A 4000-species dataset provides new insight into the evolution of ferns

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ABSTRACT

Ferns are the second-most diverse lineage of vascular plants on Earth, yet the best-sampled time-calibrated phylogeny of the group to date includes fewer than 5% of global diversity and was published seven years ago. We present a time-calibrated phylogeny that includes nearly half of extant fern diversity. Our results are evaluated in the context of previous studies and the fossil record, and we develop new hypotheses about the radiation of leptosporangiate ferns. We used sequence data from six chloroplast regions for nearly 4000 species of ferns to generate the most comprehensive phylogeny of the group ever published. We calibrate the phylogeny with twenty-six fossils and use an array of phylogenetic methods to resolve phylogenetic relationships, estimate divergence times, and infer speciation, extinction, and net diversification rates. We infer a mid-late Silurian origin for ferns (including horsetails) and an early Carboniferous origin for leptosporangiate ferns. Most derived fern families appeared in the Cretaceous and persisted for millions of years before rapidly diversifying in the Cenozoic. We find no evidence of differential rates of diversification among terrestrial and epiphytic species. Our findings challenge previous hypotheses on the evolutionary history of ferns and present a new paradigm for their Cenozoic radiation. We estimate earlier divergences for most fern lineages than were reported in previous studies and provide evidence of extended persistence of major fern lineages prior to rapid diversification in the last fifty million years.

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1. Introduction

Understanding the history of species diversification across the tree of life is a central goal of evolutionary biology. The proliferation of studies generating time-calibrated phylogenies over the past decade have provided an increasingly robust framework to test biogeographic hypotheses, identify cases of coevolution between different taxonomic groups, and estimate rates of species diversification. Such studies have presented new hypotheses about the evolution of major groups of land plants, including: bryophytes (Laenen et al., 2014; Villarreal et al., 2015), ferns (Schuettpelz and Pryer, 2009; Rothfels et al., 2015), gymnosperms (Nagalingum et al., 2011; Lu et al., 2014; Condamine et al., 2015), and flowering plants (Bell et al., 2010; Magallón, 2010; Smith et al., 2010; Magallón et al., 2015). Divergence times reported in these studies often differ considerably from estimates derived from early molecular phylogenetic studies (Wikström et al., 2001 vs. Magallón, 2010; Smith et al., 2010), the fossil record (Bromham and Penny, 2003; Smith et al., 2010), and for some groups, with other recent studies (e.g., for cycads, Nagalingum et al., 2011 and Condamine

et al., 2015; for flowering plants, Bell et al., 2010 and Smith et al., 2010). For many plant lineages, our estimates of the timing and rate of diversification remain uncertain.

In contrast to those reported for most other major plant lineages, estimates of clade ages in the ferns have been remarkably consistent across studies focusing on dating the group (Pryer et al., 2004; Schneider et al., 2004a; Schuettpelz and Pryer, 2009; Rothfels et al., 2015). The first of these studies (Schneider et al., 2004a) provided the key insight that "ferns diversified in the shadow of angiosperms," suggesting that though ferns are indeed an ancient lineage, most extant lineages are relatively young, having arisen following the emergence of angiosperm-dominated forests in the Cretaceous. This finding was consistent with evidence of diverse fern fossil floras in the age of angiosperms, as reported by earlier workers (Lovis, 1977; Rothwell, 1987). Later, Schuettpelz and Pryer (2009) largely corroborated the results of Schneider et al. (2004a), adding that diversification rates were higher in epiphytic ferns, which they interpreted as evidence of a large, recent radiation within the epiphytic niche. Like Schneider et al. (2004a), Schuettpelz and Pryer estimated divergence times using penalized likelihood analyses (Sanderson, 2003) based on a dataset of a chloroplast markers; however, their taxon sampling (400 fern taxa) was considerably denser than that of Schneider

et al. (42 taxa). Using Bayesian relaxed clock analyses on a dataset of 25 nuclear genes, Rothfels et al. (2015) recovered results that were largely congruent with those of Schuettpelz and Pryer (2009); however, this agreement was undoubtedly influenced by the use of secondary calibration points from Schuettpelz and Pryer's results (rather than direct incorporation of fossil data) to calibrate clade ages.

Since the publication of those papers, there has been a growing focus in the literature regarding approaches and challenges in estimating diversification rates (e.g., Rabosky, 2010, 2015; Brock et al., 2011; Beaulieu and O'Meara, 2015; Rabosky and Goldberg, 2015), including the advent of new and increasingly sophisticated methods (Harmon et al., 2008; Alfaro et al., 2009; Rabosky, 2014). The application of these approaches to the study of diversification has provided new insight into the magnitude (Shi and Rabosky, 2015; Condamine et al., 2015), causes (Huang and Rabosky, 2014; McGuire et al., 2014; Weber and Agrawal, 2014; Lagomarsino et al., 2016; Rose et al., 2016), and even the existence (Condamine et al., 2015; Sundue et al., 2015) of exceptional diversification across the tree of life. With these methodological advances, we can re-evaluate the finding of a post-Cretaceous radiation among the leptosporangiate ferns reported by Schneider et al. (2004a) and Schuettpelz and Pryer (2009) and characterize patterns of diversification across the entire fern phylogeny.

Here we provide new insight into the evolutionary history of ferns with new analyses and a much larger dataset than those used in earlier studies. Our study is based on a comprehensive sample of nearly four thousand fern species (~40% of extant fern diversity) from all recognized families. We infer evolutionary relationships of all major fern clades, estimate divergence dates and rates of diversification across the fern phylogeny, and revisit previous hypotheses about the Cenozoic fern radiation.

2. Materials and methods

2.1. Taxon sampling and matrix assembly

We assembled a matrix of six chloroplast markers (*atpB*, *rbcL*, *rps4* gene, *rps4-trnS* IGS, *trnL* gene, *trnL-trnF* IGS) from GenBank for a total of 4007 species (3973 ferns, 34 outgroup taxa); accession numbers and matrix statistics are given in the Supplementary Material. Sequences for each marker region were aligned using the MAFFT plugin (Katoh et al., 2002) in Geneious v. 7.0.2 (Biomatters, Ltd.); alignments were visually examined and ambiguous regions were excluded from downstream analyses. For use in phylogenetic analyses, nucleotide substitution models were chosen for each marker using the corrected Akaike information criterion in jModeltest2 (Darriba et al., 2012).

2.2. Phylogeny and divergence time estimation

To overcome computational constraints due to our large phylogeny, we used a three-step approach to develop a time-calibrated phylogeny: (1) generate a phylogeny using Maximum Likelihood (ML) analyses, (2) use this phylogeny as a starting point to develop a time-calibrated phylogeny constructed using penalized likelihood analyses and fossil data, and (3) use the time-calibrated phylogeny derived from the penalized likelihood analyses as a starting point for more comprehensive relaxed clock dating analyses.

Maximum likelihood analyses were performed using RAxML version 8.2.4 (Stamatakis, 2006) on the Cipres Science Gateway portal. The analyses were performed using the GTRGAMMA model, with separate nucleotide substitution models applied to each marker partition; node support was calculated using 100 bootstrap

replicates. The single tree with the highest likelihood score was then used in dating analyses, described below.

We developed an initial time-calibrated tree using penalized-likelihood algorithm in treePL (Smith and O'Meara, 2012). We compared eight rate-smoothing values (each differing by an order of magnitude, ranging from 0.0001 to 1000) using the program's cross-validation feature; an optimal smoothing value of 0.001 was chosen. We set the root height (MRCA of land plants) at 475 Ma, consistent with the oldest known land plant fossils (cryptospores from the Middle Ordovician of Argentina, Rubinstein et al., 2010) and other comprehensive dated land plant phylogenies (Smith et al., 2010; Magallón et al., 2013). Slightly younger fossils from the Late Ordovician (456–444 MA) of bryophyte-like sporangia (Wellman et al., 2003; Wellman, 2010) or trilete spores (Steemans et al., 2009) could also be used to constrain this node; we did not examine these alternative age choices in our dating and instead followed the conservative approach of Magallón et al. (2013). Internal nodes of the phylogeny were calibrated by incorporating minimum age constraints on 26 nodes based on an extensive paleobotanical literature review (Supplementary Materials). For the sake of clarity, ferns were defined as spore-dispersed vascular plants with megaphylls (except Psilotaceae), usually circinate vernation, and abaxial or terminal sporangia. All treePL analyses were run on the high-performance computing cluster (HiPerGator) at the University of Florida.

We used BEAST version 2.3.0 (Drummond and Rambaut, 2007) implemented on the Cipres Science Gateway portal to estimate divergence times. We used the time-calibrated phylogeny from the treePL penalized-likelihood analysis as a starting tree and fixed the tree topology to ensure convergence, as preliminary attempts at the analysis without doing so proved computationally intractable. The data set was analyzed using the uncorrelated lognormal clock model (mean = 1.0, standard deviation = 0.33) and a birth-death model of diversification (uniform prior from 0 to 10 for speciation rate and 0–1 for relative extinction rate; starting value were 1.0 for speciation and 0.5 for relative extinction). Separate nucleotide substitution models were chosen for each data partition based on the results of our model test analysis. The root age was calibrated with a minimum age of 475 Ma with a mean and standard deviation both set at 0.1. The same fossil calibrations used in the penalized-likelihood analysis were again employed as minimum age constraints using a lognormal prior, each with a mean of 5.0 and a standard deviation of 1.0; this resulted in the majority of the distribution being slightly older than the age of the fossil. We performed four separate MCMC analyses for 100 million generations and sampled every 20,000 generations, resulting in 5000 trees per run. The MCMC output was examined using Tracer (Rambaut and Drummond, 2003) to assess convergence and to evaluate if adequate effective sample sizes (ESS) had been obtained for each parameter; for each run, convergence was attained within 5–7 million generations and the ESS values for all parameters were greater than 200. We conservatively removed the first 1000 trees as burn-in and combined the remaining trees from all four runs, resulting in a total of 16,000 trees that were used to generate a maximum credibility tree.

2.3. Diversification rate analyses

We used Bayesian Analysis of Macroevolutionary Mixtures (BAMM v.2.5.0, Rabosky, 2015) to estimate speciation, extinction and net diversification rates through evolutionary time and across the fern phylogeny. This analysis uses a reverse-jump MCMC to evaluate a large number of possible diversification regimes from a given time-calibrated phylogeny, thereby estimating rates of speciation, extinction, and diversification for all branches in the

tree, along with identifying potential shifts in diversification rates across the phylogeny and evaluating statistical support for those shifts.

There has been considerable debate in the literature about the confidence with which extinction rates can effectively be estimated from molecular phylogenies (Nee et al., 1994; Purvis, 2008; Rabosky, 2010; Morlon et al., 2011; Beaulieu and O'Meara, 2015; Rabosky and Goldberg, 2015). It is evident that reasonably accurate estimates of extinction rates are difficult, but not impossible, to obtain from molecular phylogenetic data sets. Because BAMM explicitly identifies and accounts for rate heterogeneity (Rabosky, 2014), such biases stemming from rate heterogeneity can be minimized and extinction rates can be estimated with improved confidence (Beaulieu and O'Meara, 2015; Rabosky and Goldberg, 2015). Finally, recent studies in groups such as cycads (Condamine et al., 2015) and cobweb spiders (Liu et al., 2016) suggest that approaches similar to ours can effectively characterize evolutionary histories of even ancient, extinction-rich groups. Although we remain cautious regarding inferences of extinction rates from molecular phylogenies and suggest that our diversification results be interpreted with appropriate vigilance, we believe our methodology is the most robust available to address the questions raised here.

We ran our BAMM analyses using the time-calibrated phylogeny obtained from our BEAST analyses; the MCMC analysis was run for 200 million generations and sampled every 100,000 generations. Rate and rate shift priors were selected as follows: prior number of rate shifts = 5, initial lambda value = 1, lambda shift prior = 0.05, initial mu value = 1. We accounted for incomplete taxon sampling by providing estimated proportions of taxa in each family sampled (Supplementary Materials); family species richness values were taken from the following sources: Mickel and Smith (2004), Smith et al. (2006), and Flora of China (Wu et al., 2013). Post-run analyses were conducted in R v.2.3.1 (R Core Team, 2015) using the BAMMtools package. We conservatively removed the first 10% of the MCMC run as burn-in, and assessed the remaining data for convergence with an effective sample size (ESS) threshold of >200. The posterior distribution of our MCMC run was then used to calculate diversification, speciation, and extinction rates and identify shifts in rate regime across the phylogeny; different rate regimes were compared using branch-specific marginal odds ratios. We also tested for a correlation between growth habit with a structured permutation test in BAMM following Rabosky and Huang (2015); growth habit was coded as terrestrial or epiphytic (Supplementary Materials) based on the literature, examination of herbarium specimens, and images posted on www.fernsoftheworld.com.

3. Results

3.1. Data matrix and model statistics

Our complete matrix of four chloroplast markers was 8059 bp long; the percent missing data ranged from 19.7% missing for *rbcL* to 71.3% missing for *trnL* and was 48.6% overall (Supplementary Materials). For all loci, the GTR + G model was selected as the best-fit.

3.2. Topology

The ML tree was generally well-supported and recovered a topology largely congruent with other broadly sampled fern phylogenies (e.g., Schuettpelz and Pryer, 2007; Lehtonen, 2011; Rothfels et al., 2015) (Fig. 1). We recover horsetails are sister to all other ferns, with strong support (BS = 94%). Similar to most other large

scale analyses, the next three major lineages that we recover are Psilotaceae and its sister lineage Ophioglossaceae (BS = 93%), Marattiaceae (BS = 95%), and then Osmundaceae (BS = 100%). Within Osmundaceae we find *Osmundastrum* sister to the remaining genera. We then recover filmy ferns, followed by the Gleicheniales, Schizaeales, heterosporous water ferns, and the tree fern clade. Within the tree ferns, we find that *Plagiogyria*, *Culcita*, and *Loxsoma/Loxsomopsis* form a clade that is sister to all other tree ferns.

Among early divergent Polypodiales lineages, we find the first-divergent clade to be comprised of the Lindsaeaceae and their sister group, which includes *Saccoloma*, *Cystodium*, and *Lonchitis*. This clade is well-supported as monophyletic (BS = 92%); however, relationships among the taxa comprising this group remain weakly supported. We resolve the Pteridaceae as sister to eupolypod ferns with moderate support (BS = 83%).

Among eupolypod ferns, we recover two clades that correspond to the eupolypods I and Eupolypods II clades of Schneider et al. (2004a). We find the Didymochlaenaceae sister to the rest of the eupolypods I ferns, followed by the Hypodematiaceae. Among more derived families, the Dryopteridaceae is the first divergent, followed by Lomariopsidaceae, Nephrolepidaceae, then Tectariaceae. As in previous studies, Oleandraceae, Davalliaceae, and Polypodiaceae form the most derived clade among the eupolypods I ferns.

The topology of the eupolypods II clade differs from that reported in earlier studies. We recover Aspleniaceae as sister to the remaining eupolypods II families, but with weak support (BS = 74%), and the small families that form a clade with it in Rothfels et al. (2012) (Diplaziopsidaceae, Hemidictyaceae, Rhachidosoraceae) form a grade leading to the other families: Athyriaceae, Blechnaceae, Cystopteridaceae, Onocleaceae, Thelypteridaceae, and Woodsiaceae. Relationships among these families are similar to those found in earlier studies, except that the Woodsiaceae and Athyriaceae switch positions relative to the topology of Rothfels et al. (2012).

3.3. Divergence time estimates

We calculate a crown age for ferns of 431 ma (95% HDP = 420.6–482.0 ma) (Fig. 2, Fig. S1). The crown age of leptosporangiate ferns is calculated as 357 ma (95% HDP = 355.8–357.5 ma), and the crown age of Polypodiales is calculated as 260 ma. Among the eupolypod ferns, we recovered crown ages that were older than previously reported for both the eupolypods I (mean = 160.9 ma; 95% HDP = 158.6–172.5 ma) and eupolypods II (mean = 185.8 ma; 95% HDP = 183.8–196.1 ma), and also for prominent, species-rich families such as the Aspleniaceae, Dryopteridaceae, and Polypodiaceae. We recovered younger family stem ages than were previously reported for a few families, including the heterosporous water fern families Marsileaceae and Salviniaceae (for both, mean = 153.4 ma; 95% HDP = 150.2–204.3 ma) as well as the tree fern families Metaxyaceae and Dicksoniaceae (for both, mean = 131.9 ma; 95% HDP = 94.7–172.1 ma).

3.4. Diversification rates

We recover a heterogeneous pattern of species diversification across the fern phylogeny, with early divergent groups tending to diversify slowly, and the fastest-diversifying groups largely confined to the derived Polypodiales clade (Figs. 3 and 4). Family-level net diversification rates ranged between 0.0041 4.1×10^{-3} events/ma (Equisetaceae) and 0.13 events/ma (Athyriaceae) (Fig. 3). Nine of the ten fastest diversifying families reside within the eupolypod ferns.
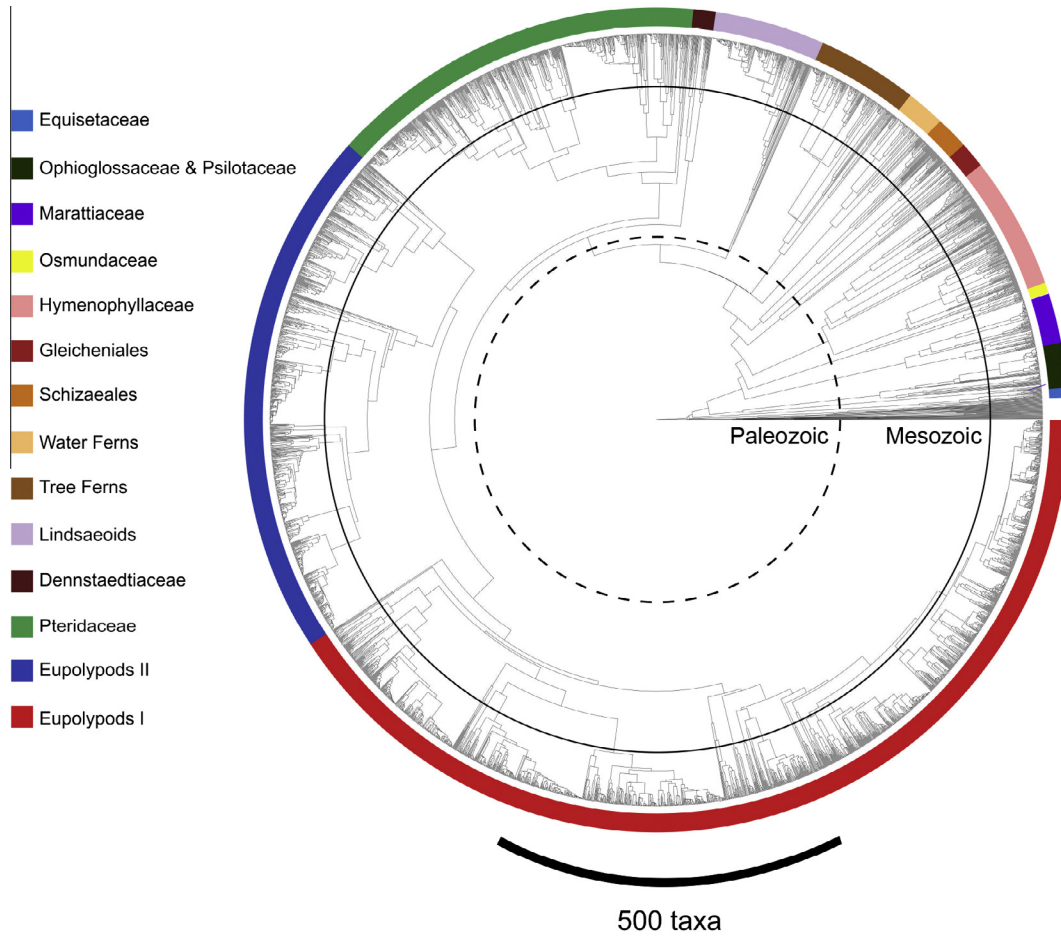
Fig. 1. Time-calibrated phylogeny of 3973 ferns and 34 outgroup taxa derived from BEAST analysis. Colors denote fern clades; concentric circles show geologic eras. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
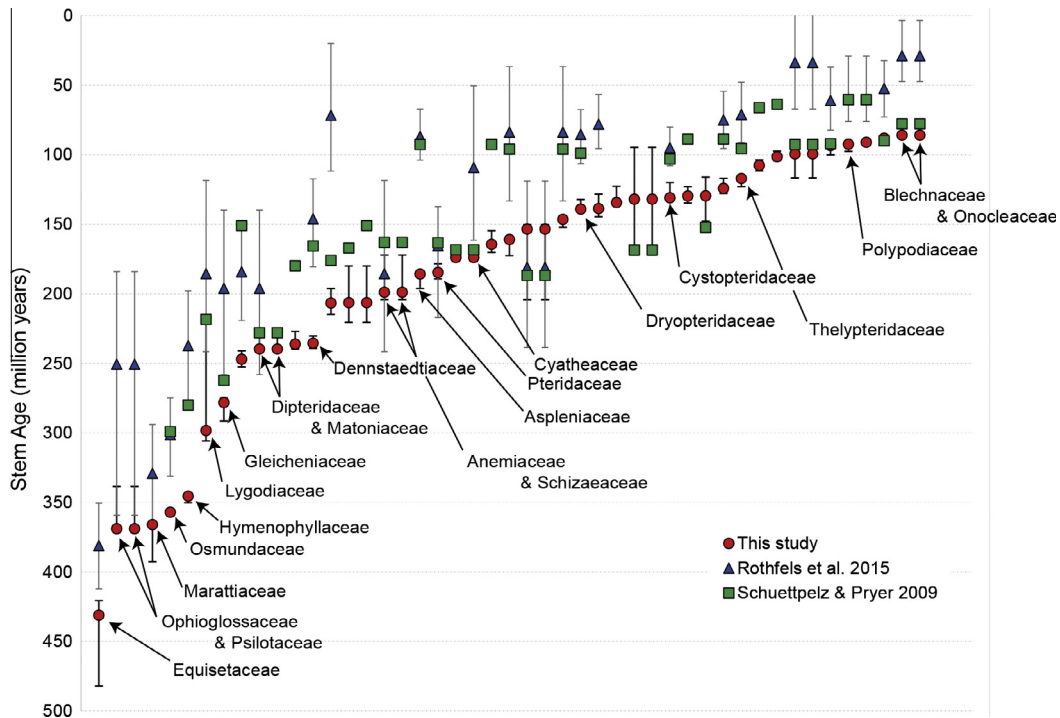
Fig. 2. Comparison of stem ages for families reported in this study, Schuettpelz and Pryer (2009), and Rothfels et al. (2015). Error bars represent age ranges reported for the given node.
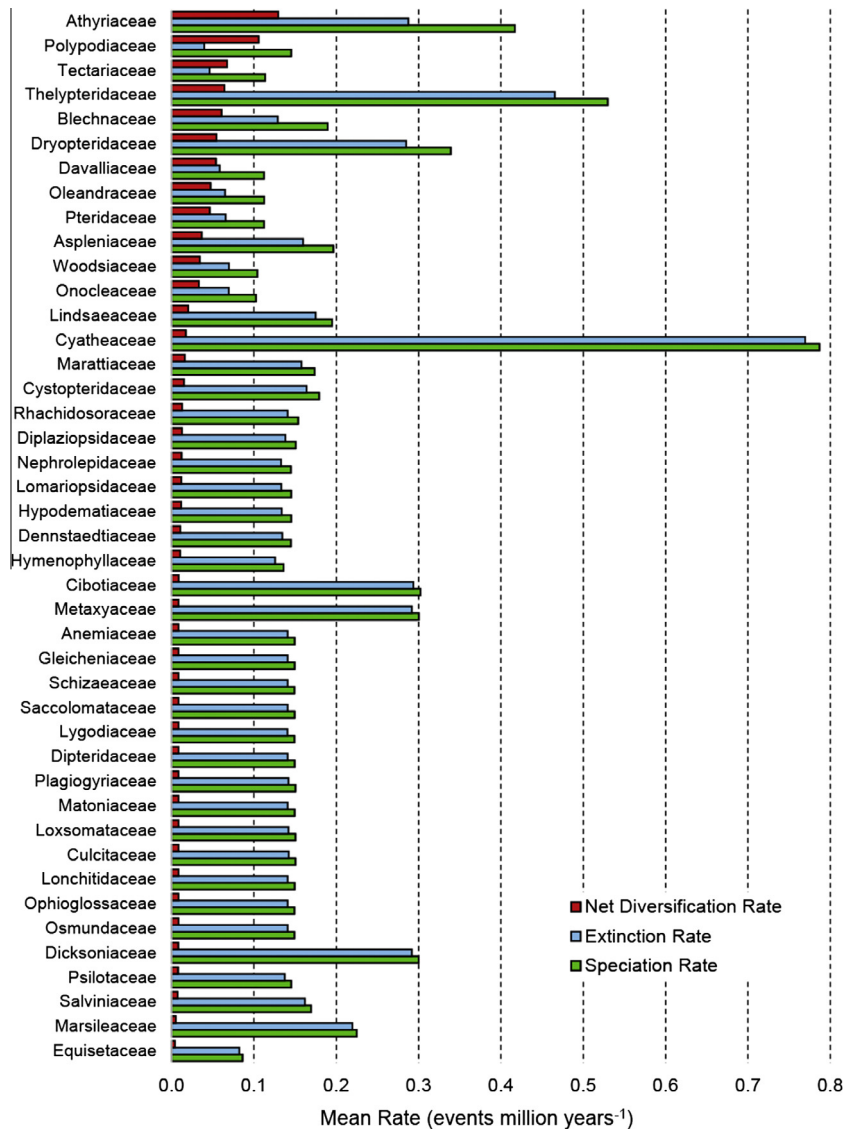
Fig. 3. Net diversification, speciation, and extinction rates estimated for each fern family using BAMM.

Across the fern phylogeny, the pattern of speciation rates generally followed that observed for net diversification rates, with the exception of high speciation rates among many families in the relatively slow-diversifying tree fern clade (Figs. 3 and 4). The family with the highest speciation rate were the scaly tree ferns, Cyatheaceae (0.79 events/ma). The lowest rate belonged to the horsetail family, Equisetaceae (0.086 events/ma). The most rapidly speciating families were distributed among the eupolypod clade (e.g., Thelypteridaceae, Athyriaceae, Dryopteridaceae) and the tree ferns (e.g., Cyatheaceae, Cibotiaceae, Metaxyaceae, Dicksoniaceae).

Extinction rates ranged from 0.04 events/ma (Polypodiaceae) to 0.77 events/ma (Cyatheaceae) (Figs. 3 and 4). Families with the highest extinction rates included many of the those found to have the highest speciation rates, including prominent eupolypod families (Thelypteridaceae, Athyriaceae, Dryopteridaceae) and some tree fern lineages (Cyatheaeae, Cibotiaceae, Metaxyaceae, Dicksoniaceae) (Fig. 4).

Analysis of diversification rates across the phylogeny demonstrate a pattern of increased diversification across numerous lineages 40–50 ma, with support for 12 shifts in diversification rate regime within this time period (Fig. 3). This pattern was most pronounced in the eupolypod ferns and some Pteridaceae lineages;

no such rate increase was detected in most early divergent lineages, with the exception of the Marattiaceae and one clade of the Lindsaeaceae.

Net diversification rate was 0.226 events/ma among terrestrial species and 0.145 events/ma among epiphytes; this difference was not significant (Mann-Whitney U-test, $P = 0.28$) (Fig. S2).

4. Discussion

4.1. Topology

The phylogenetic position of *Equisetum* has been uncertain, with conflicting conclusions reported across both morphology- and molecule-based studies (Kenrick and Crane, 1997; Rothwell, 1999; Pryer et al., 2001; Rothwell and Nixon, 2006; Knie et al., 2015). Early DNA sequence-based studies consistently placed *Equisetum* sister to the Marattiaceae (Pryer et al., 2001; Des Marais et al., 2003; Pryer et al., 2004) whereas more recent studies have resolved the group as sister to the Ophioglossales + Psilotales (Grewe et al., 2013), sister to the leptosporangiate ferns (Lehtonen, 2011), or as sister to all ferns (Kuo et al., 2011; Wickett et al., 2014;
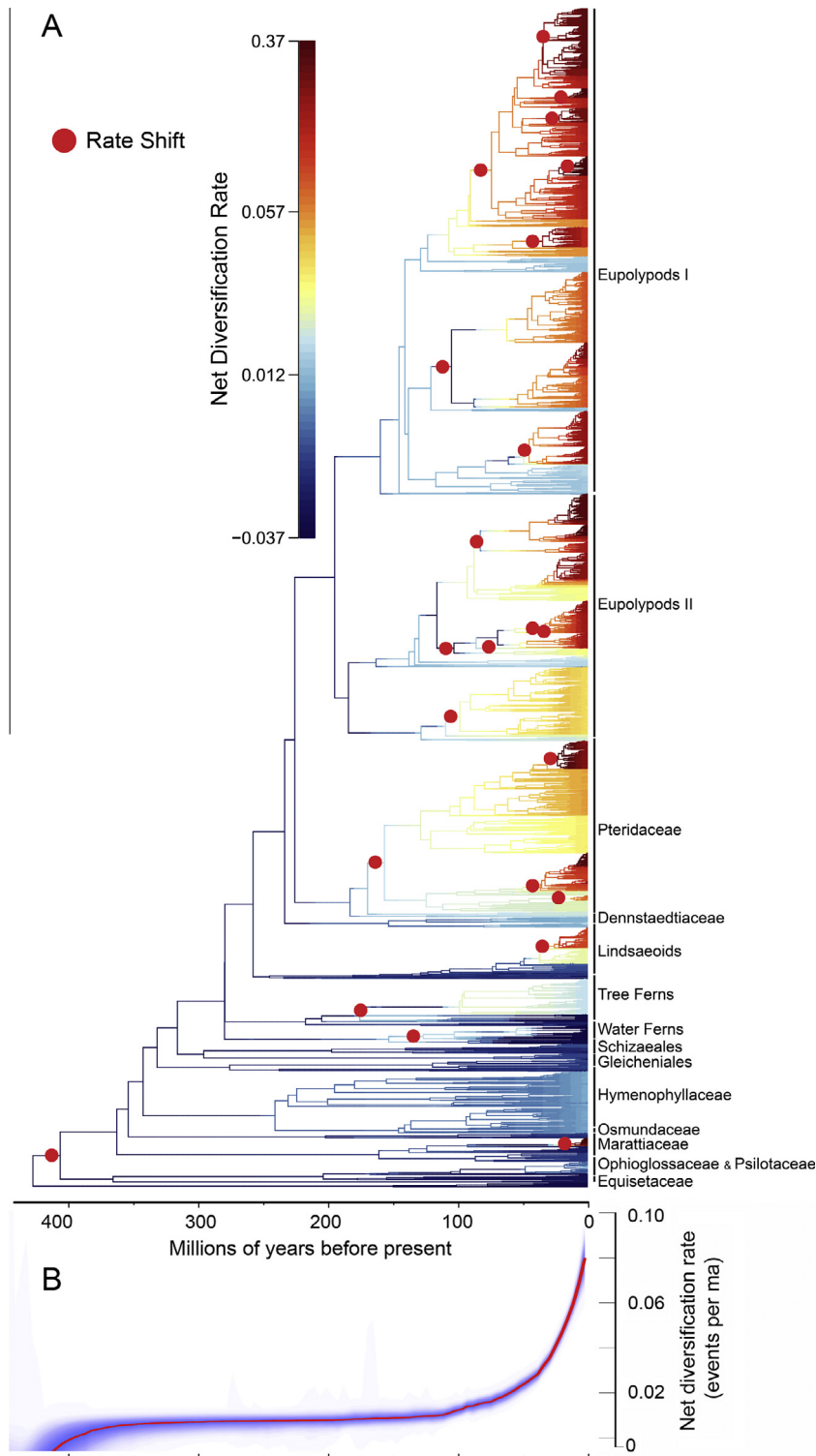
Fig. 4. (A) Phylorate plot showing the time-calibrated fern phylogeny with branches colored to depict net diversification rates and red dots indicating best-supported configuration of shifts in diversification rate. (B) Net diversification rate through time across the fern phylogeny. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Knie et al., 2015; Rothfels et al., 2015). We place *Equisetum* as sister to all ferns with strong support (Fig. 1 and Fig. S1), consistent with other recent studies, including those based on mitochondrial (Knie et al., 2015) or low-copy nuclear (Wickett et al., 2014; Rothfels et al., 2015) sequence data. We agree with the conclusion of Rothfels et al. (2015) assessment based on extant and fossil taxa that this placement fits in well with our understanding of pro-

toxylem evolution; we also consider the unique leaf homology of the Equisetaceae (Vasco et al., 2013) to support its position sister to the rest of the ferns.

The position we recover of the monotypic *Osmundastrum* sister to the rest of the Osmundaceae is consistent with most (Yatabe et al., 1999; Schuettpelz and Pryer, 2007; Metzgar et al., 2008) but not all (Grimm et al., 2014; Schneider et al., 2015) molecular

phylogenies. Evidence from the fossil record (Yatabe et al., 1999; Jud et al., 2008; Bomfleur et al., 2014) supports the distinctiveness and deep evolutionary stasis of this genus.

Relationships among early divergent Polypodiales have remained weakly supported or unresolved (Lehtonen et al., 2012); we resolve a monophyletic clade comprising the diverse family Lindsaeaceae and the small families Cystodiaceae, Lonchitidaceae, and Saccolomataceae. In our analysis, all of these families are strongly supported as monophyletic, including the Saccolomataceae, which Perrie et al. (2015) reported to be polyphyletic.

Consistent with previous studies, we recover both the Dennstaedtiaceae and Pteridaceae as monophyletic with strong support; unlike Knie et al. (2015) and Rothfels et al. (2015), we recover Pteridaceae as the sister lineage to the eupolypod ferns. The relationship found here is consistent with the findings of most other studies (e.g., Pryer et al., 2004; Schuettpelz and Pryer, 2007; Kuo et al., 2011). Further study of these relationships are necessary to ascertain which family is the sister lineage to the eupolypod ferns.

Relationships among early divergent eupolypods I families have been challenging to resolve. Our result of *Didymochlaena* as sister to the rest of the clade is consistent with some other studies (Kuo et al., 2011; Zhang and Zhang, 2015) but not others (Schuettpelz and Pryer, 2007; Lehtonen, 2011; Rothfels et al., 2015) who recovered it in the Hypodematiaceae. Among the core eupolypods I ferns, the family relationships we recover are generally consistent with those reported elsewhere, including the sister relationship of Arthropteridaceae and Tectariaceae (Liu et al., 2013). In contrast to Schuettpelz and Pryer (2007) and Zhang and Zhang (2015) both of whom found a sister relationship between the Nephrolepidaceae and Lomariopsidaceae, our analysis suggests instead that the Nephrolepidaceae are sister to a large clade comprising the Tectariaceae, Oleandraceae, Davalliaceae, and Polypodiaceae. This relationship has been reported before by Kuo et al. (2011), Lehtonen (2011), and Liu et al. (2013).

Deep relationships of the eupolypods II clade has been notoriously difficult to resolve due to short deep internodes, family-specific heterogeneity in rates of molecular evolution, and a lack of a closely related outgroup (Rothfels et al., 2012). In our phylogeny, Aspleniaceae are resolved as sister to the rest of the eupolypods II families with moderate support; this is in contrast to the position reported by Schuettpelz and Pryer (2007), Kuo et al. (2011), Rothfels et al. (2012), and Mynssen et al. (2016), all of which recovered Cystopteridaceae as sister to rest of the eupolypods II clade. In other analyses, the Aspleniaceae has been recovered either in the position found by us (Schneider et al., 2004a; Zhan et al., 2015) or as sister to all other eupolypod ferns (Hasebe et al., 1995). Our topology results in a number of small families thought to be allied to the Aspleniaceae (e.g., Diplaziopsidaceae, Hemidictyaceae, Rhachidosoraceae) forming a grade sister to the rest of the eupolypods II ferns rather than a monophyletic group, as found by Rothfels et al. (2012). Under either the topology recovered by Rothfels et al. (2012) or in this study, morphological evolution in the eupolypods II clade is complex; morphologically similar groups are surprisingly unrelated and synapomorphies for suprafamilial groups are wanting (Sundue and Rothfels, 2013). Finally, we are cautious about the effects that some of the factors discussed by Rothfels et al. (2012) may have had on our results. It is well-documented that lineages with fast rates of molecular evolution (long-branch taxa) can be difficult to place with confidence (Felsenstein, 1978; Hillis and Bull, 1993; Swofford et al., 1996; Soltis et al., 1999; Huelsenbeck et al., 2002; Nickrent et al., 2004; Rothfels et al., 2012). Among eupolypods II ferns, the Aspleniaceae exhibit by far the highest rates of molecular evolution of any family, and the crown group is subtended by a long branch. We are uncertain whether the position of the Aspleniaceae found here is an artifact of this factor; in light of this, we interpret this

with caution. Finally, though the fixed topology used in our dating analyses eliminated effects of phylogenetic uncertainty on divergence time estimation, it is important to note that alternative topologies for the placement of the Aspleniaceae would undoubtedly impact divergence time estimates for the family and other deep divergences in the phylogeny. Such effects would be especially pronounced for clades with associated fossil calibrations, as shifts in their phylogenetic position would "drag" nearby nodes deeper or shallower in evolutionary time.

4.2. Divergence time estimates

The root age of approximately 430 ma that we estimated for ferns pre-dates other recent clade age estimates by c. 40 (Magallón et al., 2013) to nearly 80 ma (Schneider et al., 2004a). This result is comparable to recent analyses conducted for angiosperms (Smith et al., 2010; Magallón et al., 2013, 2015) for which divergence time estimates (for major lineages as well as the group as whole) are generally older than were reported in earlier studies. Beaulieu et al. (2015) cautioned that rate heterogeneity and sparse taxon sampling coupled with assumptions of relaxed-clock models could be responsible for the early angiosperm crown age estimated by Smith et al. (2010); however, we find no evidence of this in our data.

Our mid-Silurian estimate for the origin of the common ancestor of horsetails and ferns is considerably older than the unequivocal fossil record, which is Devonian in age (Kenrick and Crane, 1997). Molecular divergence time estimates that are much older than the fossil record should be viewed with caution. Nonetheless, recent evidence suggests that Silurian plant life may have been more sophisticated than previously thought. Although most vascular plant fossils from the Silurian are of small taxa with simple body plans such as *Cooksonia* (Lang, 1937), it is now evident that the robust lycophyte *Baragwanathia* was likely present by the late Silurian (Kenrick and Crane, 1997; Rickards, 2000), contemporary with our estimate for the origin of the equisetophyte/fern clade and within 10 ma of the age we recover for the origin of vascular plants (Fig. S1). If lycophytes as large and specialized as *Baragwanathia longifolia*, which grew to 1 m or more and possessed 4 cm-long microphylls, were present by the late Silurian, the possibility that early representatives of the fern and horsetail lineages existed at the same time (or slightly later) should not be discounted.

Our divergence time estimates for the remaining large clades in the fern phylogeny are generally older than those reported Schuettpelz and Pryer (2009) and Rothfels et al. (2015) (Fig. 2). Here, we provide some important examples and for each, discuss potential methodological differences that may be responsible for the differences reported here and in prior studies.

Leptosporangiate ferns—We infer a crown age for leptosporangiate ferns in the early Carboniferous, approximately 55 Ma older than was reported by both Schuettpelz and Pryer (2009) and Rothfels et al. (2015), despite using the same fossil to calibrate this node in the phylogeny (Suppl. Meth.).The molecular clock dating approach used by Schuettpelz and Pryer (2009) (r8s, Sanderson, 2003) allows users to assign node calibrations as either fixed or as maximum/minimum age estimates; however, at least one node must be fixed at a given age. Schuettpelz and Pryer chose to fix the root of their tree (crown leptosporangiates) at 299 Ma, based on the Permian osmundaceous fossils *Grammatopteris* and *Rastropteris* (Skog, 2001; Rößler and Galtier, 2002). Rothfels et al. (2015) used this same age to center a truncated-normal prior distribution for the age of the node in their Bayesian dating analyses; they recovered nearly the same age. In contrast, we employed a rather broad lognormal prior distribution with a minimum age constraint equal to the age of the fossil and the majority of probability space slightly older than that constraint; thereby favoring an age for the origin of

leptosporangiate ferns that predates their appearance in the fossil record. Despite being considerably older than the fossil-based minimum age constraint used both by us and Schuettpelz and Pryer (2009), the age we estimate for the origin of leptosporangiate ferns (c. 357 Ma) is consistent with the appearance of fern fossils (*Senftenbergia*, Radforth, 1939; Eggert and Taylor, 1966) with what appear to be leptosporangia by the early Carboniferous. Although we did not use this fossil in our study due to its shared age with the fossil we used to constrain the deeper divergence between equisetophytes and ferns (*Archaeocalamites*; see Schneider et al., 2004a), its existence indicates that leptosporangiate ferns were present prior to Carboniferous-Permian boundary, consistent with our dates. Fossils of uncertain phylogenetic placement such as *Senftenbergia* may be better utilized in an integrative framework such as the total evidence dating approach described by Ronquist et al. (2012), which uses morphological data from extinct and extant taxa to infer similarity between the fossil taxon and reconstructed ancestors and then generates a branch in the phylogeny for the fossil taxon, rather than affixing it directly to a single node.

Hymenophyllaceae—Our estimate for the crown age of the filmy fern family precedes those of Schuettpelz and Pryer (2009) and Rothfels et al. (2015) by 58 and 75 ma, respectively (Fig. 2). We believe this disparity is due to: (1) a difference in fossil choice and (2) the impact of molecular rate heterogeneity on divergence times estimates made by penalized likelihood methods used by Schuettpelz and Pryer (2009). Although there is wide agreement that the Triassic fossil taxon *Hopetedia praetermissa* (Axsmith et al., 2001) belongs to the Hymenophyllaceae (Dubuisson et al., 2003; Pryer et al., 2004; Hennequin et al., 2008; Rothwell and Stockey, 2008), it has not been used in any molecular phylogenetic dating study prior to ours. On the basis of this fossil, we set a minimum age for the crown Hymenophyllaceae at 228 ma, 43 ma older than the estimate obtained by Schuettpelz and Pryer (2009), which itself was subsequently used as a calibration point by Rothfels et al. (2015). As discussed at length by numerous authors (i.e., Hug and Roger, 2007; Parham et al., 2011; Ronquist et al., 2012; Magallón et al., 2013) the impact of fossil choice on estimation of the timing of species diversification is difficult to overstate.

Rate heterogeneity among lineages was described by Smith and Peterson (2002) as "the single most fundamental obstacle to developing an accurate estimate of times of origination". It is widespread and poses significant challenges phylogeneticists (Welch and Bromham, 2005; Beaulieu et al., 2015). Along with tree ferns (Korall et al., 2010) and vittarioid ferns (Rothfels and Schuettpelz, 2014), the Hymenophyllaceae exhibit the most remarkable rate heterogeneity among the ferns, with much higher substitution rates reported for the trichomanoid clade than its sister group, *Hymenophyllum* (Hennequin et al., 2003; Schuettpelz and Pryer, 2006). Comparative studies (Ho et al., 2005; Rutschmann, 2006; Drummond and Suchard, 2010; Ho and Duchêne, 2014) have suggested that methods that assume rate autocorrelation across descendent branches in a phylogeny, including the penalized-likelihood method used previously to date divergences in the filmy ferns (Schuettpelz and Pryer, 2006; Hennequin et al., 2008) perform poorly when substitution rates change rapidly across tree space. We instead used a "relaxed clock" model (Drummond and Rambaut, 2007; Bouckaert et al., 2014) which allows rates to be drawn from a parameterized distribution and does not assume rate autocorrelation, thereby minimizing rate heterogeneity effects (Renner, 2005; Drummond and Rambaut, 2007; Ho, 2009; Sauquet, 2013). Nonetheless, we argue that these effects are likely minor compared to the disparity resulting from our inclusion of the *Hopetedia* fossil in our analysis.

Our early estimate for the origin of the Hymenophyllaceae has important implications for understanding the palaeoecology and growth habit evolution of early filmy ferns. As pointed out by Hennequin et al. (2008), these early filmy ferns probably inhabited wet forests dominated by gymnosperms, lycopsids, sphenopsids, and tree ferns. However, unlike Hennequin et al. (2008) we suggest that these habitats may have been conducive to epiphytic growth by or shortly after the middle-Triassic origin of the Hymenophyllaceae reported here. We believe that these ferns could have attained epiphytic growth earlier than previously hypothesized for three reasons: (1) recent work has provided evidence for the existence of complex forest ecosystems much earlier than previously thought (Cúneo et al., 2003; Stein et al., 2012); (2) many filmy fern species have been reported to be desiccation tolerant, especially among the primarily epiphytic *Hymenophyllum* clade (Shreve, 1911; Proctor, 2003, 2012; Cea et al., 2014); and (3) desiccation tolerance is an ancient (rather than highly derived) trait with deep homology that facilitated other major growth habit transitions in the plant kingdom, including the appearance of the first land plants (Oliver et al., 2000, 2005; Rensing et al., 2008). Together, these lines of evidence suggest that these ecosystems, although not as humid or sheltered as later angiosperm-dominated forests, were suitable for complex plant communities that included epiphytes, and that filmy ferns were likely well-suited to be among the first vascular epiphytes on Earth.

Polypodiaceae—The Polypodiaceae are the largest family of ferns after the Dryopteridaceae, and the origin and radiation of this epiphyte-rich group have been examined thoroughly in recent years (Schneider et al., 2010; Sundue et al., 2014; Sundue et al., 2015). These studies, which used secondary node calibrations obtained from the results of Schuettpelz and Pryer (2009), reported that the Polypodiaceae arose shortly after the Paleocene-Eocene Thermal Maximum (PETM, 55.5 ma) and that most major lineages underwent diversification beginning in the Oligocene and Miocene, primarily as specialized epiphytes in angiosperm-dominated tropical forests. The PETM was a climatically and ecologically transformative event in Earth's history that triggered the rapid rise of some of the earliest known angiosperm-dominated tropical rain forests (Boyce et al., 2010). As a group that diversified in such habitats shortly following this period, the Polypodiaceae have been considered one of the prime examples of the "Cenozoic radiation of ferns in an angiosperm-dominated canopy" reported by Schuettpelz and Pryer (2009). Though we agree with the assessment and others (i.e., Schneider et al., 2010; Wang et al., 2012; Sundue et al., 2015) that the majority of diversity in the Polypodiaceae arose recently, we find evidence for a rather different evolutionary timescale for the family. Our analyses support an origin of crown group Polypodiaceae in the late-Cretaceous (c. 90 ma) (Fig. 2), with most major lineages (i.e., grammitids, drynarioids, microsoroids) arising around or shortly after the PETM (55–40 ma). Our divergence date estimates place the "old, rapid radiation" (sensu Schneider et al., 2004b) starting at c. 55 ma (Fig. 4), suggesting that the earliest divergent lineages in the family would have preceded the appearance of angiosperm-dominated tropical forests, and the most species-rich lineages would have begun diversifying shortly thereafter. It is likely that early representatives of the family were adapted to xeric niches on rocks or isolated trees, as many members of the family are drought tolerant and the earliest-divergent extant lineages of Polypodiaceae include a significant proportion of species from such habitats (Sundue et al., 2015). This pattern has been shown for other forest-dwelling lineages, including salamanders (Vieites et al., 2007), birds (Ericson et al., 2006), ants (Moreau et al., 2006), and mammals (Meredith et al., 2011). Even so, it should be noted that the Cenozoic radiation of the Polypodiaceae discussed by Schuettpelz and Pryer (2009) and in subsequent studies (Schneider et al., 2010; Sundue et al., 2015) remains a generally valid paradigm under either diversification scenario.

Why do our divergence time estimates for the Polypodiaceae differ from those of Schuettpelz and Pryer (2009)? Here we high-

light two factors that are likely to have had a major effect: (1) selection and placement of fossil calibrations and (2) taxon sampling. Schuettpelz and Pryer (2009) used a single fossil calibration for the Polypodiaceae: *Protodrynaria takhtajanii*, a putative member of the Old World drynarioid clade from an Eocene deposit in Russia (Vikulin and Bobrov, 1987). Taking a conservative approach to the placement of this fossil in a phylogenetic context, Schuettpelz and Pryer (2009) used the fossil as the basis of placing a 33.9 ma minimum age constraint on the node marking the divergence of the Polypodiaceae from its sister family, the Davalliaceae. Though we share concerns about confidently assigning this fossil to a position within the extant drynarioids, we believe this fossil likely belongs to either that lineage or another derived Old World group. We felt assigning this probable member of a derived clade in the Polypodiaceae to the stem or even crown group of the family would lead to artificially young dates, and thus chose not to include it in our analyses. Rather, we incorporated three fossils which we felt we could place more precisely: (1) a *Polypodium* s. s. fossil from the Oligocene (Kvaček, 2001), (2) a well-preserved *Pleopeltis* from Dominican amber (Schneider et al., 2015), and (3) a fossil readily assignable to the extant species *Aglaomorpha heraclea* (Van Uffelen, 1991). We felt this approach allowed us to constrain the backbone of the Polypodiaceae phylogeny without unduly biasing the age of the family as a whole. Further, empirical evidence from numerous studies (Hug and Roger, 2007; Sauquet et al., 2012; Schrago and Mello, 2014; Zheng and Wiens, 2015) suggests that an increased number of (reliable) fossil calibrations is perhaps the best way to improve accuracy of divergence time estimates using current methods. Finally, we note that our estimated ages of the drynarioid or selligueid ferns—the lineages which *Protodrynaria* closely resembles—are 30 and 35 ma, straddling the age of this fossil.

Compared to other methodological concerns such as fossil integration and rate heterogeneity, the impact of taxon sampling on accuracy in molecular dating analyses has received little attention in the literature. Nonetheless, studies suggest that undersampling consistently leads to biased divergence time estimates, though the magnitude of this bias varies considerably across study groups (Yoder and Yang, 2004; Linder et al., 2005; Hug and Roger, 2007; Heath et al., 2008; Poux et al., 2008; Beaulieu et al., 2015). In an analysis of the monocot family Restionaceae, Linder et al. (2005) reported an asymptotic relationship between the proportion of taxa sampled and age estimates, with up to threefold differences between "true" ages and those obtained when taxon sampling approach 10% of total diversity. Though Schuettpelz and Pryer (2009) based their analyses on the most comprehensive fern phylogeny available at the time (based on their earlier study; Schuettpelz and Pryer, 2007), fewer than 4% of the estimated 1500 species in the Polypodiaceae were sampled. Since the publication that paper, sequence data has become available for hundreds of species; synthesizing these datasets, we included 719 representatives of the Polypodiaceae (48% of global diversity). If the relationship between taxon sampling and divergence time estimates reported by Linder et al. (2005) and others (Yoder and Yang, 2004; Poux et al., 2008) holds up for the Polypodiaceae and other fern lineages, previous attempts at molecular clock dating likely have yielded misleading results. We expect that estimates of the timing of divergence events will continually improve as future studies on fern diversification approach complete taxon sampling.

4.3. Diversification rates

Our BAMM analyses support a scenario of sharply increased diversification rates across the fern phylogeny starting c. 50 ma, including concurrent shifts in diversification rate in numerous lineages, especially among the derived eupolypod clade (Fig. 4). This

radiation coincides with the early Tertiary expansion of angiosperm-dominated tropical forests, which are thought to have facilitated fern diversification "in the shadow of angiosperms", as previously reported by Schneider et al. (2004a), Schuettpelz and Pryer (2009) and reviewed in detail by Watkins and Cardelús (2012). Though we concur with the conclusion of Schuettpelz and Pryer (2009) that the rise of primarily epiphytic families coincided with a shift in diversification rate, we do not find support for the pattern of more rapid diversification among epiphytes that they reported. Instead, our analyses indicate that diversification is actually higher among terrestrial species compared to epiphytes, though not significantly so (Fig. S2), and the majority of the fastest-diversifying families are wholly or primarily terrestrial. In light of this, we suggest that the rise of major epiphytic fern lineages was an important feature of the Cenozoic fern radiation contributing to net diversification, but this radiation was not exceptional in magnitude nor rate in comparison to that which occurred concurrently on the forest floor.

5. Conclusions

Twelve years ago, Schneider et al. (2004a) provided key insight into the evolutionary history of land plants—that "ferns diversified in the shadow of angiosperms," demonstrating that though ferns are an ancient lineage, the majority of extant diversity arose following the rise of angiosperm-dominated tropical forests in the late Cretaceous and early Cenozoic. Five years later, Schuettpelz and Pryer (2009) added that not only did ferns exploit ecological opportunity in the low-light understory, but engaged in an exceptional radiation in the epiphytic niche following the Paleocene-Eocene Thermal Maximum. We build upon the foundation laid by these studies and conclude that major fern lineages arose earlier than previously thought, suggesting that the Cenozoic radiation of ferns occurred well after the origin of these lineages. Many derived lineages persisted with low rates of diversification for tens of millions of years in the late Cretaceous before rapidly diversifying during the Cenozoic, following the "phylogenetic fuse" paradigm of Cooper and Fortey (1998). Though we are uncertain of whether these lineages were evolutionarily static during this time or if high extinction rates dominate the signal we can now extract from our phylogeny, it is evident that long periods of evolutionary innovation preceded the ferns' rise to renewed prominence during the last 60 ma. Finally, our data suggest that the relatively recent emergence and diversification of epiphytic fern lineages should be re-evaluated and considered as an important innovation and major contributor to extant fern diversity, but not an exceptional radiation.

Authors' contributions

WLT and MS designed the study, conducted analyses, and wrote the manuscript.

access to resources. The University of Florida Research Computing core was used for some analyses. We thank the coordinators of the colloquium on "The integration of ecological and phylogenetic approaches in pteridology" for support to attend the 2015 *Next Generation Pteridology* symposium at the Smithsonian Institution where an early draft of this manuscript was presented.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.09.003.

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