

Biogeography of the *Pistia* Clade (Araceae): Based on Chloroplast and Mitochondrial DNA Sequences and Bayesian Divergence Time Inference

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Abstract.—*Pistia stratiotes* (water lettuce) and *Lemna* (duckweeds) are the only free-floating aquatic Araceae. The geographic origin and phylogenetic placement of these unrelated aroids present long-standing problems because of their highly modified reproductive structures and wide geographical distributions. We sampled chloroplast (*trnL-trnF* and *rpl20-rps12* spacers, *trnL* intron) and mitochondrial sequences (*nad1* b/c intron) for all genera implicated as close relatives of *Pistia* by morphological, restriction site, and sequencing data, and present a hypothesis about its geographic origin based on the consensus of trees obtained from the combined data, using Bayesian, maximum likelihood, parsimony, and distance analyses. Of the 14 genera closest to *Pistia*, only *Alocasia*, *Arisaema*, and *Typhonium* are species-rich, and the latter two were studied previously, facilitating the choice of representatives that span the roots of these genera. Results indicate that *Pistia* and the Seychelles endemic *Protarum sechellarum* are the basalmost branches in a grade comprising the tribes Colocasieae (*Ariopsis*, *Seudnera*, *Remusatia*, *Alocasia*, *Colocasia*), Arisaemateae (*Arisaema*, *Pinellia*), and Areae (*Arum*, *Biarum*, *Dracunculus*, *Eminium*, *Helicodiceros*, *Theriophonum*, *Typhonium*). Unexpectedly, all Areae genera are embedded in *Typhonium*, which throws new light on the geographic history of Areae. A Bayesian analysis of divergence times that explores the effects of multiple fossil and geological calibration points indicates that the *Pistia* lineage is 90 to 76 million years (my) old. The oldest fossils of the *Pistia* clade, though not *Pistia* itself, are 45-my-old leaves from Germany; the closest outgroup, *Peltandraea* (comprising a few species in Florida, the Mediterranean, and Madagascar), is known from 60-my-old leaves from Europe, Kazakhstan, North Dakota, and Tennessee. Based on the geographic ranges of close relatives, *Pistia* likely originated in the Tethys region, with *Protarum* then surviving on the Seychelles, which became isolated from Madagascar and India in the Late Cretaceous (85 my ago). *Pistia* and *Protarum* provide striking examples of ancient lineages that appear to have survived in unique or isolated habitats. [Araceae; biogeography; chloroplast DNA; Bayesian divergence time estimation; mitochondrial DNA; phylogeny; *Pistia*.]

Pistia stratiotes, the water lettuce, and *Lemna*, the duckweeds, are the only free-floating aquatics among the otherwise terrestrial or epiphytic Araceae, some 3300 species in about 100 genera (Mayo et al., 1997). *Pistia* occurs in stagnant or slow-moving fresh water bodies in the Americas (North Carolina to Argentina), Africa (Egypt to the Cape), India, and Southeast Asia to northeastern Australia. The fast-multiplying leaf rosettes of water lettuce rapidly cover large surfaces, with concomitant biochemical, physical, and economic impacts, making it one of the World's worst weeds. This is reflected in over 1200 references on *Pistia* in the database of the Center for Aquatic and Invasive Plants (<http://aquat1.ifas.ufl.edu/>).

The phylogenetic placements of *Lemna* and *Pistia* have been difficult to deduce from morphology because of their much-condensed vegetative and reproductive structures (Buzgo, 1994; Mayo et al., 1997; Stockey et al., 1997; Lemon and Poslusznay, 2000). Their uniquely shared free-floating habit has resulted in numerous comparisons of the two (starting with Engler's groundbreaking morphological study [translated by Ray and Renner, 1990]; Stockey et al., 1997; Les et al., 2002, for an historical overview). However, restriction site data and se-

quencing data have shown that duckweeds are not close to *Pistia* (French et al., 1995; Renner and Weerasooriya, 2002). Rather, they appear to diverge near the base of Araceae. The relationships of *Pistia* also first became clear in the restriction site study of French et al. (1995) who sampled 87 of the family's genera and found that *Pistia* formed a clade with 14 taxa from the tribes Araceae, Ariopsidae, Arisaemateae, Colocasieae, and Pinellieae (sensu Grayum [1990]; our Table 1; the French et al. study did not include *Dracunculus*, *Eminium*, *Protarum*, which are first sampled here). This clade was among the best supported in their data set, and we henceforth refer to it as the *Pistia* clade. Well-known members are *Arisaema* (jack-in-the-pulpit, green dragon), *Arum*, and the food and ornamental plant *Colocasia esculenta* (elephant's ear or taro).

The fossil record (below) and geographic ranges (Table 1, Fig. 1) of the genera in the *Pistia* clade suggest a long history of diversification. They occur in a wide range of habitats, including many in the temperate zone, which is striking in a family that is otherwise almost restricted to warm and humid climates. Examples are *Arisaema*, *Arum*, and *Pinellia* with dozens of cold-resistant species that occur high latitudes or altitudes, for example, *Arum* in northern Europe, many *Arisaema* species in northern China, *A. ruwenzoricum* on the Ruwenzori at 3200 m, and *A. dilatatum*, *A. elephas*, and *A. propinquum* in the Himalayas well above 4000 m. Four (out of 320) species of the mainly Southeast Asian *Pistia* clade occur in North America, viz. *Pistia stratiotes*,

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TABLE 1. Taxonomic assignments of genera studied here. Geographic ranges are from Mayo et al. (1997).

Genus (no. of species)	Engler (1920)	Grayum (1990)	Mayo, Bogner, Boyce (1997)	Geographic range
Ingroup				
<i>Alocasia</i> (60)	Colocasieae/Colocasioideae	Colocasieae/Colocasioideae	Colocasieae/Aroideae	Tropical Asia
<i>Ariopsis</i> (2)	Ariopsidae/Colocasioideae	Ariopsidae/Aroideae	Colocasieae/Aroideae	India (Western Ghats, Assam, Sikkim), Bhutan, Nepal, Myanmar
<i>Arisaema</i> (150)	Areae/Aroideae	Arisaemateae/Aroideae	Arisaemateae/Aroideae	Tropical and subtropical Asia, East Africa (6-7 spp.), N. Am. (3 spp.)
<i>Arum</i> (25)	Areae/Aroideae	Areae/Aroideae	Areae/Aroideae	Himalaya to Great Britain and Norway
<i>Biarum</i> (22)	Areae/Aroideae	Areae/Aroideae	Areae/Aroideae	Portugal, Morocco to Afghanistan
<i>Colocasia</i> (8)	Colocasieae/Colocasioideae	Colocasieae/Colocasioideae	Colocasieae/Aroideae	Tropical Asia
<i>Dracunculus</i> (2)	Areae/Aroideae	Areae/Aroideae	Areae/Aroideae	Balkans, the Aegean Islands SW Turkey
<i>Eminium</i> (7)	Areae/Aroideae	Areae/Aroideae	Areae/Aroideae	Turkey to Central Asia, N Egypt
<i>Helicodicerus</i> (1)	Areae/Aroideae	Areae/Aroideae	Areae/Aroideae	Corsica, Sardinia, Minorca, Mallorca
<i>Pinellia</i> (6)	Areae/Aroideae	Pinellieae/Aroideae	Arisaemateae/Aroideae	Temperate East Asia
<i>Pistia</i> (1)	Pistieae/Pistioideae	Pistieae/Aroideae	Pistieae/Aroideae	Pantropical
<i>Protarea</i> (1)	Protareae/Aroideae	Colocasieae/Colocasioideae	Colocasieae/Aroideae	Seychelles
<i>Remusatia</i> (4)	Colocasieae/Colocasioideae	Colocasieae/Colocasioideae	Colocasieae/Aroideae	East Asia, N. Australia, Oman, Africa, Madagascar
<i>Steuðnera</i> (8)	Colocasieae/Colocasioideae	Colocasieae/Colocasioideae	Colocasieae/Aroideae	India, Myanmar, Indochina, S. China
<i>Theriophonum</i> (5)	Areae/Aroideae	Areae/Aroideae	Areae/Aroideae	South India, Sri Lanka
<i>Typhonium</i> (50) (including <i>Sauromatum</i>)	Areae/Aroideae	Areae/Aroideae	Areae/Aroideae	Tropical Africa, Yemen, East Asia, Southeast Australia
Outgroups				
<i>Caladium</i> (8)	Colocasieae/Colocasioideae	Caladieae/Colocasioideae	Caladieae/Aroideae	Neotropics
<i>Peltandra</i> (2)	Peltandreae/Philodendr	Peltandreae/Calloideae	Peltandreae/Aroideae	Eastern North America
<i>Typhonodorum</i> (1)	Typhonodorea/Philodendr	Peltandreae/Calloideae	Peltandreae/Aroideae	Comores, Madagascar, Mauritius; Pemba Is., Zanzibar
<i>Xanthosoma</i> (57)	Colocasieae/Colocasioideae	Caladieae/Colocasioideae	Caladieae/Aroideae	Neotropics

Arisaema dracontium, *A. triphyllum*, and the Mexican *A. macrospatum*. Other clade members, such as *Biarum* and *Eminium*, occur in semiarid areas in central Asia and North Africa (*Biarum* also in Southern Europe), and *E. spiculatum* is the only Araceae occurring in a desert, the Negev. In terms of numbers of species, the *Pistia* clade is centered in Eurasia, India, and Malesia, with relatively few species in Africa and just four in the New World. As shown in Table 1, with the exception of *Arisaema* (150 species), *Alocasia* (60 species), and *Typhonium* (50 species), the genera are species-poor; *Typhonium* is highly paraphyletic (Results) and its species numbers are therefore unclear.

The oldest fossils representing the *Pistia* clade are Middle Eocene leaf impressions from Messel in Germany that closely match Colocasieae (*Caladiosoma messelense*; Wilde et al., in press). *Pistia* itself is first known from seeds from the Late Oligocene/Early Miocene and mid-Miocene of Europe and Russia (Dorofeev, 1955, 1958, 1963; Mai and Walter, 1983; Friis, 1985; Kvacek, 1998). Leaf impressions from the late Cretaceous and early Paleocene of Alberta and Wyoming described as *Pistia corrugata* Lesq. have a venation quite unlike that of *Pistia*, and their familial placement is still unclear (J. Bogner, personal communication, Jan. 2003). By contrast, *Arisaema* infructescences

from the mid-Miocene (18 to 16 million years [my]) Latah Formation near Spokane (Knowlton, 1926) closely match extant North American *A. triphyllum* in the diameter, shape, and striation of the peduncle.

Here we use chloroplast (cp) and mitochondrial (mt) sequences from four markers to infer the phylogenetic relationships in the *Pistia* clade, and we use genetic branch lengths and fossils to infer the relative ages of major subclades. Of particular interest to us were (1) the likely time and region of origin of *Pistia*; (2) the age of Areae, for which a Gondwanan origin has been hypothesized (Riedl, 1980; Hay, 1992, 1993); and (3) the age and likely pathway(s) of the north-temperate disjunctions in *Arisaema*. Because the combined data violate the assumption of a strict molecular clock, we used Bayesian methods that do not rely on a clock for divergence time estimation (Thorne and Kishino, 2002; Yang and Yoder, 2003). As implemented in Thorne's software program, the approach permits multiple simultaneous calibration time windows, with upper and lower bounds set by fossils or geologic events. The resulting divergence time estimates, together with the clade's full fossil record, permit inference of the geographic waxing and waning of the lineages around the, today, pantropical *Pistia striatotes*.

TABLE 2. Species sequenced plus their status as generic types where applicable, plant sources, and GenBank accession numbers.

Species	Source	<i>TrnL</i> intron	<i>TrnL-trnF</i> spacer	<i>Rpl20-rps12</i> spacer	<i>Nad1</i> b/c intron
Ingroup					
<i>Alocasia cucullata</i> (Lour.) G. Don	MO acc. 751658	AY248983	AY248945	AY248908	AY243116
Genus type					
<i>Alocasia gageana</i> Engl. & K. Krause	MO acc. 78364	AY248984	AY248946	AY248909	
<i>Ariopsis peltata</i> J. Graham	J. Murata s.n., 16 Oct. 2001	AY248985	AY248947	AY248910	AY243120
Genus type					
<i>Arisaema amurense</i> Maxim.	J. Bogner, 18 Jul. 2001, BG Munich	AY248986	AY248948	AY248911	
<i>Arisaema aridum</i> H. Li	G. Gusman 92121	AY248987	AY248949	AY248912	AY243113
[<i>A. yunnanense</i> (H. Li) Gusman]					
<i>Arisaema ciliatum</i> H. Li	G. Gusman 92118a	AY248988	AY248950	AY248913	
<i>Arisaema dracontium</i> (L.) Schott	T. Barkman 352 (WMU)	AY248989	AY248951	AY248914	
<i>Arisaema flavum</i> (Forssk.) Schott	Hettterscheid s.n., 27.07.2001				AY243114
ssp. undetermined					
<i>A. flavum</i> ssp. <i>abbreviatum</i> (Schott)	J. Murata s.n., 02.2003	AY388618	AY388619	AY388620	
Murata					
<i>A. flavum</i> ssp. <i>flavum</i>	Kew 1983-5842: Chase 16880 (K)	AY376842	AY376843	AY376841	
Section type					
<i>A. flavum</i> ssp. <i>tibeticum</i> J. Murata	A. M. Chambers s.n., 1.6.02	AY279123	AY275601	AY279150	
<i>Arisaema heterophyllum</i> Blume	G. Gusman 92100	AY248991	AY248953	AY248916	
<i>Arisaema macrospatum</i> Benth.	G. Gusman 97229	AY248992	AY248954	AY248917	
<i>Arisaema polyphyllum</i> (Blanco) Merr.	J. Murata 30	AY248993	AY248955	AY248918	
<i>Arisaema rhizomatum</i> C.E.C. Fisher	B. Chen 06 (MO)	AY248994	AY248956	AY248919	
<i>Arisaema tortuosum</i> (Wall.) Schott	W. Hettterscheid 27 Jul 2002	AY248995	AY248957	AY248920	AY243115
<i>Arisaema triphyllum</i> (L.) Torr.	T. Barkman 351 (WMU)	AY248996	AY248958	AY248921	
<i>Arum italicum</i> Mill.	BG Mainz, 20 Jul. 2001	AY248997	AY248959	AY248922	AY243121
<i>Biarum davisii</i> Turrill	MO acc. 78231	AY248998	AY248960	AY248923	AY243122
<i>Biarum tenuifolium</i> (L.) Schott	BG Bonn 16014	AY248999	AY248961	AY248924	
Genus type					
<i>Colocasia gigantea</i> (Blume) Hook. f.	T. Croat & Dzu 78014 (MO)	AY249000	AY248962	AY248925	AY243117
<i>Dracunculus canariensis</i> Kunth	BG Bonn 13049	AY249001	AY248963	AY248926	AY243123
<i>Dracunculus vulgaris</i> Schott	T. Croat 78286 (MO)	AY249002	AY248964	AY248927	
Genus type					
<i>Eminium spiculatum</i> (Blume) Schott	BG Bonn 15031	AY249003	AY248965	AY248928	AY243124
Genus type					
<i>Helicodicerus muscivorus</i> (L. f.) Engl.	MO acc. 71821	AY249004	AY248966	AY248929	AY243125
Genus type					
<i>Pinellia cordata</i> N. E. Brown	J. McClements s.n., 30 Jul. 2001	AY249005	AY248967	AY248930	AY243111
<i>Pinellia ternata</i> (Thunb.) Breit.	J. McClements s.n., 30 Jul. 2001	AY249006	AY248968	AY248931	AY243112
Genus type					
<i>Pistia stratiotes</i> L. Genus type	J. Bogner, 18 Jul. 2001, BG Munich	AY249007	AY248969	AY248932	AY243126
<i>Protarum sechellarum</i> Engl.	J. Bogner 2545 (M)	AY249008	AY248970	AY248933	AY243127
Genus type					
<i>Remusatia vivipara</i> (Lodd.) Schott	MO acc. 69705b	AY249009	AY248971	AY248934	AY243118
Genus type					
<i>Stuednera colocasiifolia</i> K. Koch	T. Croat & Dzu 77954 (MO)	AY249010	AY248972	AY248935	AY243119
Genus type					
<i>Therophonum dalzielii</i> Schott	J. Murata s.n., 21 Aug. 2002	AY249011	AY248973	AY248936	AY243128
<i>Typhonium albidinervum</i> Tang & Li	J. Murata 1	AY249012	AY248974	AY248937	AY243129
<i>Typhonium giganteum</i> Engl.	J. W. Waddick s.n., 20 Aug. 2001	AY249013	AY248975	AY248938	AY243130
<i>Typhonium hirsutum</i> (S. Y. Hu)	W. Hettterscheid H.AR 036	AY249014	AY248976	AY248939	
Murata & Mayo					
<i>Typhonium horsfieldii</i> (Miq.) Steenis	J. Murata 4	AY249015	AY248977	AY248940	
<i>Typhonium trilobatum</i> (L.) Schott	J. Murata 5	AY249016	AY248978	AY248941	AY243131
Genus type					
Outgroups					
<i>Caladium bicolor</i> (Aiton) Vent.	T. Croat 60868 (MO)	AY249018	AY248980	AY248943	AY243134
Genus type					
<i>Peltandra virginica</i> Raf.	J. Bogner 2119 (M)	AY249017	AY248979	AY248942	AY243132
Genus type					
<i>Typhonodorum lindleyanum</i> Schott	J. Bogner s.n. (M)	AY249019	AY248981		Incomplete
Genus type					
<i>Xanthosoma sagittifolium</i> (L.) Schott & Endl.	MO acc. 850652b	AY249020	AY248982	AY248944	AY243133
Genus type					

them close to *Pistia*; instead they group with Peltandreae (French et al., 1997; G. Salazar, Royal Botanic Gardens, Kew, personal communication, Oct. 2001). To facilitate future decisions about the allocation of taxonomic names, we made an effort to include type species of genera and sections (Table 2). For a related project, 82 (out of 150) taxa of *Arisaema*, all six species of *Pinellia*, and six additional species of *Typhonium* have been sequenced, and the representation of these genera here is based on the results of that study (Renner, Zhang, and Murata, 2004).

To select appropriate outgroups, we sequenced the *trnL* region in species from nine genera variously close to *Pistia* in the French et al. (1995) restriction site-based tree and the Mayo et al. (1997) morphology-based tree, viz. *Arophyton*, *Caladium*, *Chlorospatha*, *Jasarum*, *Lemna*, *Peltandra*, *Scaphispatha*, *Syngonium*, *Typhonodorum*, and *Xanthosoma* (GenBank accessions AF521870 to AF521877 and Table 2). With the exception of the Madagascan *Typhonodorum*, all are restricted to the Americas. Based on the results, we chose genera from Peltandreae (*Peltandra*, *Typhonodorum*) and Caladieae (*Caladium*, *Xanthosoma*) to root our trees. Of the outgroups used, the more distant ones (the two Caladieae) are excluded from all divergence time estimations, where they serve only to parse substitutions among the first two descendent branches.

DNA Isolation, Amplification, Sequencing, and Alignment

Total genomic DNA was isolated from silica-dried leaves using DNeasy kits (QIAGEN Inc., Valencia, CA), NucleoSpin-Plant kits (Macherey-Nagel, Düren, Germany), or the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987), with the modification that 4% CTAB was used instead of 2%, and 8 μ L of RNase were added to each sample before incubation. DNA amplification by the polymerase chain reaction (PCR) was performed according to the protocol described in Zhang and Renner (2003). To amplify the chloroplast *trnL* intron and adjacent spacer before the *trnF* gene, we used the universal primers c, d, e, and f of Taberlet et al. (1991). The chloroplast *rpl20-5'-rps12* intergenic spacer between the ribosomal protein genes S12 and L20 was sequenced using primers 'rpl20' and 'rps12' of Hamilton (1999). Parts of exons b and c of the mt NADH dehydrogenase gene (*nad1*) and the complete intron between them were sequenced using primers 'exon B' and 'exon C' of Demesure et al. (1995).

Amplified fragments were purified either by running the entire product on a low melting-point agarose gel and then recovering the DNA with QIAquick Gel Extraction Kits (QIAGEN) or by using QIAquick PCR Purification kits directly, without a prior gel purification step. Cycle sequencing of the purified PCR products used the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems [ABI], Norwalk, CT) according to the manufacturer's suggested protocol. The dye was removed by 2 μ L 3 mol/L NaOAc (pH 4.6) and 50 μ L ethanol precipitation, and samples were then run on the ABI 377 automated sequencer of the Department of Biology at the University of Missouri-St. Louis. Both strands were

sequenced and used to generate consensus sequences in Sequencher (ver. 4.1.2, GeneCodes Corp.).

Sequence alignment was done manually (in Sequencher) and was unproblematic except for a stretch of 218 basepairs (bp) in the *trnL* intron (Results). Introns are expected to be under different selective constraints than intergenic spacers because their secondary and tertiary structure influences their successful splicing during the editing of gene products. To incorporate information on folding is not yet possible for the chloroplast *trnL* intron, which is a group I intron of problematic secondary and tertiary structure (Besendahl et al., 2000; D. Kelchner, personal communication, 2003). By contrast, information on tertiary structure is available for the *nad1* b/c intron, the second of four introns in the *nad1* gene (Fauron et al., 1995). It is a group II intron (Michel et al., 1989, Michel and Ferat, 1995), the group of introns capable of self-splicing, which usually involves open reading frames. Group II introns are characterized by a uniform structure of six major domains radiating from a central wheel, and their secondary and tertiary structure are under considerable stabilizing selection, with only a few sites free to mutate more frequently. To find and align stem regions, we compared the Araceae *nad1* b/c intron sequences to an homologous sequence from *Citrullus* (watermelon, Cucurbitaceae, GenBank accession AF453650) whose secondary structure has been predicted (Michel et al., 1989) and to a data matrix of seed plant *nad1* b/c exon and intron sequences that included information on domain regions (Won and Renner, 2003).

A total of 157 new sequences were generated for this study and have been deposited in GenBank.

Phylogenetic Analyses

Parsimony, distance (minimum evolution), and maximum likelihood (ML) searches were conducted with version 4.0b.10 of PAUP* (Swofford, 2002). Bayesian analyses relied on MrBayes version 3.0b4 (Huelsenbeck and Ronquist, 2001 and Ronquist and Huelsenbeck's, 2003 on-line manual). DNA insertions or deletions that involved the majority of positions in a character row were excluded from parsimony, maximum likelihood, and Bayesian analyses.

Parsimony and minimum evolution analyses used either branch-and-bound searches or heuristic searches with 10 random taxon addition replicates, with 100 trees in memory, and TBR swapping, with the 'multiple trees' and 'steepest descent' options in effect. Maximum likelihood searches were heuristic and used the same swapping strategy. Bayesian analyses in MrBayes used one cold and three incrementally heated Markov chain Monte Carlo (mcmc) chains run for between 100,000 and 1 million cycles, with trees sampled every 100th generation, each using a random tree as a starting point and a temperature parameter value of 0.2 (the default in MrBayes). For each data set, mcmc runs were repeated twice as a safeguard against spurious results. The first 2000 to 5000 trees were discarded as burn-in, depending on when chains appeared to have become stationary, and

the remaining trees were used to construct Bayesian consensus trees. Examination of the log-likelihoods and the observed consistency between runs suggest that these burn-in periods were sufficiently long.

Models for maximum likelihood, minimum evolution, and Bayesian analyses (in MrBayes) were selected based on two approaches, pair-wise likelihood ratio tests (LRTs) of the 56 models implemented in Modeltest (Posada and Crandall, 1998) and simultaneous evaluation of the same 56 models in DT-ModSel (Minin et al., 2003). The latter uses a Bayesian information criterion approach based on decision theory to gauge the different models' performance in terms of branch-length error and degree of over-fitting. For the concatenated cp data (40 taxa), Modeltest preferred the K81 model (Kimura, 1981) plus unequal base frequencies (uf), variable substitution rates among sites (modeled as a gamma [G] distribution with shape parameter alpha) and a proportion of sites modeled as invariable (I), while DT-ModSel chose K81 + uf + G. For the concatenated cp and mt data (27 taxa), Modeltest chose HKY85 (Hasegawa, Kishino, and Yano, 1985) + G + I, while DT-ModSel chose F81 (Felsenstein, 1981) + G + I. We opted for using the simpler models.

Parameter values for both models were estimated simultaneously in PAUP and MrBayes, using a parsimony starting tree in PAUP, a random tree in MrBayes, and four rate categories. Parameter estimation in PAUP was interrupted after the likelihood score had stopped improving for at least an hour, and the estimated parameters were then used in searches that ran to completion, again using a most parsimonious tree as the starting tree. Parameter estimation in MrBayes ran for the duration of specified mcmc runs.

Apart from comparing posterior probabilities, we assessed clade support via nonparametric bootstrapping (implemented in PAUP). We did so because it has been argued that posterior probabilities may lead to overconfidence (Suzuki et al., 2002; Cummings et al., 2003) whereas bootstrapping may provide underestimates when internal character change is low and overestimates when rates of character change are high (Hillis and Bull, 1993). Bootstrap analyses under parsimony and minimum evolution used 1000 replicates, 10 random taxon addition replicates, and one tree held in memory. DeBry and Olmstead (2000) in simulations found that bootstrap values generated with one tree retained produced results indistinguishable from those obtained when all minimal trees were retained. Bootstrap analyses under maximum likelihood used 100 replicates, starting from random taxon addition trees, holding one tree in memory, and without branch swapping (the 'fast bootstrap' option in PAUP).

Divergence Time Estimation

For divergence time estimation, we used the more variable cpDNA data and slightly denser taxon sampling (36 ingroup species) because we wanted to include a fossil of *Arisaema* as one of three available fossil calibration

points for the *Pistia* clade. The mitochondrial *nad1* b/c intron locus provides no information within *Arisaema*.

As assessed by LRTs, substitutions in the 40-taxon cpDNA data sets (individually or combined) could not be modeled as clocklike. We therefore used a Bayesian approach that does not assume a strict clock and that allows the simultaneous use of different models for data partitions as well a multiple calibration windows (Thorne, Kishino, and Painter, 1998; Thorne and Kishino, 2002). The approach is based on the assumption that simultaneous analysis of several gene loci (where these can safely be assumed to share a common set of divergence times) with multiple calibrations will overcome not only the often weak signal in single data sets but also violations of the clock in each of the individual partitions (Thorne and Kishino, 2002; Yang and Yoder, 2003). Thorne's 'multidivtime' program, freely available from his web page, uses an mcmc approach to approximate prior and posterior probabilities. We used the 'baseml' program of PAML ver. 3.14 (Yang, 1997) and the F84 + G model (with five rate categories) to estimate nucleotide substitution models for each cpDNA partition and for the concatenated cpDNA data and then used Thorne's 'paml2modelinf' program to convert the paml output into model files acceptable for 'estbranches' (part of Thorne's program package, below). The topologies used for baseml and estbranches were ones found in heuristic searches under parsimony optimality from the concatenated cpDNA data, with or without polytomies; the final estbranches run included polytomies. The F84 + G model accounts for a transition/transversion rate bias and unequal base compositions plus rate heterogeneity among sites. It is the most parameter-rich model so far implemented in estbranches, but based on our model comparisons in ModelTest and DT-ModSel (above), it should fit our data well. Estbranches estimates branch lengths of the specified evolutionary tree and it also estimates the variance-covariance structure of the branch length estimates. Approximating the prior and posterior distributions involves the following data-dependent settings in the multidivtime control file: (1) Number of genes to be analyzed and name of respective branch length data file obtained from estbranches (in our case, up to three loci); (2) length, sampling frequency, and burn-in period of the Markov chain (in our case, 1 million cycles, sampled every 100th cycle and with a burn-in of 100,000 cycles); (3) a priori expected number of time units between tip and root (in our case, 1, because we set the time unit to 100 my); (4) standard deviation of the prior for the time between tips and root, which is recommended to equal the number of time units between tips and root; (5) rate at the root node, which is calculated by taking the mean distance between the ingroup root and the ingroup tips obtained from estbranches divided by the time unit (which for the concatenated cpDNA data resulted in a prior rate of 0.009). The prior for the Brownian motion parameter ν , which determines the permitted rate change between ancestral and descendant nodes, was set to 1 following the manual's recommendation that the time units between root and tips to the power of ν be about 1. The standard

deviation on ν was also set to 1. As recommended, we repeated each analysis twice to assure that Markov chains were long enough to converge.

The multidivtime control file also requires setting number and kind (upper or lower) of constraints on node times. We used three lower bounds provided by fossils: (1) The closest outgroup, Peltandreae, is first known 60-my-old leaves from Europe, Kazakhstan, North Dakota, and Tennessee, which provided a lower bound of 60 my for node 1 in Figure 3 (for references see Introduction and Discussion). (2) Middle Eocene leaf impressions from central Germany (*Caladiosoma messelense*) that closely match modern Colocasieae such as *Alocasia guttata* provided a lower bound of 45 my for node 2 in Figure 3. (3) A 16- to 18-my-old fossil of *Arisaema* cf. *triphyllum* provided a lower bound of 18 my for node 3 in Figure 3. We also explored using a fourth and fifth constraint, viz. an upper bound of 85 my for the age of *Protarum sechellarum* based on the age of the Seychelles archipelago (Braithwaite, 1984) and an upper bound of 100 my for the age of *Pistia* clade, based on the oldest fossil record of Arales (105.5; Herendeen and Crane, 1995). Constraining the *Pistia* clade root to 100 my may be further justified if the angiosperms are indeed only around 141 to 132 my old, as suggested by their earliest fossils (Brenner, 1996; Hughes, 1994).

RESULTS

Mitochondrial and chloroplast sequences were obtained for 23 members of the *Pistia* clade and four outgroups. In addition, 13 ingroup taxa were sampled just for the chloroplast loci (Table 2). Final alignments (available from the Systematic Biology Web site) comprised 726 bp from the *trnL* intron, 479 bp from the *trnL-trnF* spacer, 871 bp from the *rpl20-rps12* spacer, and 1394 bp from the *nad1* b/c intron. Excluded from all analyses were a 223-bp section of repeated TA motifs in the *trnL* intron, three poly-A runs (together 19 bp) in the *trnL* intron, a 5-bp-long poly-T run in the *trnL-F* spacer, two poly-A runs (together 67 bp) and a poly-T run (10 bp) in the *rpl20-rps12* spacer, and one poly-T run (4 bp) in the *nad1* intron.

Chi-square tests of homogeneity of base frequencies across taxa were run in PAUP for (1) the 25-taxon mtDNA data, excluding missing or ambiguous sites and using just the 36 informative sites (chi-square = 39.80, df = 78, $P = 0.9999$), and (2) the 40-taxon concatenated cpDNA data, using just the 127 informative sites (chi-square = 35.06, df = 117, $P = 1$). Neither test revealed nucleotide bias among taxa. Although rather extreme amounts of bias seem necessary for parsimony to prefer an incorrect tree (Conant and Lewis, 2001), this has only been tested in the four-taxon case.

In terms of phylogenetic signal, the *trnL* intron data for the 27 taxa contained 12 (3%) potentially parsimony-informative sites in the ingroup plus outgroup matrix (8 just for the ingroup), the *trnL-trnF* spacer 23 (6%) informative sites (15 just for the ingroup), the *rpl20-rps12* spacer 38 (5%) informative sites (28 just for the ingroup),

and the mitochondrial *nad1* b/c intron (for 25 taxa) 32 (2%) informative sites (19 just for the ingroup). The *trnL* intron (excluding the 58 gapped characters) yielded 200 equally parsimonious trees (CI = 0.93, RI = 0.90), the *trnL-trnF* spacer (excluding 110 gapped characters) 160 (CI = 0.92, RI = 0.88), the *rpl20-rps12* spacer (excluding 72 gapped or ambiguous characters) 95 (CI = 0.86, RI = 0.86), and the mitochondrial data (excluding 99 gapped and 19 ambiguous characters) 3 (CI = 0.83, RI = 0.89). Topologies resulting from the individual datasets contained no well-supported conflicting nodes (with support >60% for contradictory nodes), and data sets were therefore combined. The concatenated data yielded 28 equally parsimonious trees (CI = 0.86, RI = 0.85).

Bayesian analyses of the combined chloroplast and mitochondrial sequences for the *Pistia* clade yielded a relatively well-supported topology (Fig. 2 shows posterior probabilities and bootstrap values under maximum likelihood, minimum evolution, and parsimony). It comprises a basal tritomy of *Pistia*, the monotypic Seychelles endemic *Protarum*, and all remaining ingroup genera. The latter fall into a grade of Colocasieae (see Table 1 and Fig. 2 for tribal assignments of genera), followed by a tritomy of *Areae*, *Arisaema*, and *Pinellia*. The *Areae* genera *Arum*, *Biarum*, *Dracunculus*, *Eminium*, *Helicodiceros*, and *Therophonum* are all embedded in *Typhonium*.

Using estbranches (part of Thorne's multidivtime software package), we compared information content among the cpDNA partitions. Estbranches estimates branch lengths on the prespecified topology and it also estimates the variance-covariance structure of the branch length estimates. Where the variances are mostly 0, there is insufficient signal to confidently estimate parameters. Because this was the case for the individual partitions, we followed a recommendation to use the concatenated data (J. Thorne, personal communication), which yielded a variance-covariance matrix that contained many fewer 0 variances.

Results of the Bayesian divergence time estimation using the concatenated cpDNA data are shown in Figure 3 and Table 3, which also shows calibration points used. The absolute age of *Pistia* must lie somewhere between that of node 4 and node A, that is, between 90 and 85 my (for confidence intervals see Table 3). Whether or not the age of the Seychelles was used as an upper bound for the age of the endemic *Protarum sechellarum* made little difference for the age of the tritomy of which *Protarum* is part (Table 3, columns 5 versus 6: 89.5 versus 80.3 my). By contrast, results obtained with three minimal age constraints fairly 'high' in the tree (nodes 1 to 3 in Fig. 3) differed greatly from results that relied on upper bounds at or near the root (whether from the Seychelles archipelago [node 4] or the oldest Arales fossils [root node]; see Table 3 columns 4 versus 5 and 6).

The initial diversification of *Areae* (node B in Fig. 3) appears to have occurred between the Upper Cretaceous and the Eocene (Table 3), and the *Arum* clade (node C), which is embedded in the Malesian-centered *Typhonium*, likely diversified sometime in the Miocene. As found with denser species sampling (Renner, Zhang,

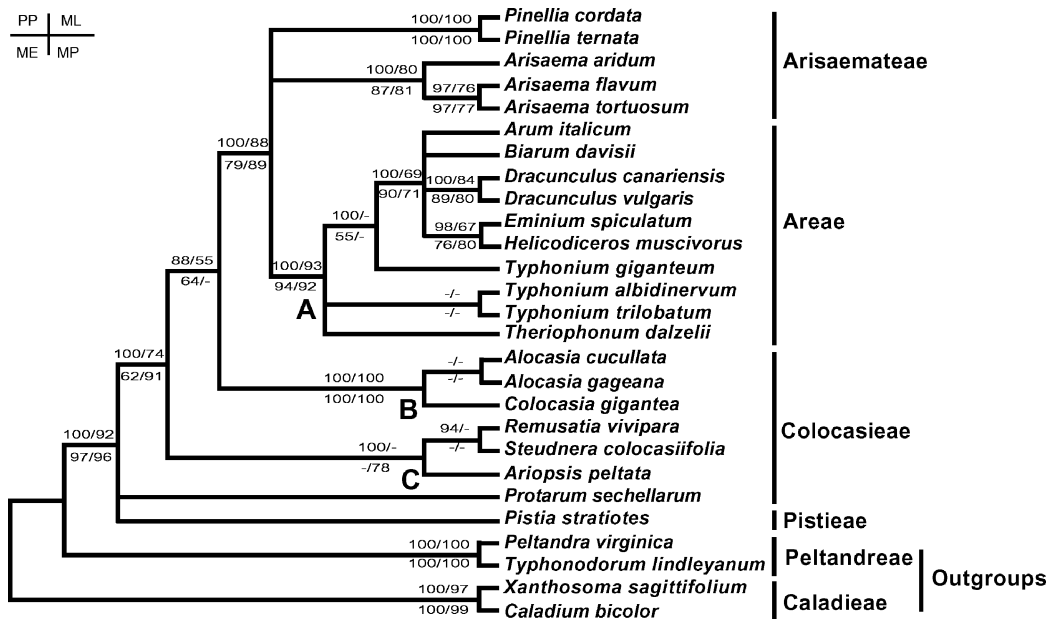


FIGURE 2. Phylogeny of the *Pistia* clade obtained from combined cp and mtDNA data under the F81 + G + I model using Bayesian inference. Above branches: Posterior probabilities (PP) followed by bootstrap support under maximum likelihood (ML); below branches: Bootstrap support under minimum evolution (ME) followed by that under parsimony (MP). Under ME, a sister group relationship between *Pistia* and *Protarum* receives 99% bootstrap support. Tribes are those of Mayo, Bogner, and Boyce (1997), and lettered nodes A to C refer to nodes in Figure 1.

and Murata, 2004), the three North American species of *Arisaema*, *A. dracontium*, *A. macrospatum*, and *A. triphyllum*, appear to stem from independent entries, one around 40 my, the other 24 my ago (Table 3), but there is overlap in the 95% credibility intervals.

DISCUSSION

Pistia is part of a clade of 15 genera that includes *Arisaema*, *Pinellia*, all Areae, and several Colocasieae (Fig. 2). This result solves a long-standing question in the understanding and classification of Araceae, where the morphological distinctness of *Pistia stratiotes* had led to it being accord the rank of a subfamily or tribe by itself because its relationships were obscure (Engler, 1920; Grayum, 1990; Bogner and Nicolson, 1991; Mayo et al., 1997). Although the restriction site data of French et al. (1995) hinted at the closeness of *Pistia* to members of Colocasieae, Areae, and Arisaemateae, these workers did not include *Dracunculus*, *Eminium*, and *Protarum*. *Dracun-*

culus and *Eminium* have always been interpreted as members of Areae, a set of genera close to *Arum* (Engler, 1920; Grayum, 1990: 682; our Table 1), and this is supported by our data. The other genus not sampled by French et al., *Protarum*, turned out to be key for the understanding of the evolution of the *Pistia* clade. *Protarum* consists of a single species endemic to the Seychelles and is the only Araceae occurring on these islands. It is not thought adapted to long-distance dispersal (Grayum, 1990). An amplified fragment length polymorphisms (AFLP) study of 11 species from five genera of Caladieae and Colocasieae, also found that *Protarum* was highly distinct and suggested that it might be 'ancestral to both New and Old World genera of Caladieae' (Loh et al., 2000). In agreement with its genetic and morphological distinctness (Grayum, 1990; Mayo et al., 1997), *Protarum sechellarum* appears to be an ancient lineage, perhaps as old as 90 my (according to an estimate that did not include the age of the Seychelles as a possible calibration point; Table 3, column 4). The Seychelles are granite islands in

TABLE 3. Bayesian estimates of divergence times (my), including 95% credibility intervals. Results in the fourth column were obtained by placing minimum age on nodes 1 to 3; those in the fifth column in addition relied on an upper bound of 100 my for the root, based on oldest fossils of Arales; those in the sixth column placed an upper bound of 85 my on node 4, based on the age of the Seychelles archipelago.

Node in Figure 3	Clade	Minimum (Mi) or Maximum (Ma) age	Constraints on nodes 1–3	Constraints on nodes 1–3 and root	Constraints on nodes 1–4 and root
1	Peltandreae	Mi = 60	112 (61.9, 234)	70.0 (60.2, 82.6)	65.7 (60.2, 79.5)
2	<i>Alocasia/Colocasia</i>	Mi = 45	71.9 (45.7, 145)	50.2 (45.2, 62.5)	50.3 (45.2, 62.6)
3	<i>Arisaema triphyllum</i>	Mi = 18	39.1 (18.7, 89.7)	24.0 (18.2, 38.4)	22.7 (18.2, 34.3)
4	<i>Protarum sechellarum</i>	Ma = 85	238 (128, 441)	89.5 (76.0, 98.4)	80.3 (70, 84.8)
A	<i>Pistia/Protarum/rest of ingroup</i>		216 (115, 405)	84.8 (70.6, 95.9)	76 (64.6, 83.5)
B	Areae		161 (81.2, 307)	65.9 (47.0, 83.4)	59.2 (42.9, 73.7)
C	<i>Arum</i>		64.1 (22.2, 142)	26.6 (10.7, 46.8)	23.9 (9.8, 41.4)
D	<i>Arisaema dracontium/A. macrospatum</i>		91.3 (37.2, 188)	39.7 (47.0, 83.4)	22.9 (18.2, 34.3)

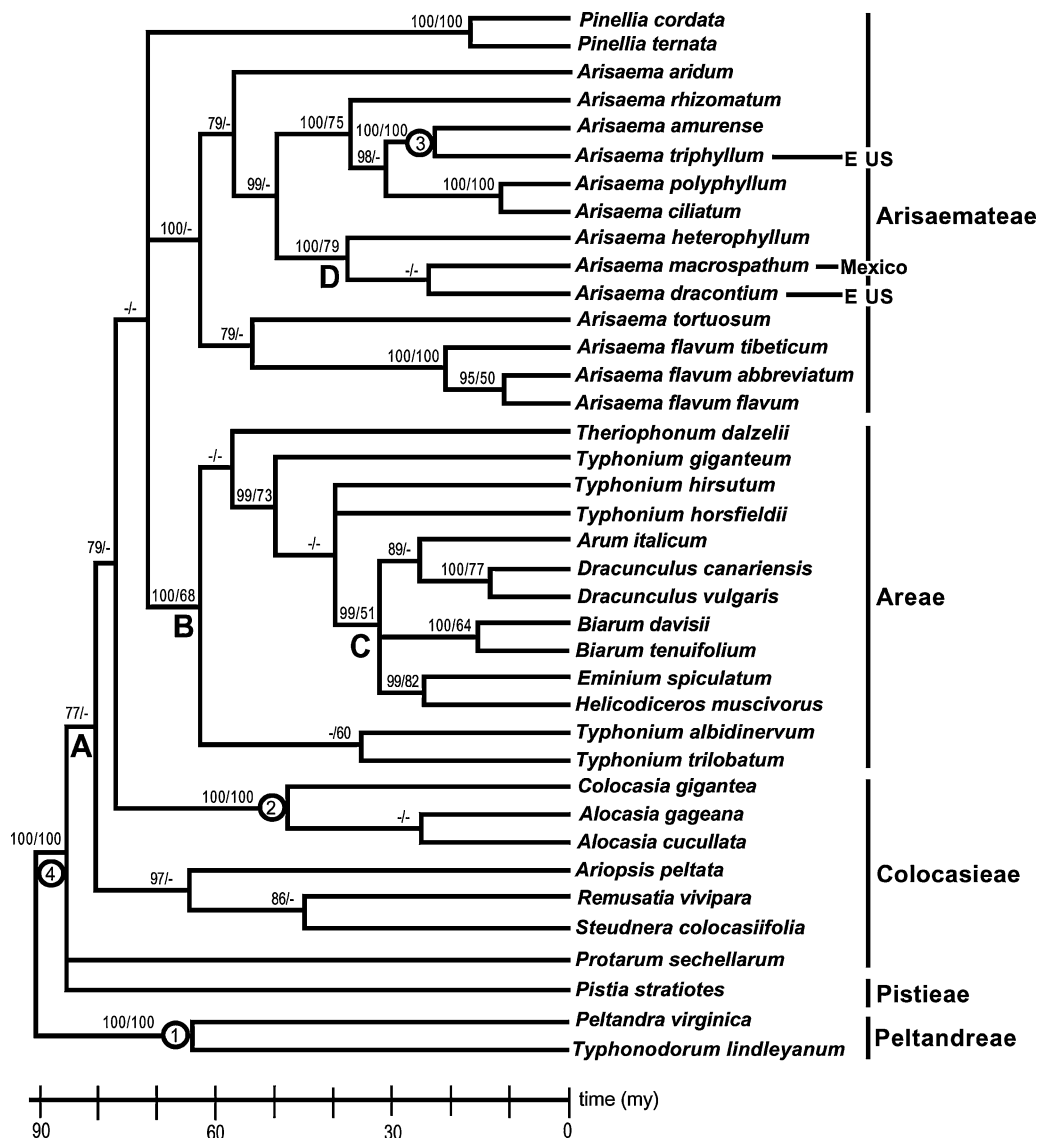


FIGURE 3. Branch lengths obtained by Bayesian divergence time estimation, using five calibrations (see Table 3). Support values at branches are posterior probabilities, followed by bootstrap percentages ($\geq 50\%$) obtained under minimum evolution (ME) with K81 + G distances. Under ME, a sister group relationship between *Pistia* and *Protarum* receives 89% bootstrap support. The tree is rooted with Caladieae as in Figures 1 and 2. Numbered nodes refer to the following minimal (Mi) or maximal (Ma) constraints based on fossils or a geologic event (not used in all analyses; see Table 3): (1) Peltandreae: Mi = 60 my; (2) Colocasieae: Mi = 45 my; (3) *Arisaema* cf. *triphillum*: Mi = 18 my; (4) age of the Seychelles archipelago: Ma = 85 my. A to D are nodes of interest discussed in the text.

the western Indian Ocean that became separated from northern Madagascar and the western coast of India in the Late Cretaceous, at least 85 my ago (Braithwaite, 1984). Another relatively basal branch in the *Pistia* clade comprises *Remusatia*/*Stuednera*/*Ariopsis*, which occur in Africa/Madagascar/India/Indochina (see maps in Fig. 1), probably partly reflecting ancient disjunctions (Mayo, 1993) and partly the fact that *Remusatia vivipara* is bird-dispersed.

The presence of one of the oldest surviving members of the *Pistia* clade on the Seychelles may point to the opening eastern Tethys as the place of early diversification of the entire group. The clade's closest relatives, the Peltandreae, comprise *Typhonodorum*, with a single species

native to Madagascar (introduced and naturalized in Mauritius, the Comores, and eastern Tanzania), *Peltandra*, with two species in subtropical to warm-temperate eastern North America, and the small Mediterranean genera *Ambrosina* and *Arisarum*. Fossil leaves of Peltandreae from the Late Paleocene and Eocene of North Bohemia, Kazakhstan, the Golden Valley Formation in North Dakota, and the Claiborne Formation in Tennessee (Wilde et al., in press) demonstrate a Cretaceous Laurasian range of Peltandreae.

A second unexpected finding with biogeographic implications (besides the placement of *Protarum* in the Pistia clade) is the paraphyly of *Typhonium*. Hay (1993: 346) hinted at such a possibility, based on overlapping

characteristics between *Typhonium* and various other *Areae*, but this is the first study to address his suggestion. The discovery that a monophyletic *Typhonium* must include *Arum*, *Biarum*, *Dracunculus*, *Eminium*, *Helicodiceros*, and *Theriophonium* (as well as *Sauromatum* [Hettterscheid and Boyce, 2000]) implies that *Areae* (node A in Fig. 1) range all around the former Tethys, from the Mediterranean north to Great Britain, west to the Canary islands, southeast to the Philippines, New Guinea, and northeastern Australia, and south to tropical Africa, India, and Sri Lanka (see maps in Fig. 1). Based on the vast and disjunct range of *Areae*, Riedl (1980) and Hay (1992) proposed a Gondwanan origin of the group, and this is supported by the divergence time estimates obtained here (Table 3). That *Areae* are not easily dispersed over sea is shown by their absence from Madagascar, which separated from Africa c. 165 my ago (Brown and Lomolino, 1998). *Arum* itself apparently evolved only during the Miocene.

Taken together, fossil and molecular evidence demonstrates that the *Pistia* clade goes far back into the Cretaceous and by the Eocene had become widespread in Laurasia. The clade's presence in Laurasia lasted well into the Miocene, as shown by Miocene *Arisaema* infructescences from Spokane (Knowlton, 1926) and by finds of *Pistia* seeds from Europe and Russia from the Late Oligocene to the mid-Miocene (Dorofeev, 1955, 1958, 1963; Mai and Walter, 1983; Friis, 1985; Kvacek, 1998). *Arisaema* apparently diversified early enough (see time scale in Fig. 3) for two lineages to attain trans-Beringean ranges. North American *A. triphyllum* groups with *A. amurense*, from a mainly Sino-Japanese clade (traditionally recognized as section *Pedatisecta* [Murata, 1990]), and *A. dracontium* and *A. macrospatum* group with *A. heterophyllum* from a predominantly Chinese clade (denser taxon sampling for these clades; Renner, Zhang, and Murata, 2004).

The diversification of the *Pistia* clade clearly relates to geological and climate events, as well as diverse habitats that became available at different times. A finer-scale analysis that would include more of the clade's 320 species would be required to test the proposed great role of ecological speciation in *Areae* and relatives (Riedl, 1980; Mayo et al., 1997). Although niche diversification may have accompanied net speciation in the *Pistia* clade, this clearly has not been the case in the *Pistia* lineage itself, which seems to have escaped competition by entering the unique niche of a free-floating freshwater aquatic sometime in the Late Cretaceous and to have persisted in that niche ever since.

Two caveats apply to our study. As shown by the analyses that used the programs *estbranches* and *DT-ModSel*, which in different ways gauge the amount of information in sequence data sets, the concatenated cpDNA data for the *Pistia* clade contained relatively little signal. Where many branches in a data set are short, estimation of divergence times is problematic, even when a molecular clock is assumed. Thus, there are large error margins on the branch length estimates and dates (Table 3). Second, the Bayesian approach to divergence time estimation from multiple loci (Thorne and Kishino, 2002), like other ap-

proaches to time inference that do not rely on a strict molecular clock, such as nonparametric rate smoothing (Sanderson, 1997) and penalized likelihood (Sanderson, 2002), both of which we have applied to our data (with results similar to those shown), seems especially sensitive to upper bounds placed at or near the root. Few simulation studies of the behavior of the various 'relaxed clock' approaches have been published, and it is therefore not clear whether this is a consistent effect. However, it is clear that credibility intervals will be wide unless nodes are constrained both from above and from below (J. Thorne, personal communication). Perhaps leaving a tree's base unconstrained allows the algorithms to assume very long basal branches to accommodate details higher up in the constrained part of the tree (see also Rodríguez-Trelles et al., 2002). In practice, constraining the root, that is, the earliest appearance of a clade, will often be highly problematic because of incomplete fossil records and because earliest fossils may not exhibit a particular clade's synapomorphies and thus go unrecognized.

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