

Overcoming among-lineage rate heterogeneity to infer the divergence times and biogeography of the clubmoss family Lycopodiaceae

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Abstract

Aim: To infer divergence times and historical biogeography of the cosmopolitan lycophyte family Lycopodiaceae.

Location: Worldwide.

Methods: We generated time-calibrated phylogenies of the Lycopodiaceae based on six regions of chloroplast DNA using a node-dating approach implemented in BEAST with eight fossil calibrations. To investigate effects of among-lineage substitution rate heterogeneity on divergence time estimation, we compared the performance of two relaxed clock models: an uncorrelated lognormal clock model and a random local clock (RLC) model. The historical biogeography of the family was inferred using two Bayesian models implemented in BioGeoBEARS.

Results: Divergence time estimates for major groups of the Lycopodiaceae obtained using the two substitution clock models differed substantially, and the RLC model was a better fit. The Lycopodiaceae crown group age is estimated to be late Devonian, and most deep divergence events date to the Carboniferous, with most extant species diversity accumulating during the Cenozoic. The timing of divergences of major clades in the Lycopodiaceae corresponds to the breakup of the Pangaeian and Gondwanan supercontinents. Long-distance dispersal events are relatively common, but generally do not appear to be followed by subsequent radiations.

Main conclusions: Accounting for among-lineage substitution rate heterogeneity improves divergence time estimates for the Lycopodiaceae. The family has a deep evolutionary history, and continent-scale vicariance events in the Mesozoic appear to have been associated with major cladogenesis events, with long-distance dispersal playing a relatively minor role.

KEYWORDS

clubmoss, Gondwana, long-distance dispersal, Pangaea, phylogenetics, vicariance

1 | INTRODUCTION

Comprising three extant families (Isoetaceae, Lycopodiaceae and Selaginellaceae), the lycophytes hold an important phylogenetic position sister to all other extant vascular plants (Wickett et al., 2014). This ancient lineage has its origins in the Silurian (Garratt,

Tims, Rickards, Chambers, & Douglas, 1984; Rickards, 2000) and was among the dominant life-forms in Earth's ecosystems through the Carboniferous (DiMichele, Stein, & Bateman, 2001). Though modern lycophyte diversity and ecological prominence pales in comparison to that of their Palaeozoic relatives, and they are today overshadowed by seed plants and ferns, understanding the

evolutionary history of the group is important because it provides insight into the timing and patterns of early land plant diversification. Recent studies have examined the divergence timing and historical biogeography of the Isoetaceae (Pereira, Labiak, Stützel, & Schulz, 2017) and Selaginellaceae (Klaus, Schulz, Bauer, & Stützel, 2017; Weststrand, 2016); however, the only dated phylogeny of the Lycopodiaceae (Wikström & Kenrick, 2001) was published more than 15 years ago and did not include a biogeographical analysis.

Though the Wikström and Kenrick study was an important early application of divergence time estimation (DTE) and has provided insight into the timing of lycophyte diversification, it does have significant limitations. First, fewer than 10% of described Lycopodiaceae species were included in the phylogeny. Sparse taxon sampling can lead to biased divergence time estimates (Duchêne, Lanfear, & Ho, 2014; Linder, Hardy, & Rutschmann, 2005; Renner, 2005; Zheng & Wiens, 2015), especially in the absence of sufficient fossils for node calibration, as is the case in the Lycopodiaceae. Furthermore, among-lineage substitution rate heterogeneity poses a significant challenge to DTE, especially when models that assume some sort of a molecular clock are used (Drummond & Suchard, 2010; Sanderson, 1997; Welch & Bromham, 2005). Since the Wikström and Kenrick study was published, methods that explicitly permit among-lineage rate heterogeneity have been developed (Drummond, Ho, Phillips, & Rambaut, 2006; Drummond & Rambaut, 2007; Drummond & Suchard, 2010; Renner, 2005); these have provided markedly different estimates of divergence times in numerous clades of organisms (Bell, Soltis, & Soltis, 2010; Douzery, Snell, Bapteste, Delsuc, & Philippe, 2004; Magallón, Gómez-Acevedo, Sánchez-Reyes, & Hernández-Hernández, 2015; Smith, Beaulieu, & Donoghue, 2010; Testo & Sundue, 2016). Given the demonstrated high rates of substitution in *Selaginella* (Korall & Kenrick, 2004) and evidence (from molecular branch lengths) of similarly elevated rates in some taxa in the Lycopodiaceae (Field, Testo, Bostock, Holtum, & Waycott, 2016), we expect that more sophisticated dating analyses may improve estimates of divergence times in the Lycopodiaceae as well.

The aim of this paper was to infer the evolutionary history of the Lycopodiaceae with improved marker and taxon sampling and more sophisticated analyses than were available to Wikström and Kenrick (2001). We increase sampling within the Lycopodiaceae to >50% of the family's diversity, use sequence data from six chloroplast markers, and incorporate eight fossil calibrations to construct our phylogeny and infer divergence times. To understand and mitigate the effects of among-lineage rate heterogeneity on our age estimates, we carry out model comparison between two relaxed molecular clock models implemented in BEAST—an uncorrelated log-normal clock (UCLN) and a random local clock (RLC)—and compare date estimates obtained using each method. Finally, we infer the historical biogeographical history of the Lycopodiaceae to place the diversification of the family within the context of Earth's complex geologic history and to elucidate the relative roles of vicariance and long-distance dispersal.

2 | MATERIALS AND METHODS

2.1 | Taxon sampling

Ingroup sampling included a total of 199 taxa, representing all 16 Lycopodiaceae genera (Pteridophyte Phylogeny Group I, 2016). Species were sampled from all regions globally where significant species richness has been described. In some cases, multiple accessions of a given named taxon were included when that species was known to be polyphyletic and comprised of multiple cryptic species; these were treated as independent taxa in our analyses. Outgroups were selected from the sister families Selaginellaceae and Isoetaceae as well as representatives of the other major land plant lineages; representatives of outgroups were selected with a focus on capturing the deepest divergences in their corresponding clade.

2.2 | DNA extraction, amplification and sequencing

Total genomic DNA was extracted from silica-dried leaf material or herbarium specimens using a standard CTAB extractions protocol (Doyle & Doyle, 1987). PCR was performed in 25 μ l mixtures of 12.5 μ l Bullseye Taq mix (MIDSCI, St. Louis), 9 μ l water, 1.25 μ l each of 10 mM forward and reverse primers, and 1 μ l of 10–20 ng \times μ l⁻¹ DNA. Six regions of chloroplast DNA (*rbcl*, *psbA-trnH*, *rps4-trnS*, *trnL*, *trnL-trnF* and *trnP-petG* IGS) were amplified; amplification primers and reaction conditions are provided in Table 1. All primers were designed for this study based on publicly available chloroplast genome sequences. PCR products were purified using shrimp alkaline phosphatase and subsequently diluted to 2 ng \times μ l⁻¹. DNA sequencing was performed in both forward and reverse directions using BigDye chemistry on an ABI 3730xl DNA analyzer at Genewiz, South Plainfield, New Jersey, USA and at Australian Genome Research Facility, University of Queensland, Australia. Sequences were assembled in Geneious 10.0.3 (Biomatters, Ltd.) and were visually inspected and manually edited as needed. Voucher information and GenBank accession numbers are provided in Appendix S1 in the Supporting Information.

2.3 | Divergence time analyses

Sequences for each region were aligned with the MAFFT (Katoh, Misawa, Kuma, & Miyata, 2002) plugin in Geneious using a gap-open penalty of 1.1–1.4 and an offset value of 0.123. We used jModelTest 2 (Darriba, Taboada, Doallo, & Posada, 2012) on each marker partition to identify best-fitting substitution models for downstream phylogenetic analyses (Table 1); the concatenated six marker dataset was used for all subsequent analyses. To facilitate computation, we generated a starting tree for all DTE analyses using a maximum likelihood approach implemented in RAxML 8.2.10 (Stamatakis, 2006) implemented in the Cipres Science Gateway portal (Miller, Pfeiffer, & Schwartz, 2010). To generate the starting tree, the GTR+ Γ substitution model was employed across all partitions following the suggestion of Stamatakis (2006), and the monophyly of seed plants, ferns

TABLE 1 Nucleotide substitution model parameters for each marker used, primers used for PCR and sequencing, and PCR conditions. PCR conditions include time in seconds on the top row and temperature in degrees Celsius on the bottom row of each cell

Marker	Aligned length	% missing	Best model	Primers	PCR conditions denat./ anneal/exten
<i>petG-trnP</i>	656	9%	TIM1+I+G	GATGTAGCGCAGCTTGGTAGC (f) ATGGTTGAAGCTCTTATCCGG (r)	45/45/60 94/55/72
<i>psbA-trnH</i>	419	23%	K80+G	GTTATGCATGAACGTAATGCTC (f) CGCGCATGGATTACAATCC (r)	45/45/60 94/48/72
<i>rbcl</i>	1,417	56%	TIM1+I+G	ATGTCACAAACGGAGACTAAAGC (f) TCAGGACTCCACTTACTAGCTTCACG (r)	45/45/90 94/53/72
<i>rps4-trnS</i>	657	19%	GTR+I+G	ATGTCCCGTTATCGAGGACCTC (f) TTACCGAGGGTTCGAATCCCTC (r)	45/45/60 94/55/72
<i>trnL</i>	510	22%	F81+G	CGAAATCGGTAGACGCTACG (f) GGGGATAGAGGGACTTGAAC (r)	45/45/60 94/52/72
<i>trnL-trnF</i>	862	30%	GTR+I+G	GGTCAAGTCCCTCTATCCC (f) ATTTGAAGTGGTGACACGAG (r)	45/45/60 94/55/72

and lycophytes was enforced. The resulting best tree was retained for use in subsequent analyses; this tree is available in Appendix S2.

We performed all DTE analyses in BEAST 2.4.5, using the Cipres Science Gateway portal (Miller et al., 2010). For all analyses, the matrix was partitioned by marker, and substitution models were assigned to each partition following the results of our model test analysis. We ran the analyses with trees linked and rates unlinked. Because of a priori evidence of significant rate heterogeneity both between the Lycopodiaceae and one of its sister families (Selaginellaceae) as well as within the Lycopodiaceae itself, special care was taken to consider the effect of molecular clock models on DTE. We compared the performance of two molecular clock models: an UCLN model (Drummond et al., 2006), in which different rates are assigned to each branch from a lognormal distribution of values, and a RLC model (Drummond & Suchard, 2010), which allows for a set of distinct substitution rate regimes to be applied locally across a phylogeny. The UCLN model allows for substitution rates for each branch in the phylogeny to be drawn from a single unimodal prior distribution, whereas the RLC model works by proposing and comparing various regimes of local strict clocks; each of these clocks can arise along any branch in the phylogeny and is applied to a set of descendent branches. Evidence from both real and simulated datasets suggests that RLC models outperform UCLN models when sudden shifts in substitution rates occur across a phylogeny (Bellot & Renner, 2014; Crisp, Hardy, & Cook, 2014; Dornburg, Brandley, McGowen, & Near, 2012), but it may be a poor fit in cases where rate variation is not punctuated along the phylogeny.

For all analyses, a birth–death tree prior was selected with a uniform prior from 0–10 for speciation rate and 0–1 for relative extinction rate; starting values were 1.0 for speciation and 0.5 for relative extinction. For each analysis, we performed three separate MCMC runs of 100 million generations and sampled every 40,000 generations, resulting in 2,500 trees per run and 7,500 trees in total. The resulting log files were inspected for convergence and adequate sampling using TRACER 1.6 (Drummond & Rambaut, 2007); ESS values

all exceeded 200 and the first 20% of trees were discarded as burn-in. A maximum credibility tree was generated from the remaining 6,000 trees. The fit of alternative clock models was compared by performing path sampling analyses (Baele, Li, Drummond, Suchard, & Lemey, 2013; Baele et al., 2012) and calculating Bayes factors. Both the UCLN and RLC trees are provided in the Supporting Information, as Appendices S3 and S4, respectively.

A node-dating approach incorporating eight fossils was used to calibrate the timing of divergences across the land plant phylogeny. In all cases, minimum ages assigned to fossils were based on the age of the oldest stratigraphic layer from which the fossil is known. To constrain the most recent common ancestor (MRCA) of land plants, we took a conservative approach and assigned a uniform distribution from 472–444 Ma, bridging the span of time between the oldest likely land plant fossils (cryptospores from the Middle Ordovician of Argentina; Rubinstein, Gerrienne, de la Puente, Astini, & Steemans, 2010) and the oldest fossils of certain affinity to the embryophyte lineage (trilete spores from the Silurian in Sweden, Steemans et al., 2009). All other fossil calibrations were provided lognormal prior distributions defined with the lower bound at the oldest age of the fossil and $\mu = 3$ and $\sigma = 1$, providing a prior shape that places the highest probability at an age just older than the fossil and 95% of the prior space within bounds of 100 Myr older than the fossil. We chose this conservative application of priors to allow the fossil record to constrain key divergence events in the land plant phylogeny, while allowing the possibility that lineages could predate their appearance in the fossil record by a considerable amount.

The node marking the divergence of lycophytes and euphyllophytes was constrained at a minimum age of 420 Ma based on the lycophyte *Baragwanathia longifolia*, which dates to the Upper Silurian (Garratt et al., 1984; Hueber, 1992). The node marking the divergence of heterosporous and homosporous lycophytes was constrained to a minimum age of 385 Ma, based on *Leclerqia complexa*, which belongs to the stem lineage subtending Selaginellaceae and Isoetaceae and is known from the Middle Devonian (Meyer-

Berthaud, Fairon-Demaret, Steemans, Talent, & Gerrienne, 2003). The crown node of the Selaginellaceae was given a minimum age of 311 Ma, based on *Selaginella suissei*, which dates to the mid-Pennsylvanian and clearly belongs to the Selaginellaceae (Thomas, 1997). The euphyllophyte crown node was assigned a minimum age of 385 Ma based on *Ibyka*, dating to the Middle Devonian (Skog & Banks, 1973), which has affinities to the fern lineage (Kenrick & Crane, 1997). The divergence of the Equisetales from the remainder of the ferns was given a minimum age of 346 Ma, given the occurrence of the Equisetalean *Archaeocalamites* in the Lower Carboniferous (Bateman, 1991). The crown node of leptosporangiate ferns was constrained at a minimum of 299 Ma, based on *Grammatopteris*, which belongs to Osmundales and is known from the Lower Permian (Skog, 2001). The seed plant crown node was given a minimum age of 323 Ma, corresponding the occurrence of Cordaitalean fossils from near the Lower–Upper Carboniferous boundary, which we interpret as members of an early divergent gymnosperm lineage, following Crane (1985).

2.4 | Ancestral range estimation

We inferred the biogeographical history of the Lycopodiaceae using the R package 'BioGeoBEARS' (Matzke, 2013) using a dispersal–extinction–cladogenesis (DEC) model (Ree & Smith, 2008) on the maximum credibility tree from our RLC DTE analysis. We ran the analysis with two variants of the DEC model, one with and one without the “jump” parameter (j) of Matzke (2014); these models were compared using a log-likelihood ratio test, and the better-fitting model was selected. Species distributions were determined through examination of floras (Mickel & Smith, 2004; Øllgaard, 1995; Wagner & Beitel, 1992; Wu, Dong, & Raven, 2013), taxonomic treatments (Øllgaard, 1989, 1992) and examination of herbarium specimens and scans from the following herbaria: AAU, B, BHC, BISH, BM, BO, BONN, BR, BRI, CANB, CHR, CNS, COL, CR, E, FI, GH, GOET, HAL, HO, HUA, JCT, KLU, K, L, LD, LE, LINN, MEL, MICH, MO, NSW, NY, P, PR, PRC, QRS, S, S-P, SING, TUD, U, UC, US, UPS, VT and W. Nine biogeographical areas were included in the analysis: North America, Central and tropical South America, south-eastern Brazil, temperate South America, Eurasia, Africa and Madagascar, Eastern Asia, South-east Asia and Oceania. These areas were chosen because they reflect the biogeographical patterns observed in the Lycopodiaceae and generally represent geologically distinct regions across most of the family's deep evolutionary history. We did not impose any limits on the maximum number of areas that could be occupied by a given taxon, and all combinations of areas were allowed, as one extant species, *Lycopodium clavatum*, occupies all of them. To account for change in geographic proximity of landmasses over time, we incorporated four different dispersal probability matrices in our analysis, with each corresponding to nonoverlapping periods of time. The time periods were defined as follows: more than 200, 200–100, 100–50 and 50–0 Ma; these time slices were chosen to reflect major motifs of continental drift, including the disintegration of Pangaea, the subsequent separation of both Laurasia and Gondwana, and the

gradual shift of the resulting continents to their current positions. The bias towards finer division among more recent time slices is due to the fact that most diversification within the family appears to have occurred within the last 100 Myr. Attempts to run the analysis with additional time slices caused the analysis to take several weeks without approaching completion. The values provided in the dispersal matrices were based on rough estimates of the distance between landmasses and, for the most recent time slice, the directions of prevailing winds and general patterns of migration exhibited by wind-dispersed plant groups. The values assigned are arbitrary (see Ree & Smith, 2008) but represent our best estimates of relative dispersal probabilities among regions at different time periods. Species distributions and our dispersal matrices are provided as in the Supporting Information as Appendices S5 and S6 respectively.

3 | RESULTS

3.1 | Divergence time estimation

Our ML best tree recovered phylogenetic relationships generally concordant with those previously reported within the Lycopodiaceae (Field et al., 2016) and within other major lineages, and the relationships among major land plant lineages were consistent with those recovered in recent large-scale analyses (Magallón, Hilu, & Quandt, 2013; Testo & Sundue, 2016; Wickett et al., 2014). Support values in both the UCLN and RLC trees are generally high, except in some recently divergent clades, where support is moderate to weak (Appendices S3 and S4). Within the Lycopodiaceae, the only prominent difference between our UCLN and RLC analyses was the position of *Phylloglossum* (Huperzioideae); it was sister to the rest of the Huperzioideae in our RLC analysis and sister to *Huperzia* in our UCLN analysis. Our path sampling analyses indicated that the best-fitting clock model was the RLC ($\Delta\ln L = 70.06$, BF = 13.7).

Our RLC analyses recovered three distinct substitution rate regimes across the Lycopodiaceae, with mean regime rates ranging from 1.3×10^{-3} (95% HPD 1.1–1.45) substitutions site⁻¹ Myr⁻¹ in *Palhinhaea* (Lycopodielloideae) to 2.1×10^{-4} (95% HPD 2.05–2.32) substitutions site⁻¹ Myr⁻¹ in *Phlegmariurus* (Huperzioideae). Apart from the background rate regime of the family, distinct elevated rate regimes were found in subfamily Lycopodielloideae and in *Phylloglossum*.

Divergence date estimates varied between the RLC and UCLN models (Figure 1). Dates obtained with the RLC model were generally older and had broader highest probability intervals, whereas the UCLN model tended to provide the younger ages and narrower highest probability intervals. Major differences in clade age estimates between these models were most evident for the crown age of the Lycopodiaceae, (mean age of 368.4 Ma [95% HPD 325–383 Ma] under the RLC model and 308.8 Ma [95% HPD 248–333 Ma] under the UCLN model) and among the subfamily Huperzioideae. The estimates generated from the best-fitting (RLC) clock model indicate that lycophytes diverged from the euphyllophyte lineage 431.5 Ma (95% HPD 426–440 Ma), that the homosporous and heterosporous

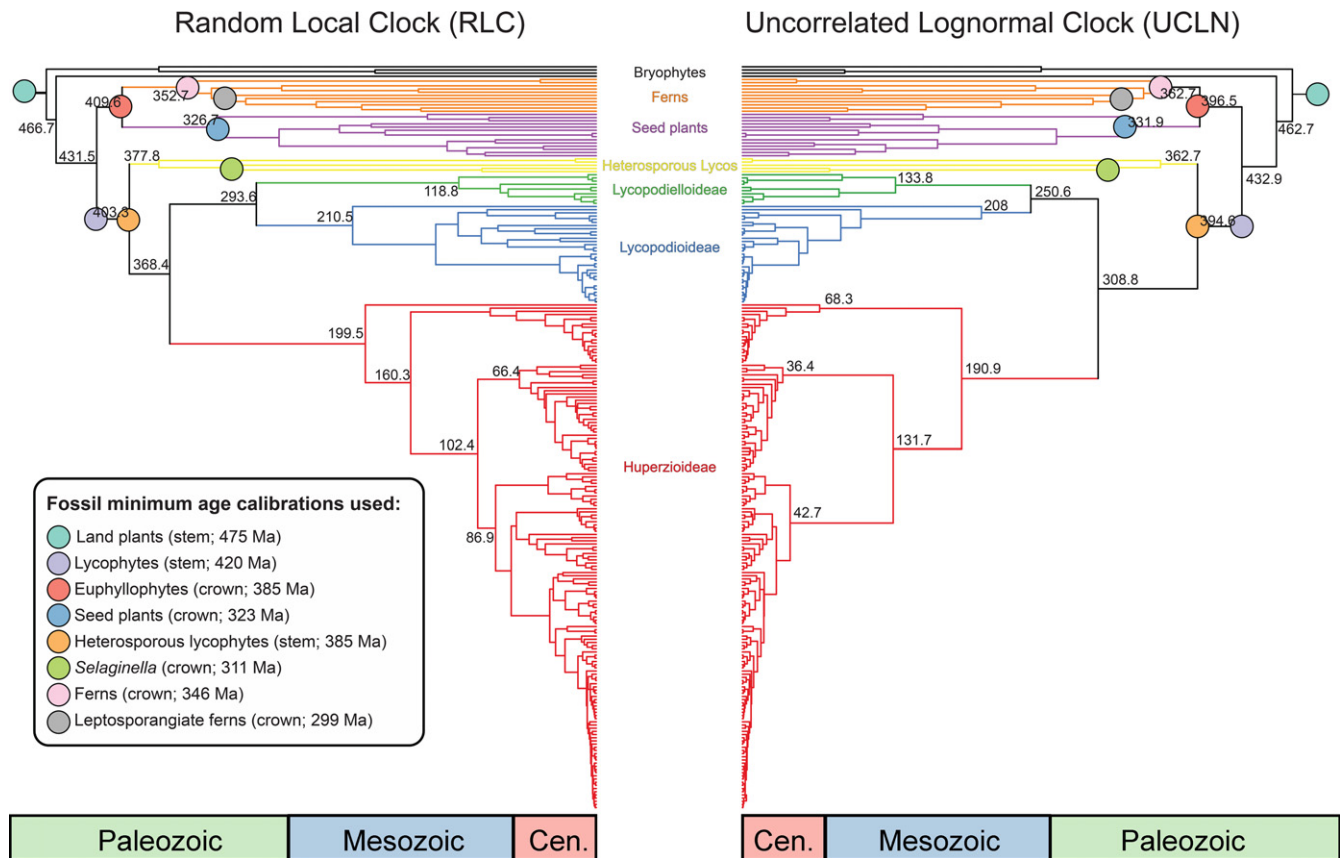


FIGURE 1 Comparison of divergence time estimates obtained using random local clock and uncorrelated lognormal clock models. Trees are maximum credibility BEAST trees obtained from each model with median dates shown. Branch colours designate major land plant clades, and coloured circles indicate the position of fossil node calibrations

lycophyte lineages diverged 403.3 Ma (95% HPD 394–414 Ma) and that the crown age of the Lycopodiaceae was 368.4 Ma (95% HPD 326–389 MYA). Within the Lycopodiaceae, we estimate that the crown age of subfamily Huperziaceae is 199.5 Ma (95% HPD 175–223 Ma) and that the divergence of subfamilies Lycopodielloideae and Lycopodiaceae occurred at 293.6 Ma (95% HPD 262–320 Ma). Divergence time estimates for genera range from 210.4 Ma (95% HPD 202–236 Ma) for the split of *Lycopodiastrum* (Lycopodiaceae) from the rest of subfamily Lycopodiaceae to 53.1 Ma (95% HPD 35–66 Ma) for the divergence between *Austrolycopodium* and *Pseudodiphasium* (Lycopodiaceae).

3.2 | Ancestral range estimation

Comparison of the DEC and DEC+*j* models implemented in BioGeoBEARS indicated that the inclusion of the *j* parameter resulted in a more likely model (DEC lnL = −447.02, DEC+*j* lnL = −429.03, $\Delta = 35.98$, $p = 0.023$). Results obtained with the DEC+*j* model indicate that the most likely ancestral range of the Lycopodiaceae is North America+Eurasia+East Asia+Oceania, that the ancestral range of subfamilies Lycopodielloideae and Lycopodiaceae were Oceania and that of subfamily Huperziaceae was East Asia+Southeast Asia+Oceania. Within the Lycopodiaceae, most lineages are widespread in the north temperate region, though a clade comprising

Pseudolycopodium, *Pseudodiphasium* and *Austrolycopodium* diversified in Oceania, with a subsequent long-distance dispersal event in *Austrolycopodium* to southern South America. Subfamily Lycopodielloideae appears to have radiated out of Oceania in the late Mesozoic, with a generally north temperate affinity among *Lycopodiella* and tropical/austral distributions in *Lateristachys*, *Palhinhaea* and *Pseudolycopodiella*. In the Huperziaceae, we recover an ancestral range of Oceania for *Phylloglossum*, East Asia+tropical Asia for *Huperzia* and Neotropics+East Asia+Africa+tropical Asia+Oceania for *Phlegmariurus*. We recover several recent long-distance dispersal events between austral regions in *Huperzia*, and many species are shared between North America and East Asia. In *Phlegmariurus*, the divergence of its two principal clades into the Neotropics and Palaeotropics occurred during the lower Cretaceous (102 Ma); several subsequent long-distance dispersal events occurred between these regions.

4 | DISCUSSION

4.1 | Substitution rate heterogeneity and clock model effects

We find evidence for two shifts in nucleotide substitution rate across the Lycopodiaceae, in the subfamily Lycopodielloideae and *Phylloglossum*. The shifts in substitution rate observed in these

groups appear to be related to a transition in growth strategy, as both taxa are characterized by short life cycles in comparison to other members of the family. In particular, members of the subfamily Lycopodielloideae and *Phylloglossum* possess partly or wholly surficial, photosynthetic gametophytes that are fast-growing in comparison to the subterranean gametophytes found in other members of the family. Though elevated rates of substitution are known to occur in groups with fast generation times in plants (Clegg, Gaut, Learn, & Morton, 1994; Gaut, Muse, Clark, & Clegg, 1992; Lanfear et al., 2013), ours is the first evidence of coordinated shifts in substitution rates and gametophyte ecology. As gametophyte growth rates and reproductive biology vary dramatically across both lycophytes (Bruce, 1976; Whittier, 1998) and ferns (Haufler et al., 2016; Nayar & Kaur, 1971; Raghavan, 1989; Sessa, Testo, & Watkins, 2016; Whittier, 1981), this finding has important implications for understanding the variation in rates of molecular evolution observed across these clades of taxa, all characterized by independent gametophyte life phases. Further study of the correlation of gametophyte longevity and nucleotide substitution rates is needed, but this effect may explain the patterns of anomalous substitution rates in some fern genera with either fast- (e.g. *Ceratopteris*; Hickok, Warne, & Fribourg, 1995) or slow-growing (e.g. *Botrychium*, *Ophioglossum*; Whittier, 1981) gametophytes.

We demonstrate a significant effect of clock model on estimates of divergence times in the Lycopodiaceae (Figures 1 and 2), with a

RLC model outperforming the UCLN clock model. Our finding of improved performance of a RLC model is consistent with findings from other groups that include lineages in which punctuated shifts in molecular evolution rates are known, such as the monocot family Xanthorrhoeaceae (Crisp et al., 2014) and cetaceans (Dornburg et al., 2012). Given the strong effect of clock model on divergence time estimates reported here and in other studies (Beaulieu, O'Meara, Crane, & Donoghue, 2015; Crisp et al., 2014; Dornburg et al., 2012; Wertheim & Sanderson, 2011), researchers should be cognizant of potentially misleading inferences of divergence times stemming from ill-fitting clock models, especially if their study group presents a priori evidence for among-lineage rate heterogeneity.

4.2 | Divergence time estimates

Considering estimates obtained from the RLC model, our divergence time estimates for the Lycopodiaceae are generally younger than those reported by Wikström and Kenrick (2001), though our estimates for the initial divergences in the family are similar. Congruence of our estimates and those of Wikström and Kenrick (2001) among deep divergences may be primarily a function of similar fossil calibrations constraining the backbone of both phylogenies; the influence of fossil calibration choice on age estimates deep in the land plant phylogeny has been demonstrated previously (Magallón, Sanderson, & Soltis, 2005; Magallón et al., 2013; Yang & Rannala, 2006). Among

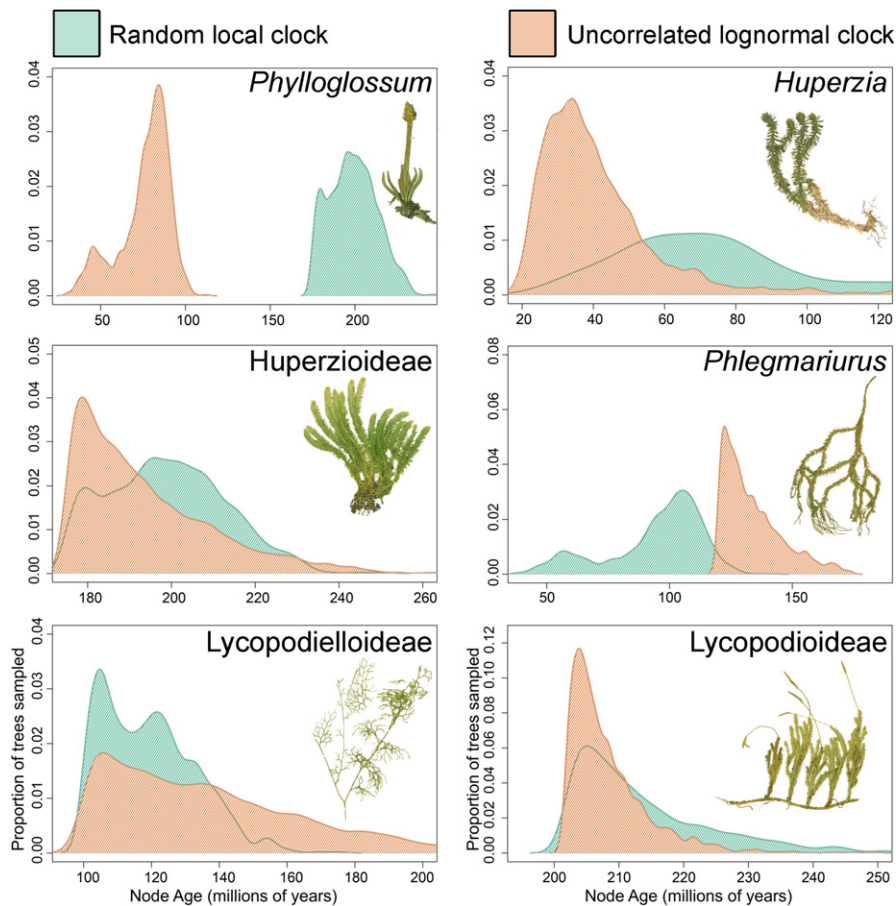


FIGURE 2 Comparison of crown age distributions from select clades in the Lycopodiaceae. Distributions are kernel density plots of mean ages obtained across the MCMC runs under each clock model



the internal nodes of the Lycopodiaceae, which were uncalibrated in Wikström and Kenrick (2001), and constrained only at a single node in our phylogeny, our dates diverge more conspicuously, though direct comparisons are difficult to make due to much denser taxon sampling in our study. Considering the crown ages for major clades that were well-sampled in both studies, we report considerably (c. 50–90 Myr) younger ages for all three subfamilies and most genera. These differences may be due to the dramatic acceleration of substitution rates found in subfamily Lycopodielloideae and *Phylloglossum*, given that major among-lineage heterogeneity in rates of molecular evolution can drive artificially old age estimates under relaxed clock models (Beaulieu et al., 2015). The difference in placement of *Phylloglossum* between our UCLN and RLC analyses may also have an impact on estimates; our analyses with fixed topologies indicated such an effect was minimal.

We recover the divergence of the homosporous and heterosporous lycophytes to be during the early Devonian (median age = 403 Ma). This age estimate is consistent with the hypothesis of a relatively rapid diversification of the major embryophyte lineages over approximately 50–75 Myr after the initial colonization of land by plants, which is corroborated both by fossil (Crane, Herendeen, & Friis, 2004; Kenrick & Crane, 1997) and molecular phylogenetic (Bateman et al., 1998; Finet, Timme, Delwiche, & Marlétaz, 2010; Magallón et al., 2013; Testo & Sundue, 2016) evidence. Consistent with the findings of Wikström and Kenrick (2001), our estimates of the divergences among subfamilies of the Lycopodiaceae date to the Carboniferous and Permian, indicating that the initial diversification of the family was underway well before the initial breakup of Pangaea at approximately 175 Ma. The divergence of the three ecologically disparate genera comprising the Huperzioidae (*Huperzia*, *Phlegmariurus* and *Phylloglossum*) coincided with the breakup of Pangaea, with these three lineages splitting during the lower to middle Jurassic. A critical innovation that appears to be attained during this initial burst of lineage diversification and specialization within the Lycopodiaceae is the evolution of epiphytism in *Phlegmariurus*, which several analyses have indicated to be the ancestral growth habit of the genus (Field et al., 2016). Assuming that epiphytism evolved in *Phlegmariurus* coincidentally with the divergence of the genus or shortly thereafter, this marks one of the earliest transitions to epiphytic growth known among vascular plants, along with filmy ferns (Hennequin, Schuettpelz, Pryer, Ebihara, & Dubuisson, 2008). Other early epiphytes, such as the ophioglossalean fern *Botryopteris*, grew as trunk epiphytes on Marattialean tree ferns (Rothwell, 1991); the earliest epiphytic *Phlegmariurus* may have exhibited a similar growth strategy. Given that vascular epiphytes were rare until the late Cretaceous (Benzing, 1990; Schuettpelz & Pryer, 2009; Taylor, Taylor, & Krings, 2009; Watkins & Cardelús, 2012), the early radiation into and persisting success in the epiphytic niche by *Phlegmariurus* is remarkable. Altogether, it appears that the early divergence events in the Lycopodiaceae correspond to a partitioning of ecological strategies among the different subfamilies and genera of subfamily Huperzioidae; much of the family's functional diversity appears to have been attained early in its evolutionary history.

Though the initial diversification of the family occurred during the Carboniferous and Permian, much of the family's generic and species richness accumulated considerably later. Apart from the early divergences in subfamily Huperzioidae and the establishment of some depauperon lineages (Donoghue & Sanderson, 2015) such as *Lycopodiastrum* and *Phylloglossum* by the upper Triassic, most groups diversified primarily during the Cretaceous and Cenozoic, coincident with the rise of modern fern (Lehtonen et al., 2017; Schneider et al., 2004; Schuettpelz & Pryer, 2009; Testo & Sundue, 2016), gymnosperm (Condamine, Nagalingum, Marshall, & Morlon, 2015; Nagalingum et al., 2011; Saladin et al., 2017; Won, Renner, & Linder, 2006) and angiosperm (Magallón et al., 2013; Xi et al., 2012) lineages. The rapid diversification of some of these groups, such as leptosporangiate ferns (Schneider et al., 2004; Schuettpelz & Pryer, 2009; Watkins & Cardelús, 2012) and the angiosperm order Malpighiales (Davis, Webb, Wurdack, Jaramillo, & Donoghue, 2005; Xi et al., 2012) are clearly closely linked to the establishment of angiosperm-dominated rain forests by the early Cretaceous; this appears to be the case for some tropical and subtropical genera such as *Phlegmariurus*, *Palhinhaea* and *Pseudodiphasium*, but not for other groups, such as *Huperzia*, *Diphasiastrum* and *Diphasium*, which appear to be adapted primarily to open temperate and tropical alpine habitats. Most species-level divergences are recent; having occurred within the last 15 Myr.

4.3 | Historical biogeography

The biogeographical history of the Lycopodiaceae is complex, and ancient vicariance events appear to have played the most important role in shaping the group's distribution (Figures 3 and 4). The initial divergences within the family occurred while Earth's landmasses were united in Pangaea, and these lineages most likely were widely distributed across the supercontinent. Early divergences within the subfamilies Huperzioidae and Lycopodioidae correspond with the initial breakup of Pangaea into the Laurasian and Gondwanan landmasses during the early Jurassic; distributions of extant genera clearly reflect this pattern (Figure 4). The primary divergence event in the Lycopodielloideae split *Lycopodiella*, which has a primarily Laurasian distribution, from the principally Gondwanan lineage comprising the genera *Lateristachys*, *Palhinhaea* and *Pseudolycopodiella*. Similarly, the divergence of the Laurasian genus *Huperzia* from the otherwise Gondwanan Huperzioidae is estimated to have occurred in the mid-Jurassic, while Laurasia and Gondwana were separated yet still proximate. Similar patterns of lineage splitting concordant with the Pangaeic breakup have been demonstrated for numerous animal groups (San Mauro, Vences, Alcobendas, Zardoya, & Meyer, 2005; Springer, Murphy, Eizirik, & O'Brien, 2003; Wildman et al., 2007), but appear to be exceedingly rare among plants; currently, robust phylogenetic evidence for this pattern is only known from the Cupressaceae (Mao et al., 2012) and Selaginellaceae (Klaus et al., 2017; Weststrand, 2016). In addition to providing a rare example of Pangaeic vicariance in a plant family, the geographic affinity of these deep divergences within the Lycopodiaceae is remarkable

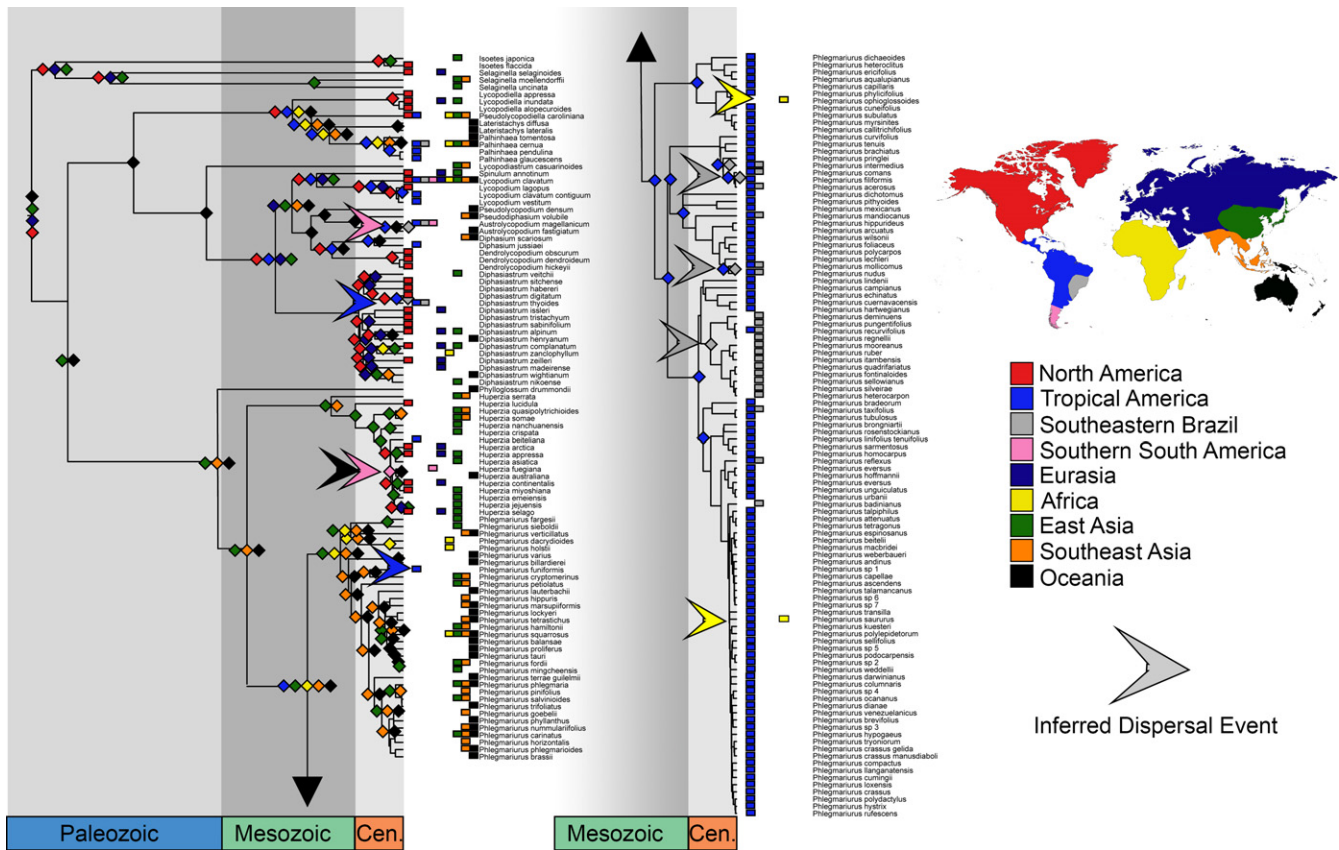


FIGURE 3 Historical biogeography of the Lycopodiaceae, as inferred using the DEC+J model implemented in BioGeoBEARS. Coloured blocks represent current (at branch tips) and inferred historical (at nodes) distributions of Lycopodiaceae taxa. Arrows represent inferred long-distance dispersal events and are colour-coded to the area to which the lineage is inferred to have dispersed

given the group's high potential for long-distance dispersal, which some authors (Vanderpoorten, Gradstein, Carine, & Devos, 2010; Wolf, Schneider, & Ranker, 2001) suggest could obscure ancient vicariance in groups of spore-dispersed plants. It is possible that long-distance dispersal may have also contributed to the early diversification history of the Lycopodiaceae, but we believe vicariance is the primary phenomenon at play, due to the coordinated timing of continent breakup and lineage divergence across the family.

The subsequent breakup of Gondwana also had an important role in shaping the geographic distribution of *Phlegmariurus*, the most species-rich genus in the Lycopodiaceae (Figure 4). *Phlegmariurus* comprises two clades—one nearly entirely Palaeotropical, the other almost entirely Neotropical—of approximately equal size, which diverged an estimated 102 Ma. Our estimate of the timing of this split is much younger than the lower Cretaceous estimate (184 Ma) reported by Wikström and Kenrick (2001) and corresponds closely with the final rifting of South America and Africa (König & Jokat, 2006; Seton et al., 2012). Both clades subsequently underwent exceptional species diversification during the Cenozoic, apparently radiating in angiosperm-dominated rain forests like most leptosporangiate fern groups (Schneider et al., 2004; Schuettpelz & Pryer, 2009; Testo & Sundue, 2016). Unlike most emblematic Gondwanan seed plant (e.g. *Podocarpus*, Quiroga, Mathiasen, Iglesias, Mill, &

Premoli, 2016; *Nothofagus*, Swenson, Hill, & McLoughlin, 2001; Proteaceae, Barker, Weston, Rutschmann, & Sauquet, 2007) and fern lineages (e.g. Cyatheaceae, Korall & Pryer, 2014; *Trichomanes*, Dubuisson et al., 2003; Dicksoniaceae, Noben et al., 2017), there is no evidence of migration between South America and Oceania through Antarctica (a connection that persisted until the middle Eocene; Wilf, Cúneo, Escapa, Pol, & Woodburne, 2013) or of intercontinental long-distance dispersal leading to subsequent diversification events in *Phlegmariurus*. The inferred pattern of ancient Gondwanan affinity within subfamily Huperzioidae may also be influenced by the position of *Phylloglossum* in the phylogeny. The phylogenetic position of this taxon is poorly supported in this and earlier studies (Field et al., 2016; Wikström & Kenrick, 2001) and if it were found to be sister to the rest of the subfamily rather than sister to *Huperzia*, it is likely that the inferred ancestral distribution of subfamily Huperzioidae would be East Asia+Oceania. This would indicate that the broad range posited here for subfamily Huperzioidae (Figure 4) may be a significant overestimate of a group with stronger affinities to Gondwana and Eurasia.

This study confirms the finding of a recent phylogeny (Gissi, 2017), which documented the presence of an endemic clade of Brazilian species nested within the Neotropical *Phlegmariurus* clade. This group is estimated to have diverged from its closest relatives

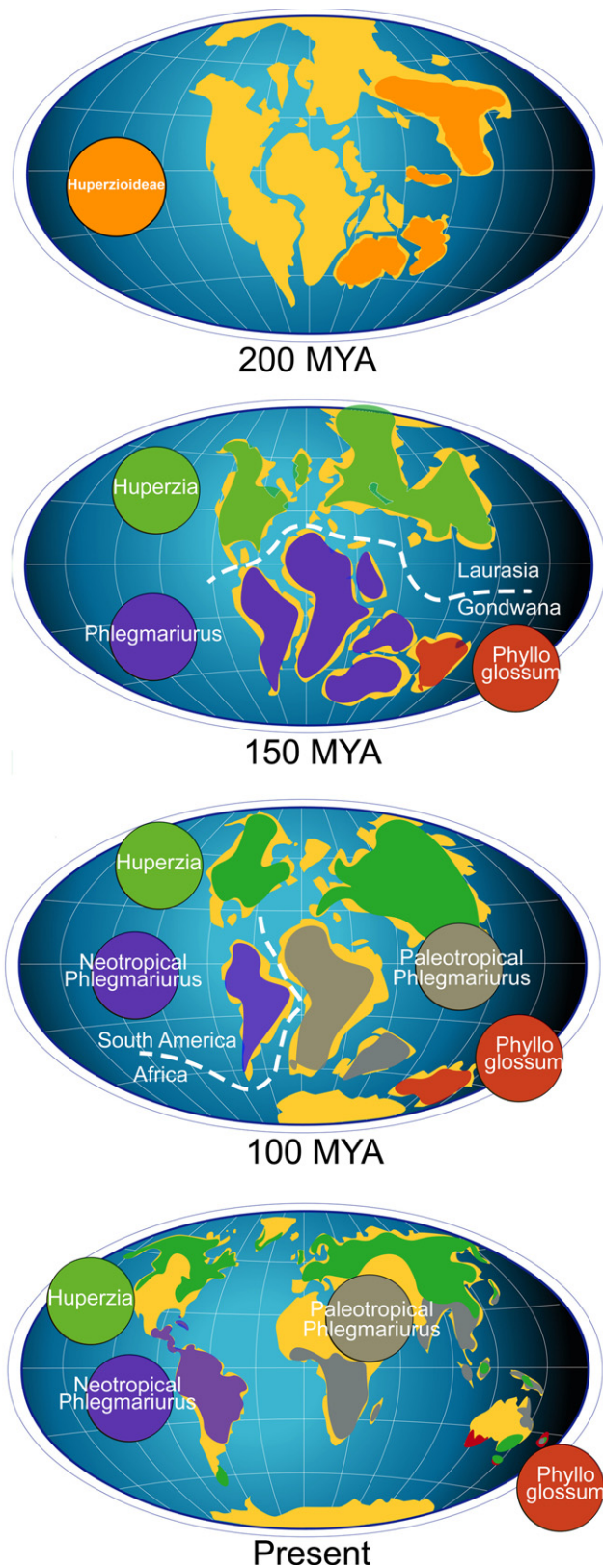


FIGURE 4 Generalized schematic of continental vicariance and cladogenesis in Lycopodiaceae subfamily Huperzioidae over the last 200 Myr. Coloured polygons represent generalized ranges of taxa, and white dotted lines identify key continental rifting events. Map from www.world-map-interactive.info

approximately 37 Ma (Figure 3), and has subsequently undergone considerable diversification in the mountains and rain forests of south-eastern Brazil. Unlike the tropical Andes, which formed primarily beginning in the Miocene (Gregory-Wodzicki, 2000), the mountain ranges of the Brazilian Highlands were established prior to the divergence of this clade, eliminating the possibility that this clade arose following a long-distance dispersal event from the Andes, as has been documented in other plant groups (Luebert & Weigend, 2014; McHenry & Barrington, 2014; Safford, 1999). Instead, it appears that this group radiated into these habitats from adjacent lowland forests and subsequently became adapted to a diversity of available habitat types in the region.

Compared to that of vicariance, the role of long-distance dispersal in shaping the diversity and distribution of the Lycopodiaceae is apparently minor. Nonetheless, several long-distance dispersal events have important implications for understanding the evolutionary history of some genera and warrant discussion. Recent trans-Austral long-distance dispersal occurred in both *Austrolycopodium* and *Huperzia* (Figure 3), fitting a pattern commonly found in Austral plant groups (Barker et al., 2007; Renner, Strijk, Strasberg, & Thébaud, 2010; Richardson, Chatrou, Mols, Erkens, & Pirie, 2004; Noben et al., 2017). Long-distance dispersal is also responsible for both species (*Phlegmariurus saururus*) and species pair (*Phlegmariurus phyllicifolius* and *P. ophioglossoides*) range disjunctions between the Neotropics and southern Africa/Madagascar, as has been reported for numerous fern and lycophyte genera by Moran and Smith (2001). Interestingly, both *P. phyllicifolius* (to at least 19° S) and *P. saururus* (to at least 31° S) have ranges that extend further south in the Andes than most other Neotropical *Phlegmariurus*; these southerly distributions may have permitted their (or their close relatives') dispersal to Africa via the prevailing westerly winds at south-temperate latitudes. Several long-distance dispersal events to south-eastern Brazil from the Andes have occurred since the Miocene; these dispersals did not result in subsequent radiations (Figure 3). Long-distance dispersal certainly has played an important role in the diversification of *Phlegmariurus* in tropical Asia and Oceania (Field et al., 2016), but the scale of these dispersal events is too fine to be detected in our analyses, as they have mostly occurred within—rather than between—the areas we defined in this study.

5 | CONCLUSIONS

This study improves our understanding of the evolutionary history of the Lycopodiaceae by providing a densely sampled, time-calibrated phylogeny and set of inferences for the family's complex biogeographical history. We demonstrate the importance of considering among-lineage rate heterogeneity when estimating lineage divergence times, and provide clade age estimates that differ considerably from previously published estimates. The Lycopodiaceae arose during the Devonian and early divergence events in the family appear to be associated with major geologic events, particularly the breakup

of Pangaea and the subsequent rifting of Gondwana; most well-supported long-distance dispersal effects are recent and were followed by limited subsequent diversification. Finally, though the family is ancient, most of its extant species diversity accumulated during the Cenozoic, coincident with the recent diversification of other major land plant lineages.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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