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Reconciling Extreme Branch Length Differences: Decoupling Time and Rate through the Evolutionary History of Filmy Ferns

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Abstract.—The rate of molecular evolution is not constant across the Tree of Life. Characterizing rate discrepancies and evaluating the relative roles of time and rate along branches through the past are both critical to a full understanding of evolutionary history. In this study, we explore the interactions of time and rate in filmy ferns (Hymenophyllaceae), a lineage with extreme branch length differences between the two major clades. We test for the presence of significant rate discrepancies within and between these clades, and we separate time and rate across the filmy fern phylogeny to simultaneously yield an evolutionary time scale of filmy fern diversification and reconstructions of ancestral rates of molecular evolution. Our results indicate that the branch length disparity observed between the major lineages of filmy ferns is indeed due to a significant difference in molecular evolutionary rate. The estimation of divergence times reveals that the timing of crown group diversification was not concurrent for the two lineages, and the reconstruction of ancestral rates of molecular evolution points to a substantial rate deceleration in one of the clades. Further analysis suggests that this may be due to a genome-wide deceleration in the rate of nucleotide substitution. [Bayesian analysis; divergence time estimates; molecular clock; molecular rate heterogeneity; monilophyte phylogeny; penalized likelihood; *rbcL*.]

Phylogenetic branch length, as estimated from DNA sequence data, is a function of both the rate of nucleotide substitution and time. Length differences among *individual* branches within a phylogeny can therefore result from discrepancies in substitution rate, time, or a combination of these factors. All of life's extant diversity, however, ultimately shares a common ancestor, and thus a common age. Any significant differences among *cumulative* evolutionary path lengths, from the root of the Tree of Life to the many extant species, must purely be the result of net rate discrepancies. This holds for any phylogenetic tree, as the sampled extant taxa will always share a common root.

Phylogenetic trees with a combination of long and short cumulative evolutionary paths are common, and the phenomenon of unequal net rates of nucleotide substitution among lineages is widespread and well recognized (Britten, 1986; Bromham and Penny, 2003; Gaut et al., 1993; Langley and Fitch, 1974; Wolfe et al., 1987). Significant deviations from the constant rate of a molecular clock have been identified across the Tree of Life (*vertebrates*: Adachi et al., 1993; Bleiweiss, 1998; Bromham, 2002; Bulmer et al., 1991; Cantatore et al., 1994; Hoegg et al., 2004; Krieger and Fuerst, 2002; Li et al., 1987, 1990; Martin and Palumbi, 1993; Martin et al., 1992; Mooers and Harvey, 1994; Springer and Kirsch, 1989; Wu and Li, 1985; *invertebrates*: Castro et al., 2002; Hebert et al., 2002; Moran et al., 1995; Schön et al., 2003; *seed plants*: Bousquet et al., 1992, Gaut et al., 1992; Nickrent and Starr, 1994; *ferns*: Des Marais et al., 2003; Schneider et al., 2004b; *liverworts*: Lewis et al., 1997; *fungi*: Lutzoni and Pagel, 1997; Zoller and Lutzoni, 2003; *algae*: Zoller and Lutzoni, 2003; *bacteria*: Moran et al., 1995).

An analysis of net rate differences, although capable of providing an informative summary, cannot possibly reveal all the intricacies involved in the evolution of the rate of evolution. Any given phylogenetic path from root to tip comprises many individual branch segments, with rate and time together determining the length of each. Time intervals between successive divergences can vary along a path, as can rates of molecular evolution, and early changes in rate in one direction (either an acceleration or a deceleration) can be masked by subsequent changes in the other direction. Characterizing the net amount and direction of change in the rate of molecular evolution is important in understanding the evolutionary history of a lineage, but equally important is an evaluation of the interaction of rate and time on individual branches through the past.

Within filmy ferns (Hymenophyllaceae), phylogenetic analyses of plastid *rbcL* sequences have revealed considerable cumulative path length differences (Pryer et al., 2001b), but the significance of these differences has not yet been addressed nor the factors responsible identified. Filmy ferns compose one of the earliest diverging families of leptosporangiate ferns (Hasebe et al., 1995; Pryer et al., 2001a, 2004; Schneider et al., 2004a). The more than 600 described species all have extremely thin leaves, with blades generally only a single cell thick, that bear unique marginal sori—reproductive structures consisting of a short to elongate sporangia-bearing receptacle subtended by a protective indusium (Iwatsuki, 1990). The species otherwise exhibit considerable levels of both morphological and ecological diversity (Dubuisson, 1996, 2003b). Recent molecular phylogenetic studies have resolved two major lineages of filmy ferns, largely corresponding to the two traditionally recognized genera—*Trichomanes* and *Hymenophyllum* (Pryer et al., 2001b). The primary and most consistent morphological differences between these groups are related to the morphology of the sori, but other less generalized differences also exist. The *Trichomanes* clade is generally characterized by having sori with campanulate (bell-shaped) indusia and exserted receptacles, tends to inhabit lower latitudes and lower altitudes, and comprises terrestrial, climbing, and epiphytic species. The *Hymenophyllum* clade usually has bivalved indusia and included receptacles, tends to be

more successful at higher latitudes and altitudes, and is composed mostly of epiphytic species, many of which are present high in the forest canopy. The aforementioned path length discrepancies are also manifested between the two major filmy fern clades, with the *Trichomanes* clade comprising species with relatively long path lengths and the *Hymenophyllum* clade comprising species with relatively short path lengths (Pryer et al., 2001b).

The major lineages of filmy ferns, as sister taxa, share a common age. Therefore, the path length differences observed in analyses of *rbcL* data, if significant, must ultimately be the result of net rate change at this locus in one or both of the filmy fern groups. Relative to the ancestral rate of molecular evolution there was either (1) a net acceleration in the *Trichomanes* lineage, (2) a net deceleration in the *Hymenophyllum* lineage, or (3) both a net acceleration in *Trichomanes* and a net deceleration in *Hymenophyllum*. However, a consideration of both time and rate through the evolutionary history of filmy ferns—rather than simply net changes in rate—yields countless plausible evolutionary scenarios, each of which has unique implications.

In this study, we explore the roles of time and rate in the evolutionary history of filmy ferns. We evaluate the significance and nature of rate differences at the *rbcL* locus and we separate time and rate across the filmy fern phylogeny to yield an evolutionary time scale of filmy fern diversification, as well as reconstructions of ancestral rates of molecular evolution.

METHODS

Taxonomic Sampling and Sequence Alignment

Fifty species were selected from the Hymenophyllaceae—25 species each from *Trichomanes* and *Hymenophyllum*—representing all of the major filmy fern lineages (Dubuisson et al., 2003a; Hennequin et al., 2003). To place this family within a broader context, 60 other vascular plant species were selected: 42 additional ferns from across the leptosporangiate phylogeny, nine species representing the four major eusporangiate fern lineages (Pryer et al., 2004), six seed plants, and three lycophytes (outgroup). DNA sequences of the plastid *rbcL* gene were obtained for each included species from GenBank (for voucher information and GenBank accession numbers, see Table 1) and aligned manually using MacClade 4.06 (Maddison and Maddison, 2000). The 5′ and 3′ ends of the resulting alignment that contained copious amounts of missing data were cropped, yielding a data matrix of 1206 base pairs (402 codons) for 110 species with no missing data (only 13 ambiguities were present within the matrix). The resulting alignment is available in TreeBASE (http://www.treebase.org; study accession number S1449).

Phylogenetic Analyses

The *rbcL* data were analyzed using a Bayesian Markov chain Monte Carlo (B/MCMC) approach, as implemented in MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003), to simultaneously yield a distribution of trees, a consensus phylogenetic hypothesis, and support values for resolved nodes. Four independent B/MCMC analyses were conducted using the model of sequence evolution most applicable to the data (GTR+Γ+I, as selected using ModelTest 3.06; Posada and Crandall, 1998), flat priors, and four chains. Chains were run for 10 million generations, and trees were sampled every 1000 generations. Following completion of each analysis, we plotted the output parameter estimates through time using Tracer 1.3 (Rambaut and Drummond, 2005) in order to recognize the point of convergence to the stationary distribution. All generations prior to this convergence (1,000,000 generations, 1000 trees, for each of the four analyses) were discarded, conservatively, as the "burn-in" phase. Through the superimposition of the parameter plots from the four analyses and a comparison of the trees resulting from these analyses, we confirmed that all four independent runs had converged to the same stationary distribution. Therefore, we pooled the post burn-in trees from each analysis (36,000 total trees), and computed a plurality consensus (using the command: sumt contype = allcompat) to obtain a fully resolved topology with average branch lengths, as well as posterior probability estimates for all nodes.

Significance Tests for the Presence of Rate Differences

To determine whether observed branch length differences were the result of a significant departure from rate constancy (i.e., a molecular clock), two models were compared. In the simpler (null) model, a molecular clock was applied such that the rates of molecular evolution for each of the branches were constrained to be equal. In the more complex (alternative) model, each branch was allowed its own unique rate of molecular evolution. These two models were contrasted across the entire Bayesian consensus tree, as well as across several partitions pruned from this tree: filmy ferns plus their resolved sister group, filmy ferns, *Hymenophyllum*, and *Trichomanes*. For each comparison, likelihoods were calculated using the program Baseml (part of the PAML 3.14b package; Yang, 1997) with the appropriate models of sequence evolution (GTR+Γ; either with or without a molecular clock constraint). The resulting likelihoods were compared using the likelihood ratio test statistic (Felsenstein, 1981).

In addition to these tests for the presence of molecular clocks, several tests were performed to determine whether significant differences in rate were present between (as opposed to within) partitions. For these analyses, the pruned tree comprising filmy ferns plus their resolved sister group was utilized and comparisons were again made between simple (null) and more complex (alternative) models. Three comparisons were made: (1) a two-rate model in which *Hymenophyllum* and *Trichomanes* had the same rate but the resolved filmy fern sister group had a different rate *versus* a three-rate model in which all three included partitions (*Hymenophyllum*, *Trichomanes*, and the resolved filmy fern sister group) had different rates; (2) a two-rate model in which the filmy fern sister

TABLE 1. Estimated divergence times and ancestral rates for all nodes resolved in this study of filmy ferns.

Internal node or terminal species[a]	N[b]	Constraint[c] (Ma)	Dates (Ma)[d]			Rates (substitutions/site/Ma)[e]			Constraint or *rbcL* sequence reference[f]
			Consensus	Mean	SD	Consensus	Mean	SD	
001 (euphyllophytes)	100	380.00	380.00	380.00	0.00				Pryer et al., 2004 (Node 01)
002 (SPE, spermatophytes)	100	310.00	310.00	310.12	0.80	0.00057741	0.00056833	0.00007235	Pryer et al., 2004 (Node 04)
003 (angiosperms)	100	121.00	121.00	121.00	0.00	0.00045885	0.00044914	0.00004419	Pryer et al., 2004 (Node 03)
004 (gymnosperms)	95		275.29	273.66	13.77	0.00039014	0.00039174	0.00004432	
005	86		254.98	254.22	16.41	0.00036231	0.00035918	0.00003517	
006	100		205.87	209.53	18.68	0.00051061	0.00050395	0.00005566	
007 (MON, monilophytes)	100	354.00	358.31	359.88	4.21	0.00072298	0.00069995	0.00008488	Pryer et al., 2004 (Node 12)
008	100		294.87	292.32	19.79	0.00060048	0.00059512	0.00005371	
009 (WHI, whisk ferns)	100		105.93	105.41	17.34	0.00045635	0.00046061	0.00005823	
010 (OPH, ophioglossoid ferns)	100		129.15	129.29	17.10	0.00063323	0.00063193	0.00006948	
011	54		352.33	353.57	4.50	0.00069601	0.00067737	0.00008888	
012 (MAR, marattioid ferns)	100		225.87	225.75	7.52	0.00056424	0.00054816	0.00005723	
013	100	206.00	206.00	206.00	0.00	0.00030430	0.00030323	0.00003592	Pryer et al., 2004 (Node 15)
014	96		337.92	339.45	7.37	0.00074659	0.00072330	0.00008886	
015 (HOR, horsetail ferns)	100		33.23	33.90	5.52	0.00081497	0.00078911	0.00010312	
016 (LEP, leptosporangiate ferns)	92		326.50	330.04	7.36	0.00063655	0.00062637	0.00006773	
017 (OSM, osmundaceous ferns)	100	206.00	206.00	206.00	0.00	0.00039357	0.00039086	0.00004473	Pryer et al., 2004 (Node 17)
018	100		56.42	57.84	14.05	0.00026591	0.00027347	0.00005089	
019	100	269.00	293.18	298.87	8.91	0.00068724	0.00068741	0.00008128	Pryer et al., 2004 (Node 19)
020	76		278.00	284.46	9.11	0.00065623	0.00066385	0.00007125	
021	100		142.99	144.82	11.43	0.00051567	0.00049255	0.00004863	
022	100		61.78	64.08	15.50	0.00037561	0.00036152	0.00006437	
023	98		121.59	123.00	8.27	0.00047495	0.00046097	0.00004822	
024	100	89.00	89.00	89.22	0.97	0.00049320	0.00047959	0.00004641	Pryer et al., 2004 (Node 28)
025	82		75.34	75.09	6.36	0.00046113	0.00045711	0.00004694	
026	35		272.74	280.00	8.24	0.00069250	0.00065786	0.00005412	
027	91		239.61	244.79	13.32	0.00057124	0.00053175	0.00004960	
028	100		129.31	135.21	18.24	0.00054589	0.00051422	0.00006339	
029	100		105.92	112.38	14.82	0.00048633	0.00045975	0.00005279	
030	100		249.93	260.75	12.79	0.00082715	0.00079590	0.00008039	
031 (SCH, schizaeoid ferns)	100	169.00	184.86	196.50	14.08	0.00095452	0.00090040	0.00010403	Pryer et al., 2004 (Node 32)
032	100	121.00	121.00	129.31	11.07	0.00106690	0.00100283	0.00012607	Pryer et al., 2004 (Node 33)
033 (COR, core leptosporangiates)	100		220.48	228.14	10.55	0.00074613	0.00071640	0.00006434	
034 (TRE, tree ferns)	100		190.83	195.99	10.72	0.00053555	0.00050451	0.00004370	
035	63		168.86	170.93	15.41	0.00039653	0.00039020	0.00004127	
036	100		47.99	49.37	12.43	0.00031553	0.00030548	0.00006235	
037	100	159.00	159.00	160.83	5.37	0.00043288	0.00039435	0.00004501	Pryer et al., 2004 (Node 42)
038	75		144.49	147.08	9.51	0.00041512	0.00038822	0.00003413	
039	100		35.72	41.49	15.86	0.00029338	0.00027207	0.00005343	
040	100		134.29	129.66	18.60	0.00028933	0.00025905	0.00005212	
041	58		118.36	112.11	22.30	0.00016915	0.00018327	0.00005428	
042	96		207.18	213.91	11.66	0.00077662	0.00074179	0.00006787	
043 (HET, heterosporous ferns)	100	137.00	161.22	166.56	11.46	0.00079688	0.00075709	0.00006682	Pryer et al., 2004 (Node 35)
044	100		71.15	74.31	11.16	0.00073129	0.00069174	0.00007109	
045	100	89.00	89.00	89.10	0.74	0.00083167	0.00079282	0.00007240	Pryer et al., 2004 (Node 37)
046 (POL, polypod ferns)	100	121.00	162.03	168.87	12.38	0.00075347	0.00071917	0.00007462	Pryer et al., 2004 (Node 47)
047	99		150.77	157.67	12.35	0.00084348	0.00079893	0.00008604	
048	63		136.10	141.12	15.58	0.00088809	0.00082413	0.00009183	
049	100	93.50	130.76	136.33	12.45	0.00086627	0.00084523	0.00010115	Pryer et al., 2004 (Node 54)
050	99		113.64	116.12	12.09	0.00071215	0.00069055	0.00008742	
051	63		104.96	107.56	11.80	0.00065195	0.00062252	0.00008392	
052	100		56.34	57.55	10.08	0.00063612	0.00061725	0.00009825	
053	81		124.24	129.33	12.56	0.00101100	0.00096134	0.00010961	

(Continued on next page)

TABLE 1. Estimated divergence times and ancestral rates for all nodes resolved in this study of filmy ferns. *(Continued)*

Internal node or terminal species[a]	N[b]	Constraint[c] (Ma)	Dates (Ma)[d]			Rates (substitutions/site/Ma)[e]			Constraint or *rbcL* sequence reference[f]
			Consensus	Mean	SD	Consensus	Mean	SD	
054 (eupolypod ferns)	100	65.00	82.51	85.12	10.57	0.00101780	0.00093481	0.00012100	Pryer et al., 2004 (Node 56)
055	66		63.97	66.84	9.19	0.00092115	0.00087342	0.00012529	
056 (pteridoid ferns)	100	65.00	86.29	92.15	11.92	0.00113530	0.00107929	0.00014082	Pryer et al., 2004 (Node 58)
057	74		79.46	83.61	11.54	0.00133610	0.00125516	0.00017972	
058 (FIL, filmy ferns)	100		202.54	210.22	20.20	0.00057692	0.00057302	0.00005140	
059 (HYM, *Hymenophyllum* clade)	100		105.06	116.36	27.23	0.00036888	0.00036521	0.00005376	
060	39		97.72	109.36	29.93	0.00025867	0.00026853	0.00008901	
061	89		85.60	100.74	25.43	0.00022521	0.00024973	0.00008468	
062	85		79.69	95.92	24.55	0.00026825	0.00026462	0.00007900	
063	86		72.83	88.49	22.92	0.00029356	0.00027545	0.00008515	
064	100		55.36	68.76	19.64	0.00029390	0.00026470	0.00009289	
065	100		23.42	29.42	9.42	0.00035830	0.00031420	0.00008992	
066	100		53.47	62.51	17.46	0.00031184	0.00028952	0.00009066	
067	100		28.53	32.60	9.61	0.00035744	0.00033073	0.00008957	
068	91		70.35	88.44	24.19	0.00027205	0.00025081	0.00008312	
069	93		53.93	68.01	20.39	0.00025988	0.00023450	0.00008822	
070	100		22.13	27.50	11.94	0.00027126	0.00023610	0.00009591	
071	87		63.57	80.95	22.05	0.00027618	0.00023986	0.00008076	
072	91		47.71	54.89	19.32	0.00026055	0.00023734	0.00009796	
073	96		33.48	41.26	15.54	0.00029522	0.00026897	0.00009979	
074	48		56.96	74.96	24.06	0.00028670	0.00025035	0.00010214	
075	100		43.09	53.98	18.95	0.00027123	0.00024171	0.00009897	
076	88		31.39	39.68	15.57	0.00027215	0.00023999	0.00010500	
077	98		27.45	34.59	14.35	0.00024899	0.00022552	0.00010799	
078	100		19.69	24.68	11.04	0.00029698	0.00026073	0.00010451	
079	17		52.44	77.71	22.07	0.00030410	0.00021123	0.00006841	
080	100		23.56	34.07	13.55	0.00031893	0.00025326	0.00009569	
081	51		45.11	64.74	21.40	0.00030284	0.00023381	0.00009852	
082	68		35.94	54.63	20.10	0.00030848	0.00021907	0.00009547	
083 (TRI, *Trichomanes* clade)	100		168.35	177.02	19.29	0.00054990	0.00054901	0.00004974	
084	89		142.71	146.26	19.17	0.00050922	0.00048701	0.00006324	
085	49		161.43	171.57	17.35	0.00051777	0.00051615	0.00006077	
086	100		108.09	111.65	21.37	0.00042011	0.00039930	0.00006557	
087	78		86.89	92.31	20.00	0.00037490	0.00036182	0.00007687	
088	59		151.33	164.27	19.34	0.00057211	0.00055299	0.00006679	
089	100		114.49	123.75	16.80	0.00055184	0.00051580	0.00006797	
090	91		101.06	108.22	15.85	0.00057358	0.00053725	0.00007146	
091	100		42.42	45.81	8.80	0.00059771	0.00055968	0.00008030	
092	100		63.75	68.32	11.15	0.00050683	0.00047416	0.00007799	
093	98		52.89	56.71	9.35	0.00053844	0.00049584	0.00008475	
094	48		48.71	53.25	9.50	0.00055211	0.00052187	0.00008941	
095	73		41.78	45.54	9.21	0.00053140	0.00049345	0.00009780	
096	100		133.15	146.90	17.44	0.00062852	0.00059294	0.00007089	
097	100		54.11	60.24	13.71	0.00054186	0.00050461	0.00008426	
098	65		41.30	44.40	9.22	0.00055344	0.00051772	0.00008566	
099	90		126.90	140.70	16.62	0.00074305	0.00068472	0.00008709	
100	100		92.02	102.61	13.21	0.00088452	0.00080775	0.00010178	
101	100		59.23	66.23	9.41	0.00086672	0.00079975	0.00011051	
102	57		54.60	58.75	8.34	0.00094028	0.00086409	0.00012230	
103	97		115.37	128.29	15.46	0.00070501	0.00063512	0.00009696	
104	100		28.67	32.45	8.45	0.00057189	0.00050491	0.00011352	
105	100		91.30	102.47	13.80	0.00079913	0.00072306	0.00010398	
106	100		55.27	61.65	10.44	0.00082219	0.00074095	0.00010938	
Chloranthus japonicus Siebold	100	0.00	0.00	0.00	0.00	0.00032613	0.00032049	0.00005449	Qiu et al., 1993 (L12640)

TABLE 1. Estimated divergence times and ancestral rates for all nodes resolved in this study of filmy ferns. *(Continued)*

Internal node or terminal species[a]	N[b]	Constraint[c] (Ma)	Dates (Ma)[d]			Rates (substitutions/site/Ma)[e]			Constraint or *rbcL* sequence reference[f]
			Consensus	Mean	SD	Consensus	Mean	SD	
Austrobaileya scandens C. T. White	100	0.00	0.00	0.00	0.00	0.00035004	0.00035106	0.00005016	Qiu et al., 1993 (L12632)
Cycas circinalis L.	100	0.00	0.00	0.00	0.00	0.00018075	0.00018767	0.00005301	Chase et al., 1993 (L12674)
Ginkgo biloba L.	100	0.00	0.00	0.00	0.00	0.00014701	0.00015553	0.00005799	Hasebe et al., 1992 (D10733)
Pinus radiata D. Don	100	0.00	0.00	0.00	0.00	0.00028854	0.00029946	0.00006114	Bousquet et al., 1992 (X58134)
Gnetum gnemon L.	100	0.00	0.00	0.00	0.00	0.00080182	0.00076932	0.00010401	Price, 1996 (U72819)
Psilotum nudum (L.) P. Beauv.	100	0.00	0.00	0.00	0.00	0.00042740	0.00043129	0.00007103	Manhart, 1994 (L11059)
Tmesipteris oblanceolata Copel.	100	0.00	0.00	0.00	0.00	0.00043148	0.00043513	0.00007449	Hasebe et al., 1995 (U30836)
Botrychium biternatum (Sav.) Underw.	100	0.00	0.00	0.00	0.00	0.00055563	0.00055956	0.00006969	Manhart, 1994 (L13474)
Ophioglossum reticulatum L.	100	0.00	0.00	0.00	0.00	0.00071404	0.00070787	0.00009286	Pryer et al., 2001a (AF313582)
Danaea elliptica Sm.	100	0.00	0.00	0.00	0.00	0.00035151	0.00034303	0.00005522	Pryer et al., 2001a (AF313578)
Angiopteris lygodiifolia Rosenst.	100	0.00	0.00	0.00	0.00	0.00012269	0.00013370	0.00005704	Yoshinaga et al., 1992 (X58429)
Marattia attenuata Labill.	100	0.00	0.00	0.00	0.00	0.00010501	0.00012000	0.00006016	Pryer et al., 2001a (AF313581)
Equisetum x ferrissii Clute	100	0.00	0.00	0.00	0.00	0.00088661	0.00085634	0.00011419	Pryer et al., 2001a (AF313579)
Equisetum telmateia Ehrh.	100	0.00	0.00	0.00	0.00	0.00073780	0.00070686	0.00012481	Pryer et al., 2001a (AF313580)
Osmunda cinnamomea L.	100	0.00	0.00	0.00	0.00	0.00008778	0.00010599	0.00007757	Hasebe et al., 1993 (D14882)
Leptopteris wilkesiana (Brack.) H. Christ	100	0.00	0.00	0.00	0.00	0.00025828	0.00026487	0.00005737	Pryer et al., 2004 (AY612678)
Todea barbara (L.) T. Moore	100	0.00	0.00	0.00	0.00	0.00023597	0.00024183	0.00006679	Pryer et al., 2004 (AY612686)
Dicranopteris linearis (Burm. f.) Underw.	100	0.00	0.00	0.00	0.00	0.00040181	0.00037703	0.00007386	Wolf, 1995 (U18626)
Gleichenella pectinata (Willd.) Ching	100	0.00	0.00	0.00	0.00	0.00027145	0.00026877	0.00009396	Pryer et al., 2004 (AY612677)
Diplopterygium glaucum (Houtt.) Nakai	100	0.00	0.00	0.00	0.00	0.00039611	0.00039202	0.00006975	Hasebe et al., 1994 (U05624)
Sticherus palmatus (Underw.) Copel.	100	0.00	0.00	0.00	0.00	0.00049742	0.00048323	0.00005767	Pryer et al., 2004 (AY612684)
Stromatopteris moniliformis Mett.	100	0.00	0.00	0.00	0.00	0.00042426	0.00042479	0.00005933	Pryer et al., 2004 (AY612685)
Gleichenia dicarpa R. Br.	100	0.00	0.00	0.00	0.00	0.00047036	0.00046983	0.00005959	Pryer et al., 2001a (AF313584)
Phanerosorus sarmentosus (Baker) Copel.	100	0.00	0.00	0.00	0.00	0.00050928	0.00047730	0.00008175	Pryer et al., 2001a (AF313583)
Matonia pectinata R. Br.	100	0.00	0.00	0.00	0.00	0.00056729	0.00053415	0.00007771	Hasebe et al., 1994 (U05634)
Dipteris conjugata Reinw.	100	0.00	0.00	0.00	0.00	0.00040430	0.00038207	0.00006991	Hasebe et al., 1994 (U05620)
Cheiropleuria integrifolia (D. C. Eaton ex Hook.) M. Kato et al.	100	0.00	0.00	0.00	0.00	0.00052166	0.00048987	0.00006430	Hasebe et al., 1994 (U05607)
Lygodium japonicum (Thunb.) Sw.	100	0.00	0.00	0.00	0.00	0.00092235	0.00085675	0.00011211	Manhart, 1994 (L13479)
Schizaea dichotoma (L.) Sm.	100	0.00	0.00	0.00	0.00	0.00117030	0.00109744	0.00014825	Pryer et al., 2004 (AY612683)
Anemia mexicana Klotzsch	100	0.00	0.00	0.00	0.00	0.00102480	0.00095625	0.00013598	Hasebe et al., 1994 (U05603)
Plagiogyria japonica Nakai	100	0.00	0.00	0.00	0.00	0.00035158	0.00034892	0.00006013	Hasebe et al., 1994 (U05643)
Loxoma cunninghamii R. Br.	100	0.00	0.00	0.00	0.00	0.00027758	0.00026519	0.00007614	Pryer et al., 2004 (AY612679)
Loxsomopsis pearcei (Baker) Maxon	100	0.00	0.00	0.00	0.00	0.00032690	0.00031350	0.00006963	Pryer et al., 2004 (AY612680)
Metaxya rostrata (Kunth) C. Presl	100	0.00	0.00	0.00	0.00	0.00051300	0.00048793	0.00004999	Smith et al., 2001 (AF317699)
Cyathea poeppigii (Hook.) Domin	100	0.00	0.00	0.00	0.00	0.00019751	0.00016587	0.00010081	Pryer et al., 2001a (AF313585)
Hymenophyllopsis dejecta (Baker) Goebel	100	0.00	0.00	0.00	0.00	0.00033179	0.00030320	0.00005359	Wolf et al., 1999 (AF101301)
Calochlaena dubia (R. Br.) M. D. Turner & R. A. White	100	0.00	0.00	0.00	0.00	0.00027335	0.00026618	0.00005252	Hasebe et al., 1994 (U05615)
Lophosoria quadripinnata (J. F. Gmel.) C. Chr.	100	0.00	0.00	0.00	0.00	0.00005481	0.00007627	0.00008026	Wolf et al., 1999 (AF101303)
Dicksonia antarctica Labill.	100	0.00	0.00	0.00	0.00	0.00016840	0.00018356	0.00006610	Wolf et al., 1994 (U05919)
Marsilea quadrifolia L.	100	0.00	0.00	0.00	0.00	0.00074533	0.00069970	0.00008115	Manhart, 1994 (L13480)
Pilularia globulifera L.	100	0.00	0.00	0.00	0.00	0.00068663	0.00065097	0.00008281	Pryer et al., 2004 (AY612681)
Azolla caroliniana Willd.	100	0.00	0.00	0.00	0.00	0.00075435	0.00073032	0.00007319	Hasebe et al., 1995 (U24185)
Salvinia cucullata Roxb. ex Bory	100	0.00	0.00	0.00	0.00	0.00084227	0.00079879	0.00008355	Hasebe et al., 1994 (U05649)
Saccoloma inaequale (Kunze) Mett.	100	0.00	0.00	0.00	0.00	0.00057658	0.00054992	0.00008820	Pryer et al., 2004 (AY612682)
Lonchitis hirsuta L.	100	0.00	0.00	0.00	0.00	0.00085721	0.00078491	0.00009348	Wolf et al., 1994 (U05929)
Sphenomeris chinensis (L.) Maxon	100	0.00	0.00	0.00	0.00	0.00095859	0.00089639	0.00010789	Wolf, 1995 (U05934)
Pteridium aquilinum (L.) Kuhn	100	0.00	0.00	0.00	0.00	0.00060736	0.00060221	0.00009754	Wolf, 1995 (U05939)
Monachosorum henryi H. Christ	100	0.00	0.00	0.00	0.00	0.00064069	0.00062204	0.00009425	Wolf et al., 1994 (U05932)
Microlepia platyphylla (D. Don) J. Sm.	100	0.00	0.00	0.00	0.00	0.00062723	0.00061180	0.00011166	Wolf, 1995 (U18642)
Dennstaedtia punctilobula (Michx.) T. Moore	100	0.00	0.00	0.00	0.00	0.00060656	0.00059202	0.00010383	Wolf et al., 1994 (U05918)
Asplenium nidus L.	100	0.00	0.00	0.00	0.00	0.00111170	0.00105643	0.00012828	Wolf et al., 1994 (U05907)
Blechnum occidentale L.	100	0.00	0.00	0.00	0.00	0.00090029	0.00086047	0.00012816	Wolf et al., 1994 (U05909)
Thelypteris palustris Schott	100	0.00	0.00	0.00	0.00	0.00086625	0.00081078	0.00012731	Wolf et al., 1994 (U05947)
Coniogramme japonica (Thunb.) Diels	100	0.00	0.00	0.00	0.00	0.00099520	0.00094007	0.00014097	Hasebe et al., 1994 (U05611)
Adiantum raddianum C. Presl	100	0.00	0.00	0.00	0.00	0.00132980	0.00121325	0.00018663	Wolf et al., 1994 (U05906)
Ceratopteris richardii Brongn.	100	0.00	0.00	0.00	0.00	0.00152510	0.00139924	0.00021337	Masuyama et al., 2002 (AB059585)
Cardiomanes reniforme (G. Forst.) C. Presl	100	0.00	0.00	0.00	0.00	0.00025583	0.00023003	0.00009336	Hasebe et al., 1995 (U30833)

(Continued on next page)

TABLE 1. Estimated divergence times and ancestral rates for all nodes resolved in this study of filmy ferns. *(Continued)*

Internal node or terminal species[a]	N[b]	Constraint[c] (Ma)	Dates (Ma)[d]			Rates (substitutions/site/Ma)[e]			Constraint or *rbcL* sequence reference[f]
			Consensus	Mean	SD	Consensus	Mean	SD	
Hymenophyllum dilatatum (G. Forst.) Sw.	100	0.00	0.00	0.00	0.00	0.00017247	0.00017915	0.00010340	Hennequin et al., 2003 (AY095111)
Hymenoglossum cruentum (Cav.) C. Presl	100	0.00	0.00	0.00	0.00	0.00012263	0.00013002	0.00010818	Hennequin et al., 2003 (AY095107)
Hymenophyllum lanceolatum (Hook. & Arn.) Copel.	100	0.00	0.00	0.00	0.00	0.00022037	0.00019482	0.00011392	Pryer et al., 2001b (AF275646)
Microtrichomanes taeniatum (Copel.) Copel.	100	0.00	0.00	0.00	0.00	0.00034169	0.00029308	0.00010256	Pryer et al., 2001b (AF275651)
Microtrichomanes digitatum (Swartz) Copel.	100	0.00	0.00	0.00	0.00	0.00039904	0.00034968	0.00008795	Hennequin et al., 2003 (AY095114)
Hymenophyllum hygrometricum (Poir.) Desv.	100	0.00	0.00	0.00	0.00	0.00027659	0.00025365	0.00010496	Hennequin et al., 2003 (AY095113)
Hymenophyllum hirsutum (L.) Sw.	100	0.00	0.00	0.00	0.00	0.00036942	0.00034162	0.00009639	Pryer et al., 2001b (AF275645)
Hymenophyllum ferrugineum Colla	100	0.00	0.00	0.00	0.00	0.00036942	0.00033893	0.00009152	Pryer et al., 2001b (AF275644)
Hymenophyllum inaequale Desv.	100	0.00	0.00	0.00	0.00	0.00023828	0.00021818	0.00009886	Hennequin et al., 2003 (AY095112)
Hymenophyllum polyanthos (Sw.) Sw.	100	0.00	0.00	0.00	0.00	0.00028722	0.00024512	0.00009884	Pryer et al., 2001b (AF275647)
Hymenophyllum apiculatum Mett. ex Kuhn	100	0.00	0.00	0.00	0.00	0.00025910	0.00022275	0.00010296	Pryer et al., 2001b (AF275642)
Serpyllopsis caespitosa (Gaudich.) C. Chr.	100	0.00	0.00	0.00	0.00	0.00021005	0.00019617	0.00011475	Pryer et al., 2001b (AF275649)
Hymenophyllum armstrongii (Baker) Kirk	100	0.00	0.00	0.00	0.00	0.00029585	0.00026573	0.00010647	Hennequin et al., 2003 (AY095109)
Hymenophyllum pectinatum Cav.	100	0.00	0.00	0.00	0.00	0.00031838	0.00029180	0.00009873	Hennequin et al., 2003 (AY095115)
Hymenophyllum deplanchei (Mett.) Copel.	100	0.00	0.00	0.00	0.00	0.00024159	0.00019968	0.00011932	Ebihara et al., 2002 (AB064288)
Hymenophyllum baileyanum Domin.	100	0.00	0.00	0.00	0.00	0.00030158	0.00026865	0.00010512	Pryer et al., 2001b (AF275643)
Rosenstockia rolandi-principis (Rosenst.) C. Presl	100	0.00	0.00	0.00	0.00	0.00017457	0.00016218	0.00012761	Hennequin et al., 2003 (AY095110)
Hymenophyllum barbatum Baker	100	0.00	0.00	0.00	0.00	0.00031392	0.00027087	0.00010750	Ebihara et al., 2002 (AB064287)
Hymenophyllum subdimidiatum Rosenst.	100	0.00	0.00	0.00	0.00	0.00031392	0.00027434	0.00010549	Ebihara et al., 2002 (AB064290)
Hymenophyllum sibthorpioides Mett.	100	0.00	0.00	0.00	0.00	0.00030116	0.00023183	0.00010686	Hennequin et al., 2003 (AY095117)
Hymenophyllum tenellum Kuhn	100	0.00	0.00	0.00	0.00	0.00034191	0.00027279	0.00009445	Hennequin et al., 2003 (AY095116)
Hymenophyllum tunbrigense (L.) Sm.	100	0.00	0.00	0.00	0.00	0.00029226	0.00021759	0.00010346	Dubuisson, 1997 (Y09203)
Hymenophyllum fucoides (Sw.) Sw.	100	0.00	0.00	0.00	0.00	0.00037203	0.00027877	0.00009467	Wolf et al., 1994 (U20933)
Hymenophyllum secundum Hook. & Grev.	100	0.00	0.00	0.00	0.00	0.00024402	0.00016181	0.00012284	Pryer et al., 2001b (AF275648)
Trichomanes apiifolium C. Presl	100	0.00	0.00	0.00	0.00	0.00045106	0.00043558	0.00007986	Dubuisson et al., 2003a (AY175801)
Trichomanes javanicum Blume	100	0.00	0.00	0.00	0.00	0.00053174	0.00051093	0.00007116	Dubuisson, 1997 (Y09195)
Trichomanes caudatum Brack.	100	0.00	0.00	0.00	0.00	0.00039245	0.00038638	0.00008451	Dubuisson et al., 2003a (AY175805)
Trichomanes elongatum A. Cunn.	100	0.00	0.00	0.00	0.00	0.00030058	0.00028543	0.00009133	Dubuisson et al., 2003a (AY175802)
Trichomanes tamarisciforme Jacq.	100	0.00	0.00	0.00	0.00	0.00040936	0.00040528	0.00007790	Dubuisson, 1997 (Y09202)
Trichomanes osmundoides D. C. ex Poir.	100	0.00	0.00	0.00	0.00	0.00056693	0.00053007	0.00008656	Dubuisson, 1997 (Y09198)
Trichomanes ankersii C. Parker	100	0.00	0.00	0.00	0.00	0.00066104	0.00061945	0.00008521	Dubuisson et al., 2003a (AY175800)
Trichomanes elegans Rich.	100	0.00	0.00	0.00	0.00	0.00053704	0.00049926	0.00009183	Dubuisson, 1997 (Y09193)
Trichomanes lucens Sw.	100	0.00	0.00	0.00	0.00	0.00044389	0.00041705	0.00009369	Dubuisson et al., 2003a (AY175792)
Trichomanes arbuscula Desv.	100	0.00	0.00	0.00	0.00	0.00055029	0.00053084	0.00008801	Dubuisson et al., 2003a (AY175791)
Trichomanes alatum Sw.	100	0.00	0.00	0.00	0.00	0.00058515	0.00055512	0.00009007	Dubuisson, 1997 (Y09189)
Trichomanes crispum L.	100	0.00	0.00	0.00	0.00	0.00051245	0.00046484	0.00010493	Dubuisson et al., 2003a (AY175789)
Trichomanes pinnatum Hedwig	100	0.00	0.00	0.00	0.00	0.00053248	0.00048983	0.00010080	Dubuisson, 1997 (Y09200)
Trichomanes capillaceum L.	100	0.00	0.00	0.00	0.00	0.00049214	0.00046068	0.00010972	Dubuisson et al., 2003a (AY175784)
Trichomanes borbonicum Bosch	100	0.00	0.00	0.00	0.00	0.00055551	0.00050196	0.00009497	Dubuisson et al., 2003a (AY175782)
Trichomanes endlicherianum C. Presl	100	0.00	0.00	0.00	0.00	0.00056020	0.00050912	0.00008780	Dubuisson et al., 2003a (AY175787)
Trichomanes ekmanii Wess. Boer	100	0.00	0.00	0.00	0.00	0.00099409	0.00089578	0.00012048	Dubuisson, 1997 (Y09192)
Trichomanes pinnatinervium Jenman	100	0.00	0.00	0.00	0.00	0.00077247	0.00069478	0.00013169	Dubuisson, 1997 (Y09199)
Trichomanes hildebrandtii Kuhn	100	0.00	0.00	0.00	0.00	0.00091952	0.00080171	0.00012778	Dubuisson et al., 2003a (AY175788)
Trichomanes membranaceum L.	100	0.00	0.00	0.00	0.00	0.00102820	0.00092406	0.00012763	Dubuisson, 1997 (Y09197)
Trichomanes radicans Sw.	100	0.00	0.00	0.00	0.00	0.00051331	0.00044539	0.00013294	Pryer et al., 2001b (AF275650)
Trichomanes speciosum Willd.	100	0.00	0.00	0.00	0.00	0.00059233	0.00052248	0.00011195	Dubuisson, 1997 (Y09201)
Trichomanes thysanostomum Makino	100	0.00	0.00	0.00	0.00	0.00084954	0.00077232	0.00011690	Hasebe et al., 1994 (U05608)
Trichomanes minutum Blume	100	0.00	0.00	0.00	0.00	0.00084833	0.00076712	0.00011115	Hasebe et al., 1994 (U05625)
Trichomanes bipunctatum Poir.	100	0.00	0.00	0.00	0.00	0.00080959	0.00072350	0.00011978	Dubuisson, 1997 (Y09190)

[a] Node numbers correspond to those in Figure 3; lineage names and abbreviations used in figures are given in parentheses where applicable. Species are listed in the order in which they appear in Figures 1, 3, and 4.
[b] Number out of 100 randomly sampled trees in which node was present; value provides a rough approximation for the posterior probability of node.
[c] All constraints were applied as minimum ages, except at the fixed calibration point (node 001).
[d] Consensus date estimates are the result of penalized likelihood analysis of the Bayesian consensus tree; means and standard deviations result from analyses of 100 randomly sampled Bayesian trees.
[e] Rate estimates provided are for internodes subtending listed nodes and terminals. Consensus rate estimates are the result of penalized likelihood analysis of the Bayesian consensus tree; means and standard deviations result from analyses of 100 randomly sampled Bayesian trees.
[f] Referenced study node numbers and GenBank accession numbers are provided in parentheses where applicable.

group and *Hymenophyllum* had the same rate but *Trichomanes* had a different rate *versus* the three-rate model described above; and (3) a two-rate model in which the filmy fern sister group and *Trichomanes* had the same rate but *Hymenophyllum* had a different rate *versus* the three-rate model described above. Again, likelihoods were determined using the program Baseml (part of the PAML 3.14b package; Yang 1997) with the appropriate models (GTR+Γ; branch rates as described above), and compared using the likelihood-ratio test statistic (Felsenstein, 1981).

To further characterize rate differences, pairwise relative rate comparisons were conducted. All included filmy fern species, as well as all species in the resolved sister lineage to filmy ferns, were evaluated relative to one another. For each pairwise comparison (3916 total), a three-taxon tree was constructed (outgroup = *Osmunda cinnamomea*) and two models were compared—one with (null) and one without (alternative) the constraint of equal rates between the two ingroup species. The likelihoods corresponding to each of these models were compared using the likelihood ratio test statistic (Felsenstein, 1981). All 3916 pairwise comparisons were made in an automated fashion using the program HyPhy 0.99 beta (Kosakovsky Pond et al., 2005), with the GTR+Γ+I model of sequence evolution and globally estimated parameters; no correction for multiple comparisons was incorporated.

Estimation of Divergence Times and Ancestral Rates

Ancestral rates of molecular evolution and divergence times were estimated using penalized likelihood (Sanderson, 2002). This semiparametric method separates rate and time from branch length by combining a likelihood model in which each branch has its own rate parameter with a roughness penalty that penalizes excessive rate change from branch to branch. The interplay between the likelihood model and the roughness penalty is controlled by a smoothing parameter that is objectively identified from the data using a cross-validation procedure (Sanderson, 2002). Penalized likelihood analyses of the Bayesian consensus tree as well as of the 100 randomly sampled trees from the Bayesian posterior (to evaluate the effects of phylogenetic uncertainty due to both topological and branch length estimation error) were conducted using the program r8s version 1.60 (Sanderson, 2003). In all analyses, the three lycophyte outgroup taxa were pruned, and the resulting root—the divergence of monilophytes (ferns) from spermatophytes (seed plants)—was used as the fixed calibration point (380 Ma, node 001, Table 1) based on the earliest appearance of fossils belonging to each of these lineages in the Middle Devonian. In addition to this fossil calibration point, 16 minimum fossil age constraints from a previous reassessment of the fern fossil record (Pryer et al., 2004; Schneider et al., 2004a) were incorporated (Table 1; fossil constraints were applied only to nodes receiving high posterior probability support, ≥0.99). The appropriate smoothing value was independently identified for each of the 101 trees (100 randomly sampled trees plus the consensus tree) using cross validation (smoothing values from 1 to 10,000 were considered; for most trees, a value of 100 was found to be the most appropriate). Searches for solutions that optimized the penalized likelihood function were conducted using the truncated Newton algorithm with 10 random starts, each with 10 random perturbations.

Examining the Influence of Selection

To identify whether significant selectional differences exist between the *Hymenophyllum* and *Trichomanes* clades, or between either of these lineages and the filmy fern sister group, a series of tests was conducted using a subtree pruned from the Bayesian plurality consensus (comprising filmy ferns plus their resolved sister group). A total of three comparisons were made, analogous to those used to test for rate differences between partitions: (1) a two-ratio model in which *Hymenophyllum* and *Trichomanes* had the same nonsynonymous/synonymous substitution (dn/ds) ratio but the filmy fern sister group had a different ratio *versus* a three-ratio model in which all three included partitions (*Hymenophyllum*, *Trichomanes*, and the resolved filmy fern sister group) had different dn/ds ratios; (2) a two-ratio model in which the filmy fern sister group and *Hymenophyllum* had the same dn/ds ratio but *Trichomanes* had a different ratio *versus* the three-ratio model described above; and (3) a two-ratio model in which the filmy fern sister group and *Trichomanes* had the same dn/ds ratio but *Hymenophyllum* had a different ratio *versus* the three-ratio model described above. Likelihoods were calculated for each of these models using the program Codeml (part of the PAML 3.14b package; Yang, 1997). Codon frequencies were estimated from the average nucleotide frequencies at the three codon positions and equal dn/ds ratios and rates were assumed among sites. Resulting likelihoods were compared using the likelihood ratio test statistic (Felsenstein, 1981).

Results and Discussion

Phylogeny

Phylogenetic analysis of the plastid *rbcL* data yielded a reasonably well-supported hypothesis of relationships (Fig. 1); 60 of 107 nodes received high posterior probability (PP) support (≥0.99). In the Bayesian consensus tree, monilophytes (MON) are strongly supported as monophyletic (PP = 1.00), consistent with earlier analyses of morphological and multigene DNA sequence data sets (Kenrick and Crane, 1997; Renzaglia et al., 2000; Nickrent et al., 2000; Pryer et al., 2001a, 2004; Wikström and Pryer, 2005). Within monilophytes, whisk ferns (WHI) and ophioglossoid ferns (OPH) form a well-supported clade (PP = 1.00), sister to the remaining monilophyte lineages. Subsequently, marattioid ferns (MAR) are resolved as sister to a clade consisting of horsetail ferns (HOR) and leptosporangiate ferns (LEP). These basal monilophyte relationships based on analyses of *rbcL* alone are not in complete agreement with those identified in other studies (Pryer et al., 2001a, 2004), but are also not supported by high posterior probability values (Fig. 1). This region

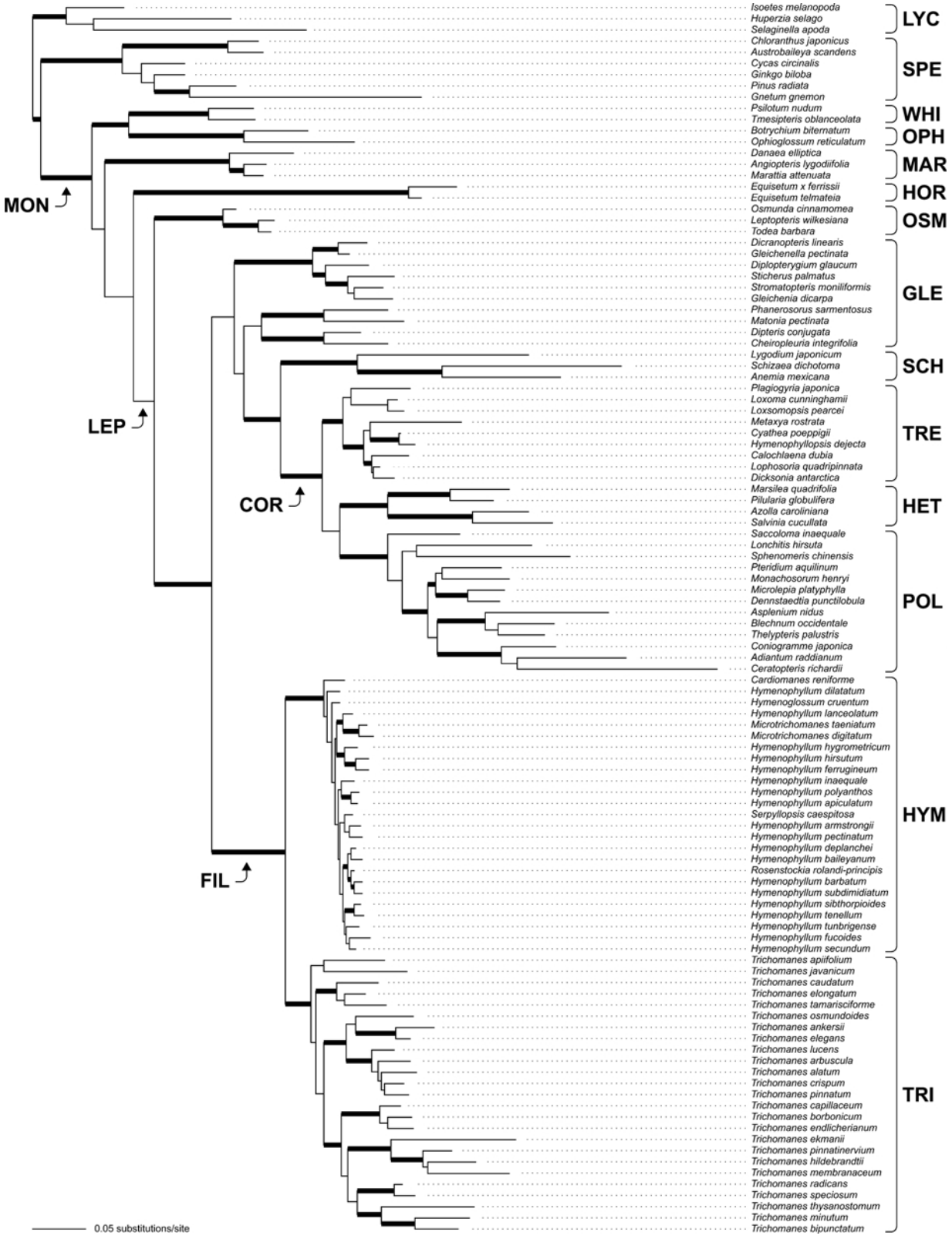

FIGURE 1. Phylogeny resulting from Bayesian analysis of *rbcL* data (plurality consensus with average branch lengths). Thickened lines identify high posterior probability support (≥0.99). Major lineages are indicated: LYC = lycophytes; MON = monilophytes (ferns); SPE = spermatophytes (seed plants); WHI = whisk ferns; OPH = ophioglossoid ferns; MAR = marattioid ferns; HOR = horsetail ferns; LEP = leptosporangiate ferns; OSM = osmundaceous ferns; GLE = gleichenioid ferns; SCH = schizaeoid ferns; COR = core leptosporangiates; TRE = tree ferns; HET = heterosporous ferns; POL = polypod ferns; FIL = filmy ferns; HYM = *Hymenophyllum* clade; TRI = *Trichomanes* clade.

of the monilophyte phylogeny has proven difficult in the past; even multigene analyses have so far failed to fully clarify these relationships (Pryer et al., 2001a, 2004; Wikström and Pryer, 2005).

Within leptosporangiate ferns (LEP), osmundaceous ferns (OSM) are sister to a large, well-supported clade (PP = 1.00) containing all other leptosporangiate lineages: gleichenioid (GLE), schizaeoid (SCH), tree (TRE), heterosporous (HET), polypod (POL), and filmy (FIL) ferns (Fig. 1). Each of these lineages, with the exception of gleichenioid ferns, is strongly supported as monophyletic by our analyses (PP = 1.00). Tree, heterosporous, and polypod ferns form a clade of core leptosporangiates (COR; PP = 1.00) sister to schizaeoid ferns (PP = 1.00); and gleichenioid ferns are paraphyletic to this core leptosporangiate + schizaeoid fern clade. Filmy ferns are resolved as sister to the assemblage of gleichenioid, schizaeoid, tree, heterosporous, and polypod ferns (Fig. 1). These results are mostly consistent with earlier analyses of leptosporangiate fern relationships (Hasebe et al., 1995; Pryer et al., 2001a, 2004), with the areas of uncertainty in this study also equivocal in the earlier studies.

Two major filmy fern lineages are resolved and well supported (PP = 1.00), corresponding to the two traditionally defined filmy fern genera: *Hymenophyllum* (HYM) and *Trichomanes* (TRI). The composition of these two clades is identical to that found in previous studies (Dubuisson et al., 2003a; Ebihara et al., 2002; Hennequin et al., 2003; Pryer et al., 2001b), with the monotypic segregate genera (*Cardiomanes*, *Hymenoglossum*, *Rosenstockia*, and *Serpyllopsis*) and species of *Microtrichomanes*, all nested within the *Hymenophyllum* clade. High posterior probability support is present for 9 of 23 nodes resolved within the *Hymenophyllum* clade and 12 of 23 nodes within the *Trichomanes* clade. The relationships of species within each of these two major clades are essentially in agreement with previous studies (Dubuisson et al., 2003a; Ebihara et al., 2002; Hennequin et al., 2003; Pryer et al., 2001b).

Significant Rate Differences

Considerable branch length differences were evident between the two major filmy fern lineages, as well as within each of these lineages and across the phylogeny as a whole (Fig. 1). Likelihood ratio test comparisons of null models of rate constancy versus alternative models with unique rates of molecular evolution for each branch revealed statistically significant departures from rate constancy across the entire tree and within all partitions examined (comparisons 1 to 5, Table 2). Even within the *Hymenophyllum* clade, where branch lengths appear to reflect clock-like evolution (Fig. 1), a molecular clock could be rejected ($P < 0.001$). Nevertheless, although these tests do indicate that the branch length differences observed are ultimately the result of significantly different rates of molecular evolution, they do not identify particular branches with aberrant rates. Within filmy ferns specifically, these tests alone do not reveal where an acceleration or deceleration in rate occurred, nor do they even distinguish between inter- and intrageneric differences.

Likelihood ratio tests to identify significant differences in rate *among*, as opposed to *within*, partitions yielded more meaningful results (Table 2). A significant rate difference between the *Trichomanes* and *Hymenophyllum* clades ($P < 0.001$; comparison 6, Table 2) was uncovered when we compared a null two-rate model in which filmy ferns were assigned a single rate of evolution (and their sister lineage a second rate) *versus* an alternative three-rate model in which the two filmy fern genera

TABLE 2. Summary of likelihood ratio test comparisons made in this study. To test for the presence of rate differences *within* various tree partitions, comparisons 1 to 5 evaluate null models of evolutionary rate constancy versus alternative models with unique rates of molecular evolution for each branch. To test for the presence of evolutionary rate differences *among* partitions, comparisons 6 to 8 evaluate two-rate models versus three-rate models. Comparisons 9 to 11 evaluate models with two nonsynonymous/synonymous substitution (dn/ds) ratios versus three-ratio models to test for significant selectional differences *among* partitions.

	Comparison (null model versus alternative model)	Tree and data set utilized	lnL (null)	lnL (alternative)	LRT	df	*P*
1	One-rate model versus many-rate model	Entire tree	−27407.69	−26948.11	919.16	108	<0.001
2	One-rate model versus many-rate model	Filmy ferns + sister lineage	−20876.41	−20595.67	561.50	87	<0.001
3	One-rate model versus many-rate model	Filmy ferns	−9317.59	−9221.21	192.76	48	<0.001
4	One-rate model versus many-rate model	*Hymenophyllum*	−3551.30	−3523.36	55.89	23	<0.001
5	One-rate model versus many-rate model	*Trichomanes*	−7302.39	−7229.28	146.23	23	<0.001
6	Two-rate model (*Hymenophyllum* = *Trichomanes* ≠ sister lineage) versus three-rate model	Filmy ferns + sister lineage	−20876.05	−20867.06	17.99	1	<0.001
7	Two-rate model (*Hymenophyllum* = sister lineage ≠ *Trichomanes*) versus three-rate model	Filmy ferns + sister lineage	−20872.18	−20867.06	10.24	1	0.001
8	Two-rate model (*Trichomanes* = sister lineage ≠ *Hymenophyllum*) versus three-rate model	Filmy ferns + sister lineage	−20867.08	−20867.06	0.05	1	0.830
9	Two-ratio model (*Hymenophyllum* = *Trichomanes* ≠ sister lineage) versus three-ratio model	Filmy ferns + sister lineage	−20618.73	−20608.49	20.48	1	<0.001
10	Two-ratio model (*Hymenophyllum* = sister lineage ≠ *Trichomanes*) versus three-ratio model	Filmy ferns + sister lineage	−20669.00	−20608.49	121.03	1	<0.001
11	Two-ratio model (*Trichomanes* = sister lineage ≠ *Hymenophyllum*) versus three-ratio model	Filmy ferns + sister lineage	−20657.68	−20608.49	98.39	1	<0.001

were each allowed to have a unique rate (and their sister lineage a third rate). Comparisons of two additional two-rate models (one in which *Hymenophyllum* was assigned the same rate as the filmy fern sister lineage and *Trichomanes* a unique rate; the other in which *Trichomanes* was assigned the same rate as the filmy fern sister lineage and *Hymenophyllum* a unique rate) *versus* the three-rate model described above, further clarified the nature of the rate differences. These tests revealed that the rate within *Hymenophyllum* is significantly different from that of the filmy fern sister lineage ($P = 0.001$; comparison 7, Table 2), whereas the rate within *Trichomanes* is not ($P = 0.830$; comparison 8, Table 2).

Pairwise relative rate comparisons among all included filmy fern species and all species in the lineage resolved as sister to filmy ferns were consistent with the above results. Of the 3916 total comparisons, 1875 were significant ($P < 0.05$; colored boxes in Fig. 2), many at higher significance thresholds ($P < 0.01$ or $P < 0.001$; darker colors in Fig. 2). Clearly, rates vary across

FIGURE 2. Results of pairwise relative rate comparisons among all included filmy fern species and all species in the lineage resolved as sister to filmy ferns. Each square in grid represents a comparison between a species in the left tree (portion of consensus tree resulting from Bayesian phylogenetic analysis, names omitted due to size) and a species in the right (mirrored) tree. A cool colored (blue) square indicates that the left taxon has a significantly slower rate than the right taxon (dark blue, $P < 0.001$; medium blue, $P < 0.01$; light blue, $P < 0.05$). A warm colored (orange) square indicates that the left taxon has a significantly faster rate than the right taxon (dark orange, $P < 0.001$; medium orange, $P < 0.01$; light orange, $P < 0.05$). White squares indicate that differences between the taxa were not statistically significant. Lineage abbreviations are as in Figure 1.

the phylogeny. Significant differences were observed within the *Trichomanes* and *Hymenophyllum* clades, as well as among species in the sister group to filmy ferns (Fig. 2). The most striking differences, however, were found in comparisons between the *Hymenophyllum* and *Trichomanes* clades (537 of 625 comparisons were significant, $P < 0.05$). These differences were consistently unidirectional; when a significant difference existed, the *Hymenophyllum* lineage evaluated was always slower than the *Trichomanes* lineage. This result indicates that considerable intergeneric rate differences exist. It does not, however, inherently reveal the phylogenetic extent of the rate differences (i.e., whether a rate discrepancy was maintained throughout a filmy fern clade or only present along the branch leading to it), nor does it reveal which of the two lineages contains the aberrant rate.

In theory, relative rate comparisons between species in the sister group to filmy ferns and species in the *Trichomanes* and *Hymenophyllum* clades could be used to identify the aberrant filmy fern lineage. This assumes that one filmy fern lineage would show a large number of significant differences in just one direction relative to the filmy fern sister group, but the other filmy fern lineage few. Our pairwise comparisons between the filmy fern and sister lineage taxa did result in a relatively large proportion of significant outcomes—876 of 1950 comparisons were significant. These differences, however, were not entirely unidirectional or restricted to a single filmy fern lineage. Of the 975 comparisons between species of *Hymenophyllum* and species from the sister group to filmy ferns, 537 were significant; all but one of the significant comparisons indicated that the net rate along the branch leading to the species of *Hymenophyllum* was slower. Of the 975 comparisons between species of *Trichomanes* and species from the sister group to filmy ferns, 339 were significant; most of these significant comparisons (261) indicated that the net rate along the branch leading to the *Trichomanes* species examined was faster, but others (78) indicated that this rate was slower. Although there are more significant comparisons relative to the *Hymenophyllum* clade, and the vast majority of these are in one direction, the lack of a uniform background rate (as evidenced by the results relative to filmy ferns and comparisons among the sister group taxa) makes it difficult to definitively identify the aberrant filmy fern group using pairwise comparisons alone.

Divergence Time Estimates and Reconstructions of Ancestral Rates of Evolution

In identifying the aberrant filmy fern lineage, ancestral rate reconstructions can be considerably more informative than the previous tests. These reconstructions more realistically assign a unique rate of molecular evolution to each branch in the phylogeny and incorporate time. Therefore, they can provide an absolute rate estimate for each internal branch, including the filmy fern stem branch (i.e., the branch immediately subtending the initial divergence between the *Hymenophyllum* and *Trichomanes* clades), that is indicative of the ancestral filmy fern rate. Such reconstructions can thus also supply a distribution of the rates outside of filmy ferns, as well as a rate distribution for each of the two filmy fern lineages for comparison. The simultaneous estimation of divergence times provides additional insight. However, it should be noted that, as with reconstructing phylogenetic relationships, reconstructing ancestral rates and divergence times are dependent on models, and the results are contingent on the use of an appropriate model with valid assumptions. Because filmy ferns themselves lack a solid fossil record to constrain our analyses, the reconstructions of rates and dates within this group are especially dependent on the penalized likelihood model of rate change. Although this model is biologically plausible, and without a doubt more realistic than simply assuming a molecular clock, the results below should be interpreted with caution.

The chronological results of our penalized likelihood analysis of the Bayesian consensus tree are presented as a chronogram (i.e., a tree in which internode lengths are proportional to time) in Figure 3. For all nodes resolved in the Bayesian consensus tree, age estimates from the analysis of the consensus phylogeny, as well as mean ages and standard deviations resulting from the 100 replicate analyses, are presented in Table 1. Because not all nodes resolved in the consensus phylogeny were present in each of the 100 randomly sampled trees, some mean ages and standard deviations are based on fewer than 100 samples. According to our analyses, the initial divergence among monilophyte lineages (node 007, Fig. 3) occurred in the Late Devonian (ca. 360 Ma). The whisk fern (WHI), ophioglossoid fern (OPH), marattioid fern (MAR), horsetail fern (HOR), and leptosporangiate fern (LEP) lineages were all present by the end of the Carboniferous (290 Ma). Within leptosporangiate ferns, we estimate the earliest divergences to have occurred in the Carboniferous and Permian. These divergences gave rise to the osmundaceous (OSM), filmy (FIL), gleichenioid (GLE), and schizaeoid (SCH) ferns, as well as to the core leptosporangiate lineage (COR, node 033, Fig. 3). A Late Triassic diversification gave rise to the three major lineages of core leptosporangiates—heterosporous ferns (HET), tree ferns (TRE), and polypod ferns (POL) (Fig. 3). Our estimates for the temporal origins of the major fern lineages are largely in accord with previous ideas (Collinson, 1996; Pryer et al., 2004; Rothwell, 1987, 1996; Skog, 2001; Soltis et al., 2002; Tidwell and Ash, 1994). The major diversification of polypod ferns is estimated to have occurred in the Cretaceous, also consistent with recent analyses (Schneider et al., 2004a).

Within filmy ferns, the initial divergence (node 058, Fig. 3), yielding the *Hymenophyllum* and *Trichomanes* clades, is estimated to have occurred near the Triassic-Jurassic boundary (206 Ma). The oldest of the scarce filmy fern fossils, which are not definitively assignable to one of the two extant clades, are from the epoch immediately preceding this boundary (Late Triassic) (Axsmith et al., 2001). Based on our analyses, the major diversification within the two extant filmy fern clades was not concurrent. The initial divergence within the *Trichomanes* clade

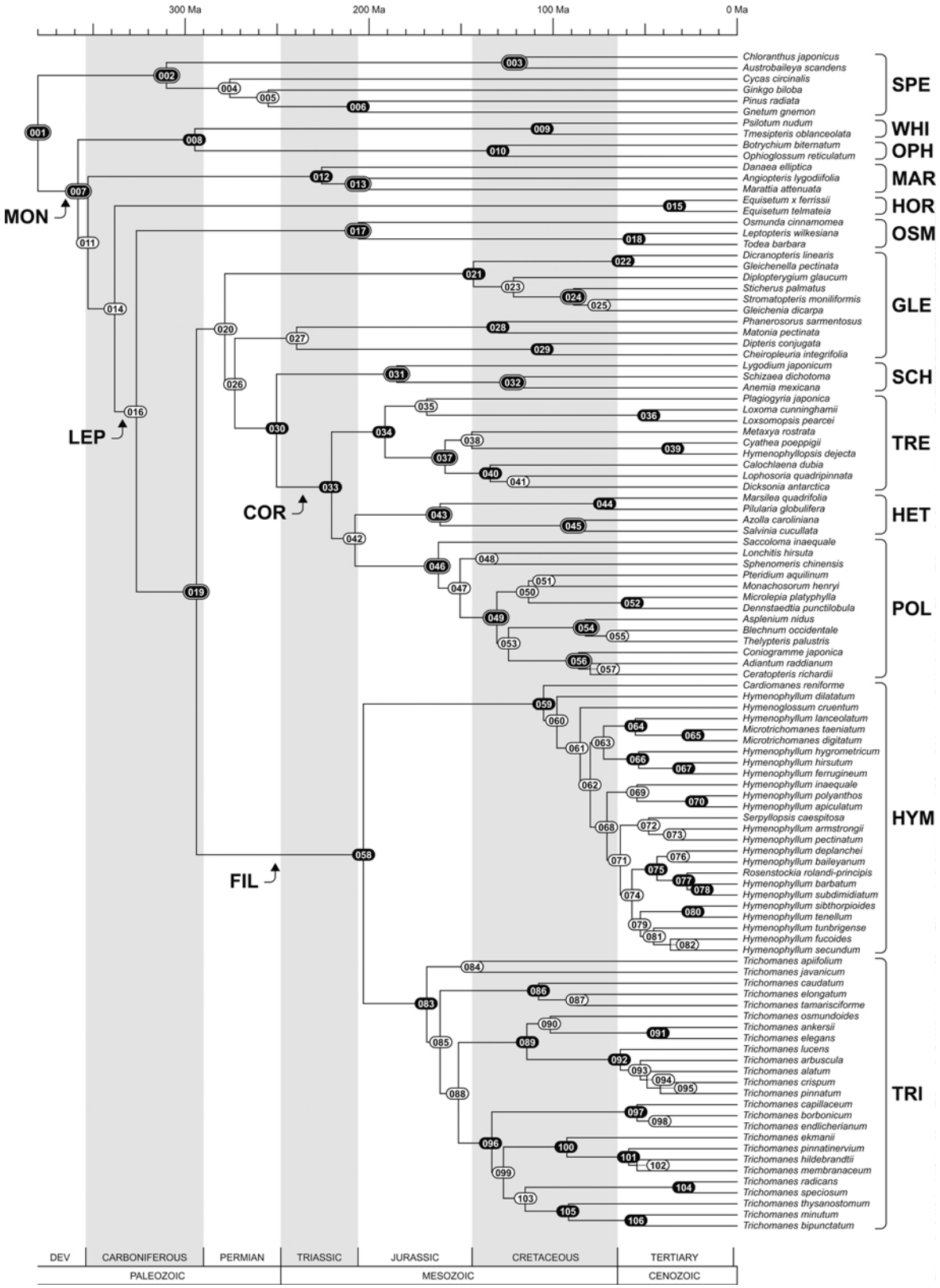

FIGURE 3. Chronogram (internode lengths are proportional to time; note scale in Ma) resulting from penalized likelihood analysis of the Bayesian consensus tree. Black ovals identify nodes with high posterior probability support (≥0.99), whereas white ovals identify nodes not receiving high support in our analysis. Circled black ovals indicate the positions of fossil constraints. Age estimates for all nodes, including means and standard deviations resulting from 100 replicate analyses, and fossil constraint information are presented in Table 1. Lineage abbreviations are as in Figure 1.

(node 083) is estimated to have occurred in the Middle Jurassic, with major diversification in the Late Jurassic and throughout the Cretaceous. Diversification within the *Hymenophyllum* clade, however, appears to be a much more recent phenomenon. The initial divergence within this clade (node 059) is estimated to have occurred in the Early Cretaceous, with subsequent major divergences in the Late Cretaceous and Tertiary.

Reconstructions of ancestral rates of molecular evolution for the *rbcL* locus are presented graphically as a ratogram (i.e., a tree in which internode lengths are proportional to rate) in Figure 4. The rate estimates for all internodes, including means and standard deviations resulting from the 100 replicate analyses are presented in Table 1. As was previously suggested by the significance tests for the presence of rate differences and the pairwise relative rate comparisons, considerable differences in absolute rate are present across the resolved phylogeny, ranging from about 0.00005 to 0.00150 substitutions/site/Ma (Table 1). Some groups are consistent in their relatively slow rate of molecular evolution, including osmundaceous ferns (OSM) and tree ferns (TRE). Others are consistent in their relatively fast rate, including schizaeoid ferns (SCH) and polypod ferns (POL). Within filmy ferns (FIL), a clear difference in rate can be observed between the *Trichomanes* (TRI) and *Hymenophyllum* (HYM) clades, with the rate within *Trichomanes* estimated to be about twice that of *Hymenophyllum* (Fig. 4). Rate heterogeneity is of course present within each of these lineages, but is much less pronounced than the resolved intergeneric differences.

When rates within the *Hymenophyllum* and *Trichomanes* crowns (i.e., for each clade, the set of branches subsequent to the initial divergence) and along the *Hymenophyllum* and *Trichomanes* stems (i.e., for each clade, the branch immediately subtending the initial divergence) are compared to the rates outside of filmy ferns, and to the rate of the filmy fern stem (i.e., the branch immediately subtending the initial divergence between *Hymenophyllum* and *Trichomanes*), it appears as though the rates within *Hymenophyllum* are aberrant (see ratogram, right side of Fig. 4). The rates within *Trichomanes*, on the other hand, appear to be rather consistent with the rates outside of filmy ferns and those along the filmy fern stem. The similarities and differences among the various partitions become even more apparent when the pools of estimates corresponding to each of the partitions (resulting from analyses of the 100 sampled trees) are graphed (left side of Fig. 4). Rates outside of filmy ferns, while varied from slow to fast, form a distribution centered at about 0.00053 substitutions/site/Ma, with 50% of the estimates falling between about 0.00036 and 0.00075 substitutions/site/Ma (Fig. 4). The distributions of rates along the filmy fern stem, the *Trichomanes* stem, and within the *Trichomanes* crown, do not show a substantial deviation from the centrality displayed in the distribution of rates outside of filmy ferns (medians of 0.00057, 0.00055, and 0.00055 substitutions/site/Ma, respectively). The rates along the *Hymenophyllum* stem and within the *Hymenophyllum* crown, however, are considerably slower (medians of 0.00035 and 0.00023 substitutions/site/Ma, respectively). The *Hymenophyllum* clade (including its stem), with a substantially different rate of molecular evolution, clearly represents the aberrant filmy fern lineage, as suggested by the tests for rate differences and the pairwise relative rate comparisons.

Based on the results of this study, the overall path length differences observed between the two major filmy fern lineages are inferred to ultimately have resulted from a rate deceleration in the *Hymenophyllum* lineage. However, to fully appreciate individual branch length similarities and differences, a consideration of both time and rate is necessary. Following the initial divergence within filmy ferns and the origin of the two extant lineages, there was maintenance of the ancestral rate of evolution along the *Trichomanes* stem, but a deceleration in rate along the *Hymenophyllum* stem (Fig. 4). Yet, because a greater amount of time elapsed between the origin of the *Hymenophyllum* lineage (node 058) and the first divergence among extant *Hymenophyllum* species (node 059) than between the origin of the *Trichomanes* lineage (node 058) and the first divergence among extant *Trichomanes* species (node 083, Fig. 3), there is only a minor length difference between these two branches (Fig. 1). The discrepancy between the initial divergence times in *Hymenophyllum* and *Trichomanes* had the opposite effect on branch lengths within the two crowns. Less time for the accumulation of substitutions in the *Hymenophyllum* crown (relative to the *Trichomanes* crown, Fig. 3), combined with the slower rate of molecular evolution (Fig. 4), resulted in strikingly shorter branches in the crown of *Hymenophyllum* (Fig. 1).

Factors Influencing Evolutionary Rate

The slowed rate of *rbcL* sequence evolution in the *Hymenophyllum* clade may have resulted from intensified purifying selection, relaxed positive selection, or simply a genome-wide deceleration in the rate of nucleotide substitution (at either the plastid or organismal level). The *rbcL* gene is protein coding, and selection would act on nonsynonymous amino-acid replacement substitutions but not on synonymous silent substitutions. Therefore, if either intensified purifying selection or relaxed positive selection were responsible for the rate deceleration in the *Hymenophyllum* clade, we would expect to find a lower frequency of nonsynonymous substitutions in *Hymenophyllum* relative to *Trichomanes* and the filmy fern sister group, but no difference among these clades in the frequency of synonymous substitutions. This bias would result in a significantly smaller nonsynonymous to synonymous (dn/ds) ratio in *Hymenophyllum*, relative to *Trichomanes* and the filmy fern sister group.

To determine whether a significant difference in selection was present between the two filmy fern lineages, or between either of these lineages and the filmy fern sister group, we conducted a series of three tests. Specifically, we compared three two-ratio models in which two of the clades were constrained to have the same dn/ds ratio to a three-ratio model in which each clade

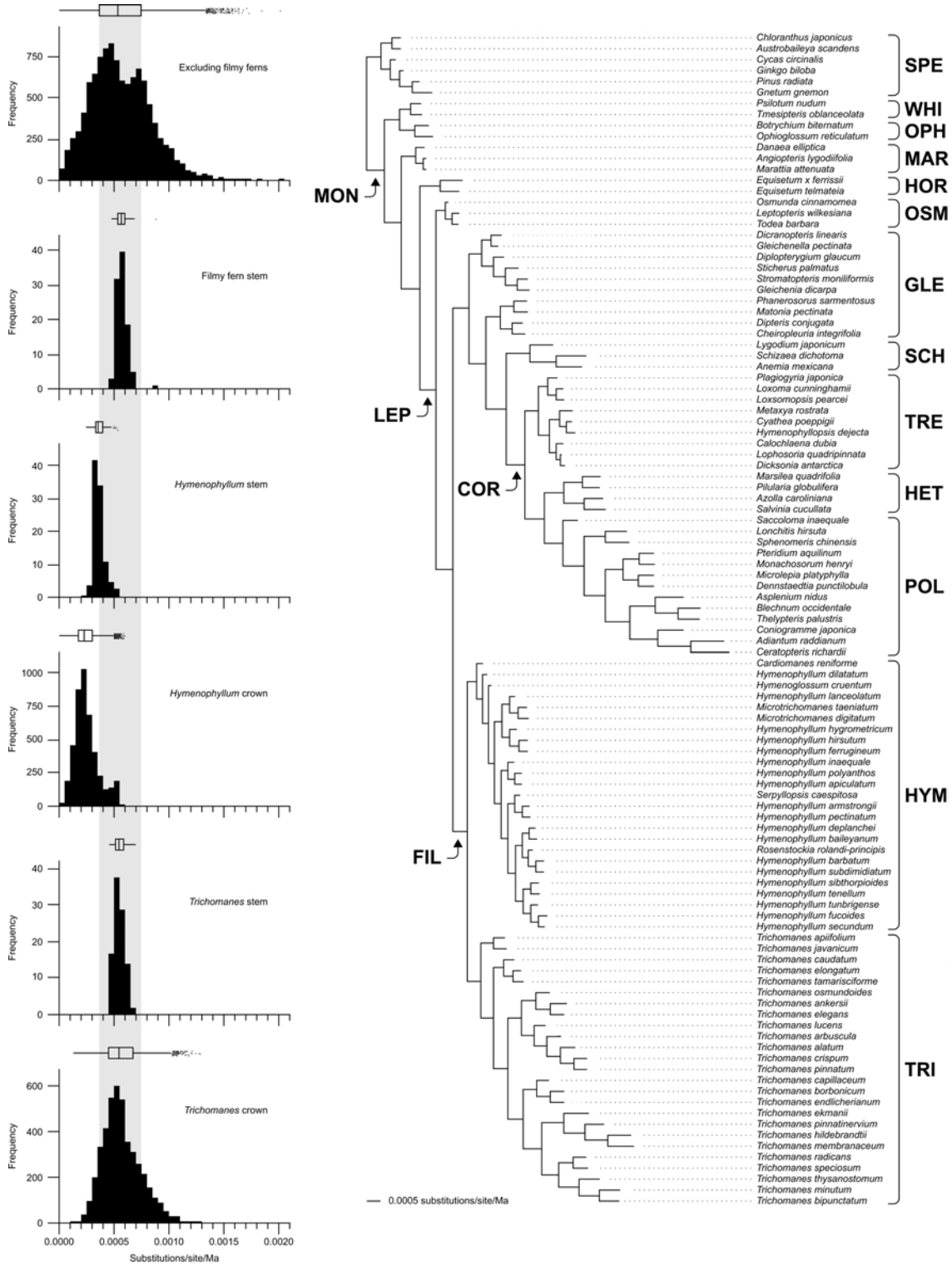

FIGURE 4. Ratogram (right side; internode lengths are proportional to rate; note scale in substitutions/site/Ma) resulting from penalized likelihood analysis of the Bayesian consensus tree; and rate distributions for critical tree partitions (left side). Lineage abbreviations in ratogram are as in Figure 1; rate estimates for all internodes, including means and standard deviations resulting from 100 replicate analyses, are presented in Table 1. Distributions of rate estimates (resulting from analyses of the 100 sampled trees) for the filmy fern stem (the branch immediately subtending the initial divergence between the *Hymenophyllum* and *Trichomanes* clades), the *Hymenophyllum* stem (the branch immediately subtending the initial divergence in *Hymenophyllum*), the *Hymenophyllum* crown (the set of branches subsequent to the initial divergence in *Hymenophyllum*), the *Trichomanes* stem (the branch immediately subtending the initial divergence in *Trichomanes*), the *Trichomanes* crown (the set of branches subsequent to the initial divergence in *Trichomanes*), and all remaining branches are presented as histograms and boxplots to the left of the ratogram.

was allowed a unique dn/ds ratio. The results of these tests revealed that dn/ds ratios were significantly different among the three clades ($P < 0.001$; comparisons 9 to 11, Table 2). However, the *Hymenophyllum* clade had the highest dn/ds ratio, contrary to what would be expected if selection were responsible for the deceleration in rate. Within *Hymenophyllum*, the deceleration in synonymous substitution rate appears to have been more substantial than the deceleration in nonsynonymous substitution rate. For this reason, a genome-wide deceleration of nucleotide substitution rate (at either the plastid or organismal level) seems more probable.

Several factors have been generally proposed to possibly influence the genome-wide rate of DNA substitution, including: (1) generation time or replication rate—more frequent replication will yield an increased number of mutations (Brunsfeld et al., 1994; Conti et al., 1993; Gaut et al., 1992, 1996, 1997; Kohne, 1970; Laird et al., 1969; Laroche and Bousquet, 1999; Laroche et al., 1997; Li et al., 1987; Wu and Li, 1985; but see Whittle and Johnston, 2003); (2) replication/repair efficiency—a less efficient system will allow more mutations to occur (Britten, 1986; Friedberg et al., 1995; Schön et al., 1998; Wu and Li, 1985); (3) exposure to mutagens—increased exposure to DNA-damaging mutagens will result in a higher mutation rate (Friedberg et al., 1995; Hebert et al., 2002; Lutzoni and Pagel, 1997; Martin and Palumbi, 1993); (4) population size—lineages with smaller effective population sizes will experience faster rates of evolution due to the amplified effects of genetic drift (Moran, 1996; Ohta, 1972, 1992); and (5) speciation rate—increased cladogenesis will result in more genetic change due to rapid evolution associated with speciation events (Barraclough and Savolainen, 2001; Bousquet et al., 1992; Mayr, 1954; Mindell et al., 1989; Webster et al., 2003). Although some of these factors involve the supply of DNA mutations, others center on the fixation of introduced mutations; many of the factors are intrinsic to evolutionary lineages, but a few are extrinsic. Overall, the hypothesized mechanisms are confounded and their contributions poorly understood.

Almost any of the proposed factors could ultimately provide an explanation for the evolutionary rate deceleration detected in *Hymenophyllum*. A slower replication rate, a more efficient replication/repair system, decreased exposure to mutagens, or larger population sizes in *Hymenophyllum*, relative to *Trichomanes*, are all plausible. However, with our current knowledge of filmy fern life history, it is impossible to even begin to discriminate among the possibilities. Filmy ferns are primarily tropical in distribution and have not been the focus of intensive ecological studies more common for plants of temperate regions. It is known that members of both the *Trichomanes* and *Hymenophyllum* clades possess long-lived gametophytes capable of vegetative reproduction (rare in ferns; usually only the sporophyte is long-lived and capable of vegetative reproduction); some populations in North America and Europe exist exclusively as gametophytes (Farrar, 1967; Rumsey et al., 1998). However, the relative contributions of the gametophyte and sporophyte stages in filmy fern life cycles have not been explored. Life history differences related to these relative contributions may certainly exist between the clades, but none have yet been described. With regard to potential mutagens, ultraviolet (UV) radiation, a leading candidate for mutagenesis (Lutzoni and Pagel 1997), cannot be invoked as being responsible for the rate discrepancy in filmy ferns. Because *Hymenophyllum* comprises species that generally inhabit more exposed niches, one would expect it to be more susceptible to UV mutagensis; yet, as we have shown here, it displays a deceleration in evolutionary rate. A speciation rate effect is also difficult to justify, as the two major extant filmy fern clades are approximately equally diverse (Pryer et al., 2001b). Virtually nothing is known about the replication/repair systems or even population size in filmy ferns.

Conclusions and Prospects

The cumulative *rbcL* branch length differences observed between the major lineages of filmy ferns are the result of significant differences in molecular evolutionary rate. Significance tests for the presence of rate differences and pairwise relative rate comparisons both revealed substantial disparity between the *Trichomanes* and *Hymenophyllum* clades, and suggested that the rate of evolution within *Hymenophyllum* is aberrant. The estimation of divergence times indicated that the *Hymenophyllum* clade diversified much more recently than did the *Trichomanes* clade, and the simultaneous reconstruction of ancestral rates of molecular evolution supported the notion that the rate of *rbcL* evolution slowed in the evolutionary history of *Hymenophyllum*. Thus, the extremely short branches in the *Hymenophyllum* crown are the result of a short duration combined with a slow evolutionary rate. Further analyses indicated that selection was not responsible for the decreased rate of *rbcL* evolution in *Hymenophyllum*, and instead implied that this observed slow-down might be due to a genome-wide deceleration in the rate of nucleotide substitution. If this deceleration is truly genome-wide, additional molecular markers, both in the chloroplast and in the other genomic compartments, should yield similar results to those of *rbcL* in this study. Although data are not currently available to properly address the universality of this hypothesis, additional studies to examine molecular evolution of the plastid, mitochondrial, and nuclear genomes in filmy ferns are underway. These, combined with detailed ecological, morphological, and ecophysiological studies to identify rate-influencing mechanisms, will provide even greater insight into the evolutionary history of this group of plants.

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Four representative species of Hymenophyllum. Note extremely thin leaves, only a single cell thick between the veins, with marginal sori consisting of sporangia-bearing receptacles subtended by protective, bivalved indusia. Members of this filmy fern clade show evidence of a genome-wide deceleration in nucleotide substitution rate. Photographs by Eric Schuettpelz.