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Systematics of the *Lemnaceae* (duckweeds): inferences from micromolecular and morphological data

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Abstract: Diminutive plants of the duckweed family have been difficult to study systematically because of reduction and character state losses that have accompanied their adaptation to aquatic habitats. Phylogenetic analysis of flavonoid and anatomical-morphological data indicate that evolution in the family has proceeded in a linear manner from complex ancestors to reduced species of subfam. *Wolffioideae*. The high probability of convergence for character reduction and loss, however, renders this conclusion tentative. Cladograms from combined data require only a slight increase in length to resolve all four genera as monophyletic. These studies indicate the need for more suitable data to evaluate hypotheses of duckweed relationships.

The *Lemnaceae* (duckweeds) comprise a small, monocotyledon family of four aquatic genera and 37 species (LANDOLT 1986, 1994). Duckweeds are particularly interesting evolutionarily because they are the world's smallest angiosperms (Fig. 1). Individuals of *Wolffia* (the smallest of the genera), seldom exceed 1 mm in size (DAHLGREN & al. 1985) and bear little similarity to other flowering plants. Despite their inconspicuous size, duckweeds are familiar inhabitants of freshwater ecosystems worldwide owing to their propensity to form extensive 'mats' on lake and pond surfaces which typically represent heterogeneous mixtures of both the various genera and species (CLARK & THIERET 1968, VOSS 1972, GODFREY & WOOTEN 1979).

Duckweeds are not simply miniature versions of larger angiosperms; rather, size reduction in the *Lemnaceae* is associated with a highly modified structural organization that results from the alteration, simplification, or loss of many morphological and anatomical features (SCULTHORPE 1967). The extreme degree of reduction has made it difficult to assess homologies with other angiosperm families and even within the duckweed family itself. The duckweed 'thallus' or 'frond' has defied characterization with conflicting interpretations as a modified stem (HEGELMAIER 1868), a modified leaf (HOFFMAN 1840), or a modified axial and leaf system (HOREN 1869, ENGLER 1877). Homologies are equally difficult to assess among the genera MCCLURE & ALSTON 1966).

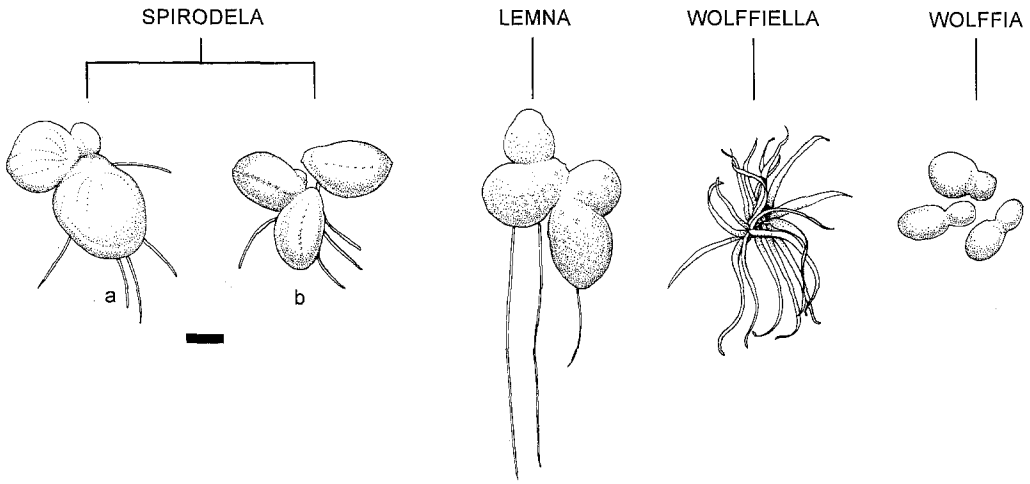

Fig. 1. Comparative habits in the *Lemnaceae*. A reduction series is evident from the most complex genus *Spirodela* (a *S. polyrhiza*; b *S. punctata*), to *Lemna* (*L. japonica*), *Wolffiella* (*W. gladiata*), and *Wolffia* (*W. australiana*). Drawn from representative plates in LANDOLT (1986). Bar: ca. 2 mm for *Spirodela*, *Lemna* and *Wolffiella*; ca. 1 mm for *Wolffia* (illustration by M. J. SPRING)

With few exceptions (see LAWALREE 1945, CROIZAT 1971), most systematists have accepted the view that evolution in *Lemnaceae* has proceeded regressively (Fig. 1) with *Spirodela* (largest; highest level of organization) representing the most primitive and *Wolffia* (smallest; simplest level of organization) the most specialized genus (HEGELMAIER 1868, DAUBS 1965, HARTOG 1975, DAHLGREN & al. 1985, LANDOLT 1986). What is less certain, however, is to what extent the many apparent reductionary 'trends' evident within the family may represent parallel or convergent evolutionary events.

Understandably, the small size, rarity of flowering, and extreme reduction of duckweeds have made them difficult subjects for systematic investigations (SCULTHORPE 1967, LANDOLT 1986). These problems inspired some of the earliest (and now classical) chemosystematic studies (e.g., MCCLURE 1964; MCCLURE & ALSTON 1964, 1966; ALSTON 1966) which were undertaken to provide more reliable taxonomic markers for this problematic group. Although such studies have established the taxonomic utility of flavonoid data for delimiting duckweed species, the phylogenetic significance of flavonoid distribution in the *Lemnaceae* has not been adequately considered.

LANDOLT (1986, 1994) assessed intergeneric and interspecific relationships of duckweeds using an "index of primitivity" derived from interpretations of polarity for 26 characteristics. This approach was used to justify a classification of the family (Table 1) that recognized two subfamilies, four genera, and 37 species. Despite the wealth of comparative data furnished by these recent monographic studies, a formal phylogenetic analysis of duckweeds has not yet been carried out.

In this study, we reexamine systematic relationships in the *Lemnaceae* using phylogenetic analyses of both micromolecular and morphological data. Our principal objective was to establish well-defined hypotheses of duckweed relationships to serve as a foundation for subsequent studies of their interrelationships.

Table 1. Classification of *Lemnaceae* (from LANDOLT 1986, 1994)

Family *Lemnaceae* DUMORT.

I. Subfamily *Lemnoideae* ENGL.

1. *Lemna* L.

A. Sect. *Alatae* HEGELM.
1. *L. aequinoctialis* WELW.
2. *L. perpusilla* TORREY

B. Sect. *Biformes* LANDOLT
1. *L. tenera* KURZ

C. Sect. *Hydrophylla* DUMORT.
1. *L. trisulca* L.

D. Sect. *Lemna*
1. *L. disperma* HEGELM.
2. *L. ecuadoriensis* LANDOLT
3. *L. gibba* L.
4. *L. japonica* LANDOLT
5. *L. minor* L.
6. *L. obscura* (AUSTIN) DAUBS
7. *L. turionifera* LANDOLT

E. Sect. *Uninerves* HEGELM.
1. *L. minuta* HUMB., BONPL. & KUNTH
2. *L. valdiviana* PHIL.

2. *Spirodela* SCHLEID.

A. Sect. *Spirodela*
1. *S. intermedia* W. KOCH
2. *S. polyrhiza* (L.) SCHLEID.

B. Sect. *Oligorrhizae* W. KOCH
1. *S. punctata* (G. MEY.) C. H. THOMPS.

II. Subfamily *Wolffioideae* ENGL.

1. *Wolffia* HORKEL ex SCHLEID.

A. Sect. *Pseudorrhizae* LANDOLT
1. *W. microscopica* (GRIFF.) KURZ

B. Sect. *Elongatae* LANDOLT
1. *W. elongata* LANDOLT

C. Sect. *Pigmentatae* LANDOLT
1. *W. borealis* (ENGELM. ex HEGELM.) LANDOLT
2. *W. brasiliensis* WEDD.

D. Sect. *Wolffia*
1. *W. angusta* LANDOLT
2. *W. arrhiza* (L.) HORKEL ex WIMM.
3. *W. australiana* (BENTH.) HARTOG & PLAS
4. *W. columbiana* KARST.
5. *W. cylindrica* LANDOLT
6. *W. globosa* (ROXB.) HARTOG
7. *W. neglecta* LANDOLT

2. *Wolffiella* (HEGELM.) HEGELM.

A. Sect. *Stipitatae* HEGELM.
1. *W. hyalina* (DELILE) MONOD
2. *W. repanda* (HEGELM.) MONOD

B. Sect. *Rotundae* LANDOLT
1. *W. rotunda* LANDOLT

C. Sect. *Wolffiella*
1. *W. caudata* LANDOLT
2. *W. denticulata* (HEGELM.) HEGELM.
3. *W. gladiata* (HEGELM.) HEGELM.
4. *W. lingulata* (HEGELM.) HEGELM.
5. *W. neotropica* LANDOLT
6. *W. oblonga* (PHIL.) HEGELM.
7. *W. welwitschii* (HEGELM.) MONOD

Material and methods

The presence or absence of forty-seven flavonoids provided binary characters for phylogenetic analysis. Data were taken from MCCLURE & ALSTON (1966) and represented the distribution of 12 glycoflavones, four anthocynins, 15 flavonols, nine flavones, and seven "undetermined" phenolic compounds.

It was first necessary to update species nomenclature by establishing the specific clones examined by MCCLURE & ALSTON (1966: table 1) and adjusting nomenclature to comply with the most recent taxonomic treatments produced by LANDOLT (1980a, b, 1986, 1994). This evaluation resulted in a number of modifications.

Data for '*Spirodela biperforata*' were merged with *Spirodela polyrhiza*; the name '*Spirodela oligorhiza*' was corrected to *Spirodela punctata*; data for '*Lemna perpusilla*' and *Lemna trinervis*' were assigned to *Lemna aequinoctialis* and merged.

Several 'species' studied by MCCLURE & ALSTON (1966) actually represented mixed accessions: '*Lemna valdiviana*' included several accessions of *L. minuta*; '*Lemna gibba*' included several accessions of *L. turionifera*; '*Lemna obscura*' included several accessions of *L. turionifera*; '*Wolffia columbiana*' included one accession of *W. globosa*. Because MCCLURE & ALSTON (1966) reported that flavonoid profiles were identical for these sets of accessions, we added the latter species in each case to the data matrix with the same coding of characters as the reported species. In addition, data for '*L. minima*' were correctly assigned to *L. valdiviana* and merged with that species; data for accessions of *L. turionifera* that were reported as '*L. obscura*' were merged with those of *L. turionifera*. Data reported for '*Wolffiella gladiata*' and '*W. lingulata*' were correctly assigned to and merged with *W. oblonga*; data for '*Wolffiella floridana*' were correctly assigned to *Wolffiella gladiata*. Data reported for '*Wolffia punctata*' were assigned to the correct name *Wolffia borealis*, and data reported for '*Wolffia papulifera*' were assigned to the correct name *Wolffia brasiliensis*. These adjustments resulted in a matrix for eight *Lemna* species, three *Spirodela* species, six *Wolffia* species, and two *Wolffiella* species (Table 2).

We selected forty-one morphological and anatomical characters (Table 3) from descriptions in several sources (COOK 1990, DAUBS 1965, LANDOLT 1986) and used these to construct a second data matrix (Table 4) for phylogenetic analysis of 35 duckweed species. The newly described species *Wolffia cylindrica* and *Wolffia neglecta* (LANDOLT 1994) were excluded from our analyses, but they do not differ from other members of sect. *Wolffia* for the characters examined. This selection included three habit, 13 vegetative morphological, eight vegetative anatomical, 11 reproductive morphological, and six reproductive anatomical characters.

Phylogenetic interpretations of the flavonoid and morphological data were obtained using PAUP 3.1.1 (SWOFFORD 1993) to perform a maximum parsimony analysis searching for shortest trees via the heuristic search option (CLOSEST addition sequence; STEEPEST DESCENT). Characters were treated as unordered in all analyses and the MULPARS option was used to retain all equally parsimonious trees (treating all characters as ordered significantly increased run times and produced comparable topologies). Strict consensus was used to depict results whenever multiple minimal length trees occurred. Bootstrap values were obtained from 200 replicates with the MULPARS and STEEPEST DESCENT options off (bootstrap estimates obtained using either additional replicates or with MULPARS on were extremely time-consuming and yielded comparable values). The decision to root cladograms at *Spirodela intermedia* was determined from several lines of evidence. This is the species that LANDOLT (1986) indicated as the most primitive in his monograph based upon his comparative index of primitivity. We also obtained this same root using a hypothetical ancestor (HTU) that contained all primitive character states based upon polarity assessments postulated on a morphological reduction series. Due to the extreme morphological divergence of the *Lemnaceae*, outgroup rooting using presumably most closely related taxa (e.g., members of *Araceae*) was impractical.

Several manipulations of the data sets were conducted (Figs. 2–6, Table 5). We first analyzed the flavonoid data using the complete set of compounds (Fig. 2A) and a subset of compounds that excluded anthocyanins and flavonoids described as "unidentified" (Fig. 2B). We also evaluated the complete set of compounds noting changes in tree statistics as genera were forced into monophyletic groups by applying topological constraints (Table 5). Because *Wolffia arrhiza*, *W. columbiana*, *W. globosa* and *Spirodela punctata* were widely misplaced from their congeners in cladograms constructed from the complete set of flavonoid data, we re-examined relationships of the remaining taxa in exclusion of these species (Fig. 2C); we then evaluated the influence of forced monophyletic topological constraints on genera for this same set of species (Fig. 2D).

Table 2. Matrix of flavonoid data reported by McCLURE & ALSTON (1966) revised to reflect current taxonomic status of duckweed clones examined (see text)

Taxon	Glycoflavones												Anthocyanins				Flavonols																	O-Flavones									Undetermined						
	1	2	7	15	16	18	20	23	24	25	26	27	8	11	12	19	3	4	5	9	10	22	36	37	38	40	41	42	43	44	45	13	14	17	21	29	32	34	35	46	47	6	28	30	31	33	39
Spirodela																																															
S. intermedia	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0
S. polyrhiza	1	1	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0
S. punctata	1	0	1	1	1	1	1	1	1	0	0	0	1	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Lemna																																														
L. aequinoctialis	0	0	0	0	0	1	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
L. gibba	1	1	0	1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. minor	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. minuta	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. obscura	0	0	0	1	0	1	1	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. trisulca	1	1	0	1	0	1	1	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. turionifera	1	1	0	1	0	1	1	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L. valdiviana	1	0	0	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	1	1	1	0
Wolffiella																																														
W. gladiata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W. oblonga	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Wolffia																																														
W. arrhiza	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0
W. borealis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W. brasiliensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W. columbiana	1	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
W. globosa	1	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
W. microscopica	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1

Table 3. Characters and states used in phylogenetic analyses of duckweeds; all characters were treated as unordered

Habit

1. Habit: terrestrial (0); aquatic (1)
2. Organization: normal (0); modified as fronds (1)
3. Frond habit: floating (0); floating or submerged (1); submerged (2)

Vegetative morphology

4. Fronds: flat (0); inflated (1); globose (2)
5. Maximum number of frond nerves: 16 (0); 7 (1); 4 (2); 3 (3); 1 (4); nerveless (5)
6. Dorsal papillae: absent (0); present (1)
7. Dorsal/ventral scales: present (0); absent (1)
8. Distal frond margin: entire (0); denticulate (1)
9. Budding pouch: 2, lateral, deltoid (0); 1, terminal, flat (1); 1, terminal, conical (2)
10. Ventral ribbon-like appendage: absent (0); present (1)
11. Stipe: elongate (0); short (1)
12. Turions: absent (0); present (1)
13. Maximum number of roots: 20 (0); 11 (1); single (2); absent (3)
14. Maximum root length: 15 cm (0); 7 cm (1); 4 cm (2); none (3)
15. Root sheath: wingless (0); winged (1); absent (2)
16. Root tip: acute (0); acute or obtuse (1); obtuse (2); absent (3)

Vegetative anatomy

17. No. of air space layers: 3–4 (0); 2–3 (1); 1–3 (2); 1 (3); none (4)
18. Guard cell plastids: present (0); absent (1)
19. Crystal cells: raphides and druses (0); raphides (1); absent (2)
20. Pigment cells: present throughout (0); absent in vegetative organs (1)
21. Anthocyanins: present (0); absent (1)
22. Frond tracheids: throughout all nerves (0); to tip of central nerve (1); to middle of central nerve (2); lowest part of central nerve only (3); absent (4)
23. Root tracheids: present (0); absent (1)
24. Epidermal cell walls: straight to slightly undulated (0); distinctly undulated (1)

Reproductive morphology

25. No. of reproductive pouches: two (0); one (1)
26. Floral position: lateral (0); upper, offcenter (1); upper, medial (2)
27. Floral organs: enclosed by prophyllum (0); prophyllum absent (1)
28. Stamen number: two (0); one (1)
29. Anther dehiscence transverse (0); apical (1)
30. Ovary insertion: above the stamens (0); at base of stamen (1)
31. Ovary shape: tapering symmetrically (0); tapering asymmetrically (1)
32. Fruits: winged (0); slightly winged (1); wingless (2)
33. Maximum ovule number: 7 (0); 2 (1); 1 (2)
34. Seed: 35–70 ribs (0); 8–22 ribs (1); smooth (2)

Reproductive anatomy

35. Anthers: 2-locular (0); 1-locular (1)
36. Anther wall formation: monocot type (0); reduced type (1)
37. Extension of filament tracheids: through connective (0); below connective (1); at base or absent (2)
38. Tracheids in ovary wall: present (0); absent (1)
39. Embryo sac: monosporic (0); disporic (1)
40. Ovule: anatropous/amphitropous (0); orthotropous (1)
41. Anther pigment cells: present (0); absent (1)

Table 4. Character state matrix used in phylogenetic analysis of duckweeds (characters and states described in Table 3; 9 = no data)

Characters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
Spirodela																																									
1. *intermedia*	1	1	0	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
2. *polyrrhiza*	1	1	0	0	0	0	0	0	0	0	0	1	0	2	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
3. *punctata*	1	1	0	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
Lemna																																									
1. *aequinoctialis*	1	1	0	0	4	1	1	0	0	0	0	1	2	2	1	0	2	1	1	1	1	3	1	1	0	0	0	0	0	0	1	2	2	1	0	0	1	1	1	1	1
2. *disperma*	1	1	0	1	2	1	1	0	0	0	0	0	2	0	0	1	2	1	1	1	0	1	1	1	0	0	0	0	0	0	0	1	1	1	0	0	1	0	1	0	1
3. *ecuadoriensis*	1	1	0	1	3	1	1	0	0	0	0	0	2	0	0	2	2	1	1	1	0	1	1	1	0	0	0	0	0	0	0	9	2	9	0	0	1	0	1	0	1
4. *gibba*	1	1	0	1	1	1	1	0	0	0	0	0	2	0	0	2	2	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	1
5. *japonica*	1	1	0	0	2	1	1	0	0	0	0	0	2	0	0	2	2	1	1	1	0	1	1	1	0	0	0	0	0	0	0	9	2	9	0	0	1	0	1	0	1
6. *minuta*	1	1	0	0	4	1	1	0	0	0	0	0	2	2	0	1	2	1	1	1	1	4	1	1	0	0	0	0	0	0	1	2	2	1	0	0	2	1	1	1	1
7. *minor*	1	1	0	0	2	1	1	0	0	0	0	0	2	0	0	2	2	1	1	1	0	0	1	1	0	0	0	0	0	0	0	1	2	1	0	0	1	0	1	0	1
8. *obscura*	1	1	0	1	3	1	1	0	0	0	0	0	2	0	0	2	2	1	1	1	0	1	1	1	0	0	0	0	0	0	0	2	2	1	0	0	1	0	1	0	1
9. *perpusilla*	1	1	0	0	3	1	1	0	0	0	0	0	2	2	1	0	2	1	1	1	1	3	1	1	0	0	0	0	0	0	1	1	2	0	0	0	1	1	1	1	1
10. *tenera*	1	1	1	0	3	0	1	0	0	0	0	0	2	2	0	0	3	1	1	1	1	4	1	1	0	0	0	0	0	0	0	9	2	9	0	0	1	1	1	1	1
11. *trisulca*	1	1	2	0	3	0	1	1	0	0	0	0	2	2	0	0	2	1	1	1	0	2	1	1	0	0	0	0	0	0	0	0	2	1	0	0	1	0	1	0	1
12. *turionifera*	1	1	0	0	3	1	1	0	0	0	0	1	2	0	0	1	2	1	1	1	0	1	1	1	0	0	0	0	0	0	0	2	2	0	0	0	1	0	1	0	1
13. *valdiviana*	1	1	1	0	4	1	1	0	0	0	0	0	2	2	0	1	3	1	1	1	1	4	1	1	0	0	0	0	0	0	1	2	2	1	0	0	2	1	1	1	1
Wolffiella																																									
1. *caudata*	1	1	1	0	5	0	1	0	1	0	1	0	3	3	2	3	3	0	2	0	1	4	1	0	1	1	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
2. *denticulata*	1	1	2	0	5	0	1	1	1	0	1	0	3	3	2	3	3	0	2	0	1	4	1	0	1	1	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
3. *gladiata*	1	1	2	0	5	0	1	0	1	0	1	0	3	3	2	3	3	0	2	0	1	4	1	0	1	1	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
4. *hyalina*	1	1	0	0	5	1	1	0	1	1	1	0	3	3	2	3	3	0	2	1	1	4	1	0	1	1	1	1	1	1	1	2	2	2	1	1	1	1	1	1	0
5. *lingulata*	1	1	2	0	5	0	1	0	1	0	1	0	3	3	2	3	3	0	2	0	1	4	1	0	1	1	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
6. *neotropica*	1	1	1	0	5	0	1	0	1	0	1	0	3	3	2	3	3	0	2	0	1	4	1	0	1	1	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
7. *oblonga*	1	1	2	0	5	0	1	0	1	0	1	0	3	3	2	3	3	0	2	0	1	4	1	0	1	1	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
8. *repanda*	1	1	0	0	5	1	1	0	1	1	1	0	3	3	2	3	3	0	2	1	1	4	1	0	1	1	1	1	1	1	1	2	2	2	1	1	1	1	1	1	0
9. *rotunda*	1	1	0	0	5	1	1	0	1	0	1	0	3	3	2	3	3	0	2	1	1	4	1	0	1	1	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
10. *welwitschii*	1	1	2	0	5	0	1	0	1	0	1	0	3	3	2	3	3	0	2	0	1	4	1	0	1	1	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
Wolffia																																									
1. *angusta*	1	1	0	2	5	0	1	0	2	0	1	1	3	3	2	3	4	0	2	1	1	4	1	0	1	2	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
2. *arrhiza*	1	1	0	2	5	0	1	0	2	0	1	1	3	3	2	3	4	0	2	1	1	4	1	0	1	2	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
3. *australiana*	1	1	0	2	5	0	1	0	2	0	1	1	3	3	2	3	4	0	2	1	1	4	1	0	1	2	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
4. *borealis*	1	1	0	2	5	0	1	0	2	0	1	1	3	3	2	3	4	0	2	0	1	4	1	0	1	2	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
5. *brasiliensis*	1	1	0	2	5	1	1	0	2	0	1	1	3	3	2	3	4	0	2	0	1	4	1	0	1	2	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
6. *columbiana*	1	1	0	2	5	0	1	0	2	0	1	1	3	3	2	3	4	0	2	1	1	4	1	0	1	2	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
7. *elongata*	1	1	0	2	5	0	1	0	2	0	1	1	3	3	2	3	4	0	2	1	1	4	1	0	1	2	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
8. *globosa*	1	1	0	2	5	0	1	0	2	0	1	1	3	3	2	3	4	0	2	1	1	4	1	0	1	2	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0
9. *microscopica*	1	1	0	2	5	0	1	0	2	0	1	0	3	3	2	3	4	0	2	1	1	4	1	0	1	2	1	1	1	1	1	2	2	2	1	1	2	1	1	1	0

Table 5. Influence of data manipulations on tree statistics; *CI* consistency index; *RI* retention index. The percent increase or decrease in tree length, CI and RI are indicated in parentheses

	Figure	No. of trees	No. of steps	CI	RI
Flavonoid data					
1. Original flavonoid matrix	2A	2	75	0.63	0.72
2. Force monophyletic genera	–	3	86(+15%)	0.55(−8)	0.61(−11)
3. Anthocyanins, unknown compounds deleted	2B	3	60	0.60	0.73
4. 'Misplaced' *Wolffia* & *Spirodela* spp. deleted	2C	1	62	0.66	0.74
5. Force monophyletic genera	2D	1	65(+5%)	0.63(−3)	0.70(−4)
Morphological data					
1. Original data matrix	3	33	98	0.66	0.92
2. Force monophyletic genera	4	153	103(+5%)	0.63(−3)	0.91(−1)
Combined flavonoid/morphological data					
1. Original data combined	5	1	173	0.64	0.82
2. Force monophyletic genera	6	14	181(+5%)	0.61(−3)	0.79(−3)

Similar analyses were conducted using the morphological and anatomical data set (Table 3). As with the flavonoid data, we evaluated the unmanipulated data set (Fig. 3) in comparison to topological constraints that imposed monophyletic genera (Fig. 4).

A final analysis was conducted on a combined data set that included all 47 flavonoid compounds and all 41 anatomical-morphological characters. In this analysis we included only those 19 species for which both data sets were available (Fig. 5); we also examined the effect of topological constraints for monophyletic genera on the combined data set (Fig. 6).

Results

Phylogenetic analysis of 47 flavonoid compounds yielded two trees of 75 steps with a consistency index of 0.63 and retention index of 0.72 (Table 5, Fig. 2A). This cladogram (Fig. 2A) depicted subfam. *Wolffioideae* as derived polyphyletically from *Lemna*, with three *Wolffia* species that were misplaced from their congeners; *Spirodela punctata* also clustered within *Lemna*. Forcing the topology of this tree to constrain all genera as monophyletic added 11 additional steps to the tree (a 15% increase in length) and reduced the CI (consistency index) and RI (retention index) considerably (Table 5). Bootstrap values indicated relatively strong support to differentiate the genera *Wolffia* and *Wolffiella*, and for subfam. *Wolffioideae*, but only if the misplaced *Wolffia* species were not considered. Fairly high bootstrap values also indicated the distinctness of all other species from *Spirodela intermedia* and *S. polyrhiza*. The fairly weak phylogenetic resolution of flavonoid data was indicated by the analysis conducted in exclusion of anthocyanin and unknown compound data (Fig. 2B). In this consensus tree, most nodes were collapsed and relatively high bootstrap values (> 70%) persisted only for a clade containing three *Wolffia* species.

Removing the species that were misplaced in the initial analysis resulted in a single tree of 62 steps (Fig. 2C) and relatively high CI and RI values (Table 5).

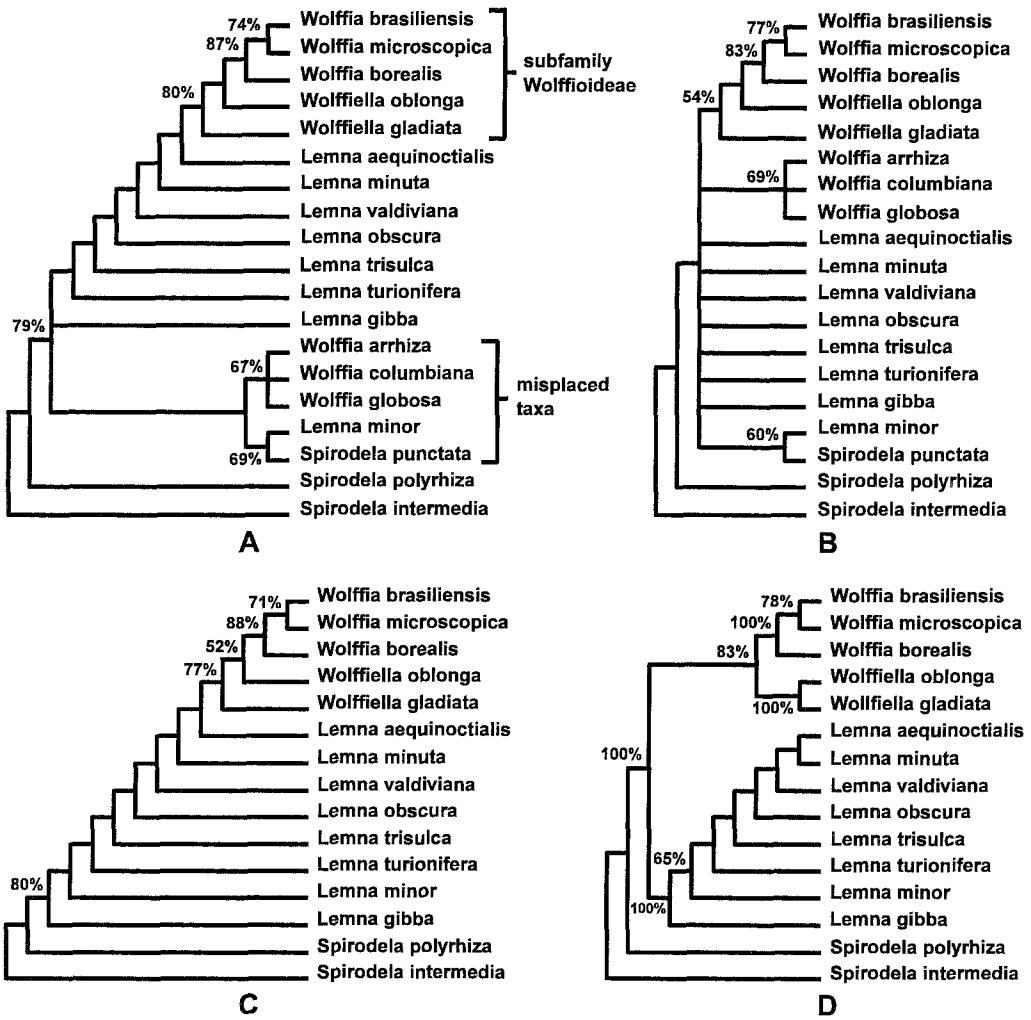

Fig. 2. Cladograms of *Lemnaceae* constructed from flavonoid data. *A* Strict consensus of two minimal length trees (75 steps) obtained from original data matrix (Table 2); *B* strict consensus of 3 minimal length trees (60 steps) obtained from exclusion of anthocyanin and unknown compounds from data set; *C* single minimal length tree (62 steps) obtained using original data matrix but excluding *Wolffia* and *Spirodela* species 'misplaced' in *A*; *D* single minimal length tree from same data set as C, but topology constrained to force monophyletic genera. Bootstrap percentages (*A–D*) are indicated for all nodes with values greater than 50%. 'Misplaced' taxa refer to species that occur outside of their assigned genera. Genera and species representing subfam. *Wolffioideae* are indicated; remaining species are in subfam. *Lemnoideae*. See also Table 5

Nodes distinguishing *Spirodela*, subfam. *Wolffioideae*, and genus *Wolffia* were strongly supported by bootstrap values. Forcing the monophyly of genera for this data set required only three additional steps (a 5% increase in length) and reduced the CI and RI by only three to four percent (Fig. 2D, Table 5). Disregarding those nodes forced by topological constraint, the only clade to be well-supported by bootstrap values was that delimiting subfam. *Wolffioideae*.

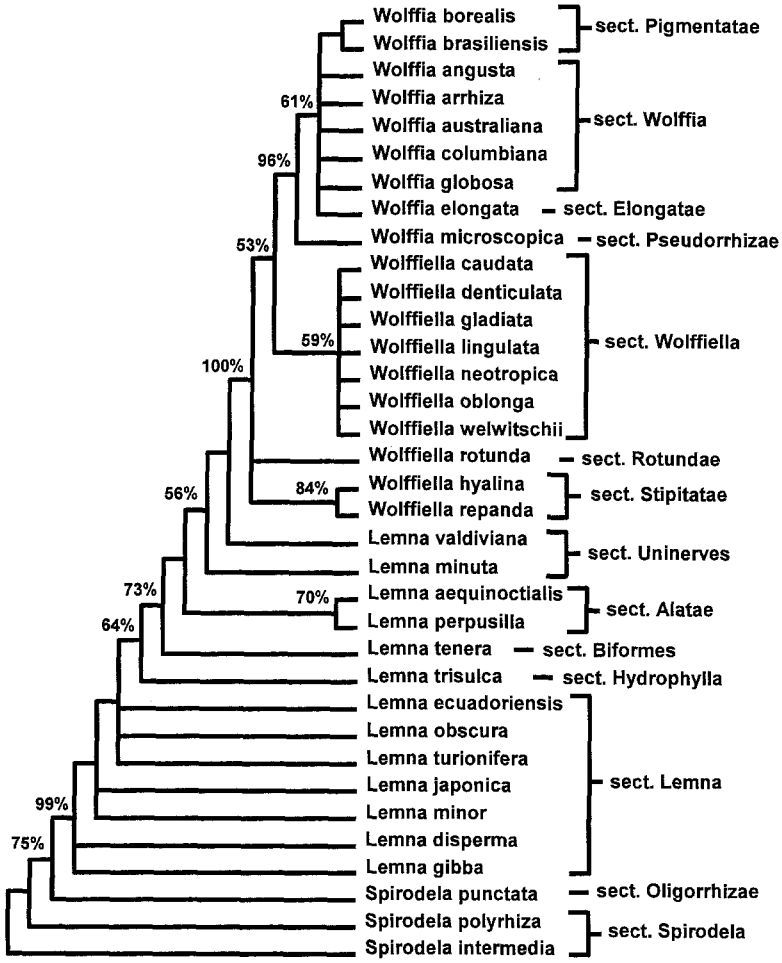

Fig. 3. Cladogram of *Lemnaceae* derived from morphological and anatomical data (Table 3). Strict consensus of 33 minimal length trees (98 steps) is shown with bootstrap percentages and taxonomic sections (Table 1) indicated. See also Table 5

Phylogenetic analysis of morphological and anatomical data generated 33 trees of 98 steps with a CI of 0.66 and RI of 0.92 (Fig. 3, Table 5). Bootstrap values for the consensus tree showed high support for subfam. *Wolffioideae*, the genus *Wolffia*, and the genus *Spirodela*. Taxonomic sections recognized by LANDOLT (Table 1) were well-supported by the arrangement of species in this analysis although often portrayed as paraphyletic groups. Species relationships of *Lemna* sect. *Lemna* were essentially unresolved using this data set. Forced monophyly of all genera added 5 steps (a 5% increase in length) and generated 120 more trees than the initial analysis (Fig. 4, Table 5); the CI and RI values decreased only moderately. Disregarding topologically constrained nodes, bootstrap values showed strong support for nodes delimiting *Wolffiella* sect. *Wolffiella* and *Stipitatae*, and *Lemna* sect. *Lemna* and *Alatae*. The association of *Lemna* sect. *Hydrophylla* and sect. *Lemna* was also well supported.

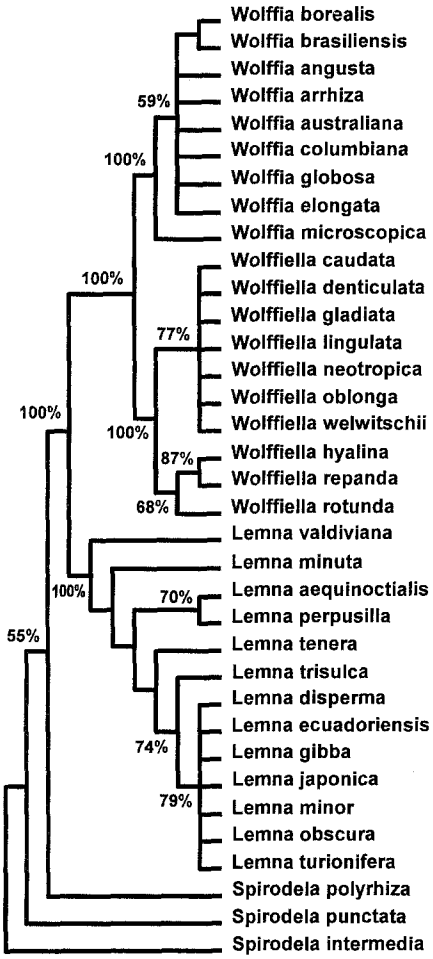

Fig. 4. Cladogram of *Lemnaceae* derived from same analysis as Fig. 3, but topology constrained to force monophyletic genera. Strict consensus of 153 minimal length trees (103 steps) is shown with bootstrap percentages (>50%) for uncollapsed nodes. See also Table 5

Use of combined flavonoid, morphological, and anatomical data yielded a single minimal length tree of 173 steps and CI and RI values comparable to those obtained for separate analyses of the data sets (Fig. 5, Table 5). The consensus tree for combined data depicted a monophyletic subfam. *Wolffioideae* that was supported by extremely high bootstraps. Strong bootstrap support also existed for the node separating *Spirodela punctata* from its congeners (and also from *Lemna*), as well as the association of three *Lemna* species with subfam. *Wolffioideae*; these species represent sections of the genus (*Uninerves*, *Alatae*) characterized by highly reduced morphology. Strong internal support was also evident for *Wolffiella* sect. *Wolffiella*, *Wolffia* sect. *Wolffia*, and the association of *Wolffia* sect. *Pigmentatae* and *Pseudorrhizae*. Forcing the monophyly of genera added 8 steps to the shortest trees (a 5% increase in length) and lowered the CI and RI values only slightly (Fig. 6, Table 5). Bootstrap values for nodes other than those forced by constraint were also relatively high for subfamily. *Wolffioideae*, *Lemna* sect. *Lemna/Hydrophylla*, *Wolffia* sect. *Pigmentatae/Pseudorrhizae*, and *Wolffia* sect. *Wolffia*.

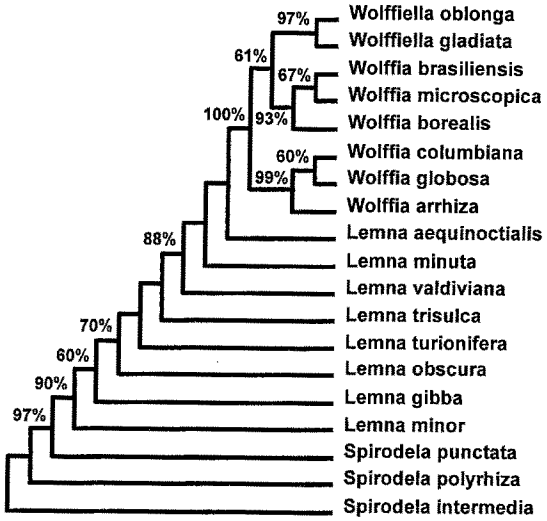

Fig. 5. Cladogram of *Lemnaceae* derived from combined data set of flavonoid, morphological and anatomical characters. Only species for which both data sets were available are included. Single minimal length tree (173 steps) is shown with bootstrap percentages (>50%) for uncollapsed nodes. See also Table 5

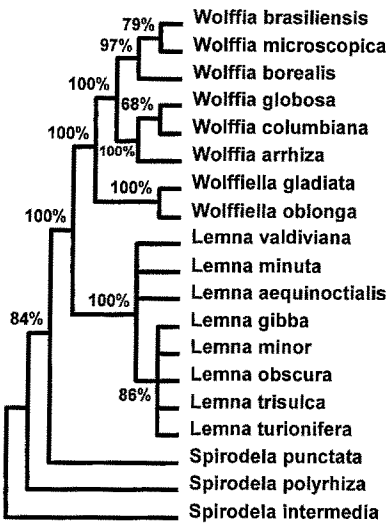

Fig. 6. Cladogram of *Lemnaceae* derived from same analysis as Fig. 5, but topology constrained to force monophyletic genera. Strict consensus of 14 minimal length trees (181 steps) is shown with bootstrap percentages (>50%) for uncollapsed nodes. See also Table 5

Discussion

In the *Lemnaceae*, phyletic losses of complex structures such as roots, tracheary elements, and stamens are simpler to rationalize than their de novo evolution. This reasoning has led to the widely accepted perception that evolution in the family has proceeded, as in most aquatic plants, largely by way of reduction (Dollo 1912, Arber 1920, Sculthorpe 1967). Circumstantial evidence of reduction is also supplemented by empirical data. For instance, vascular elaboration in *Lemna minor* L. is suppressed by a naturally occurring inhibitor present in the fronds. When effects of the inhibitor are counteracted by the addition of TIBA, the simple vascular system reverts to a more complex condition (Sargent & Wangermann 1959). In this instance it is unlikely for the inhibitor to represent a primitive characteristic.

However, not every structural feature of *Lemnaceae* has evolved towards diminution. One evident exception is the size of epidermal cells which are largest in *Wolffia* and *Wolffiella*, smaller in *Lemna*, and smallest in *Spirodela* (LANDOLT 1986). In further contrast to the reductionary trends characteristic of many conspicuous morphological and anatomical features, URBANSKA–WORYTKIEWICZ (1980) established that the largest chromosomes of *Lemnaceae* occur in *Wolffia*, and the smallest in *Spirodela*. The chromosome size of *Lemnaceae* correlates with DNA content which is highest in *Wolffia* and lowest in *Spirodela* (LANDOLT 1986). LANDOLT (1986) demonstrated that an inverse relationship exists between the DNA content and "degree of primitivity" (derived from morphological data) for duckweed species.

An inverse relationship between genome size and organismic complexity (the "C-value paradox") is not unusual, but has been observed across many groups of organisms, such as amoebas, which have C-values that are two orders of magnitude larger than those found in humans or tobacco (CAVALIER–SMITH 1985). The greater size of genomes in simpler organisms is attributed to increased amounts of non-genic rather than genic DNA.

There is no a priori reason to assume that all phylogenetic reduction or specialization in the *Lemnaceae* has occurred exclusively in a progressive, linear 'series'. It is worthy to note that the fronds of some *Wolffiella* species, e.g., *W. welwitschii*, can actually becomes as large as those of *Spirodela*. Although it is reasonable to consider that size reduction in the *Lemnaceae* evolved linearly from the largest genus *Spirodela*, then to the intermediate sized genus *Lemna*, and ultimately to *Wolffiella* and *Wollfia*, it is also possible that reductions in *Wolffia* and *Lemna*, have occurred independently since their divergence from a common ancestor. Similarly, the rootless habit could have arisen either linearly from the multi-rooted *Spirodela*, to the single-rooted *Lemna* and eventually to the rootless *Wolffia*, or directly from *Spirodela* to *Wolffia*. In both of these examples, the observation of reductionary trends (size reduction and root loss) does not clarify whether the specific evolutionary processes were strictly linear or parallel.

Phylogenetic analysis allows us to evaluate these evolutionary trends by comparing cladograms that represent linear reduction series versus independent events. In the *Lemnaceae*, this can be achieved by comparing trees showing asymmetric branches of species from *Spirodela* to *Wolffia* to symmetric trees depicting four monophyletic genera. It is for this reason that we elected to include cladograms rendered by forced topological constraints in our analyses.

The threat of parallel or convergent reduction in *Lemnaceae* is particularly serious for either flavonoid or anatomical-morphological data. Although often considered to be selectively neutral, flavonoid compounds in aquatic plants tend to be lost in submerged foliage, but retained as UV shielding compounds in floating foliage (LES & SHERIDAN 1990). No duckweed species examined is completely devoid of flavonoid compounds, but the fewest compounds are retained by the two *Wolffiella* species, both of which possess submersed habits (Table 2). The exposed, floating habit of many duckweeds may select for convergence toward efficient UV absorbing flavonoid compounds.

Flavonoid data are also problematic in systematic studies of duckweeds because of their variational patterns. VEEN (1975) demonstrated that flavonoid patterns can

vary even between conspecific duckweed clones. Flavonoid variation has also been observed between organs such as the fronds and turions of *Spirodela polyrhiza* (Reznik & Menschick 1969). In subfam. *Wolffioideae*, flavonols are probably localized in pigment cells which are common in anthers but sporadic in vegetative tissues of various species. Accordingly, flavonols are likely be detected in regularly flowering species regardless of their relationships.

Furthermore, the pattern of flavonoid variation in *Lemnaceae* does not represent compound gains, but compound losses from an ancestor similar to *Spirodela* which contains all major classes. Therefore, it is reasonable to anticipate that random, convergent associations of functionally equivalent compounds could be widespread in this family.

Although McClure & Alston (1966) could distinguish each duckweed species by their flavonoid profiles, some of their results are less conclusive given that nomenclature adjustments now indicate several instances of intraspecific polymorphisms and interspecific overlap of flavonoid profiles. Nevertheless, several generalizations remain evident. All four classes of flavonoids (glycoflavones, anthocyanins, flavonols, and O-flavones) occur in *Spirodela*. *Lemna* lacks flavonols, *Wolffiella* lacks every class except flavonols, and *Wolffia* lacks only anthocyanins. The occurrences of even these major classes of flavonoid compounds are quite sporadic in all four genera. Flavonols are both present and absent in *Spirodela* species, anthocyanins are present or absent in *Lemna* species, and both glycoflavones and flavonols are present or absent among *Wolffia* species. The particular distribution of flavonoid data indicates that most flavonoid classes were present in the common ancestors of all four genera, and that compounds have been lost independently among the genera, and in some cases, among species. This observation led Turner (1967) and later Landolt (1986) to suggest that two evolutionary lines emerged from *Spirodela*; one leading to *Lemna*, and another to *Wolffia*. If, however, one assumes instead that flavonoid evolution has paralleled the progressive, linear reduction in morphology, then the derivation of subfam. *Wolffioideae* from *Lemna* would require major reversals in flavonoid classes with a loss and gain of nearly every class of compound.

Similar interpretative problems concerning loss and reduction also occur with morphological data. The states of at least 25 of the 41 characters used in our analyses (Table 3) indicate reductions or losses. As with flavonoid data, there is a distinct possibility that many character states scored as similar in this analysis may not represent homologous comparisons. If morphological reduction is rampant in this group, then false phylogenetic associations could result from shared but convergent losses in different evolutionary lineages. Accordingly, we have approached the interpretations of these phylogenetic analyses with substantial caution.

Turner (1967) proposed that *Wolffia* is biphyletic with those species having flavonols derived from *Wolffiella* and those with glycoflavones and flavones derived from *Lemna*. A cladistic analysis of flavonoid data (Fig. 2A) supports this conclusion and also indicates the polyphyly of *Spirodela*. When anthocyanins and "unknown" flavonoids are excluded from the analysis (Fig. 2B), these associations are weakened. However, cladistic analysis of morphological data (Fig. 3) argues strongly for the monophyly of *Wolffia*, a clade with 96% bootstrap support. Analysis of combined morphological and flavonoid data (Fig. 5) also indicates

(but with less certainty) the monophyly of *Wolffia*. In each case, the species of *Wolffia* that were misplaced from their congeners in the flavonoid analysis are far removed cladistically from *Lemna minor* and *Spirodela punctata*, their indicated sister species in that analysis.

We conclude that most evidence supports the monophyly of *Wolffia*, despite the unusual results obtained from analysis of flavonoid data. Given the distribution of flavonoid compounds and the strong morphological evidence of common ancestry, it is apparent that several species of *Wolffia* have either retained ancestral flavonoid compounds, or have regained them secondarily.

A major discrepancy between these cladistic analyses and LANDOLT'S (1986) monographic conclusions is the indicated derivation of subfam. *Wolffioideae* (*Wolffiella* and *Wolffia*) from *Lemna*. Analyses of flavonoids and morphological data (Figs. 2A, C; 3) all point to reduced species of *Lemna* sect. *Uninerves* and *Alatae* as the closest sister species to subfam. *Wolffioideae* which appears to be derived from a paraphyletic *Lemna*. LANDOLT (1986) remarked on the resemblance of *L. valdiviana* (sect. *Uninerves*) to some *Wolffiella* species but believed that their "common descent" was unlikely due to inconsistencies indicated by the distribution of flavonols, guard cell plastids, epidermal cell wall types, and pigment cells.

The forcing of genera into monophyletic groups does not result in excessive tree elongation except for the original flavonoid data set; in other cases, trees elongated by only 5% and their CI, RI were reduced by 1–3% (Table 5). Although accepting these 'suboptimal' trees over the minimal length trees is not warranted under a strict parsimony criterion, we suspect that convergent losses of both micromolecular and anatomical-morphological characters strongly influence the tree topologies obtained in our analyses. In other words, cladistic analyses of micromolecular and morphological data support the hypothesis of a linear reductionary sequence leading from *Lemna* to subfam. *Wolffioideae*, but not without a great deal of reservation.

Cladograms derived from morphological data show the best agreement to taxonomic sections of the *Lemnaceae* proposed by LANDOLT (1986) regardless of whether the topology is forced to constrain monophyletic genera (Fig. 4) or not (Fig. 3). In the unconstrained analysis (Fig. 3), *Spirodela punctata* (sect. *Oligorrhizae*) is distinct from the two species of sect. *Spirodela* with a fairly high level of internal (bootstrap) support (75%) for the clade.

LANDOLT (1986) regarded *Lemna gibba* as the most primitive species of *Lemna*, and the species that was "closest to *Spirodela*" because *L. gibba* has 3–7 nerved fronds, a broadly winged fruit, and possesses several anatropous or amphitropous ovules, three layers of aerenchymatous tissue, and anthocyanins. *Lemna minor* was the "least specialized" duckweed species in the opinion of ARBER (1920). The characters used in our analysis were inadequate to confidently resolve relationships to this extent, although both species fall within the basal clades of *Lemna* (Fig. 3).

The close relationship among species of *Lemna* sect. *Lemna* is indicated by the scarcity of synapomorphies capable of resolving their interrelationships (Figs. 3, 4). The close association of *Lemna trisulca* (sect. *Hydrophylla*) with species of sect. *Lemna* supports LANDOLT'S (1986) suggestion that *L. trisulca* is "...on the same level [of advancement] as most species of the section *Lemna*". His suggestion (LANDOLT (1986) that *L. tenera* (sect. *Biformes*) is more closely related to *L. perpusilla* (sect.

Alatae) or *L. valdiviana* (sect. *Uninerves*) than to *L. trisulca* (sect. *Hydrophylla*) is also supported. The monophyly of sect. *Alatae* has reasonably high bootstrap support (Figs. 3, 4). Morphologically derived cladograms support LANDOLT's characterization of sect. *Uninerves* as the most reduced group of *Lemna* by positioning the section as the most derived in the genus (Fig. 3).

Sections of *Wolffia* and *Wolffiella* all appear to represent monophyletic groups but show weaker bootstrap support overall. However, neither *Wolffiella neotropica* nor *Wolffia brasiliensis* occupied the basal position in their respective genera although LANDOLT (1986) regarded them as the most primitive species in their sections.

Overall, the cladistic analyses of the *Lemnaceae* produced results that were largely consistent with the classification provided by LANDOLT (1986). The major discrepancy was a lack of strong support for the monophyly of each duckweed genus; rather, the micromolecular and anatomical-morphological data indicated strongly paraphyletic genera. Neither data set, nor their combined analysis, was adequate to resolve fine-scale relationships; hence, many infrasectional relationships remained quite ambiguous. Nevertheless, these studies have provided a hypothesis of duckweed phylogeny that should be tested using independent data that are not as prone to convergences (notably convergent losses) as are those associated with adaptation to aquatic conditions. DNA sequences potentially offer one such set of data, because genes lacking insertion/deletions tend to evolve by divergence rather than by losses or reduction. Sequence divergence can also be homoplasious, but is minimally (if at all) influenced by specific adaptations to aquatic life (LES & al. 1993). A project using molecular data to test the duckweed relationships hypothesized in this study is presently underway by the authors.

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