fig. 2.** Inferred phylogenetic topologies depicting the relationships of Polypodiaceae. **A,** Maximum Likelihood (ML) topology generated by IQ-tree. The values indicate a Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT, %), p-values of the Bayesian-like transformation of aLRT statistics (abayes), and ultrafast bootstrap analysis (UFBoot, %). **B,** Bayesian Inference (BI) topology generated by MrBayes; the values indicate posterior confidence values (pp). **C,** BI topology generated using Bayesian Evolutionary Analysis Sampling Trees (BEAST); the values indicate posterior confidence values (pp). **D,** Parsimony topology generated with PAUP, with the bootstrap value. An asterisk (*) indicates branches with maximum values of the indices used for all tree topologies.

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