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Phylogeny and Systematics of Lemnaceae, the Duckweed Family

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ABSTRACT. The minute, reduced plants of family Lemnaceae have presented a formidable challenge to systematic investigations. The simplified morphology of duckweeds has made it particularly difficult to reconcile their interspecific relationships. A comprehensive phylogenetic analysis of all currently recognized species of Lemnaceae has been carried out using more than 4,700 characters that include data from morphology and anatomy, flavonoids, allozymes, and DNA sequences from chloroplast genes (*rbcL, matK*) and introns (*trnK, rpl16*). All data are reasonably congruent ($I_{(MF)} < 6\%$) and contributed to strong nodal support in combined analyses. Our combined data yield a single, well-resolved, maximum parsimony tree with 30/36 nodes (83%) supported by bootstrap values that exceed 90%. Subfamily *Wolffioideae* is a monophyletic clade with 100% bootstrap support; however, subfamily *Lemnoideae* represents a paraphyletic grade comprising *Landoltia, Lemna,* and *Spirodela.* Combined data analysis confirms the monophyly of *Landoltia, Lemna, Spirodela, Wolffia,* and *Wolffiella.* Phylogenetic relationships are used to evaluate and refine the classification of duckweeds.

The duckweeds (family Lemnaceae) comprise a distinctive group of diminutive, aquatic monocotyledons whose extreme reduction, miniaturization of organs, and cosmopolitan distribution contribute to their difficult taxonomy and systematics. The world's smallest angiosperms occur within this family where some individuals may attain a width of only 0.3 mm at maturity (Landolt 1986).

Despite their minuscule size, duckweeds are important freshwater plants, especially in developing countries where they have significant aquacultural applications (Skillicorn et al. 1993). Duckweeds are also ideal experimental organisms (Hillman 1961a). Their rapid clonal growth and simple axenic culture have made them suitable laboratory subjects for studying such diverse topics as photoperiod, leaf morphogenesis, toxicology, and effects of UV irradiation and ozone damage on plants (Wangerman and Ashby 1951; Wangerman and Lacey 1952; Hillman 1959, 1961b, 1961c; Tiberg, pers. comm.). As evidenced by their common name, ''duckweeds'' are an important waterfowl food and also provide food and habitat for fish (Sculthorpe 1967).

Duckweeds have been studied taxonomically throughout the 19th and 20th centuries with important contributions by Schleiden (1839), Hegelmaier (1868), Thompson (1896, 1898), Daubs (1965), Hartog and Van der Plas (1970) and Landolt (1986). These workers greatly clarified generic and species limits in the family, which, as currently circumscribed (Table 1), contains 38 species in five genera (Landolt 1986, 1998; Les et al. 1997a; Les and Crawford 1999).

In contrast, phylogenetic relationships of duckweeds have been studied sparsely. The relationship of Lemnaceae to other monocotyledons has attracted the greatest interest (see discussion), with far fewer studies focusing on intrafamilial relationships. Hegelmaier (1868) proposed a structured classification of Lemnaceae which provided the first reasonably complete overview of interspecific relationships in the family expressed within a taxonomic framework. Ivanova (1973) proposed a phylogenetic tree of duckweed species and genera; however, the tree was not character based, and taxonomic concepts of various species were not accurately depicted. Hartog (1975) evaluated morphological and phytochemical data (mainly flavonoids and lignification) to assess some intrafamilial relationships in Lemnaceae. More detailed hypotheses of intergeneric and interspecific phylogenetic relationships in duckweeds were proposed explicitly by Landolt (1986). However, Landolt's hypotheses have only begun to be tested by formal phylogenetic analyses (see Les et al. 1997a).

The paucity of phylogenetic information on duckweeds may be attributable to the scarcity of conspicuous morphological characters, which otherwise might serve as phylogenetic markers in this structurally reduced family. Thus, as ''biochemical sytematics'' advanced in the 1960's, the taxonomically troublesome Lemnaceae were perceived as an ideal taxon upon which to test the utility and power of new micromolecular markers. Duckweeds became the subject of an intensive flavonoid survey (McClure 1964; McClure and Alston 1964, 1966; Alston 1966) and comparative chemical analysis led to novel hypotheses of interspecific phylogenetic relationships in Lemnaceae. The in-

TABLE 1. Clone identification numbers and their geographical origins used in present phylogenetic study of Lemnaceae (from Landolt 1994, 1998; Landolt and Urbanska-Worytkiewicz 1980; Crawford et al. 1996, 1997). Locality information is presented at the first mention of each clone. ‡ = clone used in phylogenetic analyses (where multiple accessions are listed). GenBank accession numbers are in square brackets.

	rbcL.	*trnK* intron [3′; 5′]	*matK*	*rpl16*
Landoltia				
L. punctata	7248 (South Africa) [AY034223]	7248 [AY034301; AY034340]	7248 [AY034185]	7248‡ [AY034262], 7429 (Japan), 7504 (USA), 7536 (Canary Islands), 8724 (Australia), 8847 (Brazil)
Lemna				
L. aequinoctialis	7671 (Brazil) [AY034228]	7671 [AY034306; AY034345]	7671 [AY034190]	8120 (USA) [AY034267]
L. disperma	7767 (Australia) [AY034236]	7767 [AY034314; AY034353]	7767 [AY034198]	8729 (Australia) [AY034275]
L. ecuadoriensis	8896 (Ecuador) [AY034231]	8896 [AY034309; AY034348]	8896 [AY034193]	8896 [AY034270]
L. gibba	6583 (USA) [AY034235]	6583 [AY034313; AY034352]	6583 [AY034197]	6751‡ (USA) [AY034274], 7377 (Egypt)
L. japonica	8653 (China) 8694‡ (Japan) [AY034233]	8653, 8694‡ [AY034311; AY034350]	8653, 8694‡ [AY034195]	8339 (China), 8693‡ (Japan) [AY034272]
L. minor	7123‡ [AY034234], 8745 (Canada)	7123‡ [AY034312; AY034351], 8745	7123‡ [AY034196], 8745	7492 (USA), 8404‡ (France) [AY034273]
L. minuta	7726 (Chile) [AY034224]	7726 [AY034302; AY034341]	7726 [AY034186]	7369 (Argentina) [AY034263]
L. obscura	7720 (USA) [AY034232]	7720 [AY034310; AY034349]	7720 [AY034194]	7144 (USA), 8107‡ (USA) [AY034271]
L. perpusilla	7507 (USA) [AY034229]	7507 [AY034307; AY034346]	7507 [AY034191]	8612 (USA) [AY034268]
L. tenera	9024 (Australia) [AY034227]	9021 (Australia) [AY034305; AY034344]	9021 [AY034189]	9023‡ (Australia) [AY034266], 9243 (Vietnam)
L. trisulca	7331 (Australia); 7875‡ (Canada) [AY034237]	7331 [AY034315; AY034354]	7331 [AY034199]	7331‡ [AY034276], 8137 (USA)
L. turionifera	7352 (USA) [AY034230]	7352 [AY034308; AY034347]	7352 [AY034192]	7686‡ (Canada) [AY034269], 8895 (Germany)
L. valdiviana	7803 (USA) [AY034225]	7803 [AY034303; AY034342]	7803 [AY034187]	8821 (Argentina) [AY034264]
L. yungensis	9208 (Bolivia) [AY034226]	9208 [AY034304; AY034343]	9208 [AY034188]	9208 [AY034265]
Spirodela				
S. intermedia	7920 (Argentina) [U68092]	7920 [AY034299; AY034338]	7920 [AY034183]	7355 (Surinam), 8410‡ (Panama) [AY034260]
S. polyrhiza	7110 (Puerto Rico) [AY034222]	7110 [AY034300; AY034339]	7110 [AY034184]	7110‡ [AY034261], 7160 (USA), 7222 (Malaysia), 7235 (Burundi), 7344 (Switzerland), 7571 (Canada), 8256 (India), 8338 (China)
Wolffia				
W. angusta	9019 (Australia) [AY034253]	9019 [AY034331; AY034370]	9019 [AY034215]	7274‡ (Australia) [AY034292], 8878 (Malaysia)
W. arrhiza	7347 (S Africa) [AY034254]	7347 [AY034332; AY034371]	7347 [AY034216]	7196 (Portugal), 7651‡ (Morocco) [AY034293]
W. australiana	7733 (Australia) [AY034251]	7733 [AY034329; AY034368]	7733 [AY034213]	7543 (New Zealand), 7631‡ (Australia) [AY034290]
W. borealis	9123‡ (USA) [AY034250], 9145 (Canada)	9123‡ [AY034328; AY034367], 9145	9123‡ [AY034212], 9145	9123‡ [AY034289], 9143 (USA)

TABLE 1. Continued.

	rbcL.	*trnK* intron [3′; 5′]	*matK*	*rpl16*
W. brasiliensis	8743 (Argentina) [AY034248]	7955 (USA), 8743‡ [AY034326; AY034365], 9134 (Brazil)	7955, 8743‡ [AY034210], 9134	7955, 9134‡ [AY034287]
W. columbiana	7467 (Columbia) [AY034255]	7467 [AY034333; AY034372]	7467 [AY034217]	7467‡ [AY034294], 7866 (USA)
W. cylindracea	9100 (Zimbabwe) [AY034256]	7233 (S Africa) [AY034334; AY034373]	7233 [AY034218]	7233‡ [AY034295], 9100
W. elongata	9197 (Columbia) [AY034258]	9197 [AY034336; AY034375]	9197 [AY034220]	9188 (Columbia) [AY034297]
W. globosa	8180 (USA) [AY034257]	8180 [AY034335; AY034374]	8180 [AY034219]	7700 (India), 8180‡ [AY034296], 8691 (Japan)
W. microscopia	8359 (India) [AY034249]	8359 [AY034327; AY034366]	8359 [AY034211]	8359 [AY034288]
W. neglecta	8917 (Pakistan) [AY034252]	8917 [AY034330; AY034369]	8917 [AY034214]	9149 (Pakistan), 9150‡ (Pakistan) [AY034291]
Wolffiella				
W. caudata	9158 (Bolivia) [AY034244]	9173 (Bolivia) [AY034322; AY034361]	9173 [AY034206]	9158‡ [AY034283], 9214 (Bolivia)
W. denticulata	8221 (S Africa) [AY034247]	8221 [AY034325; AY034364]	8221 [AY034209]	8221 [AY034286]
W. gladiata	8261 (USA) [AY034243]	8261 [AY034321; AY034360]	8261 [AY034205]	7173 (USA), 8768‡ (USA) [AY034282]
W. hyalina	8640 (Tanzania) [AY034240]	8640 [AY034318; AY034357]	8640 [AY034202]	7376 (Egypt), 8640‡ [AY034279]
W. lingulata	7289 (Brazil) [AY034241]	7289 [AY034319; AY034358]	7289 [AY034203]	7289‡ [AY034280], 7655 (Mexico)
W. neotropica	8848 (Brazil) [AY034246]	8848 [AY034324; AY034363]	8848 [AY034208]	7290 (Brazil), 8848‡ [AY034285]
W. oblonga	8984 (Columbia) [AY034242]	8984 [AY034320; AY034359]	8984 [AY034204]	7997‡ (Brazil) [AY034281], 8393 (USA)
W. repanda	9122 (Zimbabwe) [AY034239]	9122 [AY034317; AY034356]	9122 [AY034201]	9062‡ (Zimbabwe) [AY034278], 9104 (Botswana)
W. rotunda	9121 (Zimbabwe) [AY034238]	9121 [AY034316; AY034355]	9121 [AY034200]	9054 (Zimbabwe), 9072‡ (Zimbabwe) [AY034277]
W. welwitschii	7468 (Columbia) [AY034245]	7468 [AY034323; AY034362]	7468 [AY034207]	7468‡ [AY034284], 9096 (Botswana)

dependence of molecular data was viewed as a major advantage because relationships in this problematic group of plants could be evaluated independently of their morphology which was not viewed as particularly informative.

The application of molecular data to taxonomic questions in Lemnaceae has continued with emphasis shifting to macromolecular data. More recent studies have investigated allozymes (Crawford and Landolt 1993, 1995; Crawford et al. 1995, 1996, 1997; Hirahaya and Kadono 1995; Vasseur et al. 1993) and cpDNA (Beppu in Landolt 1986; Jordan et al. 1996). For the past several years, the present authors have undertaken an intensive phylogenetic study of Lemnaceae and have obtained and compiled data from a wide variety of sources including morphology and micromolecules (Les et al. 1997a), allozymes (Crawford and Landolt 1993, 1995; Crawford et al. 1995, 1996, 1997) and DNA sequences (Les et al. 1994, 1997b).

Despite these efforts, many basic systematic questions remain, ranging from the precise delimitation of taxa in Lemnaceae, to their interrelationships. These issues must be clarified before a classification of the family can be recommended with confidence. In this study, we report the results of a phylogenetic analysis of Lemnaceae that is based upon the consideration of characters derived from molecular and non-molecular data and, in the former, from both nuclear and plastid genomes. This approach has enabled us to construct a synthetic hypothesis of interspecific relationships in Lemnaceae that reflects the incorporation of most of the varied types of data routinely and historically applied to phylogenetic studies of angiosperms. These analyses have allowed us to formulate a relatively secure hypothesis of phylogenetic relationships within the Lemnaceae, which in turn serves as the foundation for a revised, evolutionary classification of the family.

Materials and Methods

Morphological, chromosome, and micromolecular data were compiled from previously published studies of Lemnaceae (Urbanska-Worytkiewicz 1980; Landolt 1986; Les and Philbrick 1993; Les et al. 1997a). Due to extensive intraspecific aneuploid and euploid polymorphisms (see Les and Philbrick 1993), chromosome numbers could not be coded reasonably and they were excluded from the phylogenetic analyses.

To enable the inclusion of allozyme data (Crawford and Landolt 1993, 1995; Crawford et al. 1995, 1996, 1997) in the phylogenetic analyses, the hierarchical ''nodes'' depicted on genetic identity dendrograms were coded as 22 binary characters. This is similar to a modification of ''Brooks Parsimony Analysis,'' and is a procedure described and recommended by Doyle (1992).

Macromolecular (DNA sequence) data were obtained from 101 clones (Table 1) representing all currently recognized (38) extant duckweed species. Five regions of the chloroplast genome were sequenced, including two protein coding loci (*rbcL, matK*) and three intron regions (5′*trnK*; 3′*trnK*; *rpl16*). Sequencing of *rbcL* followed the methods summarized by Les et al. (1993). Amplification (PCR) of the *matK* coding region and the flanking *trnK* introns used the '3914-F' and 'TRNK 2-R' primer sequences in Les et al. (1999). Amplification of the *rpl16* intron was performed using primer F71 (Jordan et al. 1996), which is located in exon 1, and primer 'R622'(5′-CCAACCCAATGAATCATTAGGATT; designed from published *rpl16* intron sequences), which binds to a site within the intron. Primer numbers refer to the 5′ position of the primer on the *rpl16* sequence of Posno et al. (1986). PCR reactions were carried out in 50–100 μl volumes (1.5 mM $MgCl_2$). For *rbcL* and *matK*, 30 cycles were used (denature @ 94°C [1 min, 15 sec], anneal @ 55°C [2 min]; extension @ 72°C [2 min, 15 sec]) with a 5 min final extension. Amplification of *rpl16* used 35 cycles (denature @ 95°C [45 sec], anneal @ 52°C [45 sec]; extension @ 72°C [45 sec]). Negative controls were run to detect contamination. PCR products were cleaned using either QIAquick™ PCR purification columns (Qiagen, Inc., Valencia, CA) or by precipitation using an equal volume of PEG:NaCl (20%:2.5M).

Cycle sequencing reactions (¼-½ volumes) were used to sequence the *matK* coding region, the *trnK* and *rpl16* introns using the BigDye™ Terminator kit (PE Applied Biosystems, Foster City, CA), and by following the standard protocol provided for ABI 377 and ABI Prism™ 310 automated sequencers (PE Applied Biosystems, Foster City, CA). Sequencing of *rpl16* was completed using the amplification primers. Sequences for the 5′ and 3′ *trnK* introns and the *matK* coding region were obtained using the PCR amplification primers (above) and these additional primer sequences: '313R': 5′-ATAGCAAAACCCTCTG; '290R': 5′-GTTCTTGTTGTG TCC; '-76F': 5′-TTCTGACCATATCGCAC; '1234R': 5′-CTGGCT TGCTAATAGGAT [except *Lemna* section *Uninerves*]; '500F': 5′-GTCCAAGATGTTCCC [*Uninerves* taxa]; '822F': 5′-GGATCCTTT CATGCATT [except *Wolffiella* section *Rotundae* and *Wolffia arrhiza, Wolffia columbiana*]; '600F': 5′-GTTGAATGCGAATCC [*Rotundae*]; '1500R': 5′-ACCTTTTTCTTCTTCC [*Wolffia arrhiza, Wolffia columbiana*]; '1241F': 5′-CCGATTTGTCAGATTC [except *Landoltia, Spirodela* and *Lemna* section *Uninerves*]; '1241F': 5′-TTGGGCCGATT-TATCAG [*Landoltia* and *Spirodela*; section *Uninerves* used 'TRNK 2-R' amplification primer].

All sequence chromatographs were edited manually and assembled into double-stranded contigs. Sequences were aligned initially in Clustal W (Thompson et al. 1994), then manually optimized by visual inspection. Alignments were trimmed to exclude highly variable regions (e.g., near exon 1 of *rpl16*) where positional homology was difficult to establish. All molecular data were entered in the GenBank database within the series of accession numbers from AY034182 to AY034375.

To facilitate comparisons, taxa were partitioned into separate blocks representing the five currently recognized genera of Lemnaceae (*Landoltia, Lemna, Spirodela, Wolffia, Wolffiella*) and the two putatively recognized subfamilies (*Lemnoideae, Wolffioideae*).

Less than 1% of the data cells were coded as missing due to incomplete regions of sequences or otherwise unavailable data. Gaps inserted during sequence alignments comprised 5.2 % of the data cells and were treated as missing data in all analyses. However, we included gap information by constructing separate multistate character matrices to score indels in all sequences except *rbcL* which lacked indels. These matrices assigned the same character state to each identical motif that occurred within a gap. This procedure is similar to the ''simple indel coding'' method of Simmons and Ochoterena (2000).

Characters were partitioned as 12 separate blocks to provide flexibility in analyses of single data sets and various data set combinations. The final partitioned data set included blocks representing anatomy and morphology (41 characters), flavonoids (47 characters), *rbcL* sequences (1,348 characters), *matK* sequences (1,557 characters), *matK* indels (7 characters), *trnK* 5′ intron sequences (817 characters), *trnK* 5′ intron indels (39 characters), *trnK* 3′ intron sequences (289 characters), *trnK* 3′ intron indels (12 characters), *rpl16* intron sequences (509 characters), *rpl16* intron indels

TABLE 2. Intraspecific variation in *rpl16* compared for 28 species of Lemnaceae (arranged in order of increasing variability). The number of substitutions represents a direct comparison from uncorrected 'p' distances (ti = transitions; tv = transversions). The seven regions for *S. polyrhiza* are Africa, Canada, China, India, Puerto Rico, Switzerland, and USA. The four regions for *L. puncata* are Africa, Brazil, Japan and USA.

Species	Regions compared	Substitutions	(ti:tv)	# Gaps
Spirodela				
S. intermedia	Panama × Surinam	0	(0:0)	0
S. polyrhiza	7 regions	0	(0:0)	0
S. polyrhiza	7 regions × Malaysia	1	(0:1)	0
Landoltia				
L. punctata	4 regions	0	(0:0)	0
L. punctata	4 regions × Australia	0	(0:0)	1
L. punctata	4 regions × Africa	1	(0:1)	1
L. punctata	Africa × Australia	1	(0:1)	1
Lemna				
L. japonica	China × Japan	0	(0:0)	0
L. minor	France × USA	0	(0:0)	0
L. obscura	USA × USA	0	(0:0)	0
L. turionifera	Canada × Germany	0	(0:0)	0
L. gibba	Egypt × USA	1	(1:0)	1
L. tenera	Australia × Vietnam	2	(0:2)	2
L. trisulca	Australia × USA	4	(4:0)	1
Wolffiella				
W. gladiata	USA × USA	0	(0:0)	0
W. lingulata	Brazil × Mexico	0	(0:0)	0
W. neotropica	Brazil × Brazil	0	(0:0)	0
W. hyalina	Egypt × Tanzania	0	(0:0)	1
W. caudata	Bolivia × Bolivia	2	(0:2)	0
W. welwitschii	Botswana × Columbia	2	(1:1)	0
W. oblonga	Brazil × USA	6	(1:5)	1
W. rotunda	Zimbabwe × Zimbabwe	6	(1:5)	3
W. repanda	Botswana × Zimbabwe	10	(1:9)	4
Wolffia				
W. arrhiza	Morocco × Portugal	0	(0:0)	0
W. australiana	Australia × New Zealand	0	(0:0)	0
W. borealis	USA × USA	0	(0:0)	0
W. brasiliensis	Brazil × USA	0	(0:0)	0
W. globosa	Japan × USA	0	(0:0)	0
W. angusta	Australia × Malaysia	1	(1:0)	0
W. neglecta	Pakistan × Pakistan	2	(0:2)	0
W. globosa	Japan / USA × India	9	(3:6)	2
W. columbiana	Columbia × USA	9	(3:6)	5
W. cylindracea	S Africa × Zimbabwe	13	(6:7)	5

(20 characters), and allozymes (22 characters) for a total of 4,708 characters.

The PAUP* 4.0 beta 4a computer program (Swofford 1998) was used for all analyses other than alignments (see above). Nucleotide bias was evaluated for all sequences using a chi-square test. Uncorrected 'p' distances were calculated in all pairwise species combinations for *rbcL*, *matK*, *trnK* 3′ and 5′ introns, and the *rpl16* intron. Intraspecific variation (substitutions, gaps, ti:tv ratios) in *rpl16* was compared for 28 species of Lemnaceae in various pairwise geographical comparisons (Table 2).

Unless indicated otherwise, all analyses were performed using equally weighted Fitch parsimony. Cladograms were rooted first using sequences from *Pistia* (*rbcL*, *matK*, *trnK*, *rpl16*, flavonoids) and seven other Araceae genera (*rbcL*). It was not practical to obtain other comparable data from these divergent outgroup genera. These analyses consistently placed a clade consisting of *Spirodela polyrhiza* and *S. intermedia* in the basal position of Lemnaceae. The remainder of analyses excluded Araceae genera and used *S. polyrhiza* and *S. intermedia* for ingroup rooting of the remainder of the Lemnaceae taxa (see Discussion).

Congruency of data partitions was evaluated by calculating the Mickevich-Farris incongruency index ($I_{(MF)}$) (Mickevich and Farris 1981; Swofford 1991). Significance of $I_{(MF)}$ (Table 3) was tested using the partition-homogeneity test of PAUP* as described in Les et al. (1999). This test was run on 100 replicates using heuristic search with NNI branch swapping (limited to holding 3,000 trees, ≥ 100 steps). Congruency was also evaluated for coding (*matK*, *rbcL*) vs. non-coding (*trnK*, *rpl16*) cpDNA data partitions. Due to missing data for various taxa, we could not evaluate $I_{(MF)}$ for data combinations involving flavonoid or allozyme data.

Preliminary analyses indicated that for sequences with gaps, inclusion of indel matrices produced an entirely congruent phylogeny compared to the same data partition for which the indel matrix was excluded; however, addition of indel characters tended to increase resolution (resolved otherwise unresolved nodes) as well as consistency and retention indices (Table 4). Consequently, we

TABLE 3. Congruency of data partitions as evaluated by Mickevich-Farris incongruence metrics and the partition-homogeneity test of PAUP*. Tree lengths (steps) are indicated for maximum parsimony solutions and minimum possible lengths. i_w = sum of extra steps in uncombined data sets; i_T = number of extra steps in combined data set; i_B = difference in extra steps between uncombined and combined data; $I_{(MF)}$ = incongruency index ($i_B = i_T - i_w$; $I_{(MF)} = i_B/i_T$). Comparisons with $p < 0.05$ (*) are considered to be significantly incongruent. All probabilities (partition-homogeneity test) based on heuristic search, 100 replicates, NNI branch swapping. Coded indel characters are included.

Data partitions	Steps (parsimony)	Steps (minimum)	i_w	i_T	i_B	$I_{(MF)}$	p
trnK 3′ intron × *trnK* 5′ intron	804	602	200	202	2	0.012	0.910
trnK intron (3′,5′ combined) × *matK*	1705	1286	409	419	10	0.024	0.160
trnK intron (3′,5′ combined) × *rbcL*	1138	802	323	336	13	0.039	0.010*
trnK intron (3′,5′ combined) × *rpl16* intron	1185	881	294	304	10	0.033	0.100
rpl16 intron × *matK*	1277	963	303	314	11	0.035	0.010*
rpl16 intron × *rbcL*	707	478	216	229	13	0.057	0.020*
matK × *rbcL*	1225	884	330	341	11	0.032	0.012*
coding cpDNA × non-coding cpDNA	2419	1764	627	655	28	0.043	0.010*

included indel matrices with their respective data partitions in all subsequent analyses.

Separate cladograms were constructed from *matK*, *rbcL*, *trnK* introns (combined 5′, 3′) and *rpl16* intron sequences. These trees were generated using heuristic search (simple addition sequence referenced to *Spirodela intermedia*) and TBR branch swapping (starting trees obtained by stepwise addition) with the ''MulTrees'' option in effect. Strict consensus trees were used in all cases to depict results where multiple, equally parsimonious solutions resulted. Bootstrap values were calculated for these trees using a heuristic search with ''fast'' stepwise-addition of taxa and 500 replicates.

A combined analysis of all data was performed using heuristic search (random addition sequence; 100 replicates) with TBR branch-swapping (options as above). The resulting tree was depicted as a phylogram with proportional branch lengths. Bootstrap values were calculated for each node from 500 replicates using a ''full'' heuristic search (simple addition sequence referenced to *Spirodela intermedia*) with TBR branch-swapping (options as above).

The genus *Wolffia* showed the least consistent phylogenetic topology among the different molecular data sets investigated. To observe the effect of taxon inclusion on non-random structure in the dataset, we calculated skewness (g_1) for *matK*, *trnK* introns, *rpl16* intron, and *rbcL* data partitions for all *Wolffia* species, and then recalculated skewness following the removal of each species, one at a time, from each data set. The resulting change in skewness was reported as '±δ' (Table 5). All skewness estimates were made from a subset of 100,000 randomly selected trees.

For *matK*, the data were analyzed using maximum likelihood. Using Jukes-Cantor, K2P, F81, and HKY85, a heuristic search with TBR was performed. For all models, the starting tree was obtained using stepwise addition, with the sequences added ''as-is''. In addition, for HKY85, trees were also obtained using stepwise addition with 10 random sequence additions. Empirical base frequencies were used, with a ti:tv ratio of 2.00.

RESULTS

All DNA sequences were AT rich with slightly lower AT bias present in *rbcL*. Nucleotide composition was homogeneous across all taxa for each sequence (χ^2 = 11.69–30.73; 111 d.f.; p = 1.00). Sequence divergence (reported as uncorrected 'p' distances to provide a general overview of species divergence across all sequences) ranged from 0.0% (all sequences) to 18.2% (*trnK* 3′ intron) among pairwise comparisons of 38 Lemnaceae species. Maximum 'p' distances were less than 13.0% for the remaining sequences and only 5.5% in the *rbcL* dataset. Identical sequences occurred in 11 pairwise comparisons: *Lemna valdiviana* × *L. yungensis* (3′ *trnK* intron; *rpl16* intron), *L. ecuadoriensis* × *L. turionifera* (3′ and 5′ *trnK* introns), *L. minuta* × *L. valdi-*

TABLE 4. Effect of inclusion/exclusion of indel information in sequences with gaps used in phylogenetic analyses of Lemnaceae. ''Indels excluded'' refers to analyses where gaps were treated as missing data; ''indel matrix included'' refers to analyses where gaps were treated as missing data, but where indels were scored as separate character states included in a separate matrix (see text). CI = consistency index; CI_{EXC} = consistency index excluding uninformative characters; RI = retention index. Because gap information tended to improve tree statistics and/or resolution, it was included in all analyses. The # of steps and # of nodes resolved in the maximum parsimony strict consensus trees are indicated.

Sequences with gaps	# steps	CI	CI_{EXC}	RI	# nodes resolved
trnK 3′ intron (indels excluded)	187	0802	0.745	0.920	24
trnK 3′ intron (indel matrix included)	239	0.824	0.786	0.925	24
trnK 5′ intron (indels excluded)	478	0.709	0.625	0.853	19
trnK 5′ intron (indel matrix included)	563	0.719	0.643	0.860	27
rpl16 intron (indels excluded)	294	0.731	0.650	0.874	26
rpl16 intron (indel matrix included)	373	0.748	0.686	0.876	27
matK (indels excluded)	884	0.766	0.703	0.911	33
matK (indel matrix included)	893	0.766	0.704	0.911	33

TABLE 5. Influence of taxon removal in *Wolffia* on skewness (g_1) and tree ''islands'' from coding (*matK*; *rbcL*) and noncoding (*trnK* introns, *rpl16*) sequences. Data were combined for 5′ and 3′ introns of *trnK*. Departure from g_1 value of all included taxa ('none') is indicated by δ. n/a = not applicable (self comparison).

	matK		*rbcL*		*trnK* introns		*rpl16* intron	
Excluded taxa	g_1	δ	g_1	δ	g_1	δ	g_1	δ
None	−0.941	n/a	−0.887	n/a	−0.985	n/a	−0.938	n/a
W. microscopica	−1.513	−0.572	−1.220	−0.333	−1.289	−0.304	−1.136	−0.198
W. brasiliensis	−1.506	−0.565	−1.261	−0.374	−1.324	−0.339	−1.169	−0.231
W. borealis	−1.488	−0.547	−1.203	−0.316	−1.297	−0.312	−1.035	−0.097
W. australiana	−1.469	−0.528	−1.027	−0.140	−1.373	−0.388	−0.987	−0.049
W. angusta	−0.787	+0.154	−0.815	+0.072	−0.887	+0.098	−0.875	+0.063
W. cylindracea	−0.787	+0.154	−0.762	+0.125	−0.871	+0.114	−0.821	+0.117
W. neglecta	−0.782	+0.159	−0.797	+0.090	−0.887	+0.098	−0.878	+0.060
W. arrhiza	−0.781	+0.160	−0.831	+0.056	−0.892	+0.093	−0.819	+0.119
W. columbiana	−0.780	+0.161	−0.786	+0.101	−0.879	+0.106	−0.873	+0.065
W. elongata	−0.777	+0.164	−0.772	+0.115	−0.890	+0.095	−0.890	+0.048
W. globosa	−0.773	+0.168	−0.813	+0.074	−0.897	+0.088	−0.846	+0.092

viana (3′ *trnK* intron), *L. turionifera* × *L. japonica* (*rpl16* intron), *L. ecuadoriensis* × *L. obscura* (*rbcL*), *Wolffia angusta* × *Wolffia neglecta* (*matK*; 3′ and 5′ *trnK* introns), and *Wolffiella lingulata* × *Wolffiella oblonga* (3′ *trnK* intron).

Intraspecific *rpl16* variation was not evaluated for 10 species (*L. aequinoctialis*, *L. disperma*, *L. ecuadoriensis*, *L. minuta*, *L. perpusilla*, *L. valdiviana*, *L. yungensis*, *Wolffia elongata*, *W. microscopica*, and *Wolffiella denticulata*) where only single accessions were sequenced. However, seven of these species (*L. disperma*, Australia, New Zealand; *L. ecuadoriensis*, Ecuador; *L. perpusilla*, eastern USA; *L. yungensis*, Bolivia; *Wolffiella denticulata*, southern Africa; *Wolffia elongata*, northern South America; *W. microscopica*, India and Pakistan) are restricted geographically (Landolt 1986). Accessions from different geographic areas were used for sequences at other loci (*matK*, *rbcL*) for the wider ranging species (*L. aequinoctialis*, *L. minuta*, *L. valdiviana*) as well as for *L. disperma*, *L. perpusilla* and *W. elongata*. While this represents very minimal sampling within species, it has the potential to detect variation between geographic areas.

Of the remaining 28 species where multiple accessions were sequenced, the *rpl16* intron was invariant ('p' distance = 0.00) in 12 (43%) species (Table 2). Intraspecific variation (manifest as nucleotide substitutions and gaps) was detected in 16 (57%) species (Table 2). Where accessions differed by nucleotide substitutions, transversions outnumbered transitions in 12 instances, transitions outnumbered transversions in three instances, and transitions equalled transversions in one case (Table 2). Multiple accessions ranged from complete uniformity over broad geographical comparisons (e.g., *Lemna minor* from France vs. USA; *L. turionifera* from Germany vs. USA; *Spirodela polyrhiza* for 7/8 regions surveyed) to relatively high variability among accessions in geographical proximity (e.g., *Wolffia cylindracea* clones from South Africa and Zimbabwe differed by 13 substitutions and five gaps; *Wolffiella repanda* clones from Botswana and Zimbabwe differed by 10 substitutions and four gaps; Table 2).

The general pattern to emerge from the admittedly small sampling within species was for *rpl16* sequences to exhibit very low intraspecific variability in *Landoltia*, *Lemna*, and *Spirodela* whereas both *Wolffia* and *Wolffiella* contained species with relatively higher levels of intraspecific variability (Table 2). In both of the latter genera, the highest intraspecific variation (*Wolffia cylindracea* and *Wolffiella repanda*) was observed among populations of African taxa (Table 2). For several of these cases, *rpl16* sequences from different accessions showed a higher similarity to closely related taxa than to conspecifics.

The congruency of different data partitions was high overall, with less than 5.7% incongruence ($I_{(MF)}$) observed among data partitions (Table 3). Partition-homogeneity tests indicated general agreement (i.e., no significant incongruency) between the 3′ and 5′ *trnK* introns and between the combined *trnK* intron data with either *matK* or *rpl16* (Table 3). However, significant differences were noted for combined partitions of *rbcL* and *trnK* intron data, and for *rpl16* when combined either with *rbcL* or *matK* (Table 3). Significant differences also occurred between coding vs. non-coding cpDNA data partitions.

Despite some disagreement among data sets indicated by the sensitive partition-homogeneity test, many groups were consistently resolved. The phylogenetic integrity of *Landoltia*, *Spirodela*, all sections of *Wolffiella* and all sections of *Lemna* except sect. *Hydrophylla* (*L. trisulca*) was indicated by all four molecular data sets (Fig. 1). Some discrepancies occurred regarding the position of *Landoltia* (*matK*, *trnK* introns vs. *rbcL*, *rpl16*) and *Wolffia brasiliensis* (placed in *Wolffiella* by *matK* data). Most of the other incongruities occurred with respect to the positions of *Wolffia* species (Fig. 1). Generally, most disagreeing nodes were supported by weak to moderate bootstrap values (Fig. 1).

We used results of the partition-homogeneity test to identify potential problems due to incongruence, but not as the sole criterion to determine combinability of data sets. Topologically, the *matK* and *rbcL* cladograms differed by relatively minor details (Fig. 1) despite the significant difference indicated by the partition-homogeneity test (Table 3). Conversely, a number of differences existed between the *trnK* intron and *rpl16* intron cladograms (Fig. 1c-d), yet these data sets were congruent statistically (Table 3). We concluded that all molecular data sets were combinable, but were alerted to possible problems in *Wolffia* (see below), particularly with respect to several basal species characterized by relatively long branches and low bootstrap support (Figs. 1, 2).

We could not effectively test the allozyme, flavonoid or morphological data sets using the partition homogeneity test. Our decision to combine these data with the DNA sequence data was made after preliminary analyses indicated that their inclusion did not materially alter the topology of the combined DNA sequence cladogram. Furthermore, the inclusion of these remaining data sets provided additional support for various nodes on the cladogram.

One maximum parsimony cladogram (2674 steps; CI = 0.71; $CI_{(exc)}$ = 0.64; RI = 0.88) resulted from a heuristic search of all combined data and provided complete resolution among the 38 species of Lemnaceae (Fig. 2). No islands of shorter trees were recovered using 100 random addition sequences. Bootstrap support for nodes was high throughout the cladogram with only two nodes (both in *Wolffia*) supported at less than 70% (Fig. 2). Twenty-two nodes (73% of total) were supported at 100%; 32 nodes (89% of total) had greater than 80% bootstrap support (Table 6).

Sectional integrity was upheld in most instances (Table 6) with six sections supported as monophyletic groups with 100% bootstrap values. The monotypic section *Rotundae* (*Wolffiella rotunda*) was also resolved as a distinct branch flanked by 100% bootstrap nodes (Fig. 2).

Discussion

Criteria for Conducting a Systematic Study of Lemnaceae. In a morphologically problematic group such as Lemnaceae, fundamental taxonomic problems such as basic species delimitation must be reconciled before systematic work can proceed effectively. The early molecular studies of duckweeds using flavonoids (McClure and Alston 1966) provide an example where a number of interpretive problems resulted because some accessions were not identified accurately (see Les et al. 1997a). The reduced and modified morphology of duckweeds requires careful attention to technical characters for proper species identification. Also, a comprehensive study of this cosmopolitan family requires the examination of collections obtained worldwide. Without access to hundreds of duckweed accessions worldwide (a reflection of more than 40 years of collecting by Landolt and others) and to living cultures of those collections, this study would not have been possible. Moreover, Landolt (1986) was able to develop a confident system for species identifications of these duckweed collections using morphological features. Species identifications and adequate collections present obstacles to nearly every systematic study and it is unusual to have access to such an extensive sample of study material, which enabled us to include every known extant genus and species in the present analysis. Because duckweed identification can be extremely difficult, it was first necessary to demonstrate the robustness of taxonomic species (Landolt 1986, 1992, 1994, 1998) limits before proceeding to the phylogenetic analyses. Only in this way could we be reasonably certain that our results would not be faulted by comparisons of data obtained from specimens that were identified inaccurately. This corroboration was obtained initially by allozyme analyses (Crawford and Landolt 1993, 1995; Crawford et al. 1995, 1996, 1997, unpublished). Allozyme studies demonstrated first, that geographical accessions identified as the same species exhibited high genetic identities at allozyme loci (thus verifying the limits indicated by the morphologically defined species), and secondly, whether certain species with subtle morphological differences represented the same or discrete taxa (i.e. whether ''cryptic species'' existed within widespread, morphologically diverse species). We also examined *rpl16* DNA sequence variation among different populations of 28 of the 38 duckweed species (Table 2). For a few species, the *rpl16* data indicated that conspecific accessions (based on morphology) may represent different, but closely related, species. Interestingly, for these same accessions, allozyme data indicated a high de-

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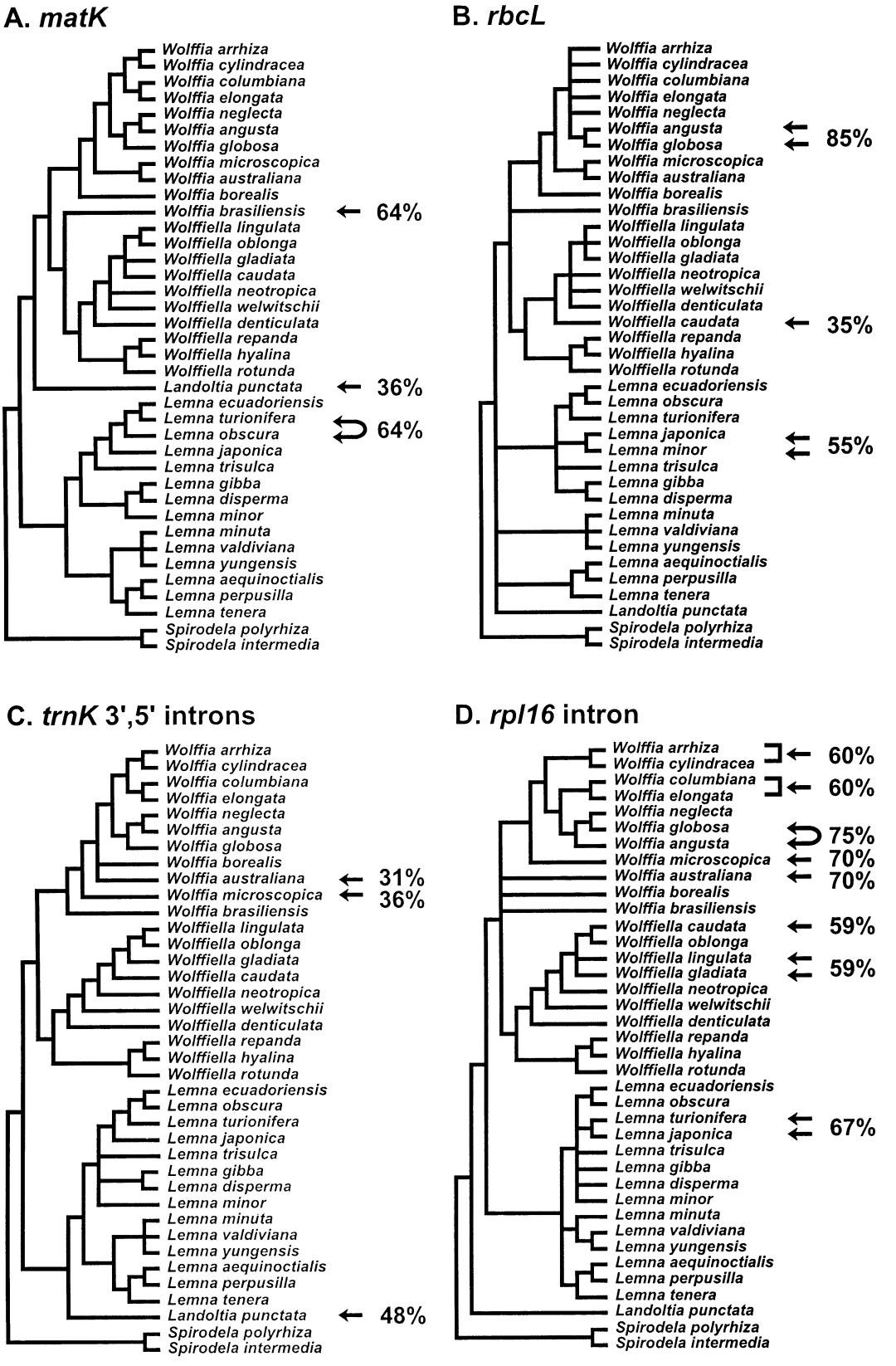
FIG. 1. A-D, Maximum parsimony cladograms of Lemnaceae species derived from four DNA sequence data sets (strict consensus trees shown). A. *matK* cladogram (6 trees @ 893 steps; CI=0.766; $CI_{(exc.)}$=0.704; RI=0.911). B. *rbcL* cladogram (3,990 trees @ 321 steps; CI=0.623; $CI_{(exc.)}$=0.538; RI=0.846); C. *trnK* 3′ and 5′ introns (36 trees @ 804 steps; CI=0.749; $CI_{(exc.)}$=0.684; RI=0.880); *rpl16* intron (75 trees @ 371 steps; CI=0.752; $CI_{(exc.)}$=0.690; RI=0.879). Arrows indicate nodes (with bootstrap support) that occur in a position different from other trees shown. Curved arrows indicate taxa reversed in orientation; square brackets indicate congruent groups of taxa resolved in different positions.


A. matK
Wolffia arrhiza
Wolffia cylindracea
Wolffia columbiana
Wolffia elongata
Wolffia neglecta
Wolffia angusta
Wolffia globosa
Wolffia microscopica
Wolffia australiana
Wolffia borealis
Wolffia brasiliensis
64%
Wolffiella lingulata
Wolffiella oblonga
Wolffiella gladiata
Wolffiella caudata
Wolffiella neotropica
Wolffiella welwitschii
Wolffiella denticulata
Wolffiella repanda
Wolffiella hyalina
Wolffiella rotunda
Landoltia punctata
36%
Lemna ecuadoriensis
Lemna turionifera
Lemna obscura
64%
Lemna japonica
Lemna trisulca
Lemna gibba
Lemna disperma
Lemna minor
Lemna minuta
Lemna valdiviana
Lemna yungensis
Lemna aequinoctialis
Lemna perpusilla
Lemna tenera
Spirodela polyrhiza
Spirodela intermedia
B. rbcL
Wolffia arrhiza
Wolffia cylindracea
Wolffia columbiana
Wolffia elongata
Wolffia neglecta
Wolffia angusta
Wolffia globosa
85%
Wolffia microscopica
Wolffia australiana
Wolffia borealis
Wolffia brasiliensis
Wolffiella lingulata
Wolffiella oblonga
Wolffiella gladiata
Wolffiella neotropica
Wolffiella welwitschii
Wolffiella denticulata
Wolffiella caudata
35%
Wolffiella repanda
Wolffiella hyalina
Wolffiella rotunda
Lemna ecuadoriensis
Lemna obscura
Lemna turionifera
Lemna japonica
Lemna minor
55%
Lemna trisulca
Lemna gibba
Lemna disperma
Lemna minuta
Lemna valdiviana
Lemna yungensis
Lemna aequinoctialis
Lemna perpusilla
Lemna tenera
Landoltia punctata
Spirodela polyrhiza
Spirodela intermedia
C. trnK 3',5' introns
Wolffia arrhiza
Wolffia cylindracea
Wolffia columbiana
Wolffia elongata
Wolffia neglecta
Wolffia angusta
Wolffia globosa
Wolffia borealis
Wolffia australiana
31%
Wolffia microscopica
36%
Wolffia brasiliensis
Wolffiella lingulata
Wolffiella oblonga
Wolffiella gladiata
Wolffiella caudata
Wolffiella neotropica
Wolffiella welwitschii
Wolffiella denticulata
Wolffiella repanda
Wolffiella hyalina
Wolffiella rotunda
Lemna ecuadoriensis
Lemna obscura
Lemna turionifera
Lemna japonica
Lemna trisulca
Lemna gibba
Lemna disperma
Lemna minor
Lemna minuta
Lemna valdiviana
Lemna yungensis
Lemna aequinoctialis
Lemna perpusilla
Lemna tenera
Landoltia punctata
48%
Spirodela polyrhiza
Spirodela intermedia
D. rpl16 intron
Wolffia arrhiza
Wolffia cylindracea
60%
Wolffia columbiana
Wolffia elongata
60%
Wolffia neglecta
Wolffia globosa
75%
Wolffia angusta
Wolffia microscopica
70%
Wolffia australiana
70%
Wolffia borealis
Wolffia brasiliensis
Wolffiella caudata
59%
Wolffiella oblonga
Wolffiella lingulata
Wolffiella gladiata
59%
Wolffiella neotropica
Wolffiella welwitschii
Wolffiella denticulata
Wolffiella repanda
Wolffiella hyalina
Wolffiella rotunda
Lemna ecuadoriensis
Lemna obscura
Lemna turionifera
Lemna japonica
67%
Lemna trisulca
Lemna gibba
Lemna disperma
Lemna minor
Lemna minuta
Lemna valdiviana
Lemna yungensis
Lemna aequinoctialis
Lemna perpusilla
Lemna tenera
Landoltia punctata
Spirodela polyrhiza
Spirodela intermedia

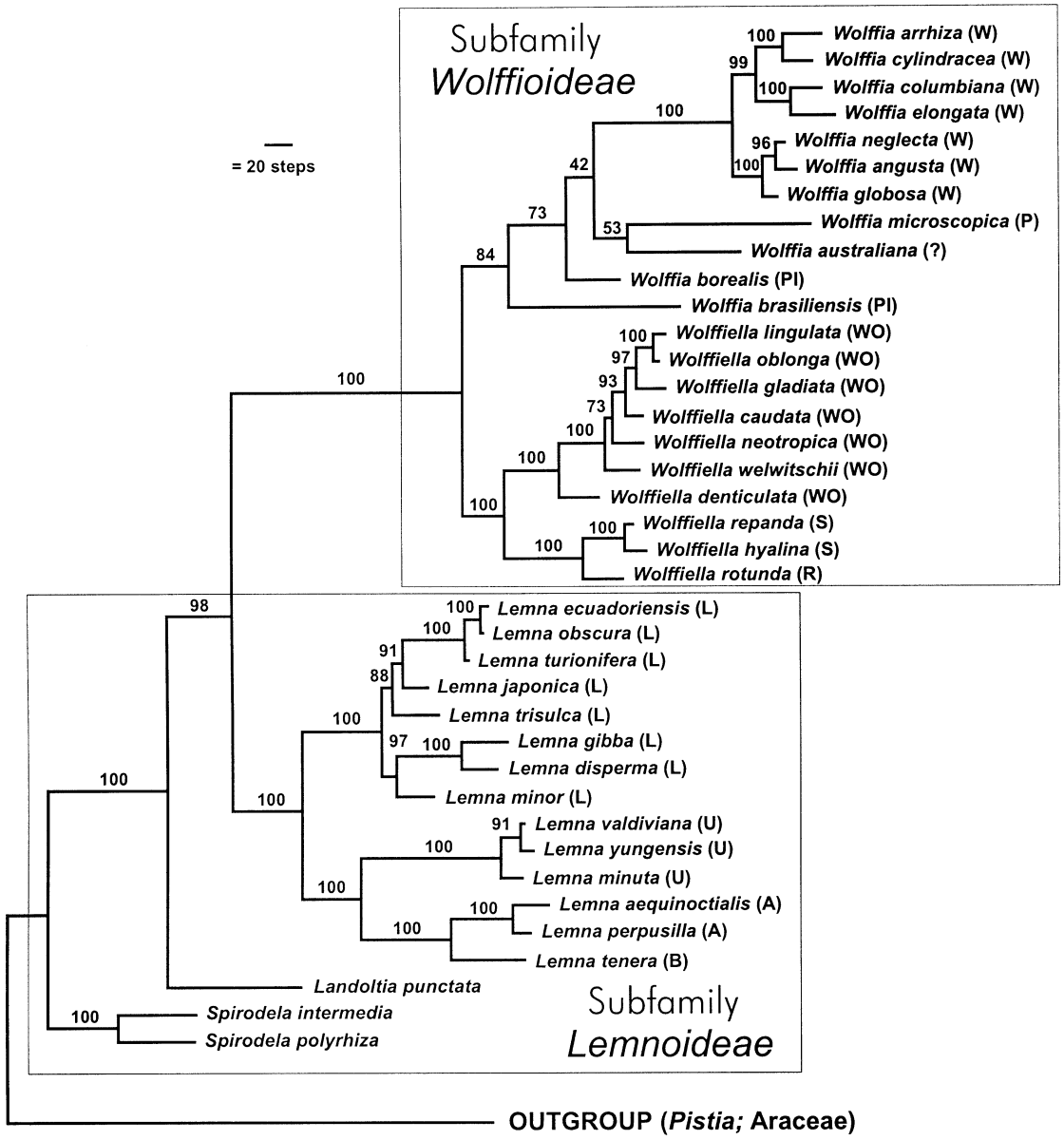


FIG. 2. Maximum parsimony cladogram of Lemnaceae species resulting from combined analysis of morphological, flavonoid, allozyme and DNA sequence data. Shown is the single tree resulting from the analysis (2,674 steps; CI=0.711; $CI_{(exc.)}$=0.643; RI=0.875). Bootstrap support for nodes is indicated above branches. The two widely accepted subfamilies of Lemnaceae are indicated; subfamily Wolffiodeae is holophyletic; subfamily *Lemnoideae* is paraphyletic. Sectional designations are given in parentheses after each species. *Lemna*: A, *Alatae*; B, *Biformes*; L, *Lemna*; U, *Uninerves*. *Wolffia*: P, *Pseudorrhizae*; PI, *Pigmentatae*; W, *Wolffia*; ?, unassigned to section. *Wolffiella*: R, *Rotundae*; S, *Stipitatae*; WO, *Wolffiella*.

gree of genetic cohesiveness, to the exclusion of other species. Overall, we feel that the combined morphological, allozyme and DNA sequence data have indicated the existence of 38 species in the collections that we examined, though we cannot discount the possibility that further study would reveal additional ''cryptic'' species.

Outgroups and Rooting. Traditionally, two hypotheses have been proposed for the phylogenetic placement of Lemnaceae in the monocotyledons. The family is assumed to be related either to *Pistia* in the Araceae (Hegelmaier 1868; Engler 1877; Velenovsky 1907; Arber 1919, 1920; Brooks 1940; Meusel 1951; Maheshwari 1956, 1958a, b; Daubs 1965;) or to the Alismatidae (Eichler 1875; Lawalree 1945; Deyl 1955). The association with Alismatidae was influenced by the er-

TABLE 6. Results of phylogenetic analysis (combined data) and recommended phylogenetic classification of Lemnaceae.

Taxon	Resolution	Bootstrap support	Branch length
Lemnaceae Dumort.	monophyletic	100%	163 steps
Subfamily *Lemnoideae* Engl.	paraphyletic	n/a	n/a
Landoltia Les & Crawford	monophyletic	monotypic	103 steps
1. *L. punctata* (G. Meyer) Les & Crawford			
Lemna L.	monophyletic	100%	52 steps
Section *Lemna*	monophyletic	100%	60 steps
2. *L. disperma* Hegelm.			
3. *L. ecuadoriensis* Landolt			
4. *L. gibba* L.			
5. *L. japonica* Landolt			
6. *L. minor* L.			
7. *L. obscura* (Austin) Daubs			
8. *L. trisulca* L.			
9. *L. turionifera* Landolt			
Section *Alatae* Hegelm.	monophyletic	100%	47 steps
10. *L. aequinoctilalis* Welw.			
11. *L. perpusilla* Torrey			
Section *Biformes* Landolt	monophyletic	monotypic	58 steps
12. *L. tenera* Kurz.			
Section *Uninerves* Hegelm.	monophyletic	100%	106 steps
13. *L. minuta* Humb., Bonpl. & Kunth			
14. *L. valdiviana* Phil.			
15. *L. yungensis* Landolt			
Spirodela Schleid.	monophyletic	100%	114 steps
16. *S. intermedia* W. Koch			
17. *S. polyrhiza* (L.) Schleid.			
Subfamily *Wolffioideae* Engl.	monophyletic	100%	174 steps
Wolffia Horkel ex Schleid.	monophyletic	84%	35 steps
Section *Wolffia*	monophyletic	100%	105 steps
18. *W. angusta* Landolt			
19. *W. arrhiza* (L.) Horkel ex Wimm.			
20. *W. columbiana* Karst.			
21. *W. cylindracea* Landolt			
22. *W. elongata* Landolt			
23. *W. globosa* (Roxb.) Hartog			
24. *W. neglecta* Landolt			
Section (unassigned)			
25. *W. australiana* (Benth.) Hartog & Plas	n/a	n/a	n/a
Section *Pigmentatae* Landolt	paraphyletic	n/a	n/a
26. *W. borealis* (Engelm. ex Hegelm) Landolt			
27. *W. brasiliensis* Wedd.			
Section *Pseudorrhizae* Landolt	monophyletic	monotypic	139 steps
28. *W. microscopica* (Griff.) Kurz			
Wolffiella	monophyletic	100%	31 steps
Section *Wolffiella*	monophyletic	100%	41 steps
29. *W. caudata* Landolt			
30. *W. denticulata* (Hegelm.) Hegelm.			
31. *W. gladiata* (Hegelm.) Hegelm.			
32. *W. lingulata* (Hegelm.) Hegelm.			
33. *W. neotropica* Landolt			
34. *W. oblonga* (Phil.) Hegelm.			
35. *W. welwitschii* (Hegelm.) Monod			
Section *Rotundae*	monophyletic	monotypic	31 steps
36. *W. rotunda* Landolt			
Section *Stipitatae*	monophyletic	100%	32 steps
37. *W. hyalina* (Delile) Monod			
38. *W. repanda* (Hegelm.) Monod			

roneous belief that Lemnaceae possessed helobial endosperm, though its formation is actually cellular (Brooks 1940). Even though Lemnaceae share certain features with Alismatidae, the similarity between these groups indicates only a distant relationship (Landolt 1986).

The relationship of Lemnaceae to Araceae was proposed more than 175 years ago by Hooker and Brown

who considered them to be ''. . . a reduced or simplified *Aroidea*, next akin to *Pistia*'' (Smith 1824). Schleiden (1838, 1839) essentially agreed with this interpretation. Largely because of their common free-floating habit, Lindley (1853) combined duckweeds and water lettuce (*Pistia*) in the family Pistiaceae which he placed with Araceae in order Arales. However, the distinctness of the family Lemnaceae (excluding *Pistia*) has been stressed repeatedly by many authors (Engler 1892; Wettstein 1901; Lotsy 1911; Hallier 1912; Bessey 1915; Skottsberg 1940; Kimura 1956; Emberger 1960; Melchior 1964; Cronquist 1968, 1988; Hutchinson 1973; Cronquist 1988; Thorne 1992).

The tradition of segregating Lemnaceae from Araceae (with *Pistia*) did not detract substantially from arguments that *Pistia* represented the sister group to duckweeds (e.g. Arber 1919). Arber (1920) identified *Pistia* as ''. . . the member of the Araceae most nearly allied to the duckweeds.'' Daubs (1965) concluded that the family was ''. . . related to Araceae through the water-lettuce *Pistia*.'' Hutchinson (1975, p. 113) speculated that the Lemnaceae were ''. . . an extreme paedomorphic reduction of a pleustonic ancestor allied to *Pistia*.'' Cronquist (1988) remarked that *Pistia* ''. . . is seen pointing the way toward *Spirodela*, the least reduced genus of Lemnaceae.'' Zennie and McClure (1977) noted similar biochemical (flavonoid) pathways in *Pistia* and Lemnaceae, but their comparison emphasized general classes of compounds rather than specific constituents.

Contrary opinions of duckweed relationships were suggested early on by Koch (1852) who emphasized many dissimilarities between *Pistia* and *Lemna*. Dahlgren et al. (1985) agreed that the ''often stated'' link between *Pistia* and Lemnaceae was unlikely, although duckweeds were probably ''. . . extremely derived offshoots from araceous ancestors (the closest ones being perhaps not necessarily *Pistia*).'' Palynological data do not indicate a close relationship between *Pistia* and the Lemnaceae (Grayum 1984) and Landolt (1986) stressed that most evidence of a common ancestry for *Spirodela* and *Pistia* was based upon morphological features that are widespread among monocots. Landolt (1986) contrasted numerous features that differ in *Spirodela* and *Pistia* concluding that, ''. . . there are too many differences between *Pistia* and Lemnaceae to make a direct descent of *Spirodela* from *Pistia* convincing.'' He attributed most of the similarities between the groups to convergent aquatic adaptations.

The closest living relative of Lemnaceae is yet to be determined. However, it is at least fairly certain that duckweeds are not closely related to Alismatidae. An *rbcL* survey of Alismatidae (Les et al. 1997c) strongly supported the monophyly of a clade containing *Lemna*, *Pistia*, and seven other genera of Araceae (98% bootstrap support), which was distinct from a clade containing 69 species (representing all families and orders) of Alismatidae (96% bootstrap support). Of the eight Araceae taxa in that analysis, *Lemna* was closest to a clade consisting of *Pistia* and *Ariopsis*, with both *Lemna* and *Pistia* having relatively long branches in the analysis.

Cladistic analyses of morphological (Mayo et al. 1995) and cpDNA RFLP (French et al. 1995) data also indicated the derivation of the Lemnaceae from within Araceae, but did not indicate a close (i.e., ''sister group'') relationship between *Pistia* and Lemnaceae. Both *Pistia* and Lemnaceae were isolated among genera of subfamily *Aroideae* in the RFLP analysis (French et al. 1995). Yet, Lemon and Posluszny (2000a, 2000b) examined the developmental biology of *Pistia* and Lemnaceae, concluding that shoots of Lemnaceae appear to have evolved from a *Pistia*-like shoot system. Thus, it may be premature to dismiss the possibility of a close relationship between *Pistia* and Lemnaceae. Inclusion of duckweeds within Araceae (e.g. Maheshwari 1958; Dahlgren et al. 1985; Mayo et al. 1995) is convincingly supported by phylogenetic analyses of morphological and molecular data, yet there is still no definitive indication of which specific aroid taxa are closest phylogenetically to the duckweed clade. If these findings are corroborated by further studies, they would support the merger of the family Lemnaceae with Araceae.

The most recent available data (French et al. 1995; Mayo et al. 1995) indicate that duckweeds are closely allied to the monoecious *Aroideae*, which arguably constitute the most suitable outgroup for Lemnaceae. Thus, *Pistia* (Araceae, *Aroideae*) is an appropriate outgroup, even though it remains to be determined whether it is the closest living aroid relative of duckweeds. In a practical sense, no aroid taxon represents a suitable outgroup for our morphological analyses where the data set contains mainly characters restricted to Lemnaceae. A similar problem in Nymphaeaceae was resolved using molecular data of various putatively related taxa to determine the rooting topology within the family; ''ingroup'' rooting was then used for subsequent analyses (Les et al. 1999). We followed a similar approach using *rbcL* sequences from eight Araceae species (including *Pistia*), along with *matK*, *trnK*, *rpl16* intron sequences and flavonoid data from *Pistia* to evaluate the root of Lemnaceae. These analyses consistently identified a clade comprising *Spirodela polyrhiza* and *S. intermedia* in the basal position of Lemnaceae. Cladograms in all subsequent analyses were rooted using *S. polyrhiza* and *S. intermedia* as the ''outgroup'' for the remainder of the family. This method of ingroup rooting alleviated problems with long branch attraction (from distant outgroups) and allowed the entire data set (including morphological characters) to be used in our phylogenetic analyses.

Influence of Data Partitions on Tree Construction in Lemnaceae. The cpDNA data showed instances of statistically significant incongruency (Table 3), despite the fact (assuming maternal inheritance of cpDNA in Lemnaceae) that they are ''linked'' genetically on the same chloroplast DNA molecule and presumably have experienced the same phylogenetic history. Most cases of incongruency involved comparisons of coding to non-coding regions (Table 3).

The source of much incongruency was readily traced to a single taxon (*Wolffia brasiliensis*), which possessed a divergent *matK* sequence. Analysis of *matK* data singularly placed *W. brasiliensis* within the genus *Wolffiella*, whereas all other data resolved this species either within *Wolffia*, or placed it unresolved between the genera (i.e., *rbcL*). Because morphological data argue persuasively for inclusion of *W. brasiliensis* in *Wolffia* (Landolt 1986; Les et al. 1997a), we do not consider an association with *Wolffiella* (as indicated by *matK* data) to be a realistic possibility.

Phylogenetic placement of *W. brasiliensis* within *Wolffia* could be achieved with *matK* data by using a step matrix to acutely downweight transitions in parsimony analysis (tv:ti = 5:1; codon positions 3:2:1 = 1:2:1.5). However, this weighting scheme also resolved *Landoltia* as a sister group to subfamily *Wolffioideae* (results not shown). The phylogeny estimated from maximum likelihood (even when using more complex models such as HKY85) was unable to resolve *Wolffia* as monophyletic. When *matK* was excluded from the analyses, bootstrap support for *Wolffia* (including *W. brasiliensis*) increased to 97%. When *Wolffia brasiliensis* was excluded from the data set (*matK* data included), incongruency between *matK* and other cpDNA data disappeared ($p = 0.24$). We resequenced *matK* in two additional accessions of *W. brasiliensis* to demonstrate that no error existed in our original sequence for this species. Aside from several minor nucleotide substitutions, all accessions of *W. brasiliensis* essentially agreed and produced the misplacement with *Wolffiella* upon phylogenetic analysis.

Although ''long branch attraction'' is a phenomenon often discussed at taxonomic levels higher than family, the branches leading to basal *Wolffia* species (*W. brasiliensis*, *W. borealis*, *W. microscopica*, *W. australiana*) are extremely long, as is the branch leading to the *Wolffia*/*Wolffiella* clade itself (Fig. 2). In the case of *W. brasiliensis*, the many DNA substitutions (notably transitions) that have taken place in this branch have effectively attenuated the phylogenetic signal in the DNA data to the extent that accurate placement of this taxon is thwarted, most noticeably using unweighted *matK* data. The other divergent *Wolffia* species are also affected by this phenomenon, but not to the extent causing their misplacement outside of the genus. It is of interest to note that overweighting of second codon positions in *matK* data (4× relative to first positions) induces the relocation of all ''divergent'' *Wolffia* species (*W. brasiliensis*, *W. borealis*, *W. microscopica*, *W. australiana*) to *Wolffiella* (results not shown).

Evidence of signal loss in divergent DNA sequences is provided by a comparison of skewness (g_1) statistics, which reflect the extent of non-random structure (arguably, phylogenetic signal) in data sets (Hillis 1991). Although divergent taxa, such as outgroups, typically increase the skewness of phylogenetic trees toward a more negative g_1 value (Les et al. 1999), the inclusion of divergent *Wolffia* species had the opposite effect and actually decreased skewness. This result is demonstrated by changes in skewness (g_1) upon single taxon removal, relative to skewness when all taxa are included (Table 5). Removal of *Wolffia microscopica*, *W. brasiliensis*, *W. borealis* or *W. australiana* increases the negativity of g_1 considerably; whereas, removal of other *Wolffia* species results in a more positive g_1 value (Table 5). Thus, inclusion of *W. microscopica*, *W. brasiliensis*, *W. borealis* or *W. australiana* sequences attenuates non-random (i.e., phylogenetic) structure in the data, as indicated by the reduced phylogenetic signal overall. The less divergent species all contribute to non-random signal as evidenced by the reduction in skewness following their removal from analyses. It is also noteworthy that signal loss accompanied the four divergent *Wolffia* species in all cpDNA data sets. However, the greatest influence on skewness was observed for *matK* coding sequences (Table 5), which also yielded much incongruency in our phylogenetic analyses. Data from the *rpl16* intron were least influenced by inclusion of the divergent *Wolffia* taxa and *trnK* produced an intermediate effect (Table 5). Thus, in the case of *Wolffia*, non-coding cpDNA regions provided superior phylogenetic signal compared to the *matK* coding region. It is not obvious why a stronger phylogenetic signal is retained in the *rpl16* and *trnK* introns (the latter physically flanking the *matK* coding region) than *matK*. This unexpected result advises against wide generalization on the utility of DNA sequence data for phylogenetic analysis.

The incomplete phylogenetic resolution of *W. brasiliensis* from *rbcL* data is not unexpected, given the relatively lower resolution expected from more slowly evolving sequences. *Wolffia* is poorly resolved in general by *rbcL* data alone (Fig. 1b). When all data are combined, the position of *W. brasiliensis* appears to be accurate; it is placed phylogenetically at the base of *Wolffia* in agreement with the hypothesis of Landolt (1986). However, the inclusion of *W. brasiliensis* reduces bootstrap support for *Wolffia* from 97% to 84%, which is directly attributable to incongruency of *matK* data in the placement of this species.

The systematic utility of morphological and micromolecular data in Lemnaceae has recently been eval-

TABLE 7. Taxonomic history of Lemnaceae showing number of species recognized in different genera as indicated in major treatments. Additional citations include Reichenbach (1828) for *Staurogeton*; Bartling (1830) for *Wolffia*; Hegelmaier (1895) for *Wolffiella*; and Les and Crawford (1999) for *Landoltia*.

		Linnaeus, 1753	Schleiden, 1839	Hegelmaier, 1868	Daubs, 1965	Hartog & Van der Plas, 1970	Landolt, 1986	Current (see text)
Lemna	(1753)	4	2	7	9	9	13	14
Staurogeton	(1828)	—	—	—	—	—	—	—
Spirodela	(1839)	—	1	2	5	4	3	2
Telmatophace	(1839)	—	1	—	—	—	—	—
Wolffia	(1844)	—	1	12	8	7	9	11
Wolffiella	(1895)	—	—	—	6	5	9	10
Wolffiopsis	(1970)	—	—	—	—	1	—	—
Pseudowolffia	(1970)	—	—	—	—	3	—	—
Landoltia	(1999)	—	—	—	—	—	—	1
TOTAL	# SPECIES:	4	5	21	28	29	34	38

uated using cladistic analyses (Les et al. 1997a). Neither data set contains enough information on its own to provide adequate species level resolution in the family (Les et al. 1997a). Problems for phylogenetic analyses also arise because flavonoids remain uninvestigated for half of the 38 duckweed species. Flavonoid compounds also show many convergent losses (particularly with anthocyanins) which lead to unreasonable phylogenetic results such as the polyphyly of the genera *Wolffia* and *Lemna* (Les et al. 1997a). Several morphological characters support the distinctness of many taxonomic sections recognized in Lemnaceae; however, they do not clearly define the genera when subjected to phylogenetic analysis (Les et al. 1997a).

Morphological and micromolecular data sets do, however, resolve the subfamily *Wolffioideae* with high internal support (100% bootstrap). Both data sets show the distinctness of *Landoltia punctata*; morphology provides good internal support for sections *Alatae* (*Lemna*), *Stipitatae*, and *Wolffiella* (*Wolffiella*) (Les et al. 1997a). Chromosome data for Lemnaceae are not helpful systematically due to the wide range in variation of euploid and aneuploid numbers that occurs even within single species (Les and Philbrick 1993). *Lemna*, *Spirodela*, *Wolffia*, and *Wolffiella* all contain species with counts ranging from $2n = 20$–80 and even the monotypic *Landoltia punctata* contains $2n = 40, 43, 44, 46, 50$ cytotypes (Landolt 1986; Les and Philbrick 1993). Thus, the power of phylogenetic resolution in Lemnaceae relies mainly on the molecular data sets that are incorporated.

Allozyme data have been invaluable for verifying species boundaries in the Lemnaceae (see above) and also for obtaining preliminary estimates of phylogenetic relationships within duckweed genera. However, duckweed allozymes diverge extensively at the genus level (where a marked reduction in genetic identity occurs), and provide essentially no information for evaluating relationships among the genera (Crawford and Landolt 1993, 1995; Crawford et al. 1995, 1996, 1997). Among the different types of molecular data considered (flavonoids, allozymes, DNA sequences), the latter have provided the best resolution and internal support for interspecific relationships. When all data are combined, a fairly clear picture of duckweed relationships emerges because each data set contributes various levels of support to different regions of the resulting phylogeny. Despite some relatively minor incongruence among the data sets, we believe that results depicted by our combined data cladogram represent a fairly accurate appraisal of duckweed phylogeny wherever the level of internal support is high (e.g., $>$ 70%). Only three nodes in this cladogram do not satisfy this criterion, and represent a group of four divergent *Wolffia* species (Fig. 2). Although the interrelationships of these four species remains uncertain (beyond their basal position in *Wolffia*), the interspecific phylogeny appears now to be well resolved for the remainder of Lemnaceae.

Prior Taxonomy and Systematics: Comparisons and Evaluations From Present Results. The first monograph of Lemnaceae was written by Schleiden (1839), who recognized only five species (Table 7). Hegelmaier (1868) contributed a thorough monograph of Lemnaceae. He regarded *Wolffiella* as a subgenus of *Wolffia*, but later segregated it as a separate genus (Hegelmaier 1895). However, he did not include *W. welwitschii*, *W. hyalina*, and *W. repanda* within the new genus. Of the 21 species recognized by Hegelmaier, 20 continue to be recognized taxonomically. Thompson (1896) treated the ''ligulate'' *Wolffias* of the United States and recognized four genera in his revision of North American Lemnaceae but provided no intrageneric classification (Thompson 1898). In 1949, Monod transferred *W. welwitschii*, *W. hyalina*, and *W. repanda* to the genus *Wolffiella*. Daubs (1965) published a monograph of Lemnaceae that recognized four genera (*Spirodela*, *Lemna*, *Wolffia*, *Wolffiella*), but he did not suggest an intrageneric classification scheme. Hartog and Van der Plas (1970) established two new genera: *Pseudowolffia*

(which included *Wolffiella hyalina* and *Wolffiella repanda*) and *Wolffiopsis* (which included *Wolffiella welwitschii*). Hartog and Van der Plas (1970) regarded Hegelmaier's two ''species groups'' in *Spirodela* as having differences ''too insignificant to regard them as different sections.'' Landolt (1986) published the most comprehensive monograph of Lemnaceae to date, which recognized 34 species in 14 sections and two subfamilies.

Daubs (1965) placed *L. perpusilla* and *L. aequinoctialis* in synonymy, but these species are quite distinct, differing by 42 steps in our combined data cladogram (Fig. 2). Landolt (1986) suggested the possible hybrid origin of *L. perpusilla* (involving *L. aequinoctialis* and *L. turionifera*); however, our results provide no evidence for this hypothesis. Rather, *L. perpusilla* is a distinct species within the section *Alatae* clade (Fig. 2). Daub's synonymy of *Wolffia arrhiza* and *W. cylindracea* is unwarranted by our results, which show them as distinct sister species that differ by 53 steps in our combined analysis tree (Figs. 1, 2). Daubs regarded *L. disperma* and *L. obscura* only doubtfully as distinct species, but they are distinct in all analyses (Figs. 1, 2).

Thompson (1898) believed *Lemna* to be more closely related to *Wolffiella* than to *Wolffia*. Our phylogenetic results do not dismiss this possibility, but equally support a close relationship of *Lemna* and *Wolffia* (Fig. 2). Lawalree (1945) accepted an evolutionary model of increasing specialization rather than reduction in Lemnaceae and suggested that *Spirodela* was derived from *Wolffia*. All of our data sets, either independently or combined, dismiss this hypothesis by placing *Wolffia* in a highly derived position relative to *Spirodela* when trees are rooted by araceous outgroups. Lawalree's hypothesis has not gained acceptance and in our view should no longer be given serious consideration.

Daubs (1965, p. 5) remarked that ''. . . it was not possible to trace a direct lineal relationship'' among the duckweed genera, and provided only sparse comments on their hypothetical relationships. He referred to *Spirodela intermedia* as ''an ancestral type,'' which is borne out by our phylogenetic analyses. He also regarded *Lemna disperma*, *L. gibba*, and *L. obscura* as ''closely allied.'' Our analyses show a close relationship between *L. disperma* and *L. gibba*, but do not show *L. obscura* to be particularly closely related to them (Fig. 2). Daubs also stated that *L. obscura* ''partakes of some of the characters of both *L. gibba* and *L. minor*.'' Our resolution of these species within the clade comprising section *Lemna* lends support to this general observation.

Flavonoids indicate a biphyletic origin of *Wolffia* (Turner 1967; Les et al. 1997a), but this is surely an artifact of convergent compound losses (Les et al. 1997a). Our analyses portray *Wolffia* either as distinct, or (*matK* data) with *Wolffia brasiliensis* misplaced to *Wolffiella* (Figs. 1–2); however, no *Wolffia* species occur with *Lemna* and *Spirodela* species as depicted by the flavonoid analyses. Our combined data (including flavonoids) cladogram (Fig. 2) resolves *Wolffia* as a monophyletic clade with good internal support.

Hartog (1975) assessed intrafamilial relationships in Lemnaceae using morphological and phytochemical data (mainly flavonoids). However, he could not confirm a phylogenetic basis for the presumed morphological reduction series typically proposed for Lemnaceae, which is depicted as a pattern of progressive reduction from *Spirodela* to *Lemna* to species of subfamily *Wolffioideae*. Hartog concluded that morphological data clearly showed a trend in reduction from *Spirodela* to *Lemna* and subfamily *Wolffioideae*, but he could not ascertain (as Thompson attempted) the most primitive taxon within subfamily *Wolffioideae*. Ivanova (1973) believed that the truly phylogenetic series for duckweeds had *Wolffiella* derived from *Wolffia*. Our results (Figs. 1, 2) provide phylogenetic support to a morphological reduction series from *Spirodela* to *Wolffia*; however, the even split of clades (genera) within subfamily *Wolffioideae* makes it impossible to determine whether *Wolffiella* or *Wolffia* is more primitive on the basis of our data alone.

Ivanova (1973) believed *Lemna* to be biphyletic with *Lemna gibba*, *L. minor*, *L. trisulca*, *L. disperma*, and *L. obscura* derived from *Spirodela intermedia* and *L. aequinoctialis*, *L. perpusilla*, *L. valdiviana*, and *L. minuta* derived from *Landoltia punctata*. Our results lend no support to this hypothesis; rather, *Lemna* is clearly a monophyletic clade within Lemnaceae (Figs. 1, 2).

SUBFAMILIES OF LEMNACEAE. Most authors have agreed on ''the distinction of two units'' within Lemnaceae (Landolt 1986). Hegelmaier (1868) recognized these groups taxonomically as the tribes *Lemneae* and *Wolffieae*. Engler (1889) and most subsequent authors have recognized this major division at the rank of subfamily (*Lemnoideae* Engler, *Wolffioideae* Engler). Landolt (1986) described Lemnaceae as ''sharply divided into two subfamilies,'' which are ''well separated.'' The taxonomic division of Lemnaceae is clearly evidenced by our results, which show an extremely long branch (174 steps) separating subfamilies *Lemnoideae* and *Wolffioideae*. However, taxonomic recognition of these subfamilies creates some interpretive problems in light of the perceived phylogenetic relationships. Acceptance of subfamily *Wolffioideae* is not controversial. It resolves as a remarkably differentiated, well supported, monophyletic clade (100% bootstrap) in all analyses of individual or combined data sets (Figs. 1, 2). On the other hand, recognition of subfamily *Lemnoideae* is problematic, given that it is resolved as a paraphyletic grade of taxa comprising *Spirodela*, *Landoltia*, and *Lemna* (Fig. 2). The taxonomic recognition of paraphyletic taxa is controversial, and we do not wish to debate this issue here. Rather, we simply clarify that as currently

defined, subfamily *Lemnoideae* represents a paraphyletic assemblage of three clades that correspond to the genera *Spirodela*, *Landoltia*, and *Lemna*. The elimination of paraphyletic subfamilies would require the further designation of *Landoltia* (monotypic) and also the genus *Spirodela* (two species) as additional subfamilies. However, doing so would subdivide the family much in the same way that the generic classification already accomplishes.

GENERA AND SECTIONS OF LEMNACEAE. From the nine genera of Lemnaceae described since 1753, only five (Table 7) can be justified as phylogenetically meaningful clades (but see comments below). Landolt (1986) remarked that ''the differences between *Spirodela* and *Lemna* are not very great'' but that the genera could be distinguished by a number of morphological and biochemical characters. Although some authors have merged *Spirodela* and *Lemna*, our phylogenetic results support their segregation. We recognize the genus *Spirodela*, but as amended to exclude *S. punctata* (Les and Crawford 1999). The remaining species (*S. polyrhiza* and *S. intermedia*) resolve as a well-supported basal clade in Lemnaceae (Figs. 1, 2). The recent transfer of *Spirodela punctata* to a new genus *Landoltia* was prompted by preliminary results that originated during the course of the current study (Les and Crawford 1999). With even more data to consider at this time, a distinct position of *Landoltia* remains secure phylogenetically in all analyses (Figs. 1, 2) although its placement varies somewhat in analyses of single data sets. The combined data cladogram (Fig. 2) necessitates the recognition of *Landoltia* in order to maintain the integrity of all other duckweed genera as holophyletic clades. The isolated position of *Landoltia* is evidenced by the high level of bootstrap support that delimits the clade both from *Spirodela* (100%) and the remainder of Lemnaceae (98%).

Lemna, the original duckweed genus of Linnaeus (1753), is well-supported as a monophyletic clade. Although morphological data depict a paraphyletic grade of genera (Les et al. 1997a), the combined molecular and non-molecular data place all *Lemna* species in a robust clade with 100% bootstrap support (Fig. 2). Two genera (*Telmatophace*, *Staurogeton*) have been proposed to subdivide *Lemna*, but neither has phylogenetic integrity. The genus *Telmatophace* was erected by Schleiden (1839) to accommodate *Lemna gibba*, one of the original species named by Linnaeus. This taxon did not gain acceptance as a genus, but was retained as a subgenus by Hegelmaier (1868). Neither Hartog and Van der Plas (1970) nor Landolt (1986) recognized *Telmatophace* at any rank. Landolt (1986) observed that multiple ovules (a defining character of *Telmatophace*) also occur in *L. disperma*, and that some plants of *L. gibba* are uniovular. *Lemna gibba* is distinct from other *Lemna* species both morphologically and phytochemically (Les et al. 1997a), but in light of our combined phylogenetic evidence, this species nests well within species belonging to *Lemna* sect. *Lemna* (Fig. 2). Consequently, the recognition of *Telmatophace* is not defensible phylogenetically from our results.

Reichenbach (1828) segregated the morphologically distinctive *Lemna trisulca* as subgenus *Staurogeton* (not validly published), which was elevated to generic status by Schur (1866). Schleiden (1839) placed *Staurogeton* in synonymy with *Lemna trisulca*. Hegelmaier (1868) later recognized *Staurogeton* (*L. trisulca*) at the sectional level with Hartog and Van der Plas (1970) eventually restoring the taxon to subgeneric rank (Hartog 1975). Landolt (1986) reduced *L. trisulca* back to a section of *Lemna*, but under the name *Hydrophylla* Dumortier (1827), which has priority over *Staurogeton* at sectional rank. Taxonomic dispositions over 150 years indicate that *Lemna trisulca* has generally been regarded as distinct within the genus, but not so distinct as to warrant status as a separate genus. Cladistically, *Lemna trisulca* nests within the genus *Lemna* where it is distinct morphologically (but not by its flavonoid profile) from species of *Lemna* sect. *Lemna* (Les et al. 1997a). Yet, Landolt (1986) admitted that *L. trisulca* was ''in many respects on the same level as most species of the section *Lemna*.'' Our results (Fig. 2) support Landolt's observations by clearly placing *L. trisulca* within the clade representing the genus *Lemna*. Phylogenetically, this well-supported monophyletic clade (100% bootstrap support) precludes the recognition of *L. trisulca* as a separate genus. Despite its few morphological peculiarities, there is almost no phylogenetic basis for the segregation of this species either as a unique subgenus (as advocated by Hartog 1975) or as a section of *Lemna* (Figs. 1, 2). The combined data cladogram clearly places *L. trisulca* within the clade of species that comprises section *Lemna* (100% bootstrap support). We recommend the transfer of *L. trisulca* to section *Lemna* where it represents a distinctive, but phylogenetically integral member of that group.

Our results call for a renewed taxonomic evaluation of sectional divisions within *Lemna*. Using only morphological characters, sects. *Alatae* and *Biformes* are distinct phylogenetically, with sect. *Uninerves* to a lesser extent; it is resolved as distinct from other *Lemna* species, but on the basis of morphological characters, it is paraphyletic (Les et al. 1997a). Other than sect. *Hydrophylla* (see above), which has no phylogenetic integrity, the remaining sectional divisions (*Lemna*, *Uninerves*, *Alatae*, *Biformes*) are exceptionally well-defined as monophyletic clades, each with 100% bootstrap support (Fig. 2; Table 6). Each section has diagnostic morphological features: *Uninerves*—a single nerve vs. 3–7 nerves in other *Lemna*; *Alatae*—root sheaths winged at base vs. unwinged in other *Lemna*; *Biformes*—sub-

merged, entire, tapering fronds vs. floating, rounded fronds in other *Lemna* species (Landolt 1986).

Relative branch lengths provide another type of comparison, although our unweighted parsimony approach did not correct branch length estimates for multiple substitutions, which must have occurred throughout the molecular data. With this in mind, several observations are yet noteworthy. The branches defining *Lemna* sects. *Lemna* (60 steps), *Uninerves* (106 steps), *Biformes* + *Alatae* (68 steps), all surpass that of *Lemna* itself (52 steps) with the branch defining sect. *Alatae* (47 steps) only slightly shorter. *Lemna* sect. *Uninerves* is particularly well differentiated, and exceeds the branch length of all duckweed genera except for *Spirodela* (114 steps). The branches defining the genera *Wolffiella* (31 steps) and *Wolffia* (35 steps) are considerably shorter than those defining any of these sections. We do not advocate the delimitation of genera on the basis of relative branch lengths, but observe that these sections are substantially differentiated and certainly warrant taxonomic subdivision. Whether this subdivision should remain at the level of section or be elevated to genus is a matter of taxonomic opinion, and the latter possibility merits serious consideration.

The precise placement of *Lemna japonica* in section *Lemna* is problematic. Landolt (1986) suggested that *L. japonica* possibly originated through hybridization of *L. minor* and *L. turionifera*. Although previous allozyme studies have supported this interpretation (Hirahaya and Kadono 1995), our own allozyme studies along with comparisons of *rpl16*, *rbcL*, *matK*, and *trnK* sequences from a variety of accessions (unpublished data) have been inconclusive. A satisfactory resolution of this issue will require much additional work beyond the scope of the present paper. There is no conflict regarding the position of *L. japonica* within *Lemna* section *Lemna*, but the position of *L. japonica* as a distinct species intermediate phylogenetically between *L. minor* and *L. turionifera* (Fig. 2) deserves further scrutiny.

The genera *Wolffia* and *Wolffiella* have been retained in all major taxonomic treatments since their inception (Table 7). *Wolffia* is particularly well defined morphologically, but exhibits unusual flavonoid heterogeneity, the latter attributable to convergent compound losses (Les et al. 1997a). In *Wolffiella*, there are three morphologically distinct sections that correspond to those delimited by Landolt (1986), but the genus is paraphyletic if morphological characters are considered alone (Les et al. 1997a). However, our individual or combined data sets resolve the genus *Wolffiella* as a robust clade with 100% bootstrap support, and also resolve each section (*Wolffiella*, *Rotundae*, *Stipitatae*) as clades with 100% bootstrap support (Figs. 1, 2).

Hartog and Van Der Plas (1970) established the genus *Wolffiopsis*, thus removing *Wolffiella welwitschii* from the genus *Wolffiella*. This taxon differs morphologically from other *Wolffiella* species by possessing two flowers per frond (Hartog and Van Der Plas 1970; Landolt 1986) and by the shape and symmetry of fronds (Hartog 1975). Landolt (1986) argued that *W. welwitschii* did not even warrant recognition as a separate section, given that it differed from other *Wolffiella* species only by minor features of symmetry, which vary similarly in the genus *Lemna*. Cladistically, morphological data place *W. welwitschii* within *Wolffiella* where it is nested within section *Wolffiella* (Les et al. 1997a). A firm placement of *W. welwitschii* within *Wolffiella* sect. *Wolffiella* is also evidenced by data from allozymes (Crawford et al. 1997) and DNA sequence data (Figs. 1, 2). In combined phylogenetic analyses, *W. welwitschii* is placed unequivocally within the genus *Wolffiella* (100% bootstrap support), and also within section *Wolffiella* (100% bootstrap support; Fig. 2). Accordingly, our phylogenetic analyses support Landolt's (1986) conclusion that there is no rationale to justify the segregation of this taxon either at the generic or intrageneric level; recognition of the monotypic *Wolffiopsis welwitschii* would necessarily result in a polyphyletic subdivision of *Wolffiella*.

Hartog (1975) stated that *Wolffiopsis* (i.e., *Wolffiella welwitschii*) and *Wolffiella* were more closely related to each other than to either *Pseudowolffia* (i.e., *Wolffiella* section *Stipitatae*) or *Wolffia*. These relationships are corroborated by our phylogenetic results. The recognition of *Pseudowolffia* as a distinct genus (Hartog and Van Der Plas 1970) presents a more contentious issue than the above example. Hegelmaier (1868) placed *Wolffia hyalina* and *Wolffia repanda* with *Wolffia microscopica* to comprise *Wolffia* section *Stipitatae*. Monod (1949) transferred Hegelmaier's *Wolffia hyalina* and *Wolffia repanda* to the genus *Wolffiella*, an opinion later endorsed by Hartog (1969). Subsequently, Hartog and Van Der Plas (1970) transferred both species to a new genus *Pseudowolffia* in light of what they perceived as a number of distinct characters. Following his discovery of *Wolffiella rotunda* (section *Rotundae*), a species intermediate to the *Stipitatae* and other *Wolffiella*, Landolt (1986) retained *W. hyalina* and *W. repanda* in *Wolffiella*, but placed them within the isolated section *Stipitatae* (transferred nomenclaturally from *Wolffia*). Morphological cladistic analyses resolve *Wolffiella* section *Stipitatae* as a well-supported (84% bootstrap support), monophyletic section in Lemnaceae (Les et al. 1997a). Allozyme data further demonstrate a close relationship between *Wolffiella hyalina* and *Wolffiella repanda* which share a genetic identity of 0.800 (Crawford et al. 1997). However, these two species share allozyme alleles only with *Wolffiella rotunda* (genetic identity = 0.504–0.538) and there is nearly a complete absence of shared alleles (genetic identity = 0.000) between any of these three species and the remainder of *Wolffiella*, having a slight genetic identity (0.012–0.013) only with *W. neotropica* (Craw-

ford et al. 1997). Thus, allozyme data would support the removal of *W. hyalina* and *W. repanda* from *Wolffiella*, but only by the inclusion of *Wolffiella rotunda*. Indeed, our combined data (Fig. 2) resolve the *Stipitatae* (100% bootstrap support) and the *Stipitatae* / *Rotundae* (100% bootstrap support) both as well-defined clades phylogenetically.

Despite the compelling phylogenetic evidence available, generic delimitation in *Wolffiella* remains a question of preference, given that the phylogenetic relationships are compatible with taxonomic classifications that recognize either one genus with two-three sections, or two distinct genera. To achieve the greatest practicality, morphological discontinuities should be persuasive when attempting to reconcile such purely taxonomic dilemmas. Morphological characters resolve section *Stipitatae* as a clade, but do not resolve a clade consisting of *Stipitatae* and *Rotundae* (Les et al. 1997a). According to Landolt (1986), the major distinctions between sect. *Wolffiella* (widespread) and *Stipitatae* / *Rotundae* (all African in distribution) are floating fronds and a lack of pigment cells in the latter. Thus, even an amended generic concept of *Pseudowolffia* (comprising *Wolffiella hyalina*, *W. repanda*, and *W. rotunda*) is difficult to define morphologically. The relative branch lengths (see above) in our analysis show that the *Stipitatae* / *Rotundae* is a well differentiated clade (59 steps), nearly twice the extent of the genus *Wolffiella* itself (31 steps).

When Landolt (1992) discovered *Wolffiella caudata*, he assigned it to section *Wolffiella* and believed that it was most closely related to *W. lingulata*. Our results support inclusion of *W. caudata* within section *Wolffiella*, but place it in a position intermediate to *W. neotropica* and *W. gladiata* rather than as a sister species to *W. lingulata*, from which it is relatively distant.

Phylogenetic analysis of multiple, diverse data sets indicate that Lemnaceae are monophyletic and contain five monophyletic clades that can be recognized taxonomically as genera: *Landoltia*, *Lemna*, *Spirodela*, *Wolffia*, and *Wolffiella*. However, these results also would support the recognition of a sixth genus, *Pseudowolffia* (as proposed by Hartog and Van Der Plas 1970), if it were amended to include *Wolffiella* section *Rotundae* (*W. rotunda*). Alternatively, sections *Stipitatae* and *Rotundae* could be retained in *Wolffiella*. *Lemna* sects. *Alatae* and *Uninerves* also are sufficiently differentiated phylogenetically to be recognized as distinct genera or retained as separate sections.

The genus *Wolffia* presents a special case with several highly divergent and apparently ancient taxa (*W. brasiliensis*, *W. borealis*, *W. microscopica*, *W. australiana*). *Wolffia* sect. *Wolffia* is clearly differentiated from these four species (Figs. 1–2) and may deserve consideration for recognition as a separate genus. However, the phylogenetic relationships of the four divergent *Wolffia* species can not be ascertained with any degree of certainty, even after our compilation of more than 4,700 characters. Section *Pigmentatae* (*W. brasiliensis* and *W. borealis*) resolves as paraphyletic in our combined analysis (Fig. 2; Table 6), but the low bootstrap support of adjacent nodes makes it difficult to readily accept this topology. Section *Pseudorrhizae* (*W. microscopica*) could be maintained in a monotypic section as originally described (Table 6), but its precise placement in *Wolffia* is not settled satisfactorily by our analyses (Fig. 2). Our results clearly support the removal of *W. australiana* from section *Wolffia* (Figs. 1, 2), but do not provide a firm placement of the species among the other divergent, basal *Wolffia* species. Sectional classification of *W. australiana* is not recommended until further study may better clarify the interrelationships of this species.

A single, strongly supported cladogram of Lemnaceae indicates the monophyly of several *Lemna* sections: *Alatae*, *Lemna*, and *Uninerves*; *Wolffiella* sections *Rotundae*, *Stipitatae*, *Wolffiella*; and *Wolffia* section *Wolffia*. Subfamily *Wolffioideae* is monophyletic, but subfamily *Lemnoideae* is paraphyletic. We present a revised classification of Lemnaceae that incorporates the results of our phylogenetic analysis of the family (Table 6). Relationships among four divergent *Wolffia* species (*W. australiana*, *W. borealis*, *W. brasiliensis*, *W. microscopica*) are difficult to reconcile, even with over 4,700 characters now examined. This difficulty precludes a decision on whether to retain *Wolffia* section *Pigmentatae* (*W. borealis*, *W. brasiliensis*) at this time; however, we cannot recommend a more suitable classification, until further clarification has been made.

Molecular data have proven indispensable in our studies of Lemnaceae phylogeny. However, even at the family level, we have observed instances where extensive divergence of taxa has complicated the construction of cladograms based upon molecular data. The potential for erroneous placement of divergent taxa by molecular data should not be underestimated in any phylogenetic study.

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