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Molecular phylogenetic relationships among Lemnaceae and Araceae using the chloroplast *trn*L–*trn*F intergenic spacer

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Abstract

We test competing hypotheses of relationships among Aroids (Araceae) and duckweeds (Lemnaceae) using sequences of the *trn*L–*trn*F spacer region of the chloroplast genome. Included in the analysis were 22 aroid genera including *Pistia* and five genera of Lemnaceae including the recently segregated genus *Landoltia*. *Aponogeton* was used as an outgroup to root the tree. A data set of 522 aligned nucleotides yielded maximum parsimony and maximum likelihood trees similar to those previously derived from restriction site data. *Pistia* and the Lemnaceae are placed in two separate and well-supported clades, suggesting at least two independent origins of the floating aquatic growth form within the aroid clade. Within the Lemnaceae there is only partial support for the paradigm of sequential morphological reduction, given that *Wolffia* is sister to *Wolffiella* + *Lemna*. As in the results of the restriction site analysis, pantropical *Pistia* is placed with *Colocasia* and *Typhonium* of southeastern Asia, indicative of Old World affinities. Branch lengths leading to duckweed terminal taxa are much longer relative to other ingroup taxa (including *Pistia*), evidently as a result of higher rates of nucleotide substitutions and insertion/deletion events. Morphological reduction within the duckweeds roughly correlates with accelerated chloroplast genome evolution.
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1. Introduction

Floating aquatic duckweeds of the monocot family Lemnaceae are clearly the most highly reduced (derived?) of all flowering plants. Although the Lemnaceae have long been associated with the Araceae (see Les et al., 2002 for historical review), relationships between the families remain uncertain (Mayo et al., 1997). Cladistic analyses using either morphological characters (Stockey et al., 1997) or molecular characters (Davis, 1995; Duvall et al., 1994; French et al., 1995) all support the hypothesis that the Lemnaceae are closely related to or embedded within the Araceae. However, the results of various studies differ in the phylogenetic position of the Lemnaceae and their relationship to other aroid genera (Fig. 1). Traditional morphological studies have commonly indicated a close relationship between the floating aroid *Pistia* and the duckweeds (e.g., Arber,

1920a,b; Engler, 1877; Hegelmaier, 1868; Mayo et al., 1997; Sculthorpe, 1967). This single origin of a floating aquatic habit is supported by published molecular analyses using the chloroplast gene *rbc*L (Duvall et al., 1993; Les et al., 1997) and morphological analyses using a combination of living and fossil species (Stockey et al., 1997; Fig. 1a). Indeed, extinct floating aroids and lemnoids provide excellent evidence for a long history of the floating aquatic habit among these plants, with the Upper Cretaceous *Pistia corrugata* Lesquereux (Fig. 2a) showing marked similarities to the living *Pistia stratiotes* L. (Fig. 2d). The smaller, morphologically reduced *Limnobiophyllum scutatum* (Dawson) Krassilov (Fig. 2b) and *Limnobiophyllum expansum* (Heer) Kvaèek occur in Paleocene and Miocene deposits respectively, forming a transformational series leading to the largest living duckweed *Spirodela* Schleiden (Fig. 2c). Continued reduction through *Lemna* L. and *Wolffiella* Hegelmaier, to *Wolffia* Horkel ex Schleiden (Fig. 2e) is considered to reflect increasing levels of specialization (Les et al., 1997; Stockey et al., 1997).

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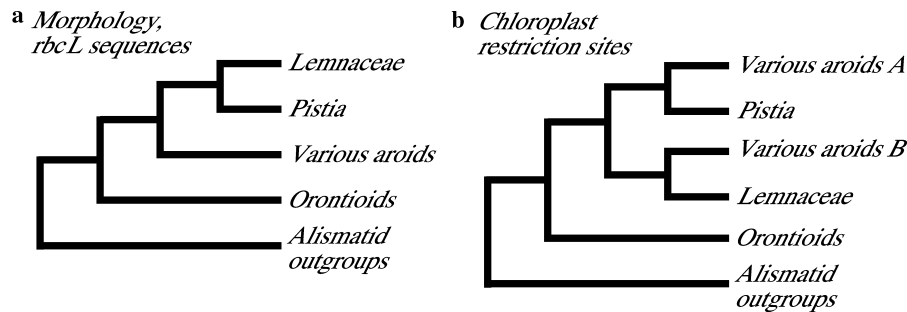*E-mail address:* rothwell@ohio.edu (G.W. Rothwell).

Fig. 1. Hypotheses of relationships among Lemnaceae, *Pistia*, and other aroids implied by previously published results of various authors. (a) Hypothesis of *Pistia* as the sister to Lemnaceae. (b) Hypothesis of much more distant relationship between *Pistia* and Lemnaceae.
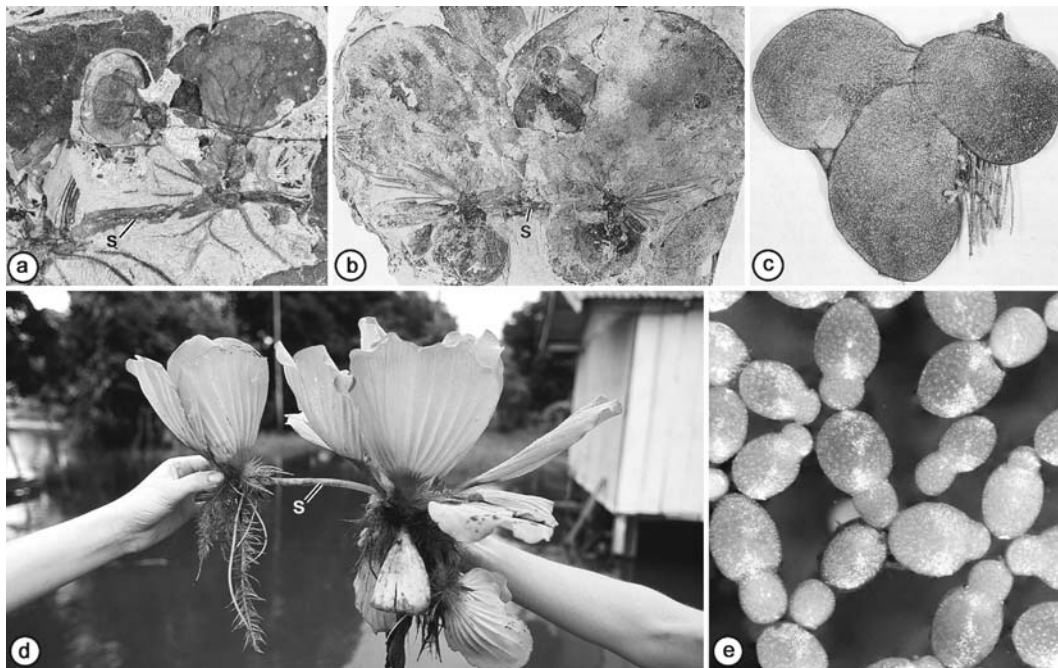

Fig. 2. Floating aquatic aroids and lemnoids showing variation in size and complexity among fossil and living species. Species that consist of plants with stems, leaves and roots are connected by stolons (s), while species with more reduced/derived morphology consist of interconnected fronds. (a) *Pistia corrugata* from 75 million year old Cretaceous deposits of Alberta, Canada. (b) *Limnobiophyllum scutatum* from 62 million year old Paleocene deposits of Alberta, Canada. (c) Living *Spirodela intermedia*. (d) Living *Pistia strateoides*. (e) Living *Wolffia brasiliensis*.

By contrast, other molecular analyses, namely those focusing on chloroplast restriction site data, remove *Pistia* and the Lemnaceae to distantly related clades and embed both within the Araceae (e.g., French et al., 1995; Mayo et al., 1997; Renner and Weerasooriya, 2002; Fig. 1b). Interestingly, the most current anatomical and palynological evidence also refutes a close relationship between the Lemnaceae and *Pistia*, suggesting instead a relatively primitive position for the Lemnaceae immediately above the basal Orontieae and sister to the remaining Araceae (Bogner, personal communication).

At least in part, these discrepancies of relationship and phylogenetic position of Lemnaceae within a broader aroid assemblage may be due to low sampling of aroid and lemnoid genera. Previously published studies supporting a close relationship of Lemnaceae and *Pistia* have included too few taxa to potentially overcome exemplar effects (i.e., too few DNA sequences used to represent major, divergent evolutionary lineages; Sytsma and Baum, 1996). In addition, the GenBank sequence for *Pistia* is inexplicably divergent at irregular points across its length, both in comparison to Lemnaceae and to other aroid genera. Its substantial divergence may be largely artifactual, and it is plausible that the apparent sister relationship of this particular *Pistia* sequence and the sole *rbc*L Lemnaceae representative in previous studies is due to "long-branch attraction" rather than a true evolutionary relationship.

To help distinguish between the competing hypotheses of affinities and phylogenetic position for the

Lemnaceae, we have sequenced a range of Araceae and Lemnaceae across both families for a moderately fast-evolving chloroplast spacer. Sequences for the *trn*L–*trn*F intergenic spacer were obtained for 35 aroid/lemnoid ingroup samples and one outgroup (*Aponogeton distachyus*, Aponogetonaceae, Alismatales), the latter family revealed by higher-level studies as one of the nearest sisters to the Araceae/Lemnaceae clade. Non-lemnoid ingroup taxa were selected from clades evenly distributed across the Araceae s. str. (Mayo et al., 1997) and comprising 11 tribes, as represented in the strict consensus tree from chloroplast restriction site analysis (French et al., 1995). Additional species of the very large genus *Anthurium* were included for species-level comparison of divergence. One or two species of four long-established genera, as well as *Landoltia punctata* (recently segregated from *Spirodela* by Les and Crawford, 1999), were included for the Lemnaceae.

Most samples were generously provided from the extensive living collections of Josef Bogner at the Munich Botanical Gardens and Thomas Croat at the Missouri Botanical Garden. The remainder were collected at the Ohio University greenhouse, from wild populations near Athens, Ohio or southeastern Missouri, or on the campus of the Universidad Nacional Autónoma de México in Mexico City, Mexico.

2. Materials and methods

2.1. DNA extraction

Samples of Lemnaceae were examined with a binocular dissecting microscope to conform single-species composition prior to DNA extraction, and identifications were made using Landolt (1986) and comparisons with specimens annotated by Landolt at the Missouri Botanical Garden herbarium. Identifications for nearly all aroids were those of J. Bogner and T. Croat; the remaining samples were identified by comparison with verified specimens annotated by these specialists. DNA extractions of the 36 samples were prepared from freshly preserved leaf tissue desiccated in silica gel (Table 1). Extractions were made using a modified SDS "mini-extraction" protocol (Edwards et al., 1991) followed by the chloroform–isoamyl alcohol extraction, alcohol precipitations and acetate salt rinses used in the standard CTAB protocol (Doyle and Doyle, 1987; Smith et al., 1991), scaled down to 1.5 ml microfuge tubes.

2.2. Amplification and sequencing of trnL–trnF spacer

The polymerase chain reaction (PCR, Mullis et al., 1986) was used to amplify the *trn*L–*trn*F spacer for sequencing using primers "E" and "F" (Taberlet et al., 1991). Reaction constituents and thermal cycler pro-gram followed those used for the Internal Transcribed Spacer nrDNA region by Ballard et al. (1999), but employing 35 rather than 30 cycles during PCR. Successful reactions were cleaned using a PCR Preps kit (Promega) and quantified with a GeneQuant II spectrophotometer (Pharmacia Biotech). Samples were cycle-sequenced with dye-terminator chemistry (Applied Biosystems) using primer "E." After ethanol–sodium acetate precipitation, products were analyzed on an ABI 310 capillary DNA Analyzer at Ohio University. Sequences have been submitted to GenBank and accession numbers are provided in Table 1. The aligned data set and trees illustrated in Figs. 3 and 4 have been submitted to TreeBASE.

2.3. Phylogenetic analysis and hypothesis testing

Sequencer trace files of *trn*L–*trn*F spacer sequences were edited in Sequencher 3.0 software (GeneCodes), then aligned using CLUSTAL X (Jeanmougin et al., 1998) with a range of incremental gap penalties from 5 to 30 specified in separate submissions. The resulting alignments proved to be identical or essentially so. Following minor manual adjustments per the strategy of Bogler and Simpson (1996), each aligned data set was subjected to preliminary maximum parsimony analysis in PAUP*, version 4.0b10 (Swofford, 2002). The data set providing the lowest number of steps and the highest consistency index (CI, Kluge and Farris, 1969) and retention index (RI, Archie, 1989; Farris, 1989) was selected for further analysis.

Phylogenetic analysis of the accepted aligned data matrix focused initially on maximum parsimony, comparing results from PAUP* on the one hand with those from Winclada beta 0.9.9 (Nixon, 1999, 2002) and Nona (Goloboff, 1999) on the other. Parsimony analysis in PAUP* was conducted on both the accepted aligned data set using nucleotide substitutions alone, and on a second data set that included binary gap codes assigned automatically by PaupGap (Cox, 1997), both with gaps as missing data. Analysis began with 100,000 random-addition replicates and NNI-swapping, saving one tree from each replicate, and ended with TBR-swapping on each replicate and saving all most-parsimonious trees. Bootstrap support (Felsenstein, 1985; Sanderson, 1989) in PAUP* was evaluated from 10,000 random-addition replicates and TBR-swapping, saving a single tree from each replicate. Parsimony jackknife analysis in PAUP*, emulating JAC resampling with 36.8% nominal deletion and "collapse = amb" under the condense trees option, also used 10,000 random-addition replicates and TBR-swapping, holding one tree per replicate. The accepted aligned data matrix was also subjected to maximum likelihood. Likelihood parameters were established using ModelTest version 3.06, and these were utilized in subsequent analysis of the entire data set with PAUP*.

Table 1
Collections used for phylogenetic analysis and GenBank accession numbers for sequence data

| Species	Family	Subfamily	Voucher	Country of origin and source	DNA #	GenBank Accession Nos.
Aglaonema rotunda N.E. Br.	Araceae	Aroideae	Croat 71742	Unknown origin	A069	AY290843
Amorphophallus glossophyllus Hett.	Araceae	Aroideae	Croat 77989	Vietnam, Ninh Binh Province	A101	AY290854
Amorphophallus variabilis Blume	Araceae	Aroideae	Croat 74114	Indonesia, Java	A001	AY290853
Amydrium humile Schott	Araceae	Monsteroideae	Croat 81406	Unknown origin	A131	AY290828
Anthurium cordifolium Kunth	Araceae	Pothoideae	Croat 81448	Jamaica, Manchester	A012	AY290831
Anthurium ochranthum K. Koch	Araceae	Pothoideae	Croat 75190	Panama, Colón	A021	AY290833
Anthurium propinquum Sodiro	Araceae	Pothoideae	Croat 80797	Colombia, Chocó	A009	AY290830
Anthurium radicans K. Koch & Haage	Araceae	Pothoideae	Croat 76139	Ecuador	A015	AY290829
Anthurium ravenii Croat & R.A. Baker	Araceae	Pothoideae	Croat 79254	Costa Rica, Puntarenas	A003	AY290835
Anthurium salgarense Croat ined.	Araceae	Pothoideae	Croat 69878	Colombia, Antioquia	A024	AY290834
Anthurium watermaliense Bailey & Nash	Araceae	Pothoideae	Croat 81449	Panama	A020	AY290832
Aponogeton distachyus L.f.	Aponogetonaceae		Stockey RS 61	S. Africa, ex Bogner coll'n, Munich Bot. Garden	437/ RS 61	AY290825
Arophyton buchetii Bogner	Araceae	Aroideae	Stockey RS 30	Madagascar, ex Bogner coll'n, Munich Bot. Garden	442/ RS 30	AY290857
Asterostigma lividum (Lodd.) Engl.	Araceae	Aroideae	Croat 79479	Brazil	A108	AY290841
Caladium picturatum K. Koch & Bouché	Araceae	Aroideae	Croat 54080	Venezuela, Bolívar	A098	AY290855
Calla palustris L.	Araceae	Calloideae	Ballard s.n. (BHO)	Washtenaw Co., MI	366	AY290837
Callopsis volkensii Engl.	Araceae	Aroideae	Croat 77245	East Africa	A092	AY290840
Colocasia esculenta (L.) Schott	Araceae	Aroideae	Rothwell & Ballard A137 (BHO)	Climatron, MO Bot. Garden	A137	AY290859
Culcasia striolata Engl.	Araceae	Aroideae	Croat 53499	Nigeria, Ogun	A096	AY290844
Cyrtosperma cuspidispathum Alderw.	Araceae	Lasoideae	Croat 81515A	Papua New Guinea	A114	AY290836
Gymnostachys anceps R. Br.	Araceae	Gymnostachyd.	Stockey RS 47	Australia, ex Bogner coll'n, Munich Bot. Garden	440/ RS 47	AY290826
Nephthytis poissonii N.E. Br.	Araceae	Aroideae	Croat 69743	Africa	A094	AY290842
Philodendron adamantinum Schott	Araceae	Aroideae	Croat 82933	Brazil	A124	AY290845
Pistia stratiotes L.	Araceae	Aroideae	Rothwell & Ballard 356 (BHO)	OU greenhouse	356	AY290860
Schismatoglottis mutata Hook.f.	Araceae	Aroideae	Croat 53521	Thailand	A087	AY290846
Symplocarpus foetidus (L.) Nutt.	Araceae	Orontioideae	Stockey RS 42	N. America, ex Bogner coll'n, Munich Bot. Garden	441/ RS 42	AY290827
Typhonium sp.	Araceae	Aroideae	Sizemore 96-425	Vietnam, ex Croat collection, MO Bot. Garden	A107	AY290856
Typhonodorum lindleyanum Schott	Araceae	Aroideae	MBG 931747	Madagascar, ex Croat collection, MO Bot. Garden	A132	AY290858
Zamioculcas zamiifolia (Lodd.) Engl.	Araceae	Aroideae	Croat 61123A	Southeast Africa	A088	AY290838
Zantedeschia albomaculata (Hook.) Baill.	Araceae	Aroideae	Rothwell & Ballard 358 (BHO)	OU greenhouse	358	AY290839
Landoltia punctata (G. Meyer) D.H. Les & D.J. Crawford	Lemnaceae		Ballard 00-023 (BHO)	Shannon Co., MO	431	AY290849
Lemna gibba L.	Lemnaceae		Ballard 00-002 (BHO)	UNAM campus, Mexico	429	AY290848
Lemna minor L.	Lemnaceae		Ballard 351 (BHO)	Athens Co., OH	351	AY290847
Spirodela polyrhiza (L.) Schleiden	Lemnaceae		Ballard 365 (BHO)	Washtenaw Co., MI	365	AY290850
Wolffia brasiliensis Weddell	Lemnaceae		MBG s.n. (BHO)	Greenhouse, MO Bot. Garden	A138	AY290852
Wolffiella gladiata (Hegelm.) Hegelm.	Lemnaceae		Ballard 00-003 (BHO)	UNAM campus, Mexico	422	AY290851
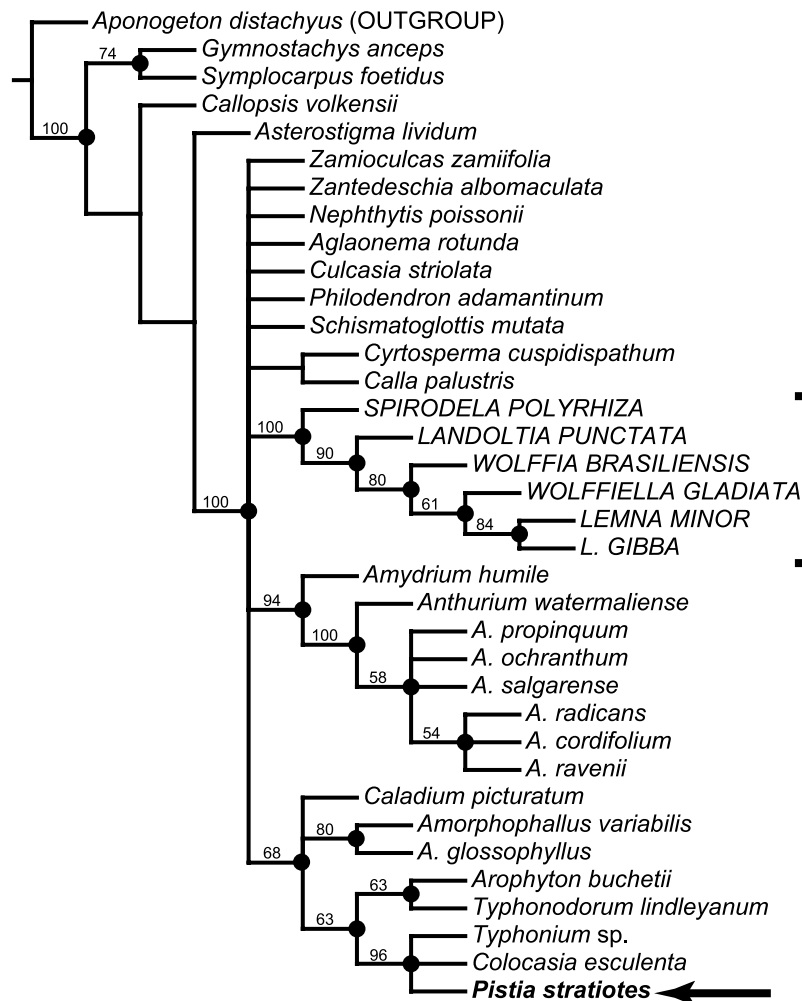
Fig. 3. Strict consensus of 409 most-parsimonious trees of 487 steps, based on maximum parsimony analysis of chloroplast DNA sequences from the *trn*L–*trn*F intergenic spacer for 29 Araceae, 6 Lemnaceae and 1 Aponogetonaceae. Bootstrap values above horizontal branches, nodes supported by 51% or greater jackknife values are indicated by black dots. Lemnaceae are capitalized and denoted by a bracket; *Pistia* is in bold face and denoted by an arrow.

Sequence divergence values between outgroup and ingroup and among various ingroup combinations were calculated within PAUP* using Jukes-Cantor distances. All ModelTest and PAUP* analyses and calculations were performed on an iMac Macintosh personal computer.

The heuristic and parsimony ratchet ("island hopping") methods were employed with Nona spawned from within Winclada on the accepted aligned data set excluding gap codes. Heuristic analysis used 100 replications with 10 maximum trees kept and one starting tree per rep; parsimony ratchet invoked 100 passes of 10,000 iterations each, 10% of characters (52) perturbed, one tree held from each iteration, random constraint level = 10, and amb = poly−. Both bootstrap and jackknife analyses used 100 replications with 10 search reps, one starting tree per rep, and "don't do max (TBR)." Winclada and Nona were performed on a NetData IBM-compatible personal computer.

3. Results

PAUP* generated 216 most-parsimonious trees of 518 steps, whereas Winclada and Nona yielded 409 most-parsimonious trees of 487 steps, with CI = 0.72 and RI = 0.69, suggesting all trees were derived from a single island. Results from both software algorithms were highly congruent, with results from Winclada and Nona giving a more highly resolved phylogeny (Fig. 2). Results from maximum likelihood analysis of the unconstrained data set were also essentially identical to the Winclada/Nona phylogeny. Therefore, our report on relationships focuses on the parsimony results from the Winclada/Nona program.

In the ingroup, the orontioid and gymnostachydoid genera are placed as sisters in a moderately well supported basal-most "proto-aroid" clade that is sister to the remainder of the aroids plus Lemnaceae (Fig. 3). The genera *Callopsis* and *Asterostigma* are placed at the
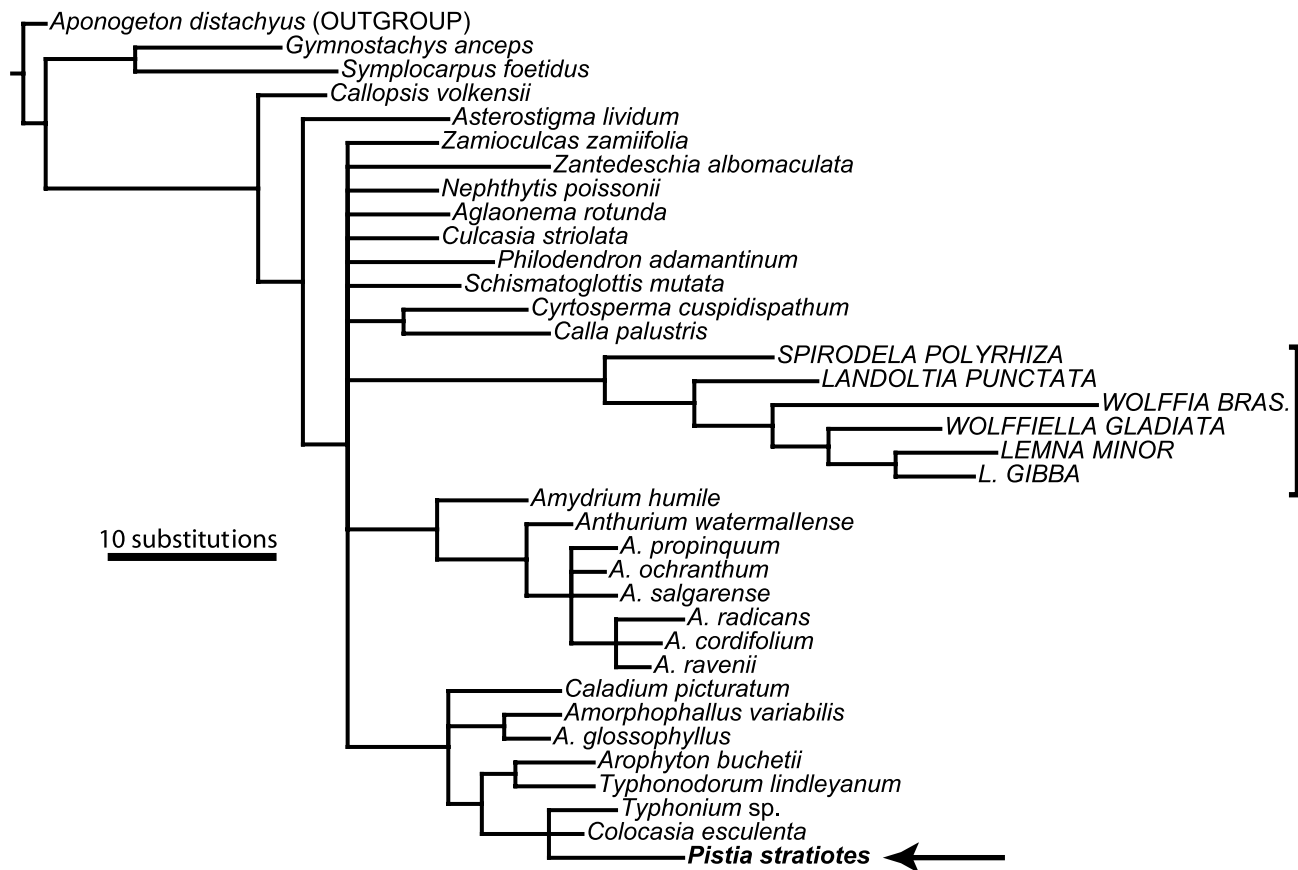
Fig. 4. Phylogram of strict consensus tree, with branch lengths proportional to the number of nucleotide substitutions. Lemnaceae are capitalized and denoted by a bracket; *Pistia* is in bold face and denoted by an arrow. Note that nucleotide substitutions represented by individual branch lengths roughly correlate with morphological reduction in the Lemnaceae (*Spirodela > Landoltia > Wolffiella > Lemna > Wolffia*), with fewer substitutions in *Pistia* and related Araceae.

next two higher nodes, but in positions that receive no bootstrap or jackknife support. The remainder of the aroid/lemnoid assemblage forms a polytomy, wherein are embedded three moderately to very strongly supported monophyletic lineages: (1) the Lemnaceae, (2) *Amydrium + Anthurium*, and (3) a third taxonomically heterogeneous clade that includes *Pistia* in one of the most derived positions. High bootstrap and jackknife values across most nodes in the clades containing Lemnaceae and *Pistia* preclude any very close relationship between the latter two taxa (Fig. 3). Within the Lemnaceae clade, *Spirodela polyrhiza* is basal-most as previously suggested by other data sets, and sister to the rest of the family. The recently segregated monotypic genus *Landoltia* is above *Spirodela* and sister to the rest of the Lemnaceae, echoing the results of Les et al. (1997). At successively higher nodes in the clade are *Wolffia brasiliensis*, *Wolffiella gladiata*, and two *Lemna* species (i.e., *L. minor + L. gibba*). Generally, relationships throughout the Lemnaceae receive moderately to very strong bootstrap and jackknife support. Ranges of sequence divergence values are presented in Table 2 for outgroup vs. ingroup, "Proto-aroids" vs. aroids/

lemnoids, aroids vs. lemnoids, within aroids s. str. and within lemnoids.

4. Discussion

4.1. Relationship of Lemnaceae and Pistia

Results of phylogenetic analysis using chloroplast *trn*L–*trn*F sequences reject the hypothesis set forth by traditional morphological evidence published *rbc*L sequences that *Pistia* is the sister to, or is very closely related to, the Lemnaceae. Our results from *trn*L–*trn*F sequences are in full agreement, however, with results of chloroplast restriction site variation (French et al., 1995) and with other, more intensive studies of the clade containing *Pistia* using *trn*L–*trn*F sequences by Renner and Weerasooriya (2002). These cumulative results support the concept of at least two independent origins of a floating aquatic habit in extant members of the aroid/lemnoid lineage. Recently studied Cretaceous (Fig. 2a) and Tertiary (Fig. 2b) fossils of floating aquatic aroids and lemnoids do not conform in venation and

Table 2
Sequence divergence values for *trn*L–*trn*F sequences of Aponogetonaceae, Araceae, and Lemnaceae, based on Jukes-Cantor distances

Groups	Range (%) and taxa compared
Outgroup (*Aponogeton*) vs. ingroup	24.7–57.6 (*Gymnostachys*; *Wolffiella*)
"Proto-Aroids" vs. aroids/lemnoids	14.0–33.3 (*Symplocarpus–Nephthytis*; *Symplocarpus–Wolffia*)
Aroids sensu lato vs. lemnoids	2.6–45.9 (*Culcasia–Zamioculcas*; *Callopsis–Lemna gibba*)
Within Aroids sensu stricto (minus lemnoids)	2.6–11.5 (*Culcasia–Zamioculcas*; *Anthurium ravenii–Pistia*)
Within lemnoids	8.8–47.2 (*Lemna minor–L. gibba*; *Lemna gibba–Wolffia*)

anatomical features to extant floating aquatic morpho-types and are probably not related to them. This raises the probability of three or four independent origins of the floating aquatic habit within the broader aroid/lemniod assemblage (Johnson et al., 1999; Stockey et al., 1997).

4.2. Relative sequence divergence in Lemnaceae

In the phylogram depicting divergence in nucleotide substitutions from the Winclada strict consensus tree (Fig. 4), molecular differentiation in the chloroplast spacer roughly parallels the degree of morphological reduction in the Lemnaceae as a whole. In contrast, however, the sequence for *Pistia* is hardly more differentiated than other related aroid genera. The very long branch lengths in the Lemnaceae suggest a heightened rate of chloroplast sequence evolution relative to other aroids sensu lato. This apparent acceleration of chloroplast evolution should be examined and tested using other chloroplast gene regions, and nuclear gene regions as well.

4.3. Morphological reduction vs. phylogenetic position in the Lemnaceae

Within the Lemnaceae clade, *Spirodela* (the basal-most taxon) shows less molecular divergence than the others, while *Wolffia* is the most divergent (Fig. 4). Nevertheless, the cladistic placement of genera does not conform precisely to the simple pattern of morphological simplification from *Spirodela*, to *Lemna*, to *Wolffiella*, and then to *Wolffia*. The relationships of *Wolffia*, *Wolffiella* and *Lemna* contradict those proposed by the traditional hypothesis of progressive morphological reduction. It also runs counter to the recent strongly supported results of Les et al. (2002) based on four other chloroplast regions, but no evidence was presented by the authors on lemnoid/aroid relationships, and the phylogenies were ultimately rooted with *Spirodela*. It is possible that the addition of more species of polytypic genera of Lemnaceae and more genera of Araceae would rearrange the topology within this lineage. Evidence from other gene regions, including the much less variable *trn*L intron, would also be valuable.

The present data set convincingly rejects the long-held hypothesis of a close relationship between *Pistia* and the Lemnaceae, supports the phylogenetic place-ment of the segregate genus *Landoltia* between *Spirodela* and other Lemnaceae, and largely supports the relationships of aroid genera indicated by prior studies. Our results also suggest an acceleration of chloroplast evolution in the Lemnaceae that is generally correlated with morphological reduction, a finding which bears further investigation and correlation with substitution rates in variable nuclear gene regions.

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