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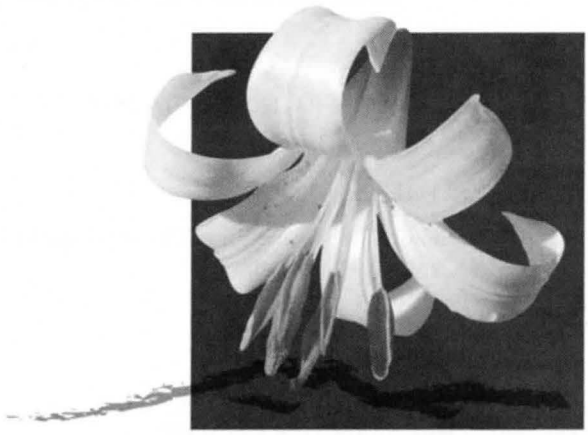

MONOCOTS

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A REAPPRAISAL OF PHYLOGENETIC RELATIONSHIPS IN THE MONOCOTYLEDON FAMILY HYDROCHARITACEAE (ALISMATIDAE)

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ABSTRACT

The diverse, aquatic Hydrocharitaceae have defied stable classification for nearly two centuries. Anatomical and morphological convergence characterize many aquatic plants and undoubtedly have hindered the ability of researchers to ascertain accurately those features representing reliable phylogenetic markers within Hydrocharitaceae. Most prior classifications of the family have emphasized few characters to define major taxonomic subdivisions (i.e., they were highly artificial). Previous studies using molecular data have shown that DNA sequences provide novel indications of phylogeny not indicated previously by morphologically based classifications; however, they have not yet recommended alterations to the classification for the family. We conducted a more comprehensive phylogenetic study of Hydrocharitaceae to better elucidate evolutionary relationships among the genera that in turn could be used to provide insight for improvements in classification. We analyzed different data sets (55 morphological characters; chloroplast *rbc*L, *mat*K, *trn*K intron sequences; nuclear ribosomal ITS region sequences) singly and in various combinations using maximum parsimony and maximum likelihood methods of phylogenetic reconstruction. Phylogenetic analysis of combined data yielded a fully resolved tree depicting four well-supported, major clades within Hydrocharitaceae. We use these results to propose a phylogenetic classification of Hydrocharitaceae recognizing four subfamilies that correspond to these clades: Anacharidoideae, Hydrilloideae, Hydrocharitoideae, and Stratioideae. Phylogenetic analysis also indicated the pattern of derivation with respect to submersed lifeforms, hydrophilous pollination, and marine habitation in the family. Character reconstructions indicated that several features, (e.g., ovule type; occurrence of detaching male flowers), once thought to provide strong phylogenetic markers in Hydrocharitaceae, actually are highly homoplasious and have acutely mislead past attempts at classification of the family.

Key words: Alismatidae, Anacharidoideae, aquatic angiosperms, convergent evolution, Hydrilloideae, Hydrocharitaceae, Hydrocharitoideae, hydrophily, molecular systematics, monocotyledons, pollination, seagrasses, Stratioideae.

INTRODUCTION

Hydrocharitaceae Juss. ("hydrocharits") are aquatic monocotyledons currently circumscribed as comprising 17 genera and approximately 75 species (Cook 1996). Despite their relatively small size, Hydrocharitaceae exhibit some of the greatest diversity of any aquatic angiosperm family, including freshwater and marine species; annual and perennial life histories; amphibious, free-floating, and submersed lifeforms; broad to narrowly linear leaves in rosettes or caulescent arrangements; showy to highly reduced flowers; wind, insect, and water pollination, and male flowers that detach and float on the water surface as some examples. The latter character is unique to this family among angiosperms.

As in many other aquatic plants, the combination of morphological reduction and convergent aquatic adaptation has made it difficult to establish phylogenetic relationships with certainty, especially using characters derived from comparative morphology and anatomy (Les and Haynes 1995). The inability of traditional morphological characters to provide data adequate for resolving credible and consistent infrafamilial relationships in Hydrocharitaceae is particularly evident in past studies that have yielded extremely volatile classifications. Notably, various authors have proposed to subdivide this small family even further into as many as nine different families including Blyxaceae (Aschers. & Gürke) Nakai, Elodeaceae Dumort., Enhalaceae Nakai, Halophilaceae J. Agardh, Hydrillaceae Prantl, Otteliaceae Chatin, Stratiotaceae Link, Thalassiaceae Nakai, and Vallisneriaceae Link (Dumortier 1829; Link and Willdenow 1829; Chatin 1855*e*; Agardh 1858; Prantl 1880; Nakai 1943, 1949) in a mosaic of circumscriptions. There also has been little agreement with respect to the grouping of subordinate genera among different classifications proposed (Table 1). Other anomalous Hydrocharitaceae classifications are compared in Tomlinson (1982), Shaffer-Fehre (1991*b*), and Tanaka et al. (1997). Despite these many previous classifications, none has yet been based on a phylogenetic analysis, thus they provide only weak hypotheses of relationships.

Another complication concerns the correct placement of *Najas* (Najadaceae Juss.) that has been shown to possess a close affinity to Hydrocharitaceae (Shaffer-Fehre 1991*a*, *b*; Les et al. 1993, 1997; Les and Haynes 1995; Tanaka 1997), but until recently had been assumed to be related quite distantly to the family (e.g., Dahlgren et al. 1985). Prior molecular studies incorporating *rbc*L and *mat*K data (Les et al.

Table 1. Contrasting historical classifications of Hydrocharitaceae.

Richard (1811)	Chatin (1855e)	Ascherson and Gürke (1889)	Hutchinson (1959)
Group I (3 stigmas)	Hydrocharitaceae	Halophiloideae	Halophiloideae
Acaules	Hydrochariteae	*Halophila*	*Halophila*
Blyxa	*Hydrocharis*	Stratiotoideae	Thalassioideae
Vallisneria	*Limnobium*	Hydrochariteae	*Thalassia*
Caulescentes	Vallisnerieae	*Hydrocharis*	Vallisnerioideae
Elodea	*Apalanthe*	*Limnobium*	Anachariteae
Hydrilla	*Elodea*	Ottelieae	*Egeria*
	Hydrilla	*Ottelia*	*Elodea*
Group II (6 stigmas)	*Vallisneria*	Stratioteae	*Hydrilla*
Folia sessilia		*Stratiotes*	*Lagarosiphon*
Enhalus	Otteliaceae	Thalassioideae	*Nechamandra*
Stratiotes	Otteliae	*Enhalus*	Blyxeae
Folia petiolata	*Ottelia*	*Thalassia*	*Blyxa*
Hydrocharis	Enhaleae	Vallisnerioideae	Enhaleae
Limnobium	*Enhalus*	Blyxeae	*Enhalus*
Ottelia	*Stratiotes*	*Blyxa*	Limnobieae
		Hydrilleae	*Hydrocharis*
		Elodea	*Limnobium*
		Hydrilla	Ottelieae
		Vallisnerieae	*Ottelia*
		Lagarosiphon	Stratioteae
		Vallisneria	*Stratiotes*
			Vallisnerieae
			Vallisneria

Cook (1982)[a]		Dahlgren et al. (1985)	Schaffer-Fehre (1991*b*)	
Group I:	*Egeria*	Halophiloideae	Halophiloideae	
	Hydrocharis	*Halophila*	*Halophila*	(group 1)
	Limnobium	Hydrilloideae	Hydrocharitoideae	
	Ottelia	*Blyxa*	*Blyxa*	(group 2)
	Stratiotes	*Egeria*	*Najas*	(group 2)
Group II:	*Blyxa*	*Elodea*	*Nechamandra*	(group 2)
Group III:		*Hydrilla*	*Stratiotes*	(group 2)
Subgroup A:	*Elodea*	*Lagarosiphon*	*Ottelia*	(group 3)
Subgroup B:	*Appertiella*	Hydrocharitoideae	*Hydrilla*	(group 4)
	Enhalus	*Hydrocharis*	*Hydrocharis*	(group 4)
	Lagarosiphon	*Limnobium*	*Limnobium*	(group 4)
	Maidenia	*Ottelia*	*Lagarosiphon*	(group 5)
	Nechamandra	*Stratiotes*	*Maidenia*	(group 6)
	Vallisneria	Thalassioideae	*Vallisneria*	(group 6)
Subgroup C:	*Hydrilla*	*Thalassia*	Thalassioideae	
Group IV:	*Halophila*	Vallisnerioideae	*Enhalus*	(group 7)
	Thalassia	*Enhalus*	*Thalassia*	(group 7)
		Vallisneria		

[a] Classification of pollination mechanisms.

1993, 1997; Tanaka 1997) all have resolved *Najas* within Hydrocharitaceae.

The fundamental importance of securing accurate phylogenetic information as a basis for further study cannot be overstated. Systematically, the diverse Hydrocharitaceae provide an excellent model system for demonstrating the evolutionary transition from terrestrial to aquatic habitats in flowering plants. Sculthorpe (1967) specifically identified three "biological trends" in the family: hermaphroditism to unisexuality and dioecy; entomophily and anemophily to hydrophily; and freshwater to marine habitation. Yet, to achieve a valid interpretation of these trends, the phylogenetic relationships within the family must be resolved thoroughly and confidently. Hydrocharitaceae also contain several invasive and notoriously weedy species (e.g., *Egeria densa, Hydrilla verticillata*) whose phyletic relationships may provide information useful for studying the evolution of invasive characteristics in aquatic plants.

The objective of this study is to provide a more comprehensive phylogenetic analysis of intergeneric relationships within Hydrocharitaceae that is based on information compiled from various sources including morphological and molecular (both chloroplast DNA and nuclear DNA) data. We use these resulting indications of phylogeny to explore some of the evolutionary trends within the family and to propose modifications to the subfamilial classification of this unusual group of aquatic angiosperms that long has defied a satisfactory systematic treatment.

Table 2. Characters and states used in morphological phylogenetic analysis of Hydrocharitaceae genera (see text for references).

Non-reproductive characters:

1: *habitat* (0 = freshwater; 1 = marine);
2: *habit* (0 = rosettes with rhizomes/stolons; 1 = rosettes with roots only; 2 = procumbent rhizomatous stems; 3 = caulescent with rhizomes/stolons; 4 = caulescent with roots only);
3: *cauline phyllotaxy* (0 = whorled; 1 = alternate, scattered; 2 = opposite/pseudowhorled; 3 = both scattered and compacted as rosettes);
4: *leaf habit* (0 = air contact; 1 = both air contact and submerged; 2 = submerged only);
5: *petiole* (0 = present; 1 = absent);
6: *lamina* (0 = broad, circular; 1 = short, linear; 2 = ribbon-like; 3 = broad and ribbon-like);
7: *leaf margins* (0 = toothed or with hard spine cells; 1 = entire or with soft fin cells);
8: *enlarged, paired, apical leaf-spines* (0 = absent; 1 = present);
9: *number of mesophyll layers* (0 = more than 3; 1 = 0–3);
10: *abaxial spongy leaf tissue* (0 = absent; 1 = present);
11: *abaxial midvein teeth* (0 = absent; 1 = present);
12: *stipules* (0 = present; 1 = absent);
13: *squamules* (0 = more than 2 per leaf axil; 1 = single or paired);
14: *squamule morphology* (0 = entire; 1 = fringed);
15: *roots* (0 = unbranched; 1 = branched);
16: *duration* (0 = perennial; 1 = annual);

Reproductive characters:

17: *flowers* (0 = all bisexual; 1 = all unisexual; 2 = bisexual [cleistogamous] and unisexual);
18: *male inflorescence* (0 = stalked; 1 = sessile or subsessile);
19: *male inflorescence bracts* (0 = free; 1 = united);
20: *male spathes* (0 ≥ 3 flowered; 1 = 1–2 flowered);
21: *male floral buds* (0 = attached; 1 = liberated under water);
22: *male petals* (0 = 3; 1 ≤ 3 [tepals considered as calyx in all cases]);
23: *male petal length* (0 = greatly exceeding the sepals; 1 = nearly equal to shorter than sepals or absent);
24: *stamens* (0 = free at base or solitary; 1 = united at base);
25: *stamen number [male or hermaphroditic flowers]* (0 = 9 or more; 1 = 6; 2 = 3 or less);
26: *staminodes [male flowers]* (0 = absent; 1 ≥ 1 staminode and >3 stamens; 2 = 1 staminode and 2 stamens; 3 = 3 sail-like staminodes and 3 stamens);
27: *filament length* (0 = some or all filaments longer than or equal to anthers; 1 = all filaments shorter than anthers; 2 = anthers sessile/subsessile [filaments < 0.1 mm]);
28: *anthers* (0 = tetrasporangiate; 1 = bi- or uni-sporangiate; 2 = tetra- and tri- or bi-sporangiate);
29: *pollen* (0 = monads; 1 = tetrads and monads; 2 = moniliform);
30: *pollen exine* (0 = echinate; 1 = baculate to spinulose; 2 = reticulate; 3 = smooth; 4 = exine lacking);
31: *floral symmetry* (0 = actinomorphic; 1 = tendency toward zygomorphy in ontogeny);
32: *pollination syndrome* (0 = entomophilous; 1 = "B" type of Cook (1982); 2 = epihydrophilous; 3 = hypohydrophilous; 4 = anemophilous);
33: *female flower number* (0 = more than 1; 1 = maximum of 1);
34: *number of bracts [female inflorescence]* (0 = 2; 1 = 1);
35: *female spathe* (0 = unfused; 1 = fused);
36: *female sepals* (0 = present; 1 = absent);
37: *female petals* (0 = present; 1 = absent);
38: *female flower hypanthium* (0 = absent; 1 = elongated);
39: *female flower staminodia* (0 = present; 1 = absent);
40: *female flower* (0 = stalked; 1 = sessile);

Table 2. Continued.

41: *nectaries* (0 = present; 1 = absent);
42: *coiling peduncle* (0 = absent; 1 = present);
43: *style morphology* (0 = bilobed/bifid [rarely trifid] less than ½ way to base; 1 = bifid to base; 2 = simple);
44: *carpel number* (0 = greater than 3; 1 = 3 or less);
45: *maximum # seeds* (0 ≥ 20; 1 ≤ 15);
46: *placental dissepiments* (0 = absent [smooth]: 1 = present);
47: *maximum seed length [mm]* (0 = 5 or less [small]; 1 = 6–9 [medium]; 2 ≥ 10 [large]);
48: *vestiture of seed surface* (0 = hairs; 1 = stiff processes; 2 〉 striate, smooth or reticulate);
49: *endotegmen tuberculae* (0 = absent; 1 = present);
50: *seed shape* (0 = cylindrical, ellipsoidal, fusiform; 1 = pyriform; 2 = globose);
51: *placentation* (0 = laminar; 1 = parietal; 2 = basal);
52: *ovules* (0 = anatropous; 1 = orthotropous);
53: *fruit type* (0 = dry; 1 = fleshy);
54: *fruit surface* (0 = smooth; 1 = hairy, scarious or spiny);
55: *mucilage in fruit* (0 = absent; 1 = present)

MATERIALS AND METHODS

Morphological Data

Characters and character states used in morphological phylogenetic analyses were compiled from numerous sources, principally: Richard (1811); Chatin (1855*a, f*); Caspary (1857*a, b*); Rohrbach (1871); Rendle (1901); Kirchner et al. (1908); Marie-Victorin (1931); Singh (1965); Kaul (1969, 1970); Tomlinson (1969, 1982); Hartog (1970); Wilder (1975); Cook (1982, 1985, 1996); Cook and Lüönd (1982*a, b, c,* 1983); Cook and Triest (1982); Lowden (1982); Triest (1982); Cook and Urmi-König (1983*a, b,* 1984*a, b,* 1985); Symoens and Triest (1983); Cook et al. (1984); Catling and Wojtas (1986); Shaffer-Fehre (1991*a, b*); Appert (1996) as well as from observations of living and preserved material. We scored a total of 55 characters representing 16 vegetative and 39 reproductive traits (Tables 2, 3). Where inconsistencies within genera existed (due to conflicting character states among congeneric species), the states were coded and analyzed as polymorphisms.

DNA Sequence Data

Chloroplast DNA (cpDNA) data.—We compiled cpDNA sequences for 18 genera including *Butomus* (used as the outgroup), *Najas,* and 16 of the 17 genera recognized within Hydrocharitaceae s.s. The cpDNA data consisted of 1183 base pairs (bp) of *rbc*L data with 17 of the sequences retrieved from GenBank as deposited by Les et al. (1997) and one sequence newly generated for *Maidenia rubra.* The *rbc*L sequence for *M. rubra* was produced using the same methods as described in Les et al. (2002*a*). Our *mat*K data set consisted of 1582 bp with 12 sequences retrieved from GenBank as deposited by Tanaka et al. (1997) and sequences newly generated for *Apalanthe granatensis, Butomus umbellatus, Lagarosiphon major* (Ridl.) Moss, *Maidenia rubra,* and *Vallisneria americana* that we obtained using the methods described in Les et al. (2002*b*). An unusually long insertion/deletion (indel) and high variability near the 3′-end of *mat*K impaired sequencing of the complete *mat*K region,

Table 3. Matrix of characters and character states (see Table 2) used in morphological phylogenetic analysis of Hydrocharitaceae genera and *Butomus* (outgroup). Polymorphisms are indicated in parentheses. ? = data missing; — = data not applicable.

Character number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Apalanthe	0	4	0	2	1	1	0	0	?	0	0	1	1	0	0	0
Appertiella	0	4	1	2	1	1	0	1	1	0	0	1	1	0	0	1
Blyxa	0	(124)	3	2	1	2	0	0	0	0	0	1	1	0	0	0
Butomus	0	2	—	0	1	2	0	0	0	0	0	1	0	0	1	0
Egeria	0	4	0	2	1	1	0	0	1	0	0	1	1	0	0	0
Elodea	0	4	0	2	1	1	0	0	1	0	0	1	1	0	0	0
Enhalus	1	2	—	2	1	2	1	0	0	0	0	1	0	0	0	0
Halophila	1	3	2	2	(01)	0	0	0	0	0	0	1	1	0	0	0
Hydrilla	0	3	0	2	1	1	0	0	1	0	1	1	1	1	0	0
Hydrocharis	0	0	—	0	0	0	1	0	0	1	0	0	1	0	1	0
Lagarosiphon	0	4	(01)	2	1	1	0	1	1	0	0	1	1	0	0	0
Limnobium	0	0	—	0	0	0	1	0	0	1	0	0	0	0	1	0
Maidenia	0	3	1	2	1	1	0	0	1	0	1	1	1	0	0	0
Najas	0	4	2	2	0	1	0	0	1	0	0	1	1	0	0	1
Nechamandra	0	4	1	2	1	1	0	0	1	0	0	1	1	0	0	(01)
Ottelia	0	(12)	3	1	1	(023)	(01)	0	0	0	0	1	0	0	0	0
Stratiotes	0	1	—	1	1	1	0	0	0	0	0	1	0	0	0	0
Thalassia	1	2	1	2	1	2	0	0	0	0	0	1	1	0	0	0
Vallisneria	0	(03)	1	2	1	(12)	0	0	0	0	0	1	0	0	0	(01)

resulting in shorter sequences in the latter two genera. As a result, our data set for *Vallisneria americana* and *Maidenia rubra* contained only 616 bp of *mat*K and *Nechamandra* lacked *mat*K data entirely. Following the methods reported in Les et al. (1997) we obtained *trn*K intron data for 13 genera consisting of 1546 aligned sites for the 5′ region and 207 aligned sites for the 3′ region. The 5′ intron sequence for *Nechamandra alternifolia* was somewhat shorter at 1335 bp. We were unable to obtain 3′ *trn*K intron region sequences for *Halophila stipulacea* (Forssk.) Acherson, *Maidenia rubra, Nechamandra alternifolia, Najas marina,* and *Vallisneria americana.*

Nuclear ribosomal DNA (nrDNA).—We obtained DNA sequences of the nrITS region (ITS-1, ITS-2, 5.8S) for all 18 genera included in the study following the methods described in Les et al. (2002*b*). All new cpDNA and nrITS sequences have been deposited in GenBank (Table 4).

Data Analyses

Phylogenetic analyses of morphological data were carried out using maximum parsimony as implemented by the program PAUP* vers. 4.0 beta 4a (Swofford 1998). Character states were treated as unordered and heuristic searches were conducted using a simple addition sequence referenced to *Butomus* which retained one tree at each step, and tree-bisection-reconnection (TBR) branch swapping restricted to best trees using stepwise addition for starting trees while saving multiple trees (MULPARS option). Trees were rooted using *Butomus* as the outgroup following the *rbc*L analysis of Les et al. (1997). Strict consensus was used to depict results yielding multiple, equally parsimonious trees. Internal support was determined from 1000 bootstrap replicates using a "full" heuristic search with search options as described above.

Indels were treated as missing data. For *mat*K, we scored the presence or absence of distinctive indel motifs to produce a separate binary character set (gaps in other regions were too variable to score confidently). The resulting indel matrix for *mat*K consisted of 23 characters and was included only in maximum parsimony analyses (see below). Data from all cpDNA regions were combined and analyzed as a block partitioned separately from the morphological and nrDNA data. In some cases (*Blyxa, Halophila, Hydrocharis, Limnobium, Najas, Thalassia*) we combined different molecular loci sequenced from different but congeneric species under the same genus for analysis.

Molecular data were analyzed using maximum parsimony (MP) and maximum likelihood (ML) methods. The MP analyses of cpDNA and nrDNA essentially followed the same approach used to analyze the morphological data (see above). Results yielding equally minimal length trees were depicted as strict consensus trees with internal nodal support calculated from 1000 replicates as described above. Sequences from *Butomus* were used to root the trees.

The cpDNA data matrix for ML analyses used the same data set included in the parsimony analyses except for the removal of large indel regions (>10 base pairs) in the *mat*K and *trn*K regions. After aligning the complete set of ITS sequences, it was apparent that many regions were too divergent to reasonably ensure the maintenance of nucleotide site homologies. We identified those regions where excessive divergence occurred and removed questionably aligned regions prior to our analyses of the nrITS data. Although preliminary analyses indicated that removal of the highly divergent regions did not influence the resulting tree topologies to any great extent, we analyzed only the nrITS data from the less divergent regions.

For each molecular data set (cpDNA and nrDNA), we employed an iterative search strategy (Swofford et al. 1996; Sullivan et al. 1997) to evaluate different models of molecular evolution using likelihood ratio tests. The data sets were examined initially under 24 models of substitution to deter-

Table 3. Extended.

17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
0	—	—	—	—	—	—	0	2	—	0	?	0	0	0	0	—	—	—	—
1	1	1	0	1	1	1	0	1	0	0	1	0	?	0	1	1	0	1	0
1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
0	—	0	—	—	—	—	0	0	—	0	0	0	2	0	0	—	—	—	—
1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0
1	1	1	1	(01)	0	0	1	0	0	1	1	1	0	0	2	1	0	1	0
1	1	1	0	1	0	1	0	2	0	2	1	0	2	0	1	1	0	0	0
1	1	0	1	0	1	1	0	2	0	2	2	2	4	0	3	1	0	0	1
1	1	1	1	1	0	1	0	2	0	1	0	0	1	0	4	0	0	1	0
1	1	0	0	0	0	0	1	1	1	0	0	0	1	0	0	1	1	0	0
1	1	1	0	1	0	1	0	1	1	0	?	0	?	0	1	1	0	1	0
1	1	0	0	0	0	1	1	1	1	0	0	0	1	0	0	0	0	0	0
1	1	1	0	1	1	1	0	2	2	1	1	0	0	1	1	1	0	1	0
1	0	1	1	0	1	1	0	2	0	2	0	0	4	—	3	1	1	?	1
1	1	1	0	1	1	1	0	2	2	1	1	0	3	1	1	1	0	1	0
2	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0
1	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
1	1	1	0	0	1	?	0	0	0	2	2	2	1	0	3	1	0	1	0
1	1	1	0	1	1	0	0	2	2	1	1	0	1	1	1	1	0	1	0

mine the best-fit model using MrModeltest vers. 1.1b (Nylander 2003).

Model parameters were optimized on sets of the ten most parsimonious trees found in searches using equally weighted parsimony for the nrITS and cpDNA data sets, respectively. ML searches (heuristic searches with TBR branch-swapping) subsequently were conducted using the best fit substitution model (GTR + I + Γ for both data sets) fully defined. Model parameters were as follows: nrITS data (AC-0.90678, AG-1.8306, AT-1.2144, CG-0.8699, CT-4.8948, I = 0.1368, α = 0.5080); cpDNA data (AC-1.8934, AG-2.1376, AT-0.2620, CG-0.9488, CT-2.5945, I = 0.2426, α = 0.9424). Nodal support was estimated by bootstrap analyses (200 replicates, heuristic searches and TBR branch-swapping) under the best-fit model.

Data set congruency was estimated by implementing the ILD/partition-homogeneity test of PAUP* (Swofford 1998). The ILD test was used in the six pairwise comparisons of nrITS, *mat*K, *rbc*L, and *trn*K intron data sets as well as in an overall comparison of cpDNA and nrITS data. In addition, the morphological data set was compared to each molecular data set. Considering the precautions stated by Yoder et al. (2001) regarding the ILD test, we selected a value of $P < 0.005$ as the level where potential data incongruence might be indicated.

Observing no major incongruence among trees generated from cpDNA or nrDNA (see Results), we combined the molecular data for analysis. Because the pattern of molecular evolution for these data sets differed conspicuously as demonstrated by their distinct model parameters (see above), we restricted the analysis of the combined molecular data to maximum parsimony methods. Where both approaches were used on individual data sets, we observed that ML and MP analyses produced very similar results.

Although the topology resulting from analysis of morphological data alone differed somewhat from the molecular analyses (see Results), it varied only at nodes that were supported poorly. Thus, the overall agreement between the morphological and molecular trees warranted their combined analysis. We first conducted separate combined analyses of the morphological data with either the cpDNA or nrDNA sequences, using the MP methods described above. With identical topologies resulting from those analyses, we combined the morphological data with all molecular data and analyzed the combined data set using the MP methods described above. Bootstrap support was obtained in all instances from 1000 replicates and a full heuristic search.

Because of its unique occurrence within Hydrocharitaceae, we mapped the character of detached male flowers on the combined data phylogeny using both ACCTRAN and DELTRAN optimizations to determine the number of origins for the trait within the family. We used the same optimizations to explore the number of origins for orthotropous ovules, given their importance in early classifications of the family. Codings for pollination types and life-forms were provided for the terminal taxa on the final combined data tree.

RESULTS

Morphological Analyses

Character state distributions for 55 characters scored for 19 study genera and analyzed using maximum parsimony produced four equally minimal length trees of 164 steps with a consistency index (CI) = 0.482, a consistency index excluding uninformative characters (CI_{exc}) = 0.465, and retention index (RI) = 0.587 (Fig. 1). Internal bootstrap support exceeded 50% for only a few nodes, notably for clades consisting of *Hydrocharis* and *Limnobium* (100%), *Vallisneria* and *Maidenia* (71%), *Vallisneria, Maidenia,* and *Nechamandra* (62%), and genera with a submersed life-form (63%). Lower internal support (41%) was obtained for clades consisting of the marine genera (*Enhalus, Halophila, Thalassia*), for *Apalanthe, Egeria,* and *Elodea* (34%), and for genera

Table 3. Extended.

37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
—	—	—	—	0	0	1	1	1	1	0	0	1	0	1	1	0	0	0
1	0	1	1	1	0	2	1	1	1	0	2	1	0	1	?	0	0	0
0	0	0	1	1	0	0	1	0	1	0	1	1	0	1	0	0	0	0
—	—	—	—	0	0	0	0	0	?	0	2	0	0	0	0	0	0	0
0	0	0	1	0	0	1	1	1	1	1	0	1	0	1	1	0	0	0
0	0	0	1	1	0	(012)	1	1	1	1	0	1	0	1	1	0	0	0
0	0	0	0	1	1	2	0	1	0	2	2	1	1	1	0	1	1	1
1	0	1	1	1	0	(01)	0	0	1	0	2	1	2	1	0	1	1	0
0	0	0	1	1	0	0	1	1	1	0	2	1	0	1	0	0	(01)	0
0	1	0	0	0	0	1	0	0	0	0	0	1	0	1	1	1	0	1
0	0	0	1	1	0	1	1	0	1	0	?	1	0	1	1	0	?	0
0	1	0	0	1	0	1	0	0	0	0	0	1	0	1	1	1	0	1
1	1	(01)	1	1	1	1	1	0	1	0	2	1	0	1	1	0	0	0
1	1	1	1	1	0	0	1	1	?	0	2	1	0	2	0	0	0	0
1	0	1	1	1	0	0	1	0	1	0	2	1	0	1	1	0	0	0
0	1	0	1	0	0	1	0	0	0	0	0	1	0	1	0	1	0	0
0	0	0	1	0	0	1	0	0	0	2	0	1	0	1	0	1	0	1
1	0	1	1	1	0	1	0	1	0	2	2	1	1	1	0	1	0	0
0	1	0	1	1	1	1	1	0	1	0	2	1	0	1	1	1	0	1

with emergent or floating-leaved life-forms (34%). *Najas* resolved in a position somewhat distant from *Hydrilla* in a clade comprising the marine genera and *Vallisneria* clade; however, it was placed in this part of the tree with poor internal support (12%).

Although material of the rare *Appertiella hexandra* C. D. K. Cook & Triest was unavailable for molecular analysis, morphological data placed the genus within a clade comprising the marine genera, *Vallisneria* clade, *Najas,* and *Lagarosiphon.* Placement within this clade was not supported strongly, and its relationship to other members of the clade was otherwise unresolved.

Molecular Analyses

Chloroplast DNA (cpDNA).—DNA sequence data from *rbc*L, *mat*K, *trn*K 3′ region, *trn*K 5′ region, and the binary indel matrix for *mat*K combined to produce a matrix of 4541 cpDNA characters for the 18 genera analyzed. Maximum parsimony analysis yielded three equally minimal length trees of 2405 steps with a CI = 0.796, CI_{exc} = 0.683, and RI = 0.718. Maximum likelihood (ML) analysis (excluding the indel matrix) employing a GTR + I + Γ model generated one optimal tree having a log likelihood (lnL) score of −14,964.04352 (Fig. 3).

Both likelihood and parsimony methods recovered a clade of the marine genera (*Enhalus, Halophila, Thalassia*) and a monophyletic group comprising *Maidenia, Nechamandra,* and *Vallisneria* with strong support (100%). *Hydrilla* and *Najas* resolved as a clade (MP) or as a basal grade (ML) within a larger clade containing the groups mentioned previously (Fig. 3). The association of *Najas* within this hydrocharit clade was supported strongly (96–98%) regardless of the method of analysis. Other clades supported strongly by both methods of analysis consisted of *Hydrocharis* and *Limnobium* (100%), *Apalanthe, Egeria,* and *Elodea* (100%), *Blyxa* and *Ottelia* (100%), and the placement of *Lagarosiphon* sister to the two previously mentioned clades (100%). The precise position of *Stratiotes* and interrelationships of the larger clades remained unresolved or weakly supported (MP = 45%) in the cpDNA analyses (Fig. 3).

Nuclear ribosomal DNA (nrDNA).—The nrITS data produced an alignment of 947 sites for the 18 genera analyzed. After removing from the analysis those regions of questionable alignment (see above), the resulting data set consisted of 414 sites. Parsimony analysis of this reduced data set generated a single minimal length tree of 573 steps with a CI = 0.716, CI_{exc} = 0.602, and RI = 0.577. Maximum likelihood analysis employing a GTR + I + Γ model generated one optimal tree having a lnL score of −2552.7346 (Fig. 4).

Both ML and MP analyses of the ITS data produced similar topologies and comparable levels of internal support (Fig. 4). Each analysis resolved the marine genera (*Enhalus, Halophila, Thalassia*) as a relatively well-supported clade (72–86%). A clade consisting of *Apalanthe, Egeria,* and *Elodea* also was well supported (94–99%) and positioned sister to a relatively well-supported clade of *Blyxa* and *Ottelia* (79–83%) and in succession, sister to *Lagarosiphon* (68–70%). Clades consisting of *Maidenia, Nechamandra,* and *Vallisneria,* and of *Hydrocharis* and *Limnobium* also were strongly supported (100%) in both analyses. Maximum parsimony reversed the position of *Maidenia* and *Nechamandra* as resolved by ML analysis and cpDNA analyses; however, that minor discrepancy was supported only moderately (38%). The positions of *Halophila* and *Thalassia* were reversed by the ML analysis compared to the parsimony analysis that agreed with the cpDNA analyses; again, this relatively minor inconsistency had only moderate support (58%). Both analyses placed *Najas* near *Hydrilla* and the *Vallisneria* clade (*Maidenia, Nechamandra, Vallisneria*), but somewhat distant (however, with weak support) from the clade of marine genera (*Enhalus, Halophila, Thalassia*). Each analysis resolved *Hydrocharis* and *Limnobium* as the basal clade in the family.

Table 4. Vouchers and GenBank accession numbers for 18 genera surveyed. GenBank accession numbers reported in following order of loci: nrITS, *mat*K, *trn*K 5′ intron, *trn*K 3′ intron, *rbc*L; na = not applicable (sequence not included); [a] vouchers specified in Tanaka et al. (1997).

1. *Butomus* L. [*B. umbellatus* L.]: *Les 499* (CONN); AY870346, AY870364, AY870371, AY874442, U80685.
2. *Apalanthe* Planch. [*A. granatensis* (Humb. & Bonpl.) C. D. K. Cook & Urmi-König]: *Cook s. n.* (Z); AY870362, AY870367, AY870387, AY874453, U80693.
3. *Blyxa* Naronha ex Thouars [*B. aubertii* L. C. Richard]: *Charlton s. n.* (MANCH); AY870359, na, AY870384, AY874450, U80694; [*B. japonica* (Miq.) Maxim. ex Asch. & Gürke]: *Tanaka 95101*[a]; AB002566, na, na, na.
4. *Egeria* Planch. [*E. densa* Planch.]: *Les s. n.* (CONN); AY870360, AB002567[a], AY870385, AY874451, U80695.
5. *Elodea* Michx. [*E. nuttallii* (Planch.) H. St. John]: *Les s. n.* (CONN); AY870361, AB002568[a], AY870386, AY874452, U80696.
6. *Enhalus* Rich. [*E. acoroides* (L. f.) Rich. ex Steud.]: *Walker 1611942* (UWA); AY870347, AB002569[a], AY870372, AY874443, U80697.
7. *Halophila* Thouars [*H. engelmannii* Asch.]: *Wimpee s. n.* (CONN); AY870349, na, AY870374, na, U80699; [*H. ovalis* (R. Br.) Hook. f.]: *Tanaka 95138*[a]; na, AB002570, na, na, na.
8. *Hydrilla* Rich. [*H. verticillata* (L. f.) Casp.]: *Cook s. n.* (Z); AY870353, AB002571[a], AY870378, AY874447, U80700.
9. *Hydrocharis* L. [*H. morsus-ranae* L.]: *Les & Waycott s. n.* (CONN); AY870350, na, AY870375, AY874445, U80701; [*H. dubia* (Blume) Backer]: *Tanaka 95122*[a]; na, AB002572, na, na, na.
10. *Lagarosiphon* Harv. [*L. muscoides* Harv.]: *Cook s. n.* (Z); AY870363, AY870368, AY870388, AY874454, U80702.
11. *Limnobium* Rich. [*L. spongia* (Bosc.) Steud.]: *Cook s. n.* (Z); AY870351, na, AY870376, AY874446, U80704; [*L. laevigatum* (Humb. & Bonpl. ex Willd.) Heine]: *Tanaka 95152*[a]; na, AB002574, na, na, na.
12. *Maidenia* Rendle [*M. rubra* Rendle]: *Jacobs 8872* (NSW); AY870354, AY870365, AY870379, na, AY870370.
13. *Najas* L. [*N. marina* L.]: *Wakeman s. n.* (CONN); AY870352, AY870369, AY870377, na, U80705.
14. *Nechamandra* Planch. [*N. alternifolia* (Roxburgh ex Wight) Thwaites]: *Cook s. n.* (Z); AY870356, na, AY870381, na, U80706.
15. *Ottelia* Pers. [*O. alismoides* (L.) Pers.]: *Bogner s. n.* (M); AY870358, AB002575[a], AY870383, AY874449, U80707.
16. *Stratiotes* L. [*S. aloides* L.]: *Les s. n.* (CONN); AY870357, AB002576[a], AY870382, AY874448, U80709.
17. *Thalassia* K. Koenig [*T. testudinum* Banks ex K. Koenig]: *Wimpee s. n.* (CONN); AY870348, na, AY870373, AY874444, U80711; [*T. hemprichii* (Ehrenb.) Asch.]: *Tanaka 95134*[a]; na, AB002577, na, na, na.
18. *Vallisneria* L. [*V. americana* Michx.]: *Les s. n.* (CONN); AY870355; AY870366; AY8703808; na, U03726.

Combined molecular data.—The ILD test indicated no significant incongruency among the molecular data partitions when using $P < 0.005$ as a threshold value. The following comparisons: nrITS–*mat*K, nrITS–*trn*K, *rbc*L–*trn*K, *trn*K–*mat*K yielded *P*-values in the range of 0.288–0.721. Although *rbc*L data were less congruent with nrITS ($P = 0.028$) and *mat*K ($P = 0.010$), the different data sets produced very similar (even identical) topologies and the overall agreement between the cpDNA and nrITS data was high ($P = 0.461$). Most discrepancy among molecular data sets presumably was due to the inclusion of the divergent genus *Najas*. Excluding *Najas* from the ILD tests raised all *P*-values above 0.116. Yet, the resolution of *Najas* embedded well within the family (a major question of this study) was indicated in every data set analyzed. From these results, we concluded that the combined analysis of our molecular data was warranted.

Maximum parsimony analysis of the combined 4955 molecular characters produced three equally minimal length trees of 2984 steps with a CI = 0.779, CI_{exc} = 0.664, and RI = 0.690. The resulting topology of the strict consensus tree (Fig. 5) retained the same basic groups as those resolved by the previous molecular analyses (Fig. 2–4). Most resolved nodes had strong support (11 nodes >98%). Support for the association of *Hydrilla* and *Najas* was elevated to 98% and the inclusion of that clade with the marine genera and *Vallisneria* clade received comparable support (98%).

Combined molecular and morphological data.—The ILD test showed significant incongruency between the morphological data set and every molecular data partition ($P = 0.001$ in all instances). However, the inclusion of morphological data only slightly altered the tree topologies produced using each single molecular data set (either cpDNA or nrITS data), and in each case, the same topology was produced in the combined analysis (see below). Furthermore, the addition of the morphological data to the combined molecular data resulted only in the resolution of two additional nodes and did not otherwise alter the tree topology produced by the analysis of combined molecular data. Because of these results, and the understanding that even a *P*-value of 0.001 does not necessarily preclude the combinability of data (Yoder et al. 2001), we concluded that the combination of our morphological and molecular data was warranted.

Parsimony analysis of combined morphological and cpDNA data (total of 4596 characters) yielded a single minimal length tree of 2242 steps with a CI = 0.737, CI_{exc} = 0.637, and RI = 0.696 (Fig. 6). Parsimony analysis of combined morphological and nrITS data (total of 469 characters) yielded a single minimal length tree of 627 steps with a CI = 0.584, CI_{exc} = 0.514, and RI = 0.550 (Fig. 6). The combination of morphological data either with cpDNA data or nrDNA data produced an identical topology that differed only by the degree of internal support provided by each different molecular data set (Fig. 6). Parsimony analysis of all data combined (total of 5010 characters) yielded a single minimal length tree (identical in topology to those shown in Fig. 6) of 2695 steps with a CI = 0.720, CI_{exc} = 0.621, and RI = 0.675 (Fig. 7). Overall, the combined molecular/morphological data showed results similar to previous analyses; however, these trees were completely resolved with moderate support (72%) for the position of *Stratiotes* between the *Hydrocharis–Limnobium* clade and remaining genera, and low support (56%) associating the *Lagarosiphon–Egeria* clade with the clade containing *Hydrilla, Najas, Vallisneria* and the marine genera. Eleven of the 15 nodes (73%) were supported with bootstrap values exceeding 96%.

ACCTRAN and DELTRAN optimizations indicated multiple origins of orthotropous ovules (ACCTRAN = 3 gains, 1 loss; DELTRAN = 4 gains) and detached male flowers

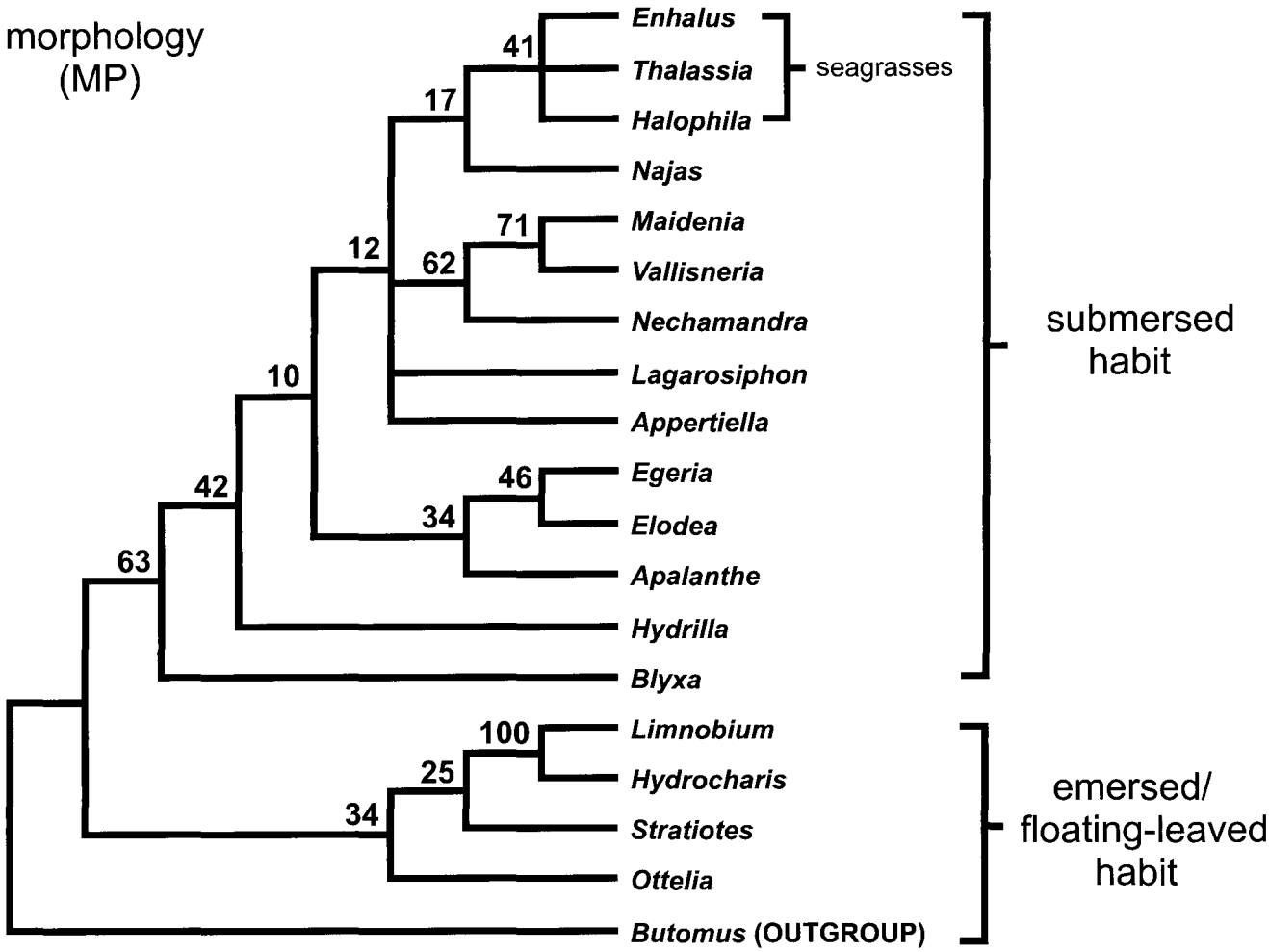

Fig. 1.—Strict consensus of four maximum parsimony (MP) trees (164 steps) generated from state distributions of 55 morphological characters (Table 2, 3). Numbers above branches represent internal support of the tree as provided by bootstrap analysis (1000 replicates).

(ACCTRAN/DELTRAN = 4 gains) in the family. If the occurrence within some species of *Elodea* (which is polymorphic for the latter trait) also is considered, then five independent origins would be indicated for the characteristic within the family (Fig. 7).

Emergent and floating life-forms occurred in the outgroup (*Butomus*) and basal genera (*Hydrocharis, Limnobium, Stratiotes*) when mapped on the combined data cladogram, with submersed life-forms representing a conspicuously derived condition in Hydrocharitaceae (Fig. 7). When pollination types (as classified by Cook 1982) were mapped on the same cladogram, entomophily was plesiomorphic with hydrophily being derived and homoplasious (Fig. 7).

DISCUSSION

Morphological Studies and Inferences

Botanists long have strived to produce a classification of Hydrocharitaceae that would arrange the highly diverse genera into natural subordinate groups. One of the earliest taxonomic studies of Hydrocharitaceae was made by Richard (1811) who divided the family into two groups according to stigma number (3 or 6) with further subdivision based on conspicuous vegetative characters (Table 1). However, Richard's highly artificial scheme produced unlikely associations such as the grouping of the marine *Enhalus* with the freshwater *Stratiotes,* and the association of the highly specialized *Vallisneria* with the relatively unspecialized *Blyxa.* Endlicher (1836–40) modified Richard's treatment by subdividing his first group (which he recognized as tribe Vallisnerieae Dumort.) to yield yet another tribe Anacharideae Endl.

The French botanist Adolf Chatin evaluated anatomical evidence to clarify taxonomic relationships within Hydrocharitaceae (Chatin 1855*a–g*). Chatin's survey of ovule types and other anatomical features persuaded him to follow Richard's subdivision of the family, and he recognized two tribes: the "true" Hydrochariteae (comparable to Richard's second group) and Vallisnerieae (which reincorporated Endlicher's Anacharideae), defined primarily by the presence of vascular elements in the former and their absence in the latter (Chatin 1855*c, d*). By that circumscription, tribe Hydrochariteae included *Enhalus, Hydrocharis, Limnobium, Ottelia,* and *Stratiotes*; whereas, tribe Vallisnerieae contained *Apalanthe, Elodea, Hydrilla,* and *Vallisneria* (Chatin 1855*c, d*).

Because Chatin determined previously that *Vallisneria* possessed orthotropous ovules (Chatin 1855*e*), he concluded that *Ottelia,* with anatropous ovules, was worthy of transfer to a distinct tribe, a distinct family, or possibly even to some

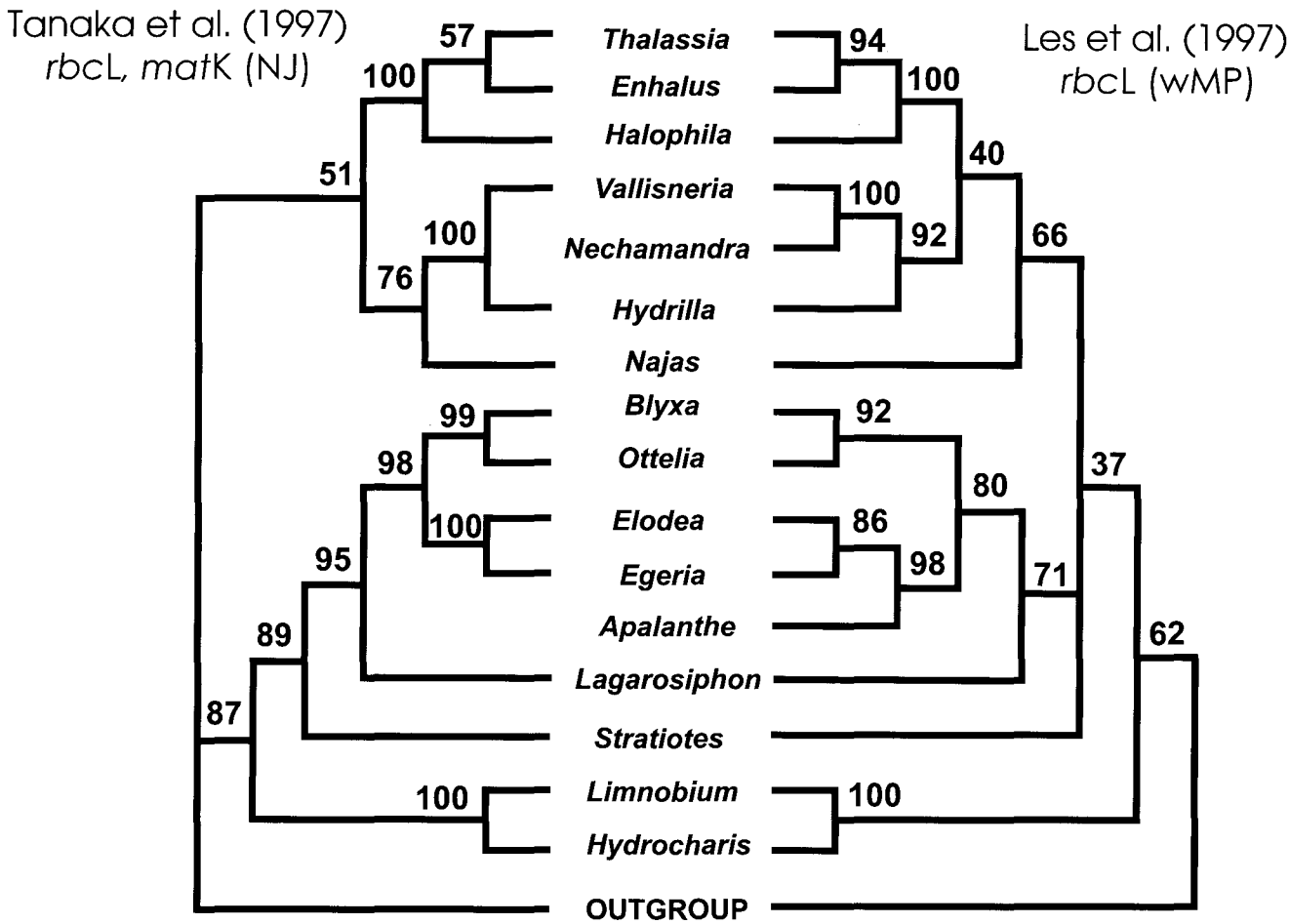

Fig. 2.—Comparison of cladograms from previous studies using cpDNA to study phylogenetic relationships in Hydrocharitaceae. Left: Cladogram redrawn from Tanaka et al. (1997) from *mat*K and *rbc*L sequence data analyzed by neighbor-joining (NJ) and rooted using *Hydrocleys* as the outgroup. Right: Cladogram adapted from Les et al. (1997) generated with *rbc*L data analyzed by weighted maximum parsimony (wMP) and rooted using multiple Alismatidae outgroups. The trees are highly congruent in topology and show similar levels of internal support. Numbers above branches in both trees represent bootstrap values.

closely related family (Chatin 1855*d*). Subsequently, a more thorough survey convinced Chatin to recognize Otteliaceae as a distinct family (Chatin 1855*e*) to accommodate those genera with anatropous ovules (*Enhalus, Ottelia, Stratiotes*). This realignment required Chatin to refine his earlier classification, which was based on the presence or absence of vascular elements. He used vasculature differences and integument number (which he perceived to differ in the family) to define tribes within each of the newly circumscribed families (Chatin 1855*e*).

Chatin's classification was criticized by Caspary (1857*a*, *b*) who challenged the accuracy of his anatomical observations and corrected several misconceptions regarding ovule anatomy such as Parlatore's (1855) misinterpretation of *Stratiotes* as having orthotropous ovules and Chatin's misconceptions concerning integument numbers. Caspary argued convincingly that various anatomical discrepancies did not warrant the acceptance of Chatin's proposed classification. He published a revised treatment of the tribe Hydrilleae that included *Elodea, Hydrilla,* and *Lagarosiphon* by virtue of their similarly reduced anatomy (Caspary 1858). This tribe (often recognized as Anacharideae) of anatomically reduced, submersed Hydrocharitaceae has been retained by contemporary authors such as Tomlinson (1982) who tentatively also included *Nechamandra*; noting, however, that instead it may belong with *Vallisneria.*

Ovule morphology remains misunderstood in Hydrocharitaceae and deserves a more thorough examination. Cronquist (1981) and Dahlgren et al. (1985) remarked that ovules of Hydrocharitaceae usually were anatropous, and Schmidt-Mumm (1996) simply described the family as having anatropous ovules. However, our survey found that orthotropous ovules actually predominate, occurring in 9 of the 17 hydrocharit genera where ovule type has been reported (it remains unknown in *Appertiella*; Tables 2, 3).

From these examples, it is apparent that much of the previous taxonomic history of Hydrocharitaceae has been influenced by highly artificial approaches where modifications to classification have been made on the basis of the distributions of a small number of characters as well as misinformation regarding their character states. Even fairly recent studies (e.g., Shaffer-Fehre 1991*a*, *b*) have attempted to define infrafamilial taxa using relatively few characters. It is understandable that this approach has not yet produced a stable, more natural classification.

Over the years, many morphological studies have provid-

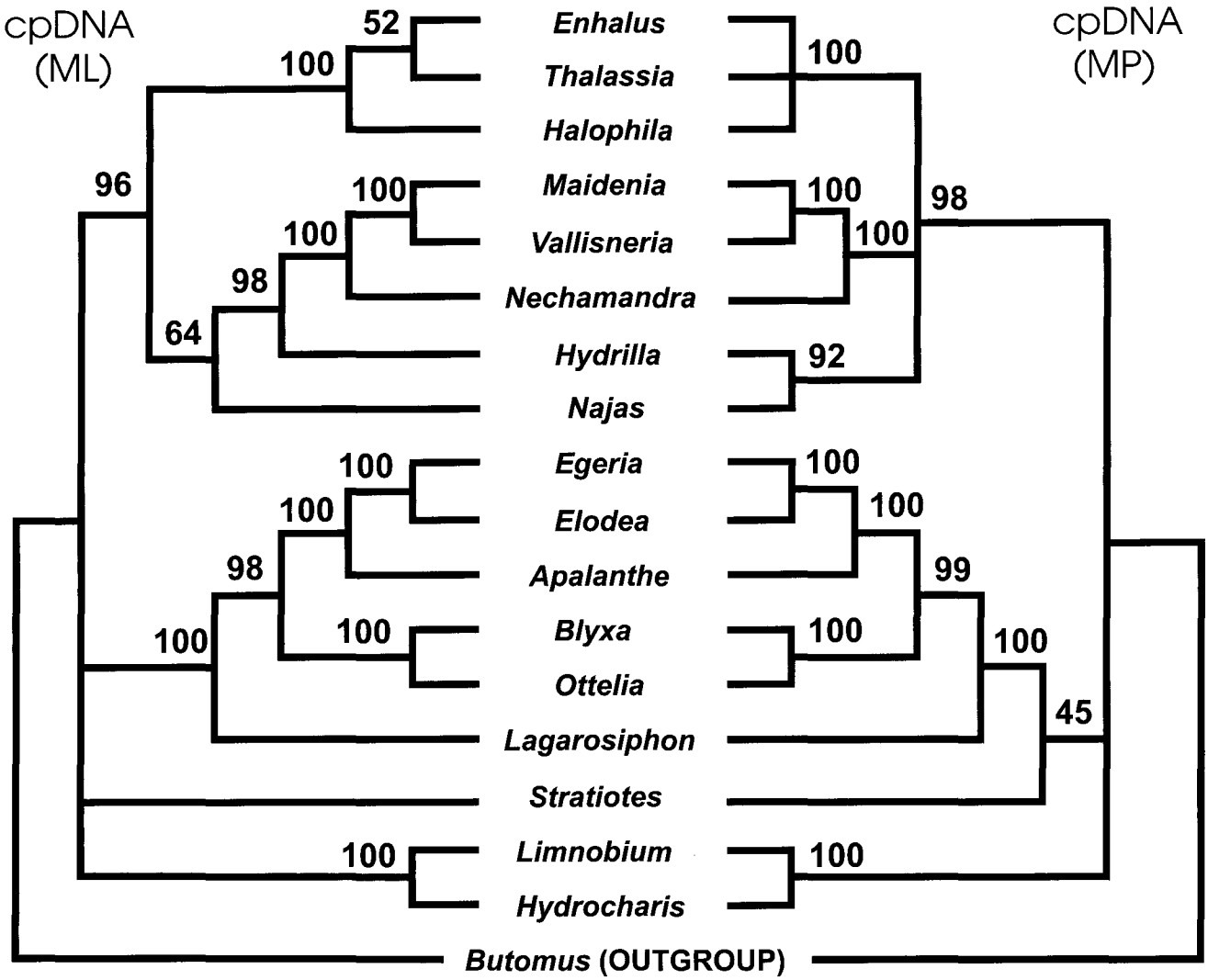

Fig. 3.—Cladograms depicting results of combined cpDNA analysis (present study) using maximum likelihood (ML) and maximum parsimony (MP) methods. Left: Best cladogram recovered (lnL = −14,964.04352) using maximum likelihood analysis (GTR + I + Γ model). Numbers above branches represent bootstrap values obtained from 200 replicates. Right: Strict consensus of three trees (2405 steps) generated from maximum parsimony analysis. Numbers above branches represent bootstrap values obtained from 1000 replicates. Both methods of analysis recovered similar clades with comparable levels of internal (bootstrap) support.

ed further insight into structural similarities and differences among hydrocharit genera. However, the extensive level of morphological and anatomical diversity found within Hydrocharitaceae does not readily disclose a conspicuous pattern of relationships within the family. Furthermore, most aquatic taxa are well known for reductions in vegetative and floral structures leading to convergence in form (Sculthorpe 1967) that generally makes it difficult to interpret morphological characters for phylogenetic analysis. The extent to which parallel reduction and convergent adaptations have influenced interpretations of character state homologies in Hydrocharitaceae has not yet been evaluated adequately. Only recently, some progress in clarifying this issue has been made using more empirical approaches to evaluate distributions of larger numbers of morphological characters in the family.

Several years ago, Dr. C. D. K. Cook (Botanic Gardens, Zürich) sent to us his unpublished results of various morphological cluster analyses that he had used to analyze phenetic relationships among Hydrocharitaceae genera (C. D. K. Cook pers. comm.). Although some of those analyses produced several consistent clusters (e.g., *Hydrocharis* and *Limnobium*; *Egeria* and *Elodea*; *Maidenia* and *Vallisneria*) other associations of genera varied among the analyses or were too diffuse to provide much insight. Although this approach was not phylogenetic, it was the first analytical attempt to obtain a more natural perspective of relationships in the family that was based on more than just a small subset of characters.

Our cladistic analysis of 55 morphological and anatomical characters for 18 hydrocharit genera represents the first comprehensive phylogenetic analysis of morphological data conducted at the generic level for Hydrocharitaceae. Parsimony analysis (Fig. 1) indicated that some of the same phenetic groupings obtained by Cook also were resolved cladistically. We obtained high bootstrap support (100%) for a clade containing *Hydrocharis* and *Limnobium*, and moderate support (66%) for a clade uniting *Vallisneria* with *Maidenia*. The

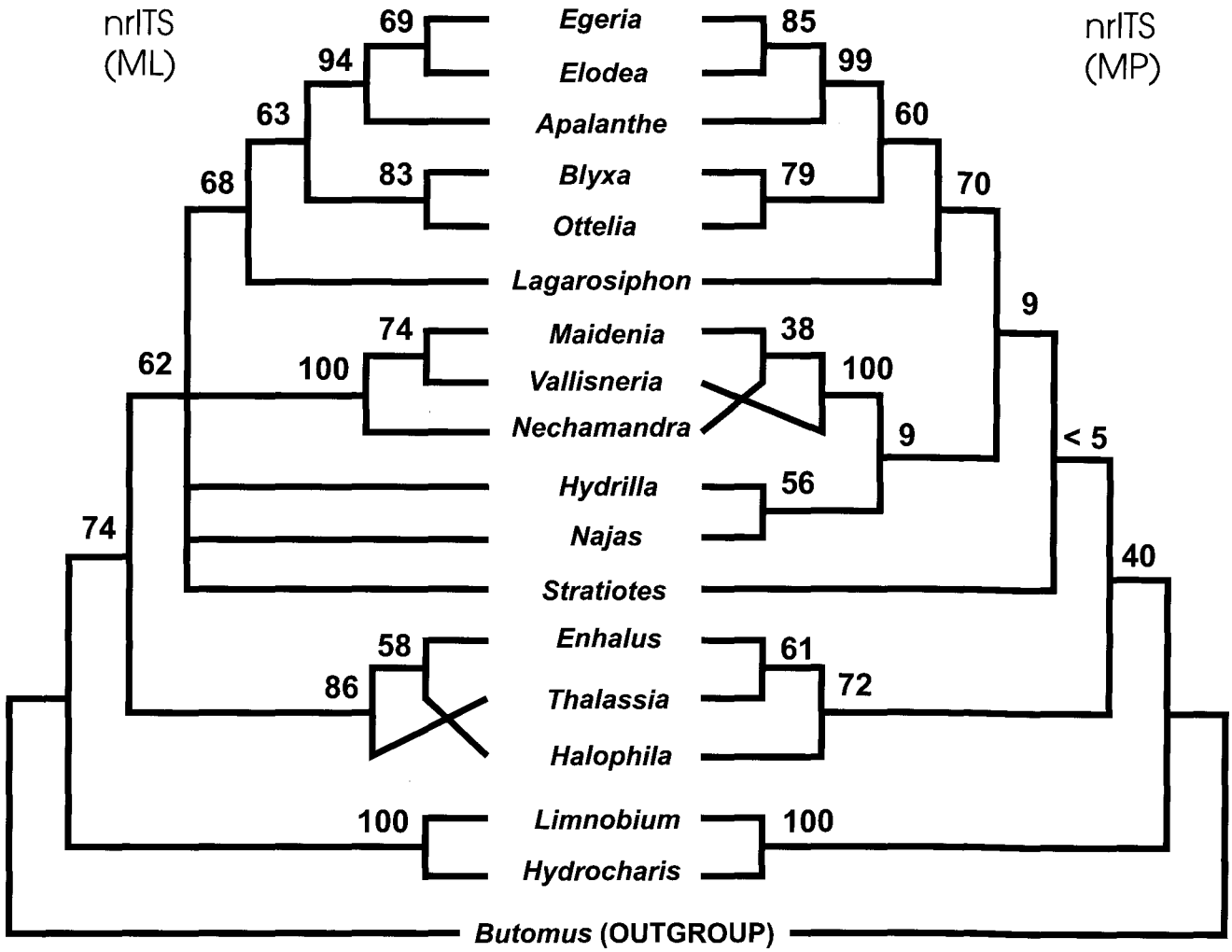

Fig. 4.—Cladograms showing results of nrITS data analysis using maximum likelihood (ML) and maximum parsimony (MP) methods. Left: Best cladogram recovered (lnL = −2552.7346) using maximum likelihood analysis (GTR + I + Γ model). Numbers above branches represent bootstrap values obtained from 200 replicates. Right: single, minimal length tree (573 steps) recovered from maximum parsimony analysis. Numbers above branches represent bootstrap values obtained from 1000 replicates. Both methods of analysis recovered similar clades with comparable levels of internal (bootstrap) support. Minor discrepancies occurred in the positions of *Halophila* (likelihood) and *Vallisneria* (parsimony), deviations that were not supported strongly.

latter result is particularly informative given that other work (Les et al. unpubl. data) shows clearly that *Maidenia* is nested within *Vallisneria*; yet strong cladistic support for these genera still is not forthcoming from these morphological data alone.

The vegetatively similar *Hydrocharis* and *Limnobium* are believed to represent the most primitive elements within Hydrocharitaceae (Kaul 1969, 1970; Wilder 1975; Cook and Urmi-König 1983*b*) and resolve as a sister group to the remainder of the family (Fig. 1). Richard (1811) remarked on the difficulty of distinguishing these genera in absence of their fairly distinctive flowers. Shaffer-Fehre (1991*a*, *b*) found the testa structure of *Hydrocharis dubia* to be so similar to *Limnobium* that she merged the species with that genus. Cook and Urmi-König (1983*b*) also found it difficult to separate *Hydrocharis* and *Limnobium* vegetatively stating that the "affinities of *Limnobium* sensu lato are clearly with the genus *Hydrocharis*." The high degree of internal support for the clade containing *Hydrocharis* and *Limnobium* (Fig. 1) indicates that these genera are indeed closely related using morphological criteria.

As in Cook's phenetic analyses, our morphological phylogenetic analysis resolved *Egeria* and *Elodea* as sister genera; we also resolved *Apalanthe* in a position adjacent to that clade (Fig. 1). Although weakly supported (35–47% bootstrap), the association of these three similar genera is not surprising given their taxonomic history. St. John (1962, 1965) reduced *Apalanthe* to a subgenus of *Elodea*; whereas, Hauman-Merck (1912, 1915) merged both *Apalanthe* and *Egeria* with *Elodea* on the basis of their pollination system. Cook (1985) remarked that *Apalanthe* shared many features in common with *Egeria* or *Elodea* and was "patristically related" to *Egeria*. Cook and Urmi-König (1985) concluded that *Elodea* probably was related most closely to *Apalanthe*. Despite their similarities, the distinctness of all three genera is evidenced by a number of features unique to *Apalanthe* and the fact that *Elodea* is unable to hybridize with *Egeria* (Cook and Urmi-König 1985).

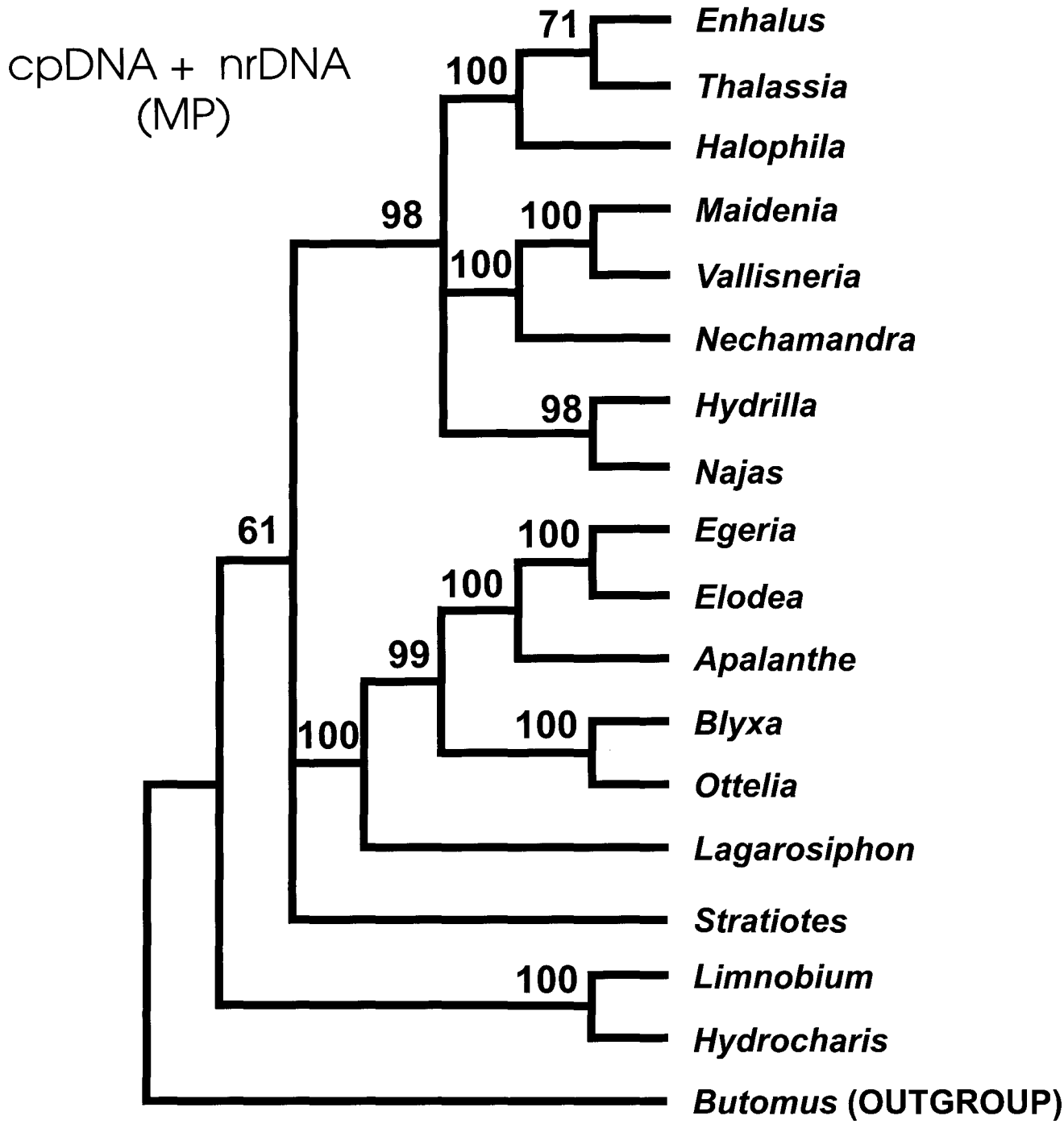

Fig. 5.—Maximum parsimony (MP) analysis of combined cpDNA and nrITS data. The strict consensus of three trees (2984 steps) generated from maximum parsimony analysis is shown. Numbers above branches represent bootstrap values obtained from 1000 replicates. All but two resolved nodes show strong internal support (≥98%) from bootstrap analysis.

A close phylogenetic relationship between *Maidenia* and *Vallisneria* always has been quite evident morphologically. Aston (1973) viewed the two genera to be very closely related because of their similar floral morphology. Hutchinson (1959) merged *Maidenia* with *Vallisneria,* and Cook (pers. comm.) essentially regards *Maidenia* to be a modified *Vallisneria.* Shaffer-Fehre (1991*a*) also placed *Maidenia* with *Vallisneria* on the basis of similarities in their seed coat structure. However, Tomlinson (1982) suggested that the vegetative morphology of *Maidenia* might indicate its relationship to Anacharideae. As mentioned above, this possibility is untenable given the results of a recent molecular study of *Vallisneria* indicating that *Maidenia* is embedded within the genus phylogenetically (Les et al. unpubl. data).

Our morphological analysis shows weak support for an alliance of *Nechamandra* with *Vallisneria* and *Maidenia* (Fig. 1). Yet, this result is not surprising given that *Nechamandra* was once included in *Vallisneria* (Symoens and

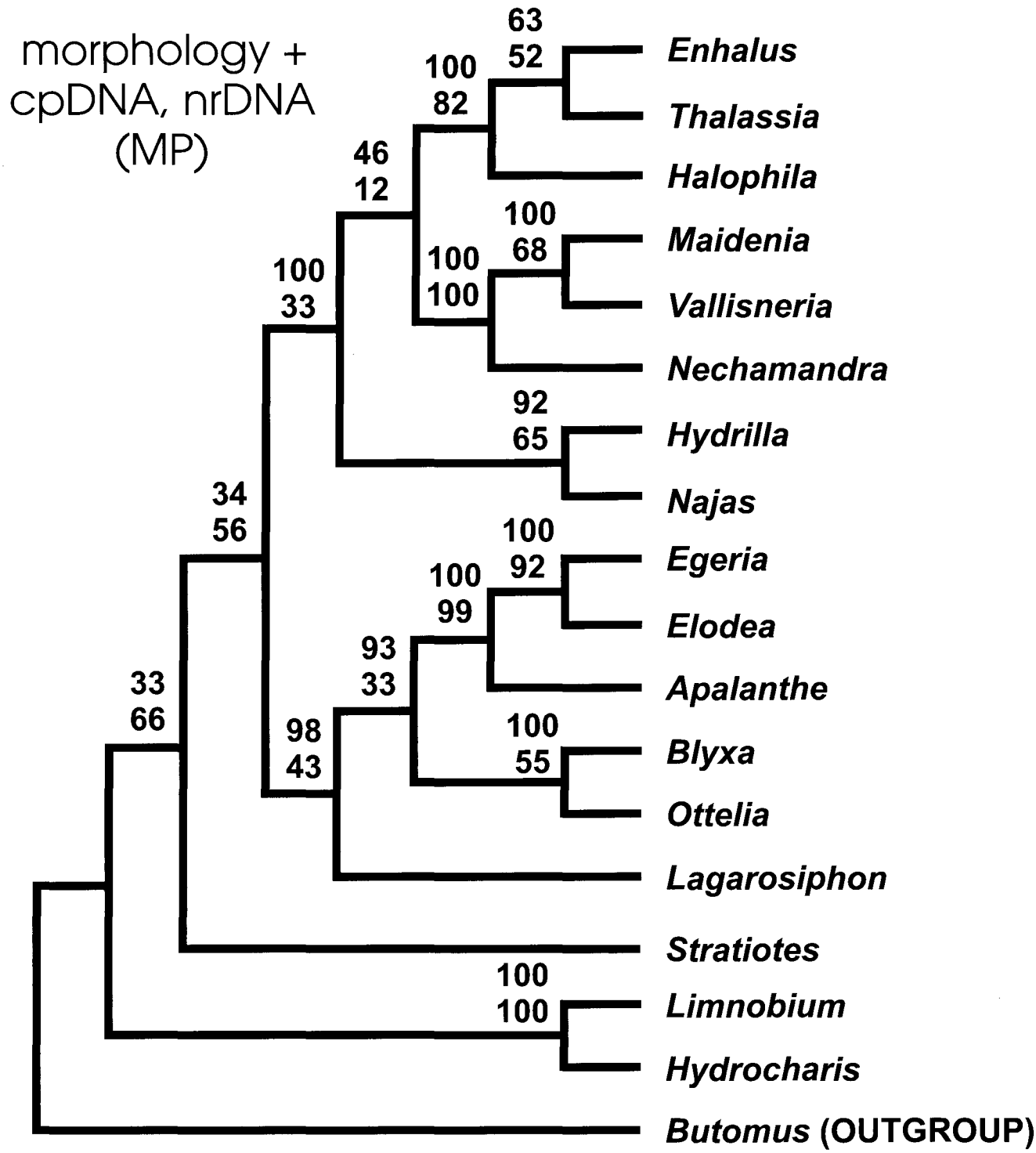

Fig. 6.—Cladogram resulting from maximum parsimony (MP) analysis of combined morphological and molecular data. The same tree was recovered whether morphological data were combined either with cpDNA data (1 tree @ 2242 steps), or with nrITS data (1 tree @ 627 steps). Numbers above branches represent bootstrap values obtained from 1000 replicates. Levels of bootstrap support provided by combined morphology and cpDNA data are shown by the upper numbers above each branch; those from combined morphology and nrITS data are shown by the lower numbers.

Triest 1983). Cook also has suggested that *Nechamandra* is related closely to *Vallisneria* (Tomlinson 1982) but Shaffer-Fehre (1991*a*, *b*) concluded that the seed coat structure of *Nechamandra* placed it closer to *Blyxa, Najas,* and *Stratiotes*. However, our analysis (Fig. 1) indicates that these four genera show no close association whatsoever based on phylogenetic inferences using parsimony analysis of morphological characters that include seed coat features.

Among other Hydrocharitaceae, the endemic Madagascar genus *Appertiella* has been placed closest phylogenetically

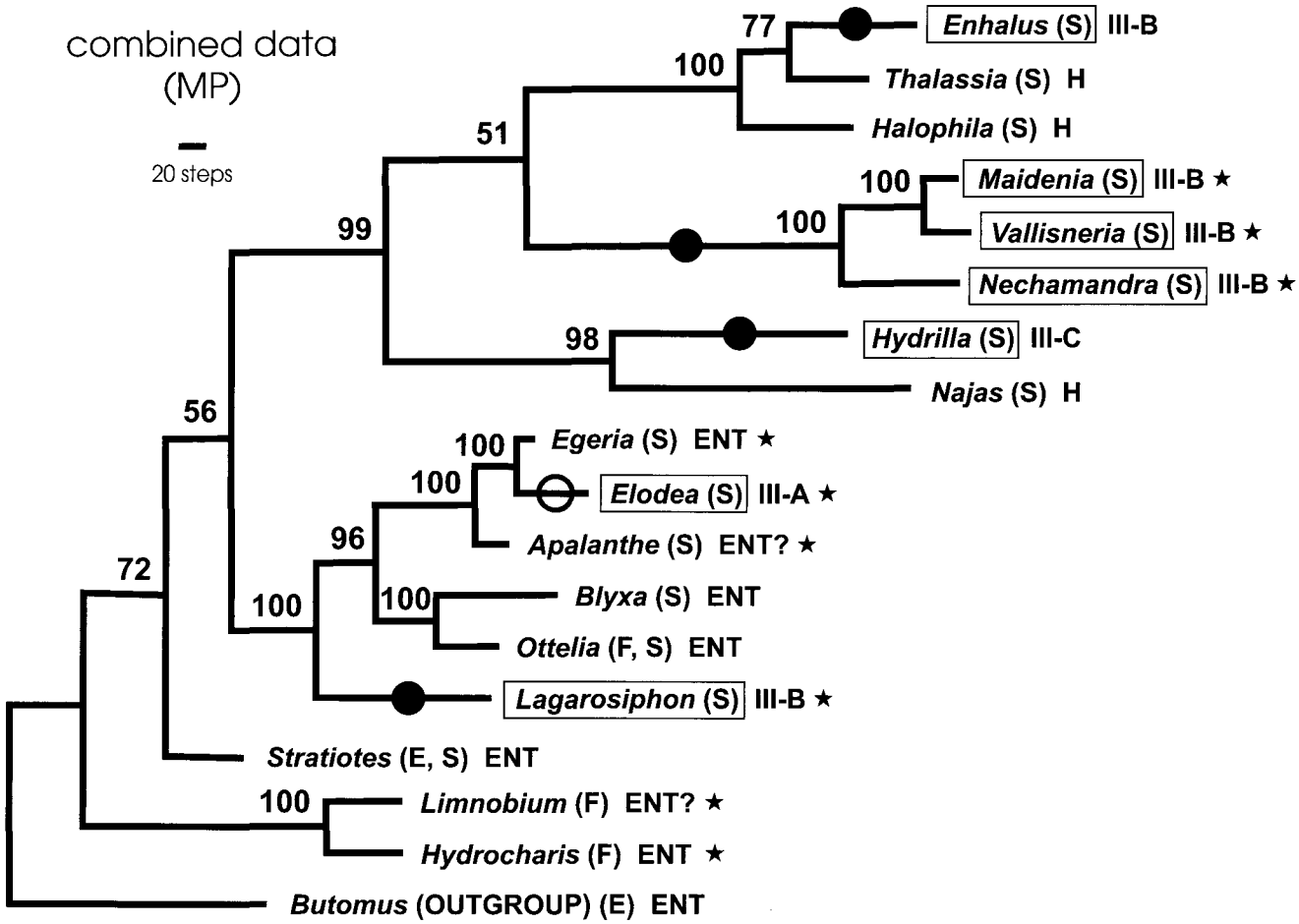

Fig. 7.—Single most parsimonious tree (2695 steps) recovered from MP analysis of all data combined. The tree is shown as a phylogram depicting relative branch lengths (scale bar = 20 steps). Numbers above branches represent bootstrap values obtained from 1000 replicates. Life-forms of genera are indicated by symbols in parentheses: (E) = emergent; (F) = floating-leaved; (S) = submersed. Boxes indicate genera where male flowers detach from plants and float on the water surface (category III pollination types). Pollination types (from Cook 1982) are indicated as: ENT (entomophilous); H (hypohydrophilous); III-A (pollen released on water surface); III-B (anthers making direct contact with stigma); III-C (pollen shed ballistically). Closed circles indicate four independent origins of type III pollination as shown by character state reconstructions using either ACCTRAN or DELTRAN optimizations. The open circle indicates a fifth origin of type III pollination in *Elodea* (which is polymorphic, possessing species having both attached and detached male flowers). Ovule type also is highly convergent on this tree, with ACCTRAN and DELTRAN optimized reconstructions indicating 3–4 separate derivations of orthotropous ovules (indicated by stars), respectively.

to *Lagarosiphon* (Cook and Triest 1982). Although not definitive, our morphological cladogram (Fig. 1) resolved both genera in a topology consistent with that interpretation.

A major difference between our phylogenetic analyses and Cook's earlier phenetic results concerns the interrelationships of the marine genera *Enhalus, Halophila,* and *Thalassia.* Cook's phenetic analyses consistently split these three genera into 2–3 different groups, but never grouped them together, a result reminiscent of taxonomic schemes (e.g., Hartog 1970) that segregated these genera among different subfamilies of Hydrocharitaceae. Conversely, our phylogenetic analysis resolved the marine Hydrocharitaceae as a clade, albeit with weak support (Fig. 1). Tomlinson's (1982) remark that *Thalassia* was "much more specialized" than most Hydrocharitaceae is supported by our morphological cladistic analysis (Fig. 1) that shows the genus to occupy a position quite derived in the family.

Few specific relationships have been postulated between and among other hydrocharit genera. Triest (1982) suggested that *Lagarosiphon* was closely related to *Elodea* and *Hydrilla,* a result inconsistent with relationships depicted in the morphological cladogram (Fig. 1) as well as results from analysis of combined data (Fig. 7). Shaffer-Fehre (1991a) noted a similarity in the seed coat anatomy of *Blyxa* and *Lagarosiphon,* taxa that also did not resolve closely in our morphological analysis. Aston (1973) remarked that *Blyxa* resembled juvenile plants of *Ottelia ovalifolia* (R. Br.) Rich. where their degree of similarity could lead to confused identifications. Although *Blyxa* and *Ottelia* did not associate as a clade in the morphological cladogram (Fig. 1), they were not far removed in that analysis.

Most nodes were not supported strongly in the morphological cladogram, yet our phylogenetic analysis did indicate that hydrocharit taxa having large, showy flowers and more complex anatomy and morphology (e.g., *Hydrocharis, Ottelia, Stratiotes*) tended to occur more basally than did those

taxa with highly reduced floral and vegetative morphologies. All genera represented by submersed life-forms occurred more distally in the tree than did those having either emergent or floating-leaved life-forms (Fig. 1). The marine genera (*Enhalus, Halophila, Thalassia*) resolved in a relatively specialized phylogenetic position with respect to most of the freshwater genera (Fig. 1). These results support the common perception that evolution in Hydrocharitaceae has proceeded generally via transitions from emergent to submersed life-forms and from freshwater to marine taxa.

The position of Najas.—Believing that morphological homologies generally were poorly understood in Alismatidae, Sculthorpe (1967) regarded any discussion on relationships of *Najas* as "phylogenetic speculation." However, he did conclude that the genus was not primitively simple as earlier authors had believed. Although Najadaceae long have been regarded as allied phylogenetically to Potamogetonaceae, some morphological data have indicated a possible association with Hydrocharitaceae.

Rendle (1901) and Singh (1965) interpreted the outer floral "envelope" of *Najas* as similar to the spathe found in Hydrocharitaceae. Although Rendle (1901) believed that *Najas* was most closely allied to *Zannichellia* L. (Zannichelliaceae), he also remarked on the similarity of the genus to *Elodea, Hydrilla,* and *Lagarosiphon* of Hydrocharitaceae. Miki (1937) suggested that "an intimate affinity" existed between *Najas* and Hydrocharitaceae based upon his evaluation of various morphological characters. He believed that *Najas* was derived from Hydrocharitaceae and was remote phylogenetically from Potamogetonaceae. Wilder (1975) remarked that unlike most Alismatidae, *Najas* and Hydrocharitaceae similarly lack the ability to produce nonprecocious buds. Compelling morphological evidence of a close relationship between *Najas* and Hydrocharitaceae was provided by Shaffer-Fehre (1991*a*, *b*) who discovered unique seed coat features that linked together these taxa. Les et al. (1993) and Les and Haynes (1995) showed that *Najas* and Hydrocharitaceae could be resolved as a sister group by phylogenetic analysis of cpDNA data as well as morphological data.

Our morphological analysis supports the inclusion of *Najas* within Hydrocharitaceae (Fig. 1), with a moderate degree of internal support (64%). Parsimony analysis embedded *Najas* rather deeply within the family, close to the marine genera. Although not definitive, this result shows overall that *Najas* is not so distinct morphologically as to preclude its placement within Hydrocharitaceae. However, because *Najas* is modified for hydrophilous pollination and is highly reduced otherwise, it is difficult to ascertain the influence of convergent character states attributable to morphological reduction.

Molecular Studies

Chloroplast DNA (cpDNA).—Hydrocharitaceae have been fairly well studied taxonomically, yet few efforts focused specifically on the elucidation of phylogenetic relationships within the family until the relatively recent advent of molecular data. Les et al. (1993) and Les and Haynes (1995) evaluated higher level relationships within subclass Alismatidae, using cladistic analysis of morphological and cpDNA sequence data, which indicated a close relationship between Najadaceae and Hydrocharitaceae (as noted above). Soon afterward, two studies appeared using cpDNA sequences that evaluated intergeneric relationships in Hydrocharitaceae. Tanaka et al. (1997) examined relationships among *Najas* and 13 hydrocharit genera using *rbc*L and *mat*K sequence data analyzed by neighbor-joining methods. Les et al. (1997) included two *Najas* species and 20 species from 15 hydrocharit genera in a survey of relationships in subclass Alismatidae using a weighted parsimony analysis of *rbc*L data. The results of these analyses produced topologies that differed in several details including the precise placement of *Najas* within the family (Fig. 2). However, the topologies generated by the two studies were extremely similar overall considering that different outgroups, taxon sampling, data sets, and analytical methods were used. Most nodes were supported quite well in both trees indicating that DNA sequence data showed potential for resolving at least some questions of relationships within the group. However, a number of branches (notably those leading to *Najas, Hydrilla,* and the marine genera) were long, a situation where it would have been desirable to use a likelihood analysis that typically performs better than either neighbor-joining or weighted parsimony methods in such instances (Page and Holmes 1998).

Our present cpDNA sequence analyses improved on these earlier studies in several ways. We increased the sample of hydrocharit genera (including *Najas*) to 18, lacking only the rare *Appertiella,* which has not been relocated in the field in recent years. We have added *rbc*L and *mat*K sequences for those genera not surveyed for these loci in the prior studies. We also have added to the analysis two additional cpDNA loci, namely the 3′ and 5′ *trn*K intron regions. These modifications increased the extent of cpDNA sequence data nearly twofold over previous studies. Furthermore, we have analyzed the cpDNA data using maximum likelihood as well as maximum parsimony to better assess the presence and influence of long internal branches in the phylogenetic trees. Another refinement was the use of *Butomus* (Butomaceae) as the outgroup. This genus is closest phylogenetically to Hydrocharitaceae (Les et al. 1997) and thus better suited than *Hydrocleys* Rich., the outgroup used by Tanaka et al. (1997).

It is satisfying that the trees resulting from our expanded cpDNA data (Fig. 3) retain topologies very similar to those recovered in the earlier studies (Fig. 2). In particular, all associations strongly supported (>90%) in the earlier cpDNA analyses were retained in the results from our expanded analyses. A notable improvement was the increased level of internal support (96–98%) for the placement of *Najas* within the clade including *Hydrilla,* the three marine genera, and *Vallisneria.* This result inspires confidence in accepting the merger of *Najas* within Hydrocharitaceae.

Nuclear ribosomal DNA (nrDNA).—The nrITS sequences of hydrocharit genera display a highly mosaic pattern of evolution. In some regions there is relatively high similarity among certain groups (arguably the most closely related genera) yet extreme divergence among others; whereas, in other regions, there is fairly high similarity across all genera, or in some cases, extreme divergence among all genera. Much variation in the nrITS region was expressed as indels that

made alignment quite difficult. As expected, the 5.8S region was strongly conserved and aligned easily. Several other regions conserved across genera also existed in the spacers (ITS-1; ITS-2) and could be aligned readily. Exclusion of the highly variable sites in our analyses improved the suitability of the region for phylogenetic analysis, but the smaller number of characters reduced the degree of internal support that could be provided by the ITS data. However, the inclusion of a nuclear DNA marker not only provides additional characters for phylogenetic reconstruction, but also serves to evaluate the possibility of different histories for maternally inherited (e.g., cpDNA) vs. biparentally inherited nuclear DNA that can arise through hybridization, lineage sorting, etc. (Page and Holmes 1998). The extent of even our reduced nrITS data was adequate to serve both purposes.

A common cpDNA and nrDNA history for hydrocharit taxa was indicated by the highly congruent topologies of cladograms obtained for each data set (Fig. 3, 4). Notably, these different data sets produced several of the same associations such as a large clade consisting of *Apalanthe, Blyxa, Egeria, Elodea, Lagarosiphon,* and *Ottelia* that resolved in the same topology. A monophyletic marine clade and clades consisting of *Hydrocharis* and *Limnobium* and also *Maidenia, Nechamandra,* and *Vallisneria* also were resolved by both sets of sequences. The nrITS data also provided additional internal support for the phylogenetic analysis of the family (68–100% for the clades mentioned). We observed no major inconsistencies that would warrant against combining the nrITS data with the other data sets for parsimony analysis.

Phylogenetic insights from the combined data analysis.—We view the cladogram generated from our combined data analysis (Fig. 7) to reasonably represent the best currently available estimate of phylogenetic relationships among Hydrocharitaceae genera. This cladogram is based on data from several sources representing both maternally and nuclear encoded characters, and shows relatively high internal support for most clades. Neither the different data sets nor the method of analysis influenced the resolution of any strongly supported groups except for *Hydrilla* and *Najas,* which were not always supported as a clade.

Relationships depicted in the combined data cladogram provide insight into the futility of previous attempts at classification for the family. In no instance did any of the classifications, based on a small subset of morphological characters, provide an appraisal of relationships that resembled those depicted in the combined data tree. Classifications such as those proposed by Richard (1811), Chatin (1855*e*, *g*), and Caspary (1857*a*) suggested generic associations that are at odds with the combined cladogram because they relied extensively on few, highly homoplasious characters. Anatomical characters such as those emphasized by these earlier authors are problematic in aquatic plants, which typically show strongly convergent patterns of reduction (Sculthorpe 1967; Dahlgren et al. 1985). Although this complicating factor now is generally recognized, it was poorly understood in the early to mid-nineteenth century when these classifications appeared.

Even those anatomical features such as ovule type (anatropous vs. orthotropous), which typically are regarded as having strong phylogenetic utility (e.g., Dahlgren et al. 1985), are highly homoplasious in the family (3–4 separate origins of orthotropous ovules were indicated by the combined analysis) and singularly are unsuitable for determining natural clades.

Perhaps the most striking example of a misleading convergent character in Hydrocharitaceae is illustrated by some taxa having highly unusual male flowers that are released from submersed plants in bud and float to the surface where they open and drift as independent units. Although this bizarre floral mechanism occurs nowhere else in the flowering plants, the trait has evolved as many as five separate times within Hydrocharitaceae as indicated by the combined data tree (Fig. 7). By its uniqueness, it is understandable why this unusual feature has been considered to represent a strong indication of infrafamilial relationships in the past; however, it is now apparent that the feature is extremely homoplasious. Some indication of the repeated evolution of this condition is given by Cook (1982) who recognized different pollination subcategories among the taxa having detached male flowers. Cook's unique designations for *Elodea* ("III-A" where pollen floats on the water surface) and *Hydrilla* ("III-C" where pollen is discharged through the air) correspond to independent origins of the trait as indicated by our phylogenetic analysis (Fig. 7). A third group (III-B) where anthers directly contact stigmas, unites *Maidenia, Nechamandra,* and *Vallisneria* (a strongly supported clade), yet is convergent for *Enhalus* and *Lagarosiphon.* Interestingly, the occurrence of similar pollination systems in *Enhalus* and *Vallisneria* is responsible for their placement together in many previous classifications (e.g., Hartog 1970).

Even fairly recent classifications of Hydrocharitaceae (e.g., Shaffer-Fehre 1991*a*), have relied principally on the distribution of few character states and portray groups that are inconsistent with the phyletic relationships depicted in our combined data cladogram. Dahlgren et al. (1985) considered distributions of larger numbers of characters, but their classification of Hydrocharitaceae ultimately was biased by their emphasis on relatively few features such as perianth structure. Although their classification (Table 1) is fairly compatible with the results of our combined analysis, it misplaced various genera such as *Enhalus, Hydrilla, Ottelia,* and *Thalassia.*

We believe that results of our combined data analysis inspire much greater confidence by minimizing effects of small numbers of convergent characters in constructing our phylogeny. Analyses of larger morphological data sets, either by phenetic or phylogenetic approaches have consistently indicated the same associations of certain genera as those also resolved by phylogenetic analysis of molecular data. Examples of clades recovered consistently by either approach include *Hydrocharis* and *Limnobium* (100% bootstrap support in all analyses), *Egeria* and *Elodea* (47–100% in all analyses), and *Maidenia* and *Vallisneria* (66–100% in all analyses except MP analysis of nrITS data). Other clades resolved by all phylogenetic analyses included *Maidenia, Nechamandra,* and *Vallisneria* (57–100%), the marine genera (41–100%), and *Apalanthe, Egeria,* and *Elodea* (35–100%). These results are difficult to question given the relationships indicated are consistently mirrored by various data sets and analyses. In particular, we view the relatively

isolated, well-supported, basal clade comprising *Hydrocharis* and *Limnobium* to represent a distinct subfamily to which the name Hydrocharitoideae Eaton should be applied.

Although the clade containing *Apalanthe, Blyxa, Egeria, Elodea, Lagarosiphon,* and *Ottelia* was not resolved fully using morphological data alone, it was recovered using all other data sets and is depicted as a strongly supported clade (100%) in our combined analysis. Consequently, we now regard this group to represent a fundamental phyletic subdivision of Hydrocharitaceae warranting taxonomic recognition at the rank of subfamily. This clade contains most genera once placed together in tribe Anacharideae (also known as Hydrilleae Horan.); however, it also includes genera (*Blyxa, Ottelia*) not associated previously with the group and also excludes *Hydrilla,* which was placed formerly in the tribe. This well-supported clade has not been recognized by any prior classification system. The oldest available name at the rank of subfamily appears to be Anacharidoideae Thomé. Although based on the genus *Anacharis* (a later synonym of *Elodea*), this name retains priority as a subfamily in accordance with article 11.3 of the International Code of Botanical Nomenclature (Greuter et al. 2000). Presently, we recognize subfamily Anacharidoideae to include *Apalanthe, Appertiella, Blyxa, Egeria, Elodea, Lagarosiphon,* and *Ottelia.*

Our results indicate that *Lagarosiphon* is not most closely related to *Elodea* and *Hydrilla* as Triest (1982) concluded, but is relatively close to *Blyxa* as Shaffer-Fehre (1991*a*) observed. The vegetative similarity of *Blyxa* and *Ottelia* observed by Aston (1973) indicates their close relationship as depicted by the combined data cladogram (Fig. 7) despite our inability to resolve this clade in our morphological analysis (Fig. 1).

Another fundamental clade within Hydrocharitaceae includes the three marine genera along with *Hydrilla, Maidenia, Najas, Nechamandra,* and *Vallisneria* that associate with high internal support (99%) in the combined analysis (Fig. 7). Although the morphological (Fig. 1) and nrDNA trees (Fig. 4) excluded some taxa (*Hydrilla* in the former, the marine clade in the latter), the exclusions were not supported strongly. This clade is of particular significance because it includes the genus *Najas,* once placed in a distinct and quite distantly related family by some. Although several previous analyses (Les et al. 1997; Tanaka et al. 1997) have placed *Najas* within Hydrocharitaceae, internal support for its inclusion was only moderate (51–66%) leaving the merger of these taxa to be questionable. The existence of long branches (Fig. 7) characterizing *Najas* (and also *Hydrilla,* with which it is sometimes associated) also raises the issue whether their relationship may be spurious due to the effects of long-branch attraction. Using ML approaches that provide some correction for this problem (Page and Holmes 1998), we still recovered the placement of *Najas* within the family with strong support (96%) for cpDNA data and with moderate support (74%) for nrDNA data. The combined (MP) analysis also shows *Najas* to associate strongly (98%) with *Hydrilla,* a result also recovered in MP analysis (92%) of cpDNA data and nrITS data (56%). Although the phylogenetic association of these genera is well supported using MP, the long branch-lengths of both taxa show them to be quite divergent from one another at the molecular level. We recommend that this clade of eight genera be recognized as a separate subfamily to which we assign the previously published name of Hydrilloideae Luerss.

Table 5. Phylogenetic classification proposed for Hydrocharitaceae.

Hydrocharitaceae Juss.	
I.	subfamily Hydrocharitoideae Eaton
1.	*Hydrocharis* L.
2.	*Limnobium* Rich.
II.	subfamily Stratiotoideae Luerss.
3.	*Stratiotes* L.
III.	subfamily Anacharidoideae Thomé
4.	*Apalanthe* Planch.
5.	*Appertiella* C. D. K. Cook & Triest
6.	*Blyxa* Noronha ex Thouars
7.	*Egeria* Planch.
8.	*Elodea* Michx.
9.	*Lagarosiphon* Harv.
10.	*Ottelia* Pers.
IV.	subfamily Hydrilloideae Luerss.
11.	*Enhalus* Rich.
12.	*Halophila* Thouars
13.	*Hydrilla* Rich.
14.	*Maidenia* Rendle
15.	*Najas* L.
16.	*Nechamandra* Planch.
17.	*Thalassia* Banks ex K. D. Koenig
18.	*Vallisneria* L.

Our results have compelled us to endorse the merger of Najadaceae and Hydrocharitaceae that must take into account the nomenclatural issue of priority to apply the correct family name. Both names originate from the same publication date: (Hydrocharitaceae Juss., *Genera Plantarum,* vol. 67. 4 Aug 1789; Najadaceae Juss., *Genera Plantarum,* vol. 18. 4 Aug 1789). According to the most recent International Code of Botanical Nomenclature (Greuter et al. 2000), they both are legitimate names of equal priority. In such instances, article 11.5 establishes priority based on the first choice to be effectively published (Greuter et al. 2000). As far as we can ascertain, Shaffer-Fehre (1991*b*) was first to merge the families formally under the name Hydrocharitaceae; thus, we accept her decision to retain this name and accept it with priority over the name Najadaceae.

All data support the monophyly of the marine "seagrasses" (*Enhalus, Halophila,* and *Thalassia*), which have not been classified together previously (Table 1). The monophyly of these genera indicates that a single evolutionary colonization of the marine habitat involved taxa having different pollination mechanisms, i.e., surface-pollination in *Enhalus* vs. hydrophily in *Halophila* and *Thalassia.* The marine clade is derived within Hydrocharitaceae, supporting the major biological trend ascribed previously by Sculthorpe (1967).

Our phylogeny of Hydrocharitaceae shows hydrophily to be derived relative to entomophily, which occurs in the outgroup and most genera resolved basally (Fig. 7). This result supports another of Sculthorpe's (1967) biological trends attributed to Hydrocharitaceae. Hydrophily in *Najas* and its position distant from the two hydrophilous marine genera indicates several derivations of underwater pollination within the family (see also Les et al. 1997).

The genus *Stratiotes* has an extremely unusual life-history, spending the vegetative portion of its life-cycle as a submersed plant at the bottom of ponds, but then rising up to the surface where it produces a floating rosette of emergent leaves and showy, aerial flowers (Sculthorpe 1967). Morphologically, this genus could be regarded as an evolutionary intermediate between the floating-leaved life-form that characterizes *Hydrocharis* and *Limnobium,* and the submersed life-form that occurs in all other hydrocharit genera. It is intriguing to hypothesize that the biphasic life-form of *Stratiotes* may have enabled plants to gradually acquire adaptations to a submersed existence over evolutionary time, without necessitating an abrupt abandonment of terrestrial adaptations such as entomophilous flowers. The intermediate phylogenetic placement of *Stratiotes* between the floating-leaved and submersed genera of Hydrocharitaceae (Fig. 7) is consistent with this interpretation. We propose that *Stratiotes* should be placed within a separate subfamily (published previously as Stratioideae Luerss.) to reflect its distinct position in the family.

One remaining question concerns the precise phylogenetic position of *Appertiella,* which could not be obtained for molecular analyses. Our best estimate at present is to tentatively regard it as being related most closely to *Lagarosiphon* (Cook and Triest 1982), a conclusion consistent with, but not supported unambiguously by the results of our morphological analysis (Fig. 1). This proposed relationship should be tested once material becomes available for DNA analysis and comparison with the molecular data compiled for other genera surveyed in this study.

Combined phylogenetic data analysis has significantly improved our understanding of intergeneric relationships in Hydrocharitaceae by producing a well-resolved and well-supported cladogram that lends credibility to our suggested improvements in the classification of the family. We recommend the taxonomic division of Hydrocharitaceae into four subfamilies, which correspond to the major clades depicted in our combined data analysis (Fig. 7). A synopsis of our proposed classification is presented in Table 5.

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