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PHYLOGENY AND EVOLUTION OF FERNS (MONILOPHYTES) WITH A FOCUS ON THE EARLY LEPTOSPORANGIATE DIVERGENCES[1]

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The phylogenetic structure of ferns (= monilophytes) is explored here, with a special focus on the early divergences among leptosporangiate lineages. Despite considerable progress in our understanding of fern relationships, a rigorous and comprehensive analysis of the early leptosporangiate divergences was lacking. Therefore, a data set was designed here to include critical taxa that were not included in earlier studies. More than 5000 bp from the plastid (*rbcL, atpB, rps4*) and the nuclear (18S rDNA) genomes were sequenced for 62 taxa. Phylogenetic analyses of these data (1) confirm that Osmundaceae are sister to the rest of the leptosporangiates, (2) resolve a diverse set of ferns formerly thought to be a subsequent grade as possibly monophyletic (((Dipteridaceae, Matoniaceae), Gleicheniaceae), Hymenophyllaceae), and (3) place schizaeoid ferns as sister to a large clade of ''core leptosporangiates'' that includes heterosporous ferns, tree ferns, and polypods. Divergence time estimates for ferns are reported from penalized likelihood analyses of our molecular data, with constraints from a reassessment of the fossil record.

Key words: Bayesian inference; divergence time estimates; ferns; fossil record; molecular systematics; monilophytes; penalized likelihood; vascular plant evolution.

Among green plant lineages, none are as species-rich as vascular plants (tracheophytes). The various members of this lineage, such as clubmosses, ferns, horsetails, gymnosperms, and angiosperms, are easy to recognize as monophyletic because of their shared derived features. Vascular plants are characterized by the presence of tracheids and sieve elements (vascular tissue) for structural support and long-distance movement of water and nutrients throughout the plant body, a highly structured and dominant (or co-dominant) sporophyte phase, and branched (polysporangiate) sporophytes. This combination of features has allowed vascular plants to become the most conspicuous organisms on the planet and the dominant primary producers in terrestrial ecosystems.

Traditionally, the vascular plant tree of life has been viewed as consisting of several grades of taxa. More explicitly, vascular plant evolution was seen as a successive series of incremental increases in complexity, from simple bryophytic ancestors through vascularized spore producers, more complex seed plants, and ultimately to angiosperms. In the past 20 years, however, we have witnessed unprecedented interest and insight into the phylogenetic relationships among major groups of living (and extinct) vascular plants, and the former picture of vascular plant evolution, one of predominant paraphyly, is changing as the tracheophyte tree of life begins to come into focus (Crane, 1985a, b; Mishler and Churchill, 1985; Doyle and Donoghue, 1986a, b, 1992; Loconte and Stevenson, 1990, 1991; Raubeson and Jansen, 1992; Chase et al., 1993; Garbary et al., 1993; Doyle et al., 1994; Manhart, 1994; Mishler et al., 1994; Nixon et al., 1994; Rothwell and Serbet, 1994; Hasebe et al., 1995; Pryer et al., 1995, 2001a, 2004; Kranz and Huss, 1996; Kenrick and Crane, 1997; Doyle, 1998; Wolf et al., 1998; Mathews and Donoghue, 1999, 2000; Qiu et al., 1999, 2000; P. S. Soltis et al., 1999, 2004; Barkman et al., 2000; Chaw et al., 2000; Kenrick, 2000; Nickrent et al., 2000; Renzaglia et al., 2000; D. E. Soltis et al., 2000, 2002; Gensel and Berry, 2001; Pigg, 2001; Rydin et al., 2002; Burleigh and Mathews, 2004; Soltis and Soltis, 2004). These advances toward a more complete understanding of vascular plant relationships have not been tied to the use of any one type of evidence, but instead have attempted to ''make sense'' of all the available data, molecular and morphological (including both fossil and extant organisms).

It is now commonly agreed that a deep phylogenetic dichotomy occurred in the early-mid Devonian (ca. 400 million years ago [mya]), separating a group that includes the modern lycophytes (less than 1% of extant vascular plants) from a group that contains all other living vascular plant lineages, the euphyllophytes (Fig. 1; Raubeson and Jansen, 1992; Kenrick and Crane, 1997; Doyle, 1998; Nickrent et al., 2000; Pryer et al., 2001a, 2004). The extant lycophytes all possess lycophylls (leaves with an intercalary meristem) and comprise three main

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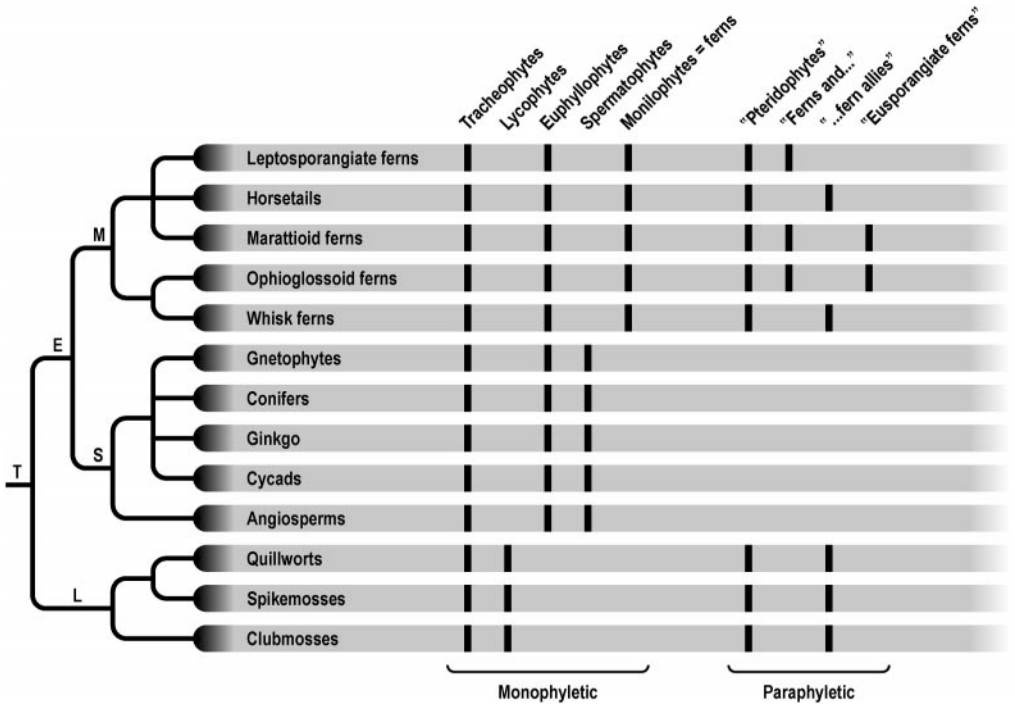

Fig. 1. Consensus tree showing relationships among the major lineages of vascular plants (based on Pryer et al., 2001a). Key clades are indicated on the tree: T = tracheophytes, L = lycophytes, E = euphyllophytes, S = spermatophytes, M = monilophytes. A black band following a lineage name indicates membership of that lineage within a particular taxon listed across the top of the figure (taxa that are not monophyletic are in quotes).

clades: homosporous Lycopodiales (clubmosses), and heterosporous Isoëtales (quillworts) and Selaginellales (spikemosses). Although living lycophytes are almost all relatively diminutive plants, many fossil members of this lineage, including such well-known examples as *Lepidodendron*, were large arborescent forms that dominated the Carboniferous landscape and that today are the major component of coal deposits (Stewart and Rothwell, 1993).

Euphyllophytes—the sister group to lycophytes—are characterized by euphylls (leaves with marginal or apical meristems and an associated leaf gap in the vascular stele), lateral branches that terminate in sporangia, and a distinctively lobed primary xylem strand (Stein, 1993; Kenrick and Crane, 1997). Extant members also possess a 30-kilobase inversion in the large single-copy region of the plastid genome (Raubeson and Jansen, 1992). Living euphyllophytes belong to two major clades (Fig. 1): seed plants (spermatophytes) and monilophytes (Kenrick and Crane, 1997; Nickrent et al., 2000; Pryer et al., 2001). Spermatophytes are united by the presence of seeds (megasporangia surrounded by integument tissue), wood produced through the activity of a secondary meristem (cambium), and axillary branching. Extant seed plants likely number between 250 000 and 300 000 species (Thorne, 2002; but see Scotland and Wortley, 2003) distributed unequally among five major clades: cycads, *Ginkgo*, conifers, gnetophytes, and angiosperms. Several extinct fossil lineages also belong to this group, including pteridosperms, Bennettitales, and glossopterids. Recent phylogenetic analyses, including those based on a combination of genes from all three genomes, have revealed conflicting signals regarding the relationships among extant spermatophyte lineages (Bowe et al., 2000; Chaw et al., 2000; Magallón and Sanderson, 2002; Rydin and Källersjö, 2002; Rydin et al., 2002; D. E. Soltis et al., 2002). Other papers in this special issue deal specifically with seed plant relationships (Burleigh and Mathews, 2004; Crane et al., 2004; Soltis and Soltis, 2004); the remainder of this paper will focus on the sister group to the spermatophytes, the monilophytes (Fig. 1).

The monilophytes (= Infradivision Moniliformopses, sensu Kenrick and Crane, 1997) share a distinctive vasculature, having protoxylem confined to lobes of the xylem strand (Stein, 1993), therefore the Latin *moniliformis* appellation for ''necklace-like.'' The monophyly of this clade has been inferred from cladistic analyses of morphology including fossil taxa (Kenrick and Crane, 1997), studies of sperm ultrastructure (Renzaglia et al., 2000, 2001, 2002), and analyses of DNA sequence data (Nickrent et al., 2000; Renzaglia et al., 2000; Pryer et al., 2001a). Extant members, which number more than 11 500 species, have a three-residue (nine-nucleotide) insertion in the plastid *rps4* gene (Pryer et al., 2001a) and belong to five major lineages: whisk ferns (Psilotales), ophioglossoid ferns (Ophioglossales), horsetails (Equisetopsida), marattioid ferns (Marattiales), and leptosporangiate ferns (Polypodiales) (Fig. 1). The Late Devonian to Early Carboniferous cladoxylopsids (e.g., Iridopteridales and Pseudosporochnales), which have characteristics of both ferns and horsetails, are almost certainly among the stem groups (Fig. 2) of the monilophytes (Skog and Banks, 1973; Stein et al., 1984; Berry and Stein, 2000; Hilton et al., 2003).

Monilophytes, like lycophytes, are all spore bearing and ''seed-free.'' Because of this, members of these two lineages were traditionally lumped under various terms, such as ''pteridophytes'' or ''ferns and fern allies'' (Fig. 1). These terms served the botanical community well while there was little resolution or understanding of the relationships among these taxa. Now, however, we have considerable confidence in the broad-scale phylogenetic relationships of vascular plants; we

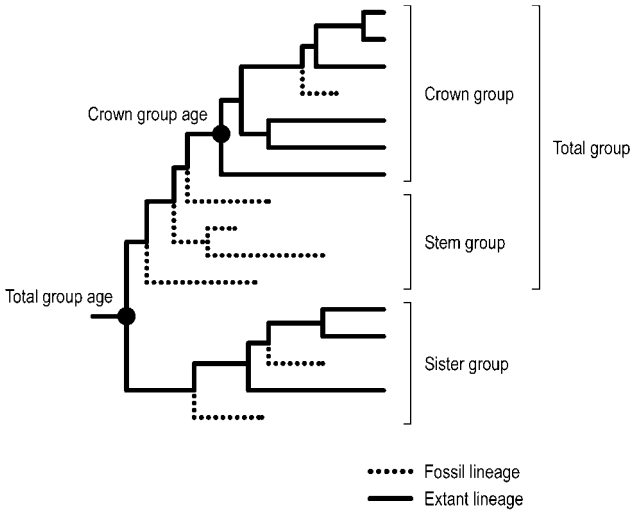

Fig. 2. Crown group and total group age definitions, as utilized in this study. Crown group age refers to the time of the deepest divergence among the extant taxa in the total group of interest. Total group age refers to the time of divergence of the total group (crown group + stem group fossils) from its sister group.

therefore prefer to use the terms monilophytes (or quite simply, a more inclusive, ferns) and lycophytes, which specify clade membership, to the terms ''pteridophytes'' and ''ferns and fern allies'' that unite paraphyletic assemblages of plants (Fig. 1). Likewise, the term ''eusporangiate ferns'' circumscribes a nonmonophyletic unit and should be avoided (although using the word ''eusporangiate'' in a descriptive sense, such as ''a eusporangiate lineage,'' may still be useful in describing sporangial morphology and development). On the other hand, ''leptosporangiate ferns'' is a long-standing and familiar term that does define a monophyletic group and continues to be informative.

Within ferns (monilophytes), the first dichotomy separates a clade consisting of whisk ferns and ophioglossoid ferns from a clade comprising horsetails, marattioid ferns, and leptosporangiate ferns (Fig. 1). Whisk ferns and ophioglossoids are both relatively small lineages (just over 100 species total in two families and about four genera—more if one recognizes the segregates of *Botrychium* at generic rank; see Hauk et al., 2003) and both have a poor fossil record. The sister relationship between these two lineages has only been identified recently (Manhart, 1995; Pahnke et al., 1996; Wolf, 1997; Nickrent et al., 2000; Pryer et al., 2001a; but see Rothwell, 1999), and unique synapomorphies are cryptic because of the extent of morphological simplification present in both families. Some whisk ferns, in fact, have such strikingly simplified body plans (pseudodichotomously branched sporophytes, no roots, and highly reduced leaves) that they were long thought to be related to some of the earliest fossil lineages of vascular plants (Parenti, 1980; Bremer, 1985), such as *Rhynia*. Reduction of the root system, however, does appear to be a shared trait. Ophioglossoids have simple, unbranched roots that lack root hairs, and whisk ferns lack roots altogether (Schneider et al., 2002). In addition, both groups have axial (and subterranean) gametophytes, sporangia that are adaxially attached, and a eusporangiate pattern of sporangial development, although these are not unique synapomorphies.

Relationships among the horsetail, marattioid, and leptosporangiate fern lineages remain elusive (Pryer et al., 2001a) and are shown in Fig. 1 as a polytomy. Horsetails are an ancient group of plants with fossil relatives dating back to the Late Devonian. Members of this clade have whorled appendages, a characteristic stele, highly reduced leaves, and sporangia borne on sporangiophores that are clustered into strobili in extant members (Kenrick and Crane, 1997). Fossil horsetails are diverse and include some arborescent representatives, such as *Calamites*, with secondary xylem. These larger forms became extinct in the Permian, but some herbaceous representatives survived, including the Mesozoic *Equisetites*. There are 15 species of living horsetails (*Equisetum*); all are relatively small in stature compared to their woody ancestors and have a worldwide distribution, mostly in temperate regions. A recent molecular phylogenetic analysis of horsetails (Des Marais et al., 2003) estimated that the *Equisetum* crown group (Fig. 2) diversified in the early Cenozoic, which is in agreement with dates estimated from fossils (Stewart and Rothwell, 1993). The long branch leading to the crown group (with no other living taxa to sample) is a complicating factor in determining the exact relationship of *Equisetum* to other monilophyte lineages (Wikström and Pryer, unpublished manuscript).

The marattioid ferns (Fig. 1) first appeared in the middle Carboniferous. In the Late Carboniferous and Permian several large marattioid representatives had evolved, including *Psaronius*, which reached heights of about 8 m. Many extant members are also treelike, but they do not possess secondary meristematic tissues. They have distinctive polycyclic dictyosteles and sporangia borne abaxially on the blades. This clade is represented by more than 200 species in about four genera (including *Marattia*, *Danaea*, and *Angiopteris*) and is almost exclusively restricted to tropical regions (Hill and Camus, 1986).

The most familiar of the monilophytes are the leptosporangiate ferns (Fig. 1), a monophyletic group of more than 11 000 species. These ferns are characterized by sporangia that develop from a single cell and have mature sporangial walls only one cell thick; most possess a distinctive annulus that serves to eject the spores (usually 64). Features of the sporangia—including the shape and position of the annulus, the structure and shape of sporangial groups (sori), and whether or not a flap of tissue (indusium) protects the sori—have figured prominently in the taxonomy and classification of these ferns. The earliest-known occurrence of leptosporangiate ferns is in the Early Carboniferous (Galtier and Scott, 1985; Galtier and Phillips, 1996); by the end of the Carboniferous six families were present. In subsequent major filicalean radiations in the Permian, Triassic, and Jurassic, several families (e.g., Osmundaceae, Schizaeaceae, Matoniaceae, and Dipteridaceae) with extant representatives replaced these Carboniferous families (Rothwell, 1987). The more-derived polypod ferns, which comprise more than 80% of living fern species, recently were shown to have diversified in the Cretaceous (on the basis of molecular age estimates), suggesting an ecological opportunistic response to the diversification of angiosperms (Schneider et al., 2004b).

Increasingly robust phylogenetic hypotheses, broadly inclusive of ferns and utilizing data from single or multiple sources (e.g., morphology; plastid, nuclear, and/or mitochondrial genes), have improved confidence in the composition of and the relationships among many taxa historically treated at familial and ordinal ranks (Hasebe et al., 1994, 1995; Manhart, 1995; Pryer et al., 1995, 2001a; Kranz and Huss, 1996; Pahnke

et al., 1996; Rothwell, 1996, 1999; Schneider, 1996; Stevenson and Loconte, 1996; Wolf, 1997; Wolf et al., 1998; Vangerow et al., 1999; Schneider et al., 2004b). In this paper, we build on our current, best estimate of monilophyte relationships (Renzaglia et al., 2000; Pryer et al., 2001a; Schneider et al., 2004b), with our primary aim to focus attention on the basal nodes of the leptosporangiate fern tree, for which we provide new DNA data and phylogenetic analyses. It should be noted that for many of the more derived leptosporangiates (polypods), the overall phylogenetic picture is still equivocal (Wolf et al., 1994; Wolf, 1995; Murakami et al., 1999; Cranfill, 2001; Smith and Cranfill, 2002; Ranker et al., 2004; Schneider et al., 2004a, c) and in need of further sampling and study. Nevertheless, providing a robust overall framework for ferns, especially at the base of the tree, will ultimately enable us to answer some long-standing systematic questions and work toward understanding the patterns of character evolution that gave rise to the Cretaceous radiation and diversification of polypod ferns (Schneider et al., 2004b).

MATERIALS AND METHODS

Taxonomic sampling—Sixty-two taxa were selected to represent all major vascular plant lineages (see Appendix 1 in Supplemental Data accompanying the online version of this article). Our sampling included three lycophytes (outgroup), each representing a different lineage, and six seed plants, including at least one representative from each of the five major lineages. Within monilophytes, the primary focus of this study, sampling was more extensive and included at least two taxa from each of the eusporangiate lineages. Within leptosporangiate ferns, 44 taxa were chosen to represent the major lineages, and we focused our sampling toward the basal nodes, with only a few exemplars from the hyperdiverse polypods.

DNA isolation, amplification, and sequencing—Genomic DNA was extracted from fresh, silica-dried, or herbarium leaf material using a modified Doyle and Doyle (1987) CTAB (cetyltrimethylammonium bromide) procedure (Dubuisson, 1997; Pryer et al., 2001b) or a DNeasy kit (Qiagen, Valencia, California, USA). For each taxon, four genes (plastid *rbcL, atpB, rps4*; nuclear small-subunit ribosomal DNA [18S]) were amplified separately using the polymerase chain reaction (PCR), following established protocols (see Pryer et al., 2001b). PCR products were cleaned using QIAquick columns (Qiagen) according to the manufacturer's protocol. Sequencing reactions were carried out for both strands of the purified PCR products using Dye Terminator Cycle Sequencing or Big Dye Terminator Cycle Sequencing reagents (Applied Biosystems, Foster City, California, USA). For information on amplification and sequencing primers see Appendix 2 in Supplemental Data accompanying the online version of this article. All sequencing reactions were processed using either ABI 377 or ABI 3700 automated sequencers (Applied Biosystems), and each sequencing read was evaluated for possible contamination using the NCBI nucleotide-nucleotide BLAST (blastn) tool (Altschul et al., 1997). More than one-third of the sequence data (97 new sequences) used in the analyses described in this article were generated specifically for this study; all other data were obtained from GenBank (see Appendix 1 in Supplemental Data accompanying the online version of this article).

Sequence alignment—Sequence fragments obtained as chromatograms were edited and assembled into contiguous alignments using Sequencher (Gene Codes, Ann Arbor, Michigan, USA). The resulting consensus sequences for each gene were aligned manually using MacClade version 4.05 (Maddison and Maddison, 2000). The alignments for *rbcL* and *atpB* were straightforward because no insertions or deletions (indels) were present. Indels, however, existed in both the *rps4* and 18S nrDNA alignments; translated amino acid sequences (*rps4*) and rRNA secondary structure (18S) were used as alignment guides. Regions of ambiguous alignment (within *rps4* and 18S nrDNA) were excluded from the subsequent analyses, as were portions of alignments at the 5′ and 3′ ends that contained copious amounts of missing data.

Phylogenetic analyses—To assess the combinability of the four single-gene data sets, a procedure was invoked in which topological conflict among trees resulting from analyses of the individual data sets was examined. Each single-gene data set was analyzed independently with PAUP* version 4.0b10 (Swofford, 2002) using an equally weighted maximum parsimony bootstrap approach to assess clade support (Felsenstein, 1985). For the plastid genes, the bootstrap analysis consisted of 1000 replicates, each with 10 random-addition-sequence replicates and tree bisection and reconnection (TBR) branch swapping. For 18S nrDNA, the bootstrap analysis consisted of 1000 replicates, each with one random-addition-sequence and TBR branch swapping, saving a maximum of 1000 trees per replicate (these modifications were necessary here to limit search time). The bootstrap consensus trees resulting from each of the four analyses were compared visually for conflict (high support for incongruent relationships; see Appendix 3 in Supplemental Data accompanying the online version of this article). Using a significance threshold of 70%, some topological conflict was detected with regard to the lycophyte and seed plant lineages. However, no significant conflict among the four genes was detected within the study group (monilophytes), and for this reason, the single-gene molecular data sets were combined and analyzed in unison.

The combined data set was analyzed using a Bayesian Markov chain Monte Carlo (B/MCMC) approach, as implemented in MrBayes version 3.0b (Huelsenbeck and Ronquist, 2001). Each gene was assigned its own model of sequence evolution (GTR + I + Γ for each), as determined using a hierarchical likelihood ratio test in Modeltest (Posada and Crandall, 1998). Two independent B/MCMC analyses were conducted using these four models, flat priors, and four chains. Chains were run for 10 million generations, and trees were sampled every 1000 generations. Following completion, the sampled trees from each analysis were plotted against their likelihood to recognize the point where the likelihoods converged on a maximum value. All trees prior to this convergence (500 trees; 500 000 generations, for each of the two analyses) were discarded as the ''burn-in'' phase. Because both analyses converged on the same maximum, the post burn-in trees from each (19 000 total trees) were pooled, and a majority-rule consensus was calculated to obtain a topology with average branch lengths, as well as posterior probabilities for all resolved nodes.

Phylogenetic analyses of the combined data set were also conducted using maximum likelihood and equally weighted maximum parsimony approaches, in PAUP* (Swofford, 2002). A heuristic search for the most likely tree was conducted using a single model of sequence evolution (GTR + I + Γ; as identified in Modeltest, using parameters as estimated in the program), 100 random-addition-sequence replicates, and TBR branch swapping. A heuristic search for the most parsimonious tree was conducted using 1000 random-addition-sequence replicates and TBR branch swapping. Maximum likelihood and maximum parsimony bootstrap analyses also were conducted. The maximum parsimony bootstrap analysis consisted of 1000 replicates, each with 10 random-addition-sequence replicates and TBR branch swapping. The maximum likelihood bootstrap analysis utilized the same model of evolution selected for the original maximum likelihood search and consisted of 100 replicates, each with one random-addition-sequence and nearest neighbor interchange (NNI) branch swapping (to limit the search time).

Divergence time estimation—Divergence times were estimated using a penalized likelihood approach (Sanderson, 2002) that does not require an assumption of rate constancy (i.e., a molecular clock). Instead, this method combines a parameter-rich model, allowing for a different rate of substitution on every branch, with a roughness penalty that constrains rate fluctuations from branch to branch. The relative contributions of these two components are controlled by a smoothing parameter that can be objectively selected using a cross-validation procedure (Sanderson, 2002). We obtained divergence time estimates for ferns through a penalized likelihood analysis (using the computer program r8s, version 1.60; Sanderson, 2003) of our Bayesian consensus tree, incorporating 21 fossil constraints from a reassessment of the fern fossil record (see Table 1). In our analysis, the three lycophyte taxa were pruned from the tree, and the root of the resulting tree (i.e., the divergence of monilophytes from spermatophytes) was used as a calibration point based on the concurrent appearance of fossils belonging to each of these lineages in the Middle De-

vonian (see Table 1). The appropriate smoothing value was determined using cross validation; we considered values from 0.1 to 10 000, and the value 10 received the best (i.e., lowest) cross validation score. Using this smoothing value, the Bayesian consensus tree, and the 21 fossil constraints, we searched for the solution that optimized the penalized likelihood function (10 random starts, each with 10 random perturbations; truncated Newton algorithm).

To evaluate the effects of phylogenetic uncertainty (Pagel and Lutzoni, 2002), due to both topological and branch length estimation error, divergence times were also estimated for each of 100 Bayesian trees randomly sampled from among the 16 952 trees that contained all nodes with significant support (posterior probability ≥95%). For each tree, we identified the appropriate smoothing value through cross validation and used penalized likelihood to estimate divergence times (as before). For these analyses, the fossil constraints were applied only to well-supported nodes (posterior probability ≥95%); the three fossil constraints that were applied to poorly supported nodes (posterior probability <95%) in the consensus analysis were, for the 100 replicate analyses, applied to the next deeper well-supported node. The 100 molecular age estimates for each well-supported node were averaged and their standard deviation calculated.

RESULTS

Alignments—The mean sequence length, alignment length, number of characters included after pruning regions of ambiguous alignment, numbers of variable and parsimony informative characters, and percentage missing data are given for *rbcL*, *atpB*, *rps4*, and 18S nrDNA in Table 2. For some taxa, *rps4* and/or *atpB* had premature stop codons within the gene, which are likely to be corrected with RNA editing (Wolf et al., in press).

Phylogenetic analyses—The Bayesian analysis of the four-gene combined data set yielded a well-resolved and well-supported topology (Fig. 3). The maximum likelihood (ML) analysis of the combined data set resulted in a single most-likely tree ($-\ln L = 58\,123.47526$; tree not shown); and likewise, the maximum parsimony (MP) analysis of the combined data set resulted in a single most parsimonious tree (11 975 steps; CI = 0.273; RI = 0.561; tree not shown). The ML tree is identical in topology to the Bayesian tree (shown in Fig. 3), with one exception within polypod ferns. The MP tree is similar to these two trees, but with some minor topological differences within the polypod and tree fern clades, a conflicting placement of *Gnetum*, and a different relationship among the lycophyte outgroup taxa. In the tree resulting from the Bayesian analysis, 43 of the 52 nodes in the monilophyte study group (Fig. 3) receive significant support from all three measures—Bayesian posterior probabilities (PP ≥ 95), and maximum likelihood and maximum parsimony bootstrap percentages ($BP^{ML} \geq 70$ and $BP^{MP} \geq 70$, respectively).

Seed plants and monilophytes are strongly supported sister groups (PP = 100; $BP^{ML} = 100$; $BP^{MP} = 100$). Within monilophytes, each of the eusporangiate lineages (whisk ferns, ophioglossoid ferns, horsetails, and marattioid ferns), as well as the leptosporangiate ferns, are strongly supported (PP = 100; $BP^{ML} = 100$; $BP^{MP} = 100$). Likewise, whisk ferns together with ophioglossoid ferns are well supported (PP = 100; $BP^{ML} = 99$; $BP^{MP} = 100$) and sister to the remaining monilophytes. Horsetails are resolved as sister to the marattioid ferns regardless of the optimization criterion used, but this relationship is weakly supported by all three measures (PP = 82; $BP^{ML} < 50$; $BP^{MP} = 76$). These two eusporangiate lineages are always together with leptosporangiate ferns in a well-supported clade (PP = 100; $BP^{ML} = 88$; $BP^{MP} = 87$).

All analyses provide exceptionally robust support (PP = 100; $BP^{ML} = 100$; $BP^{MP} = 100$) for the following major groups of leptosporangiate ferns: osmundaceous ferns, filmy ferns, schizaeoid ferns, core leptosporangiates, heterosporous ferns, and polypod ferns (Fig. 3). Two remaining major groups of leptosporangiate ferns were consistently resolved as monophyletic, but with somewhat reduced overall support: tree ferns (PP = 100; $BP^{ML} = 84$; $BP^{MP} = 85$) and gleichenioid ferns (sensu Jarrett, 1980) (PP = 88; $BP^{ML} = 86$; $BP^{MP} = 88$). Within leptosporangiates, the osmundaceous ferns are sister to the rest (PP = 100; $BP^{ML} = 100$; $BP^{MP} = 100$).

As sister to all other leptosporangiates (minus Osmundaceae), our analyses identified a clade consisting of filmy ferns together with gleichenioid ferns, and the Bayesian analysis provided strong support (PP = 96) for this relationship. Although this clade also was resolved consistently with maximum parsimony and maximum likelihood, these two methods provided only weak support ($BP^{ML} = 55$; $BP^{MP} = 57$). Within the gleichenioid ferns, there is robust support (PP = 100; $BP^{ML} = 100$; $BP^{MP} = 100$) for the family Gleicheniaceae (*Dicranopteris*, *Gleichenella*, *Diplopterygium*, *Gleichenia*, and *Stichenus*) to include the often-separated genus *Stromatopteris* (Stromatopteridaceae of Bierhorst, 1977; Wagner, 1977). There is also strong support for a sister relationship between *Cheiropleuria* + *Dipteris* and *Matonia* + *Phanerosorus* (PP = 100; $BP^{ML} = 97$; $BP^{MP} = 95$), with that clade in turn sister to Gleicheniaceae (PP = 88; $BP^{ML} = 86$; $BP^{MP} = 88$).

The schizaeoid ferns are sister to a robustly supported clade that we here refer to as the ''core leptosporangiates'' (PP = 100; $BP^{ML} = 100$; $BP^{MP} = 100$), which includes the heterosporous ferns, tree ferns, and polypods. Each of these lineages is clearly monophyletic, but the relationships among them are equivocal. Within heterosporous ferns, two clades are well supported (PP = 100; $BP^{ML} = 100$; $BP^{MP} = 100$); within tree ferns, some clades are also well supported, but the relationships among these remain ambiguous. Within polypods is a grade of enigmatic and species-poor genera (*Saccoloma*, *Lonchitis*, *Sphenomeris*) leading to a well-supported (PP = 100; $BP^{ML} = 100$; $BP^{MP} = 97$), hyperdiverse clade that contains over 80% of all living fern species.

Divergence time estimates—The results of our penalized likelihood analysis of the Bayesian consensus tree are presented as a chronogram plotted against the geologic time scale in Fig. 4. Age estimates for all nodes, as well as mean ages and standard deviations (resulting from the 100 replicate analyses) for all well-supported nodes (nodes with black symbols in Fig. 4), are presented in Table 1. Our divergence time estimates are generally older than those implied by the fossil record, but they show relatively small standard deviations (Table 1).

According to our analyses, the initial divergence among monilophyte lineages (node 07, Fig. 4) occurred in the Late Devonian (~364 mya). All four eusporangiate lineages, as well as the leptosporangiate fern lineage, were present by the end of the Carboniferous. We estimate that the whisk and ophioglossoid fern lineages diverged from one another in the Late Carboniferous (~306 mya, node 08), with their crown group divergences in the Late Cretaceous (~88 mya, node 09) and Middle Jurassic (~162 mya, node 10), respectively (Fig. 4; Table 1). As indicated by the fossil record, horsetails and marattioid ferns had diverged from one another by the end of the Devonian (~354 mya, node 12, Fig. 4); however, the

crown group divergences within these groups appear to be more recent phenomena. Extant horsetails are estimated to have diversified in the Tertiary (~38 mya, node 13); extant members of the marattioid ferns (node 14) began to diversify in the Middle Triassic (~237 mya, Fig. 4; Table 1).

Within leptosporangiate ferns, we estimate the earliest divergences to have occurred in the Carboniferous and Permian. These divergences gave rise to the osmundaceous, filmy, gleichenioid, and schizaeoid ferns, as well as to the core leptosporangiates (Fig. 4). The initial divergence within the osmundaceous ferns is estimated to have occurred by the end of the Triassic, and our analyses support an origin of the two major filmy fern lineages in the Jurassic (~163 mya, node 21, Fig. 4). The earliest divergences within the gleichenioid ferns (nodes 22 and 23, Fig. 4), giving rise to the three extant gleichenioid families (Gleicheniaceae, Dipteridaceae, and Matoniaceae), occurred in the Permian (~263 mya) and Triassic (227 mya), but we estimate divergences within each of these families to be more recent (Cretaceous). The initial divergence within schizaeoid ferns (node 32, Fig. 4) is estimated to have occurred in the Triassic (~212 mya).

A Late Triassic diversification gave rise to the three major lineages of ''core leptosporangiates'' (Fig. 4)—heterosporous ferns, tree ferns, and polypod ferns. The earliest divergences within each of these lineages occurred in the Jurassic. The most species-rich groups of polypod ferns, namely the eupolypods and pteridoids (nodes 55 and 57, respectively, Fig. 4), comprise more than 80% of extant fern species and are estimated to have diversified in the Cretaceous (consistent with the findings of Schneider et al., 2004b).

DISCUSSION

Phylogenetic relationships—There has been a steady trend in recent years of increasing resolution at the base of the fern phylogeny. The results of our analyses of a four-gene (*rbcL, atpB, rps4*, 18S) combined data set provide the highest levels of resolution and support to date across the backbone of the leptosporangiate tree (Fig. 3). This, combined with our extensive sampling within the early leptosporangiate divergences (including representatives from the majority of currently recognized genera), allows us to draw several important conclusions.

Monilophytes—Our sampling of the four major eusporangiate lineages of monilophytes in this study was identical to that of Pryer et al. (2001a). However, increased taxonomic sampling within the early leptosporangiate divergences, and the addition of Bayesian analytical tools to the maximum parsimony and maximum likelihood methods used in Pryer et al. (2001a), allow for improved understanding of the relationships among the eusporangiate lineages. In agreement with that earlier study, all measures used here provided the highest degree of support for the monophyly of monilophytes, for each of the four lineages of eusporangiate monilophytes (whisk ferns, ophioglossoid ferns, horsetails, and marattioid ferns), and for leptosporangiate ferns (Fig. 3). As was demonstrated previously (Nickrent et al., 2000; Pryer et al., 2001a), whisk ferns are most closely related to ophioglossoids, which contradicts previously accepted relationships (Wagner, 1977; Rothwell, 1999), and together they form the sister group to the rest of monilophytes with robust support. Horsetails are resolved as sister to marattioid ferns in both the current study and Pryer et al. (2001a), as well as in Wikström and Pryer (unpublished manuscript), but usually with weak support. The precise relationship of horsetails does remain subject to further study.

Leptosporangiate ferns—Establishing a robust phylogenetic hypothesis of monilophyte relationships, especially among the basal leptosporangiate nodes, is critical to understanding character evolution and early diversification in ferns. Recent studies (Hasebe et al., 1995; Pryer et al., 1995; Schneider, 1996; Stevenson and Loconte, 1996) focused on leptosporangiate ferns have provided a framework of higher-level relationships, replacing former intuitive estimates founded largely on concepts of overall similarity (see Smith, 1995 for a review). In those studies, however, only a few internal nodes received robust support, and most nodes at the base of the fern topology (especially those along the backbone) were weakly supported, making it impossible to say with certainty how any of these groups are related to one another. Our current study improves greatly on this situation.

Osmundaceous ferns—These ferns were considered by nearly all earlier workers to be an ''isolated and basal'' group, or even a ''transitional form,'' between the eusporangiate and leptosporangiate condition because they possess a mixture of sporangial features from both types (Smith, 1995). In all analyses that have included a reasonable sampling of leptosporangiate ferns, the osmundaceous ferns have been shown to be sister to the rest of the leptosporangiates (Fig. 3; Hasebe et al., 1994, 1995; Pryer et al., 1995; Wolf et al., 1998), although not always with strong support. This phylogenetic position is consistent with the fossil record for Osmundaceae (Fig. 4; Table 1) and is resolved here with clear support (PP = 100; BP^{ML} = 100; BP^{MP} = 100).

Gleichenioid ferns—The phylogenetic affinities of the peculiar New Caledonian genus *Stromatopteris* have been hotly contested (Bierhorst, 1977; Wagner, 1977). Most recently, the genus was recognized as a member of Gleicheniaceae, but in a separate subfamily (Stromatopteridoideae) from the other genera (subfamily Gleichenioideae: *Dicranopteris*, *Gleichenella*, *Diplopterygium*, *Gleichenia*, *Sticherus*) (Kramer and Green in Kubitzki, 1990). Our sampling includes a representative from each of these six genera, and our analyses clearly establish that *Stromatopteris* nests well within Gleicheniaceae, sister to *Gleichenia*. Several earlier studies, most importantly Jarrett (1980), suggested that Cheiropleuriaceae, Dipteridaceae, and Matoniaceae might be allied to one another and to Gleicheniaceae. Nonetheless, in previous single-gene studies (Hasebe et al., 1995; Pryer et al., 1995), representative taxa from these groups were usually variously displayed as a grade at the base of the leptosporangiate ferns. We show here (Fig. 3) that *Cheiropleuria* + *Dipteris* and *Matonia* + *Phanerosorus* are strongly supported as sister taxa (PP = 100; BP^{ML} = 97; BP^{MP} = 95) and that there is substantial support for a sister relationship between this group and Gleicheniaceae (PP = 88; BP^{ML} = 86; BP^{MP} = 88). Thus, we see considerable merit in referring to this entire clade as the gleichenioid ferns. This resolves the long-running controversy surrounding the relationships of Matoniaceae, a family once thought to be taxonomically isolated from all other fern groups (Klavins et al., 2004), and *Cheiropleuria* and *Dipteris*—once incorrectly assumed to be more closely related to more-derived members of Polypodiaceae (see Smith, 1995 for a review). Gleichenioid

TABLE 1. Molecular age estimates and fossil age constraints for vascular plant nodes shown in Fig. 4. Lineage names, both crown group (CG) and/or total group (TG), are provided where applicable (see Fig. 2 for terminology). A molecular age estimate, derived from the penalized likelihood analysis of the consensus tree, as well as a mean molecular age ± standard deviation (SD), derived from the penalized likelihood analysis of 100 replicate Bayesian trees, are also provided for each node, as applicable. Fossil age constraints were applied to nodes using an apomorphy-based approach. A fossil constraint was assigned to a node only if the applicable fossil shared a derived feature with a lineage descended from that node (crown group age); if we were unable to reject the possibility that a reported character state was a synapomorphy for the total group (crown group + stem group), the fossil age constraint was assigned to the next deeper node (total group age). mya = million years ago, NA = fossil constraint not applied.

Node	Lineage name(s)	Molecular age estimate (mya)	Mean molecular age ± SD (mya)	Fossil age constraint	Comments
01	Euphyllophytes (CG) Spermatophytes (TG) Monilophytes (TG)	380.00	380.00 ± 0.00	380.00	The appearance of *Ibyka* and *Crossia* marks the divergence of the two extant lineages of euphyllophytes in the Middle Devonian (Eifelian). We accept *Ibyka* to be the oldest monilophyte fossil (but not assignable to the horsetail lineage) and *Crossia* to be the oldest spermatophyte fossil, based on the protoxylem position in the mature stele (mesarch in monilophytes, endarch in spermatophytes (Kenrick and Crane, 1997).
02	Spermatophytes (CG)	321.66	321.56 ± 2.92	NA	—
03	Angiosperms (CG)	121.00	121.20 ± 2.04	121.00	The oldest angiosperm crown group fossils date to the Early Cretaceous (Valanginian; Brenner, 1996; Magallón and Sanderson, 2001), but these are not assignable to either lineage sampled here. We therefore used the oldest fossils assignable to Chloranthaceae (Early Cretaceous: Barremian; Friis et al., 1994, 1999) to constrain this node.
04	Gymnosperms (CG) Conifers (TG)	310.00	310.00 ± 0.00	310.00	Conifer cones and twigs from the Carboniferous (Pennsylvanian; Miller, 1999) are the oldest unequivocal remains of an extant spermatophyte lineage and indicate the time of divergence of conifers from other seed plant lineages.
05	—	271.98	272.16 ± 6.85	NA	—
06	—	292.44	284.95 ± 17.90	NA	—
07	Monilophytes (CG)	364.43	359.97 ± 1.82	NA	—
08	Whisk ferns (TG) Ophioglossoid ferns (TG)	305.57	292.62 ± 13.15	NA	A fossil record for the whisk ferns has not yet been documented (Kenrick and Crane, 1997). The oldest fossil assignable to the ophioglossoid ferns is *Botrychium wightonii* (Late Paleocene: Thanetian; Rothwell and Stockey, 1989).
09	Whisk ferns (CG)	88.47	81.66 ± 9.84	NA	—
10	Ophioglossoid ferns (CG)	161.96	153.45 ± 11.98	NA	—
11	Leptosporangiate ferns (TG)	359.56	354.00 ± 0.04	NA	We recognize members of Tedeleaceae and Botryopteridaceae, dating back to the Early Carboniferous (Galtier and Phillips, 1996), as among the oldest leptosporangiate ferns. Their relationships to any extant lineage are unclear. The fossil constraint from node 12 (354 mya) was applied to node 11 for the 100 replicate analyses used to calculate the molecular age mean and standard deviation.
12	Horsetails (TG) Marattioid ferns (TG)	354.00	—	354.00	*Archaeocalamites* and relatives from the Late Devonian (Famennian; Stein et al., 1984; Bateman, 1991; Kenrick and Crane, 1997) are accepted here to be the oldest unequivocal members of the horsetail lineage, based on stem anatomical characters. Marattioid ferns, such as *Psaronius* and *Scolecopteris*, were abundant in the Late Carboniferous (Pennsylvanian) and Permian (Hill and Camus, 1986; Liu et al., 2000); therefore, this lineage was likely present in the Early Carboniferous (Mississippian).
13	Horsetails (CG)	37.51	37.65 ± 3.87	NA	—
14	Marattioid ferns (CG)	236.61	230.52 ± 5.45	NA	—
15	—	206.00	206.00 ± 0.00	206.00	Fossils assignable to *Marattia* (i.e., bearing synangia similar to those of extant *Marattia*) date back to the Late Triassic (Hill and Camus, 1986; Liu et al., 2000).
16	Leptosporangiate ferns (CG) Osmundaceous ferns (TG)	323.10	319.60 ± 5.08	282.00	The oldest osmundaceous fern fossil, assignable based on stele organization, is *Grammatopteris* from the Early Permian (Asselian; Skog, 2001; Roessler and Galtier, 2002).
17	Osmundaceous ferns (CG)	206.00	206.00 ± 0.00	206.00	Based on stem anatomical characters, Miller (1971) considered *Osmundacaulis* from the Late Triassic to be a member of the osmundaceous crown group (Tidwell and Ash, 1994; Collinson, 1996).
18	—	98.57	81.25 ± 22.97	NA	—

TABLE 1. Continued.

Node	Lineage name(s)	Molecular age estimate (mya)	Mean molecular age ± SD (mya)	Fossil age constraint	Comments
19	—	286.24	284.54 ± 6.58	269.00	The oblique annulus and spore wall ultrastructure of *Oligocarpia* and *Szea* (Permian: Sakmarian; Wang et al., 1999; Yao and Taylor, 1988) indicate that these fossils are members of the lineage that includes filmy and gleichenioid ferns. The leaf morphology of these fossils does not indicate a close relationship to any extant member of this lineage; therefore, we consider them to be stem group members.
20	Gleichenioid ferns (TG) Filmy ferns (TG)	272.89	271.16 ± 6.06	NA	The oldest gleichenioid fern fossils are Middle Triassic in age (Collinson, 1996; Skog, 2001) and belong to the dipteridoid/matonioid lineage (see node 23). The oldest filmy fern fossil, *Hopetedia*, is from the Late Triassic (Carnian; Axsmith et al., 2001).
21	Filmy ferns (CG)	163.23	157.07 ± 11.26	NA	—
22	Gleichenioid ferns (CG) Gleicheniaceae (TG)	263.29	—	NA	Fossils assignable to Gleicheniaceae (e.g., *Gleichenites*, *Gleichenoides*, *Gleichenopsis*; Collinson, 1996; Skog, 2001) are known from the Jurassic and Cretaceous, but these cannot be assigned unequivocally to any extant lineage and are likely stem group members. Permian to Triassic fossils (*Oligocarpia*, *Szea*) that are often discussed as Gleicheniaceae (Yao and Taylor, 1988; Tidwell and Ash, 1994; Collinson, 1996; Wang et al., 1999) may in fact be stem group members of Gleicheniaceae but could also be stem group members of Matoniaceae, Dipteridaceae, or filmy ferns (see node 19).
23	Matoniaceae (TG) Dipteridaceae (TG)	227.00	227.00 ± 0.00	227.00	Dipteridoids and matonioids are abundant in the fossil record from the Late Triassic to Early Cretaceous (Skog, 2001). The oldest fossils, including *Phlebopteris* and *Tomaniopteris*, date back to the Middle Triassic and share leaf and soral characters with extant genera such as *Dipteris* and *Matonia* (Tidwell and Ash, 1994; Collinson, 1996; Klavins et al., 2004).
24	Matoniaceae (CG)	114.77	113.75 ± 8.92	NA	—
25	Dipteridaceae (CG)	70.89	68.67 ± 6.62	NA	—
26	Gleicheniaceae (CG)	124.22	120.61 ± 6.15	NA	—
27	—	103.53	101.98 ± 4.60	NA	—
28	—	89.00	89.04 ± 0.45	89.00	Gandolfo et al. (1997) demonstrated a sister group relationship between the fossil *Boodlepteris* (Late Cretaceous: Turonian) and the extant genus *Stromatopteris*; therefore, a minimum age of 89 mya was assigned to the divergence of *Stromatopteris* from other extant taxa.
29	—	99.63	95.42 ± 9.07	NA	—
30	—	36.58	34.11 ± 5.22	NA	—
31	Schizaeoid ferns (TG)	266.25	265.30 ± 7.89	NA	The oldest schizaeoid fern fossils are from the Middle Jurassic (see node 32).
32	Schizaeoid ferns (CG)	211.61	212.50 ± 12.60	169.00	Wikström et al. (2002) demonstrated a sister group relationship between the fossil *Stachypteris* (Middle Jurassic: Bajocian) and the extant genus *Lygodium*; therefore, a minimum age of 169 mya was applied to the divergence of *Lygodium* from other extant schizaeoid genera. The relationships of other Jurassic schizaeoid ferns, such as *Klukia* and *Klukiopsis*, are unknown.
33	—	135.29	138.18 ± 11.86	121.00	The Early Cretaceous (Neocomian) genus *Ruffordia* is generally accepted to be a relative of the extant genus *Anemia* (Wikström et al., 2002); therefore, a minimum age of 121 mya was assigned to the divergence of *Anemia* from *Schizaea*.
34	Heterosporous ferns (TG)	220.02	219.23 ± 6.52	NA	The oldest fossil assignable to the heterosporous fern lineage is *Crybelosporites berberioides* from the Late Jurassic (Lupia et al., 2000).
35	Heterosporous ferns (CG) Marsileaceae (TG) Salviniaceae (TG)	173.32	171.27 ± 7.76	137.00	Based on leaf and stem morphology, Yamada and Kato (2002) demonstrated that the fossil *Regnellites nagashimae* (Early Cretaceous: Berriasian) is assignable to Marsileaceae; therefore, we accept this fossil as a minimum constraint for the time of divergence between Marsileaceae and Salviniaceae.
36	Marsileaceae (CG)	93.02	89.64 ± 7.41	NA	—
37	Salviniaceae (CG)	89.00	89.17 ± 0.69	89.00	Megaspores of *Azolla* date back to the Late Cretaceous: Turonian (Collinson, 1991); we use these to mark the time of divergence between *Azolla* and *Salvinia*.

TABLE 1. Continued.

Node	Lineage name(s)	Molecular age estimate (mya)	Mean molecular age ± SD (mya)	Fossil age constraint	Comments
38	Tree ferns (TG) Polypod ferns (TG)	210.79	—	NA	We accept *Cyathocaulis* and related fossils (Middle Jurassic; Lantz et al., 1999) as the oldest unequivocal tree ferns (see node 42). Assignments of Triassic and Early Jurassic fern fossils to tree fern genera such as *Dicksonia* are ambiguous and cannot be accepted without further critical evaluation. The oldest unequivocal polypod fern fossils are from the Lower Cretaceous (Neocomian; Chen et al., 1997; Deng, 2002) of northern China (see node 47).
39	Tree ferns (CG)	182.86	181.78 ± 6.58	NA	—
40	Loxomataceae (TG)	154.82	150.52 ± 11.03	112.00	Based on rhizome anatomy and hair structure, *Loxsomopteris* (Early Cretaceous: Aptian; Skog, 2001) is considered to be a member of Loxomataceae and is used to constrain its time of divergence from *Plagiogyria*.
41	Loxomataceae (CG)	34.61	32.52 ± 5.96	NA	—
42	—	159.00	159.89 ± 3.07	159.00	We accept *Cyathocaulis* and related fossils (Middle Jurassic; Lantz et al., 1999; Skog, 2001) as belonging to the *Cyathea* + *Hymenophyllopsis* lineage. We use these fossils to constrain the time of divergence of *Cyathea* + *Hymenophyllopsis* from other tree fern genera.
43	—	50.23	41.80 ± 11.79	NA	—
44	—	152.73	—	NA	—
45	—	133.50	129.47 ± 15.54	NA	—
46	—	123.85	—	NA	—
47	Polypods (CG)	159.54	162.68 ± 8.96	121.00	Various crown group polypod fossils (with well-preserved polypod sporangia) are known from the Lower Cretaceous (Neocomian; Chen et al., 1997; Deng, 2002) of Northern China and are used here to constrain the age of this node.
48	—	153.48	—	NA	—
49	Lindsaeoid ferns (TG)	133.17	136.75 ± 8.78	99.00	Based on characteristic root anatomy, a fossil from the Early Cretaceous (Albian; Schneider and Kenrick, 2001) provides unequivocal evidence for the presence of lindsaeoid ferns (*Sphenomeris* in Fig. 4) at this time.
50	Dennstaedtiaceae (TG)	124.92	128.79 ± 9.85	NA	The fossil constraint from node 54 (93.5 mya) was applied to this node for the 100 replicate analyses used to calculate the molecular age mean and standard deviation.
51	Dennstaedtiaceae (CG)	102.23	98.59 ± 8.33	NA	—
52	—	92.03	—	NA	—
53	—	47.11	45.02 ± 6.29	NA	—
54	Pteridaceae (TG) Eupolypods (TG)	119.85	—	93.50	Leaf shape and the presence of pseudoindusia allow for the assignment of a fossil *Pteris* from the Late Cretaceous (Cenomanian; Krassilov and Bacchia, 2000) to the pteridoid ferns. Because this fossil is not readily assignable to any crown group pteridoid lineage, we use it here to constrain the time of divergence between pteridoid and eupolypod ferns.
55	Eupolypods (CG)	77.19	75.49 ± 7.66	NA	The fossil constraint from node 56 (65 mya) was applied to this node for the 100 replicate analyses used to calculate the molecular age mean and standard deviation.
56	—	65.00	—	65.00	Leaf impressions showing key features of both *Onoclea* and *Woodwardia* (blechnoid ferns) are known from the Late Cretaceous (Maastrichtian; Rothwell and Stockey, 1991; Upchurch and Mack, 1998; Pigg and Rothwell, 2001) and are used here to constrain the time of divergence of blechnoid and thelypteridoid ferns.
57	Pteridaceae (CG)	76.62	83.85 ± 9.45	NA	—
58	—	69.88	77.02 ± 9.15	65.00	Fossils assignable to *Acrostichum* (the sister genus to *Ceratopteris*; Schneider et al., 2004b) are present in the Late Cretaceous (Maastrichtian; Bonde and Kumaran, 2002) and are used to constrain the time of divergence between *Ceratopteris* and *Adiantum*.

TABLE 2. Summary of DNA sequence data and alignments for each molecular region used in this study of monilophyte relationships.

Marker	Mean sequence length[a]	Alignment length	Included characters	Variable characters	Parsimony informative characters[b]	% Missing data[c]
rbcL	1320	1402	1320	680	558	1.15
atpB	1141	1150	1150	635	562	0.80
rps4	591	768	474	368	312	5.55
18S	1695	1729	1670	371	225	2.90
Total	4747	5049	4614	2054	1657	2.15

[a] Completely missing sequences (one 18S; two *rps4*) were not included in this calculation.
[b] Number of characters informative for equally weighted parsimony analyses.
[c] Completely missing sequences (one 18S; two *rps4*) were included in this calculation.

ferns were diverse and abundant in the Mesozoic, and our new understanding of the relationships among extant members is important to consider when interpreting the fossil record (Skog, 2001; Klavins et al., 2004).

Gleichenioid/filmy ferns—A sister relationship between gleichenioid and filmy ferns was resolved here regardless of the optimization criterion applied; however, the support for this relationship was strong only in the Bayesian analysis (Fig. 3; PP = 96; BP^{ML} = 55; BP^{MP} = 57). To the best of our knowledge, this sister relationship has never before been proposed, despite both gleichenioid and filmy ferns having sporangia that possess an annulus with an oblique to transverse aspect. Although the interpretation of the annulus aspect in *Cheiropleuria* and *Dipteris* (only slightly oblique) is somewhat ambiguous, it appears reasonable to suggest this as a morphological synapomorphy uniting the gleichenioid + filmy fern clade. At least one interesting evolutionary and ecological implication should be considered if this sister relationship holds true. Many extant gleichenioids are scrambling or subscandent and preferentially grow in open habitats and readily colonize secondary habitats. Most filmy ferns are epiphytes, a trait that may have evolved in this lineage through a transitional step involving scrambling or hemi-epiphytism (Dubuisson et al., 2003b). A scrambling habit as the putative plesiomorphic condition (as suggested by the sister group relationship of filmy and gleichenioid ferns) is consistent with this hypothesis.

Although it remains to be seen if our grouping of gleichenioid and filmy ferns will stand the test of time, it is worth noting that what were once thought to be distantly related and successive grades of taxa at the base of the leptosporangiate ferns, may in fact be closely related members of a single clade that diversified in the Late Paleozoic/Early Mesozoic (Fig. 4). The interpretation of the fossil record needs to be reevaluated, taking these results into account. Several Late Paleozoic/Early Mesozoic fossils assumed to be related to Gleicheniaceae, such as *Szea* and *Oligocarpia*, are more likely to be stem group (Fig. 2) members of the gleichenioid + filmy fern lineage because critical character states displayed by the fossils, such as sporangial type and spore wall ultrastructure (Wang et al., 1998; Yao and Taylor, 1988), are plesiomorphic for this clade.

Schizaeoid ferns—The genera *Lygodium*, *Schizaea*, and *Anemia* (including *Mohria*) have almost always been considered to represent a related group. Although this group of ferns has sometimes been considered to be among the more basal leptosporangiate nodes by some workers, Smith (1995) pointed out that several researchers in the latter part of the last century had suggested, largely on the basis of rather tenuous characters, that the schizaeoid ferns may have been ''a possible point of origin for several higher leptosporangiate families, such as Pteridaceae, . . . and Marsileaceae.'' This was a rather astute observation by these earlier workers, given that we have relatively strong support (PP = 100; BP^{ML} = 83; BP^{MP} = 88) for stating that the schizaeoid ferns are the sister to the core leptosporangiates (Fig. 3).

Core leptosporangiates—This clade includes heterosporous ferns, tree ferns, and polypods, each of which is clearly monophyletic (Fig. 3). The relationships among these three lineages remain equivocal, and their circumscriptions, as presented here, differ considerably from traditional views. In the past, the two groups of heterosporous ferns (*Marsilea*/*Pilularia* and *Salvinia*/*Azolla*) were considered to be independently derived, but a sister group relationship has been confirmed here once again (Rothwell and Stockey, 1994; Hasebe et al., 1995; Pryer et al., 1995, 2001a).

The association of several genera, such as *Plagiogyria*, *Hymenophyllopsis*, *Loxoma*, and *Loxsomopsis*, all lacking distinct trunks, among the tree ferns also conflicts with previously proposed relationships (Smith, 1995). These four genera had been variously aligned with families now seen to be only remotely related: *Hymenophyllopsis*, *Loxoma*, and *Loxsomopsis* with the filmy ferns (Hymenophyllaceae) or dennstaedtioid ferns (Dennstaedtiaceae) and *Plagiogyria* with the osmundaceous ferns (Osmundaceae). Other tree fern genera often treated in monogeneric families, e.g., *Metaxya* (Metaxyaceae) and *Lophosoria* (Lophosoriaceae), are shown here to be closely allied to, or embedded within, dicksonioid tree ferns (Fig. 3). To some fern systematists, one of the biggest surprises of all may be the monophyly of the polypod ferns as circumscribed here (Fig. 3), which was not generally appreciated or accepted in earlier phylogenetic studies (Holttum, 1973; Mickel, 1974; Pichi Sermolli, 1977; see also Smith, 1995), despite the presence of an obvious synapomorphy—the vertical annulus of the sporanagium. Polypods comprise anywhere from 15–30 families (depending on the classification followed) and account for greater than 80% of extant species diversity.

Divergence time estimates and the fossil record—The divergence time estimates resulting from our penalized likelihood analyses (Fig. 4; Table 1) are largely in accord with previous ideas about the times of origin and diversification of major fern clades (Rothwell, 1987; Tidwell and Ash, 1994; Collinson, 1996; Rothwell, 1996; Skog, 2001; P. S. Soltis et al., 2002). However, several clades with sparse fossil records are seen with this approach to have originated much earlier than their meager fossil data would imply.

The earliest diverging of the 11 major lineages of monilophytes also have the oldest fossil records, with the exception

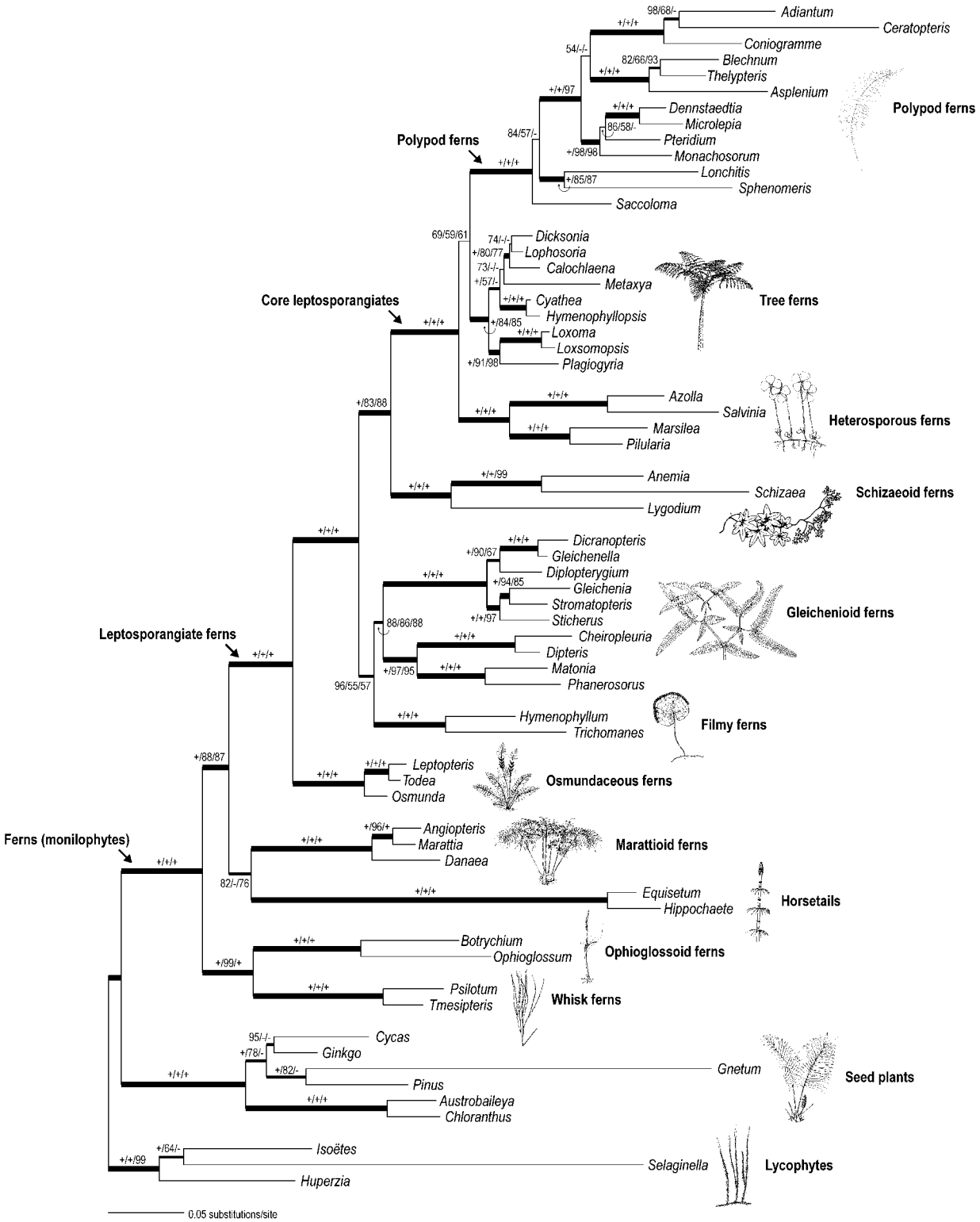

Fig. 3. Phylogenetic relationships at basal nodes of the monilophyte tree. The topology is the result of a Bayesian analysis of four genes (plastid *rbcL*, *atpB*, *rps4*, and nuclear 18S rDNA); average branch lengths are shown. Measures of support are given at the nodes: Bayesian posterior probability/maximum likelihood bootstrap/maximum parsimony bootstrap; support values equal to 100 are abbreviated (+) and support values less than 50 are not reported (−). Moderately thickened lines indicate significant support from one or two measures (posterior probability ≥ 95; maximum likelihood bootstrap ≥ 70; or maximum parsimony bootstrap ≥ 70). Heavily thickened lines (most nodes) indicate significant support from all three measures.

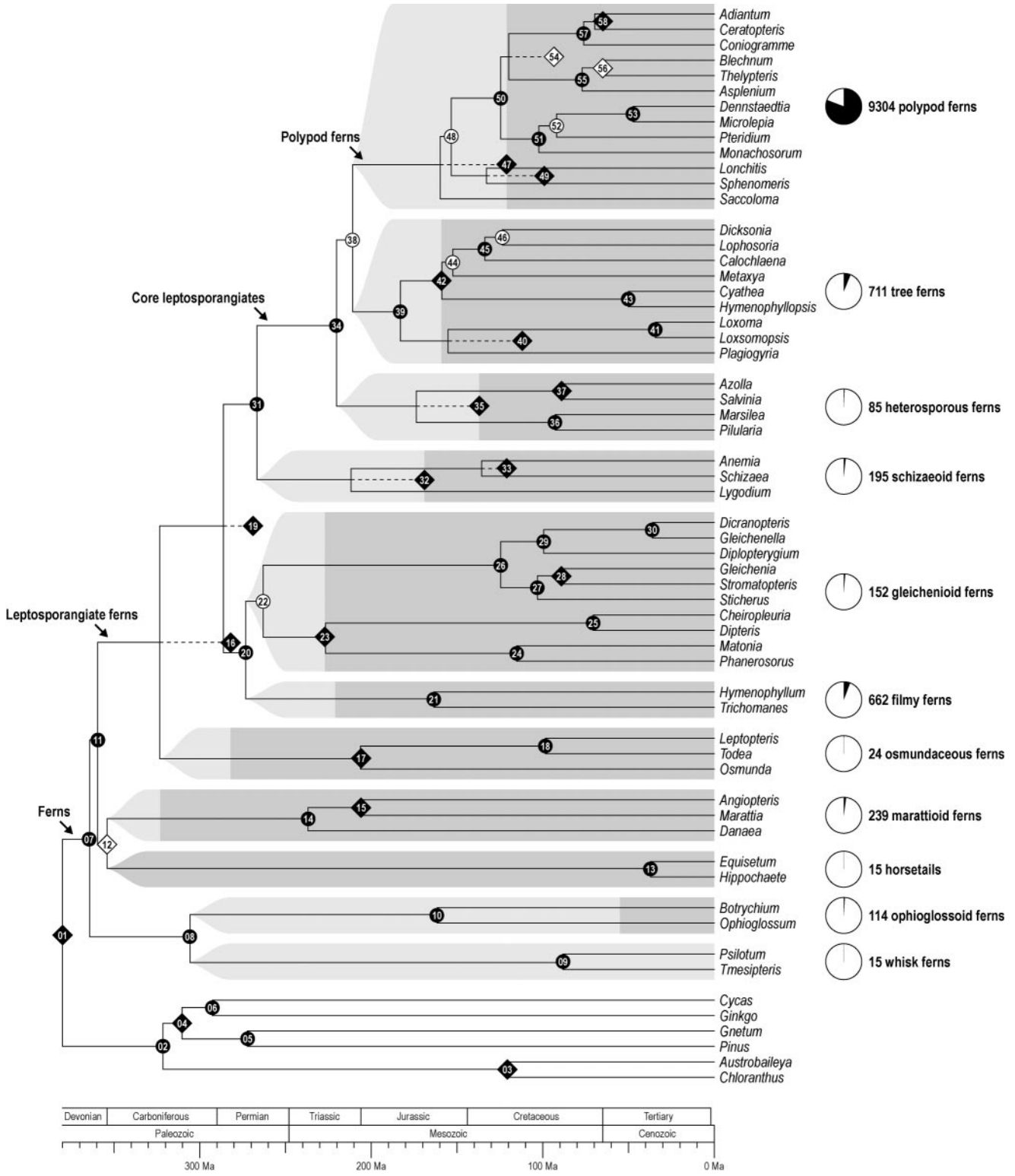

Fig. 4. Phylogenetic chronogram for monilophytes, plotted against the geologic time scale. The topology is the result of a Bayesian analysis of four genes (cf., Fig. 3). Molecular age estimates are indicated by node positions and were estimated by means of a penalized likelihood analysis of the Bayesian consensus tree (with average branch lengths), incorporating 21 fossil constraints (see Table 1). Black symbols indicate nodes with significant support (posterior probability $\geq$ 95%), and white symbols indicate nodes without significant support; circles indicate nodes that were not constrained, whereas diamonds indicate that a fossil constraint was applied at that node (position of the diamond corresponds to the age of the fossil constraint applied). All fossil ages were applied as minimum age constraints, except at node 01, which was fixed at 380 million years ago (mya). Node numbers correspond to those in Table 1, where lineage names, molecular age estimates, molecular age means, and standard deviations (for well-supported nodes), as well as fossil age constraints, are provided. Gray shadowing on branches highlights 11 major monilophyte lineages; the molecular age estimate for a lineage is indicated by the tapered point on the left side, whereas the oldest unambiguous fossil age for the lineage is indicated by the juncture line between dark gray and light gray (note that there is no fossil record for whisk ferns, and the fossil record for horsetails coincides with the molecular age estimate). Pie charts show the relative contributions of the various lineages to total extant monilophyte species diversity (>11 500 species); approximate species numbers (modified from Hassler and Swale, 2004) precede the lineage names.

of the whisk ferns and ophioglossoids (Fig. 4). A fossil record for whisk ferns is lacking, and what is available for ophioglossoids is only Cenozoic in age. We estimate that these two lineages diverged from one another in the Late Carboniferous (~306 mya, node 08, Fig. 4; Table 1), whereas the extant members diversified only in the mid to Late Mesozoic (~88 mya and 162 mya, nodes 09 and 10, Fig. 4; Table 1). One of our remaining challenges will be to identify the Carboniferous ancestors of the whisk fern + ophioglossoid lineage. The remaining eusporangiate monilophyte lineages, namely marattioid ferns and horsetails, have excellent fossil records extending into the Carboniferous (Fig. 4; Table 1). Living horsetails in the genus *Equisetum* appear to have diversified in the early Cenozoic (~38 mya), which is in agreement with Des Marais et al. (2003), whereas the extant lineages of the marattioid ferns date back to the Triassic (~237 mya and 206 mya, nodes 14 and 15, Fig. 4; Table 1).

The fossil record indicates that leptosporangiate ferns first appeared in the earliest Carboniferous (Tournaisian) and soon after diversified to give rise to roughly six independent lineages, all of which have ambiguous relationships to extant lineages. Some of these Carboniferous leptosporangiate ferns have been put forward as putative stem groups (Fig. 2) of extant lineages (Stewart and Rothwell, 1993; Galtier and Phillips, 1996; Rothwell, 1999); for example, Anachoropteridaceae for osmundaceous ferns, Tedeleaceae for schizaeoid ferns, and Sermyaceae for gleichenioid ferns (Collinson, 1996; but see Rothwell, 1999). Further studies are needed to elucidate relationships among Carboniferous and extant leptosporangiate ferns. Our divergence time estimates are consistent with the hypothesis of a major replacement of the Carboniferous leptosporangiate ferns with new (extant) lineages at the end of the Paleozoic (Rothwell, 1987; Stewart and Rothwell, 1993; Rothwell, 1996).

According to our estimates, the osmundaceous ferns, sister group to all other leptosporangiate ferns, arose in the Late Carboniferous (~323 mya, node 16, Fig. 4; Table 1). The oldest fossils unequivocally assignable to this lineage are from the Permian. The gleichenioid + filmy fern total lineage (node 20) and the schizaeoid fern + core leptosporangiates total lineage (node 31) are estimated to have originated in the Permian (~273 mya and ~266 mya, respectively); the oldest fossils assignable to extant families of these lineages are from the Triassic and Jurassic, respectively (Fig. 4, Table 1; Collinson, 1996; Skog, 2001).

The major lineages within the ''core leptosporangiates'' (node 34, Fig. 4)—heterosporous ferns, tree ferns, and polypod ferns—are shown here to have diverged in the Triassic (~220 mya and 211 mya), even though the oldest fossils unequivocally assignable to one of these lineages only date from the Middle Jurassic. There are older fossils that have been assigned to the tree fern lineage, especially Dicksoniaceae (Stewart and Rothwell, 1993; Skog, 2001), but these fossils are more likely to be stem group (Fig. 2) members of the core leptosporangiates. Therefore, these earlier assignments need reevaluation in the light of our improved understanding of the evolution of leptosporangiate ferns. The two living families of heterosporous ferns are estimated here to have diverged in the Middle Jurassic (~173 mya, node 35, Fig. 4), although the oldest fossil we can attribute to them is from the Early Cretaceous (Table 1). The tree fern and polypod lineages (nodes 39 and 47, respectively, Fig. 4, Table 1) also began to diversify in the Jurassic (~183 mya and ~160 mya). The most species-rich groups of living polypods, the pteridoid ferns and the eupolypods (nodes 57 and 55, respectively, Fig. 4, Table 1), are shown here to have diversified in the Late Cretaceous, which is consistent with the dates of divergence provided by Schneider et al. (2004b). This observation of a more recent fern diversification in the Late Mesozoic/Early Cenozoic, coinciding with the radiation of angiosperms, is echoed in Gleicheniaceae (node 26, Fig. 4, Table 1) and suggests that ferns may have experienced successive replacement events, rather than an absolute decline, after the appearance of angiosperms (Schneider et al., 2004b). Further evidence is needed to explore the implications of these findings, especially for those lineages for which we have limited available phylogenetic data.

CONCLUSIONS AND PROSPECTS

All generally recognized extant fern families and nearly all monilophyte genera at the early-diverging nodes have now been sampled in published molecular phylogenetic studies, with few exceptions. Although our study has an extensive sampling from the major basal fern nodes, the sampling is still inadequate with respect to the diversity within these groups. Some of these lineages have received some attention in this regard; e.g., Ophioglossaceae (Hauk et al., 2003), Equisetaceae (Des Marais et al., 2003), Osmundaceae (Yatabe et al., 1999), filmy ferns (Dubuisson, 1997; Pryer et al., 2001b; Dubuisson et al., 2003a; Hennequin et al., 2003), Cheiropleuriaceae (Kato et al., 2001), Matoniaceae (Kato and Setoguchi, 1998), and Schizaeaceae (Skog et al., 2002; Wikström et al., 2002). Many other important groups, such as Gleicheniaceae and tree ferns, are critically in need of further study. The polypods, the most diverse fern lineage, are especially in need of additional attention, although several ongoing studies promise much progress in the near future. Furthermore, better resolution of relationships among and within certain clades, is still possible with additional molecular data, including data on genome structure (Pryer et al., 2002). Better estimates of divergence times will likely come with new fossil findings, and improved interpretations of the fossil record will occur in light of additional evidence from gene sequencing.

Our study is one of several that provide a foundation for future family and genus level taxonomic studies in ferns, which will, in turn, allow for improved biogeographical, ecological, and evolutionary interpretations. Reinterpretation of morphological data and developmental information can now be attempted on a sounder footing, especially as they pertain to deep branches in the vascular plant tree. Using the best estimate of phylogeny available at the time (Pryer et al., 2001a), Schneider et al. (2002) were able to provide several critical insights into morphological evolution within ferns. The increased taxonomic sampling presented here will allow us to reconstruct the evolution of other critical characters (e.g., sporangium structure) and to determine their implications for the biology and systematics of ferns. Another promising field will be the reconstruction of the evolution of genome size in ferns and the correlation of size, if any, with the variation in chromosome number, or other features, exhibited by ferns. We can now begin to ask what key events might have led to the many large, species-rich radiations in the long history of fern life on Earth.

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