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ABRUPT DECELERATION OF MOLECULAR EVOLUTION LINKED TO THE ORIGIN OF ARBORESCENCE IN FERNS

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Molecular rate heterogeneity, whereby rates of molecular evolution vary among groups of organisms, is a well-documented phenomenon. Nonetheless, its causes are poorly understood. For animals, generation time is frequently cited because longer-lived species tend to have slower rates of molecular evolution than their shorter-lived counterparts. Although a similar pattern has been uncovered in flowering plants, using proxies such as growth form, the underlying process has remained elusive. Here, we find a deceleration of molecular evolutionary rate to be coupled with the origin of arborescence in ferns. Phylogenetic branch lengths within the "tree fern" clade are considerably shorter than those of closely related lineages, and our analyses demonstrate that this is due to a significant difference in molecular evolutionary rate. Reconstructions reveal that an abrupt rate deceleration coincided with the evolution of the long-lived tree-like habit at the base of the tree fern clade. This suggests that a generation time effect may well be ubiquitous across the green tree of life, and that the search for a responsible mechanism must focus on characteristics shared by all vascular plants. Discriminating among the possibilities will require contributions from various biological disciplines, but will be necessary for a full appreciation of molecular evolution.

KEY WORDS: **Generation time, leptosporangiates, life history, molecular rate heterogeneity, tree ferns.**

Rates of molecular evolution are known to be highly variable across the tree of life (Bromham and Penny 2003; Jansa et al. 2006; Johnston et al. 2006; Schuettpelz and Pryer 2006; Thomas et al. 2006; Lumbsch et al. 2008; Smith and Donoghue 2008; Stenøien 2008; Welch et al. 2008). For animals, this phenomenon is frequently attributed to differences in generation time, whereby species with shorter life cycles have more germ line replications, and hence are prone to more mutations, per unit time (Laird et al. 1969; Wu and Li 1985). A similar generation time effect has been put forward as a possible explanation for the molecular rate heterogeneity observed in plants (Gaut et al. 1992, 1996; Aïnouche and Bayer 1999; Andreasen and Baldwin 2001), but the paucity of life-history information available has necessitated a focus on such indirect measures of generation time as perenniality or growth form. Studies comparing annuals to perennials and herbaceous to arborescent taxa have uncovered striking

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differences in their rates of molecular evolution. In most cases (Gaut et al. 1996; Aïnouche and Bayer 1999; Andreasen and Baldwin 2001; Smith and Donoghue 2008; Soria-Hernanz et al. 2008), annuals have been shown to evolve faster than perennials, and herbaceous taxa faster than those that are arborescent. However, these studies focused almost exclusively on flowering plants and it remains to be seen whether this pattern is more widespread—a critical next step if we are to ever fully understand the link between generation time and rates of molecular evolution in plants.

Outside the extant seed plants, arborescence is today only present in ferns, where it is mostly restricted to the "tree fern" clade (Korall et al. 2006; 2007). Strongly supported as monophyletic, this group comprises eight families, 15 genera, and more than 600 species (Smith et al. 2006). Tree ferns are a conspicuous component of tropical, subtropical, and even south temperate floras, where closely related clades are also especially diverse (Smith et al. 2006). As the name implies, tree ferns generally have tall, erect stems, frequently reaching heights up to 20 m. Investigations of generation times, along with those of phenology and demography, have shown that the members of this primarily arborescent clade have longer generation times than closely related nonarborescent lineages (Stein 1971; Windisch and Pereira-Noronha 1983; Prange and von Aderkas 1985; Ash 1987; Cousens et al. 1988; Bittner and Breckle 1995; Mehltreter and Palacios-Rios 2003; Mehltreter and García-Franco 2008). In this study, we investigate whether the origin of arborescence in ferns is coupled with a slowdown in molecular evolutionary rate, thereby determining whether or not a generation time effect exists outside of flowering plants. To this end, we employed a multifaceted approach, combining phylogenetic inference with tests of molecular rate differences and ancestral state reconstructions, to explore the links between changes in rate and the evolution of arborescence.

Material and Methods

DATA

We assembled a four-gene (plastid *atpA*, *atpB*, *rbcL*, and *rps4*) by 106-taxon dataset, including 57 tree fern species and 49 other vascular plants (Table S1). Outside the tree fern clade, our sampling mirrored that of two previously published studies (Pryer et al. 2004; Schuettpelz et al. 2006), although *Gleichenella* and *Saccoloma* were excluded here due to missing data. Most plastid gene sequences were obtained from GenBank, but 38 tree fern sequences (Table S1) were newly generated following established protocols (Korall et al. 2006, 2007). The new DNA sequences were deposited in GenBank (accession numbers FN667541-FN667578, Table S1).

PHYLOGENETIC ANALYSES

Phylogenetic analyses were conducted using MrBayes 3.1.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), with models of sequence evolution identified using mrAIC (Nylander 2004) in combination with PHYML version 2.4.4 (Guindon and Gascuel 2003). For each gene, two independent Bayesian analyses were run for 5 million generations, employing the best-fitting model ($GTR+I+\Gamma$ for *atpA*, *atpB*, and *rbcL*, and $GTR+\Gamma$ for *rps4*), eight parallel chains, and a temperature parameter of 0.1. Trees were sampled every 1000 generations and the first 1000 trees from each analysis were discarded (conservatively) as the "burnin." The remaining $4000 + 4000$ trees were pooled and a majority-rule consensus was calculated. These single-gene consensus trees were then examined for conflict (i.e., well-supported incongruence). Finding none, the four genes were analyzed in unison following the approach above, with each gene retaining its independent model of sequence evolution.

TESTS FOR UNEQUAL RATES

Using the combined dataset and a pruned topology, we tested for molecular rate heterogeneity among the three groups of core leptosporangiates (heterosporous ferns, polypods, and tree ferns; Fig. 1). Employing the likelihood ratio test statistic (Felsenstein 1981), we compared three different null two-rate models (heterosporous fern rate = polypod rate ≠ tree fern rate; heterosporous fern rate = tree fern rate ≠ polypod rate; and polypod rate = tree fern rate ≠ heterosporous fern rate) to an alternative three-rate model (heterosporous fern rate ≠ polypod rate ≠ tree fern rate). All likelihoods were calculated in Baseml (Yang 2007) with the $GTR+\Gamma$ model of sequence evolution.

ANCESTRAL RATE RECONSTRUCTIONS

To estimate ancestral rates of molecular evolution, we used a penalized likelihood approach, as implemented in r8s (Sanderson 2003). We combined 23 fossil age constraints (Mohr and Lazarus 1994; Cantrill 1998; Lantz et al. 1999; Pryer et al. 2004) (Table S2) with a set of 2000 trees obtained in the combined Bayesian analysis. To simplify the compilation of results (Eriksson 2008), these 2000 trees were drawn at random from the subpool of trees that contained each of the well-supported ($PP \geq 0.95$) nodes.

ANCESTRAL STATE RECONSTRUCTIONS

Using MrBayes 3.1.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) and a reduced dataset of all sampled core leptosporangiates and schizaeoids (76 total taxa), we reconstructed arborescence for all well-supported ($PP \geq 0.95$) nodes within the core leptosporangiate clade. One morphological character with two states (arborescent or not) was scored from the literature (Fig. 2A) and added to the combined DNA sequence dataset. This character was given its own partition and assigned

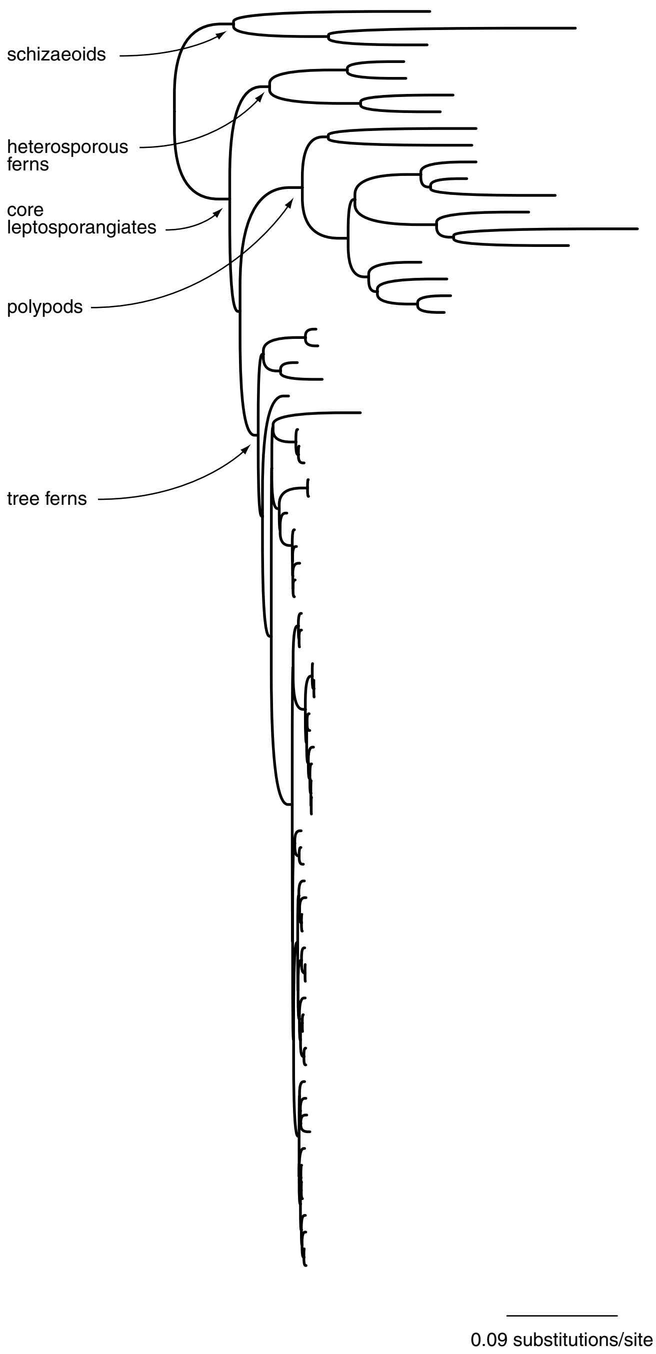

Figure 1. **Phylogenetic branch length estimates for core leptosporangiate ferns. This tree was randomly selected from the pool of trees obtained via Bayesian analysis of the combined (plastid *atpA*, *atpB*, *rbcL*, and *rps4*) data set. Tree ferns and closely related leptosporangiate lineages are indicated; more distantly related lineages (Table S1) have been pruned.**

the standard discrete model. A separate Bayesian analysis was performed for each node (using the settings described above for the phylogenetic analyses) with the node constrained and the ancestral state reconstruction requested.

Results and Discussion

Phylogenetic analysis of our combined dataset yielded a well-resolved and well-supported tree (Figs. 1 and 2A, S1; dataset and topology are deposited in TreeBASE) that is congruent with previously published phylogenetic hypotheses (Pryer et al. 2004; Korall et al. 2006, 2007). The tree fern clade is strongly supported as monophyletic (posterior probability, PP = 1.00) and is sister to the polypod ferns (PP = 0.99). Tree ferns and polypods, together with the heterosporous ferns, compose a well-supported (PP = 1.00) core leptosporangiate clade (Pryer et al. 2004).

Branches within the tree fern clade are notably shorter (i.e., have far fewer substitutions per site) than those of closely related lineages (Fig. 1). The pattern is similar in all four single-gene analyses (Fig. S2), indicating that the apparent rate difference is plastid-genome wide. Although our analyses are restricted here to the plastid genome, preliminary mitochondrial and nuclear data (Pryer et al. 2001; Wikström and Pryer 2005) do suggest that this relative rate pattern is actually consistent across all three genomic compartments. Our likelihood ratio tests confirmed that the observed branch length variation in the combined plastid tree was in fact due to a significant difference in molecular evolutionary rate. Tree ferns were found to evolve more slowly than both polypods ($P < 0.001$) and heterosporous ferns ($P = 0.002$).

By reconstructing ancestral rates of molecular evolution, we were better able to characterize the magnitude, position, and tempo of the rate deceleration. Our penalized likelihood (Sanderson 2002) analyses indicate that every branch in the tree fern clade had a slower substitution rate than any branch in the closely related polypod and heterosporous fern lineages (Fig. 2B, Table S2). Furthermore, the average rate (calculated as a simple mean of means; Table S2) for tree ferns (0.000135 substitutions per site per million years) was only about 15% of that for polypod ferns (0.000895) and only about 19% of that for heterosporous ferns (0.000701). Evidently, these differences primarily stem from a rather abrupt rate deceleration that occurred along the branch leading to tree ferns (Fig. 2B). This slowdown does continue well into the tree fern clade; however, it becomes more gradual.

Our reconstructions of arborescence reveal what appears to be one transition to a tree-like habit at the base of the tree fern clade (Fig. 2A), which coincides with an abrupt rate deceleration revealed by our penalized likelihood analyses (Fig. 2B). Although effectively a single datapoint, it is important to note that this particular transition did eventually result in the slowest rates of molecular evolution found within the core leptosporangiate ferns (Table S2). Within tree ferns, reversals to nonarborescence are not always associated with increases in rate, but the retention of a long generation time by some nonarborescent tree ferns may

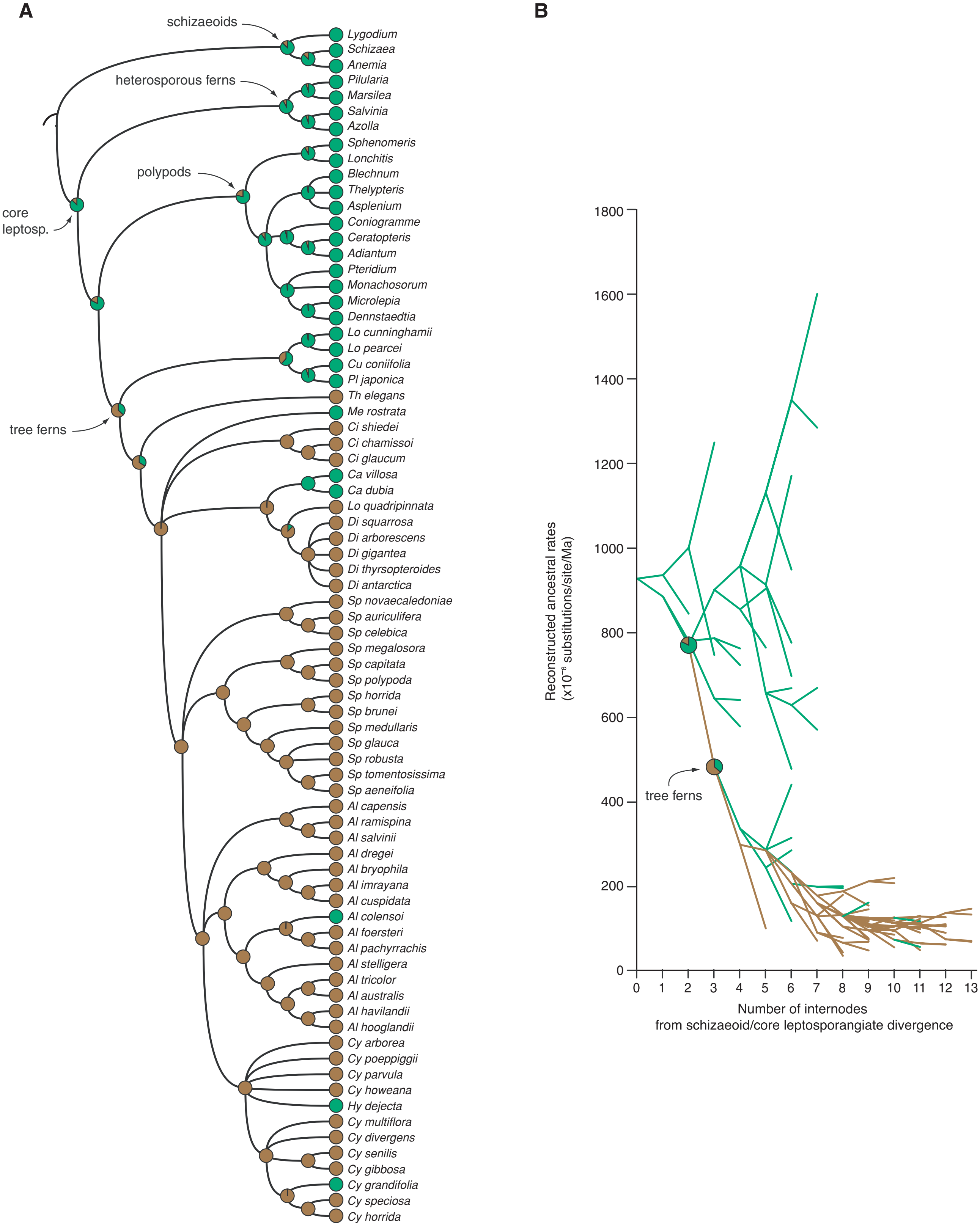

Figure 2. (A) Reconstruction of arborescence within the core leptosporangiate ferns. This 95% majority-rule consensus phylogeny results from Bayesian analysis of the combined (plastid *atpA*, *atpB*, *rbcL*, and *rps4*) dataset; all resolved nodes are supported by a Bayesian posterior probability ≥ 0.95. Ancestral state reconstructions, based on Bayesian analyses, are presented as pie charts, with the posterior probability of the ancestor being arborescent shown in brown and the posterior probability of being nonarborescent shown in green

be a complicating factor (e.g., *Plagiogyria* is reported to have a generation time of 15 years; Windisch and Pereira-Noronha 1983).

The link we discover in ferns between the origin of arborescence and a deceleration of rate implies that a generation time effect may be a more widespread phenomenon in plants than previously recognized. Our results also strengthen the notion that the relationship between generation time and molecular evolutionary rate is consistent (whereby a decrease in generation time results in an increase in rate and an increase in generation time results in a decrease in rate). Using five large datasets, Smith and Donoghue (2008) demonstrated that life-history traits and molecular rates in angiosperms were correlated, but only one of their datasets focused on a transition from an herbaceous to a woody condition (i.e., from a short generation time to a long generation time, as recovered here).

Unfortunately, there is no strong rationale for why a generation time effect should even exist in plants (Bousquet et al. 1992; Whittle and Johnston 2003; Soria-Hernanz et al. 2008). Because animals have determinate germ lines, with a fixed number of replications per generation, a species with a long generation time will undergo fewer germ line replications (and will be prone to fewer replication-induced mutations) per unit time than will a species with a short generation time. In plants, however, a germ line is not sequestered early in development. Instead, germ cells arise directly from somatic tissue, and mutations arising from both somatic and germ cell replications can be passed on to the next generation. Because somatic cell division is indeterminate and occurs continuously throughout the plant life cycle, short-lived and long-lived species could have the same overall number of “germ line” replications per unit time. Therefore, some process other than germ line replication rate must be invoked for the generation time effect we observe.

Based on our findings, we propose that the search for an underlying cause for this effect should focus on life-history attributes that are common to all vascular plants. It may be that both tree ferns and arborescent angiosperms share a lower frequency of somatic cell replication (Soria-Hernanz et al. 2008), and therefore have a lower replication-induced mutation rate. Alternatively, the mechanism may be related to the cyclical alternation between haploid gametophyte and diploid sporophyte phases, occurring once per generation in all land plants. If these two life-history phases are not equally susceptible to factors influencing mutation rates, then plants with different ratios of gametophyte:sporophyte longevity will accumulate different numbers of substitutions per unit time. Arborescent vascular plants usually have exceptionally long sporophytic phases, but possess gametophytic phases that are comparable in length to their nonarborescent counterparts. If gametophytes are somehow more prone to mutation than are sporophytes, then arborescent taxa should experience slower rates of molecular evolution. Discriminating among these and other possible explanations will require detailed studies of physiology, development, and population biology, but will ultimately provide fundamental insight into the evolutionary processes responsible for the molecular rate heterogeneity we observe in plants.

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(see Table S2 for exact figures). Character state codings for extant taxa are indicated at the terminals. Results for tree ferns and closely related leptosporangiate lineages are shown; more distantly related lineages (Table S1) have been pruned. *Ca, Calochlaena*; *Ci, Cibotium*; *Cu, Culcita*; *Cy, Cyathea*; *Al, Alsophila*; *Di, Dicksonia*; *Hy, Hymenophyllopsis*; *Lo quadripinnata, Lophosoria quadripinnata*; *Lo cunninghamii, Loxoma cunninghamii*; *Lo pearcei, Loxsomopsis pearcei*; *Me, Metaxya*; *Pl, Plagiogyria*; *Sp, Sphaeropteris*; *Th, Thyrsopteris*. (B) Changes in molecular substitution rate across the core leptosporangiate fern phylogeny. Each node from the phylogenetic tree shown in Figure 2A is plotted here according to its estimated rate (y-axis; Table S2) and the number of internodes separating it from the schizaeoid/core leptosporangiate split (x-axis). The divergence between schizaeoids and core leptosporangiates appears at internode “0”; more distantly related lineages have been pruned. Each connecting branch is colored based on the growth form reconstructed for the node it subtends (Fig. 2A): brown branches have a posterior probability > 0.5 of being arborescent; green branches have a posterior probability > 0.5 of being nonarborescent. Pie charts bound the transition from a nonarborescent to an arborescent growth form (Fig. 2A). Note that the slope in this figure is largely sampling dependent, with the sparser sampling outside of tree ferns resulting in steeper slopes than the denser sampling within tree ferns; absolute rate changes are more informative.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Fern phylogeny.
Figure S2. Phylogenetic branch length estimates for core leptosporangiate ferns.
Table S1. Taxonomic sampling, voucher information, and GenBank accession numbers for our 106-taxon dataset.
Table S2. Reconstructed ancestral rates and states for branches and nodes (respectively) appearing in Fig. S1.

Supporting Information may be found in the online version of this article.