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Phylogenetic Studies in the Monocot Subclass Alismatidae: Evidence for a Reappraisal of the Aquatic Order Najadales

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Within the angiosperm subclass Alismatidae (= superorder Alismatiflorae), contemporary taxonomists have often assigned the families Hydrocharitaceae and Najadaceae to different orders. The Najadaceae are presumably allied to a variety of aquatic families in the order Najadales, whereas the Hydrocharitaceae have been segregated as the order Hydrocharitales or placed within the order Alismatales. Analyses of DNA sequence data from the chloroplast gene *rbc*L, however, indicate that Najadaceae have a much closer phylogenetic relationship to Hydrocharitaceae than to families of the "Najadales" (Cymodoceaceae, Potamogetonaceae, Ruppiaceae, Scheuchzeriaceae, Zannichelliaceae, Zosteraceae). This association supports previous studies based upon examination of floral structure and seed coat anatomy. The *rbc*L sequence data also indicate that the Zosteraceae and Potamogetonaceae are closely related families. The *rbc*L sequence of *Zostera* is actually more similar to that of *Potamogeton richardsonii* than is the sequence of the latter to a congener, *Potamogeton amplifolius*. The marine, dioecious, hydrophilous genus *Zostera* has acquired a number of distinctive adaptations, but probably diverged relatively rapidly from freshwater Potamogetonaceae. Molecular data place Ruppiaceae as a sister group to the marine Cymodoceaceae and do not support the commonly accepted merger of Ruppiaceae and Potamogetonaceae.

INTRODUCTION

Peculiarities associated with the adaptative history of aquatic angiosperms have presented great difficulty with the classification of these plants. A consequence of the severe structural reduction of aquatic angiosperms (Arber, 1920) is the scarcity not only of morphological and anatomical features (Sculthorpe, 1967), but also biochemical compounds such as flavonoids (Les and Sheridan, 1990) that have provided useful characters for comparative phylogenetic studies of terrestrial plants. The aquatic habitat also selects strongly for structural convergences that are adaptive for survival in water. Natant leaves are an obvious example with occurrences in distantly related monocots (e.g., Araceae, Limnocharitaceae Pontederiaceae) and dicots (e.g., Nymphaeaceae, Callitrichaceae, Menyanthaceae). Greatly diminished sexuality in many aquatic angiosperms (Les and Philbrick, 1993) also renders them difficult to study for purposes of classification, owing to the fact that angiosperm systematics relies heavily on comparisons of technical floral traits.

Nowhere are these factors more evident than in the subclass Alismatidae (superorder Alismatiflorae). This is the only angiosperm subclass in which all of the orders (excluding the parasitic, terrestrial Triuridales) and families are dominated by aquatic species. The Alismatidae includes some of the most structurally reduced angiosperms (e.g., *Zannichellia, Najas*) and offers numerous instances of convergent evolution. Among monocots, water-pollination (hydrophily) is found only in the Alismatidae; however, here its occurrence in six different families (Les, 1988) indicates several independent origins. Associated with hydrophily are a number of specializations that are convergent adaptations (Dahlgren and Rasmussen, 1983; Cox and Knox, 1989). In angiosperms, the marine habit ("seagrasses") is confined to the Alismatidae, yet it is expressed among several alismatid lineages (all seagrasses are also hydrophilous). Distributions of many structural features (e.g., floating leaves) are uninformative phylogenetically due to their sporadic occurrence among various families of the subclass.

It is understandable that a number of different classifications have been proposed for the Alismatidae in attempts to unite and delimit closely related groups of species (see summary by Schaffer-Fehre, 1991). Aside from the general uncertainty regarding any relationships in the Alismatidae (Dahlgren and Rasmussen,

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. L08759, L08762, L08765, L08767, L08768; U03724–U03731.

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1983), a specific controversy concerns the position of the Najadaceae. Most contemporary classifications recognize either two or three orders (Alismatales, Hydrocharitales, Najadales) within the subclass (Takhtajan, 1980; Cronquist, 1988; Dahlgren *et al.*, 1985). The cladistic analysis of the Alismatiflorae by Dahlgren and Rasmussen (1983) is congruent with most contemporary classifications by positioning the Najadaceae and Hydrocharitaceae in different monophyletic lineages. Recent anatomical evidence by Shaffer-Fehre (1991), however, has revived the hypothesis of Miki (1937) that these two families are, in fact, quite closely related (Fig. 1).

Some reconciliation of evolutionary relationships among aquatic angiosperms has emerged recently from the systematic application of molecular data. DNA sequence data are suitable for the phylogenetic analysis of aquatic angiosperms because of their universal "presence" among aquatic taxa, and the extremely low probability that specific nucleotide substitutions in coding genes are selected as aquatic adaptations (Les *et al.*, 1991).

In this study, we have followed an approach similar to that described in our phylogenetic analysis of aquatic dicots (Les *et al.*, 1991). Specifically, we have used sequence data from the chloroplast gene *rbc*L to examine phylogenetic relationships in a selection of genera comprising three orders and nine families of the monocot subclass Alismatidae. This is the first report of an ongoing project focused on the study of evolutionary relationships among all extant families of Alismatidae.

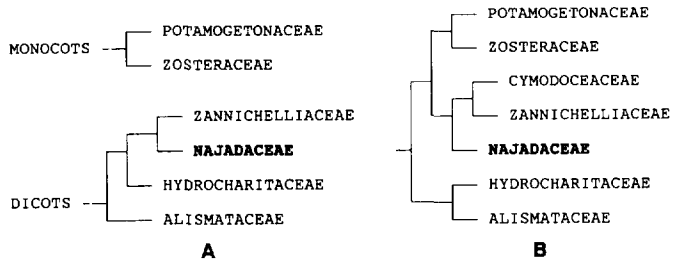

FIG. 1. Contrasting phylogenies of alismatid families (A) by Miki (1937) and (B) by Dahlgren and Rasmussen (1983) differ primarily with respect to the relationship of Najadaceae and Zannichelliaceae (simplified from original diagrams).

MATERIALS AND METHODS

Total genomic DNA was extracted from fresh leaf material using a modified CTAB technique (Doyle and Doyle, 1987). Two sequencing approaches were used. For all genera excluding *Ruppia, Syringodium,* and *Zannichellia* (Table 1), an 1183-base pair fragment of the *rbc*L gene was amplified (Saiki *et al.*, 1988) using a thermostable polymerase and two internal primers: 5F (5′-AAAGCGGCCGCACCACAAACAGA$^{G/A}$ACTAAAGC-3′) and 1210R (5′-AAAAGCGGCCGCAAGG$^{G/A}$TG$^{T/C}$CCTAAAGTTCCTCC-3′) (Fig. 2). Double-stranded DNA was amplified in 100-μl reaction volumes containing approximately 100 ng of genomic DNA isolate. Thirty to forty-five cycles comprising 1 min denaturation at 94°C, 2 min annealing at 37°C, and 2 min 15-s extension at 72°C were performed for all amplifications. Amplified products were end polished by adding 2 units of T4 DNA polymerase followed by a 20-min incubation at 37°C. Products were purified by gel isolation in low melting point agarose followed by a secondary "Gene Clean" purification (Bio 101, La Jolla, CA).

Purified amplification products were blunt-end ligated into an *Eco*RV-cut SKM13 vector (Stratagene, La Jolla, CA). Cloning of amplification products was facilitated using DH5αF′ competent cells (Bethesda Research Laboratories, Gaithersburg, MD). Recombinant plaques were screened for orientation and 5 to 10 of each strand were pooled. The single-stranded phage DNA was sequenced by standard dideoxy chain termination procedures (Sanger *et al.*, 1977) using modified T7 DNA polymerase (Sequenase, Version 2.0; United States Biochemical, Cleveland, OH) and a set of synthetic primers based on the *rbc*L sequence of maize (provided by G. Zurawski, DNAX, Palo Alto, CA). Clones of both strands of amplified DNA were compared.

For *Ruppia, Syringodium,* and *Zannichellia,* DNA amplifications were performed in a similar fashion except that the 1210R internal primer was replaced by a primer external to the 3′ end of the gene: (5′-TTTTAGTAAAAGATTGGGCCGAG-3′).

This external primer enabled the amplification and sequencing of the entire *rbc*L gene for these three genera, beyond the first 26 bp of the forward primer sequence. The external primer did not work, however, in attempted amplifications of DNA from several of the other families examined in the analysis (notably Hydrocharitaceae). Accordingly, we restricted our analysis to the 1183-bp region of the gene that was complete for all taxa studied. Data for *Ruppia, Syringodium,* and *Zannichellia* were obtained by direct sequencing of double-stranded PCR products. Rapid immersion of denatured DNA/primer mixtures in an ice bath facilitated primer annealing and limited the reassociation of double-stranded DNA. Otherwise, sequencing reactions were carried out as described above.

Nucleotide substitutions were evaluated for the number of phylogenetically informative sites represented and the relative percentage substitution at codon positions. Percentage divergence and transition: transversion ratios were calculated for all pairwise comparisons and were also calculated relative to the outgroup taxon *Saururus cernuus*. A χ-square test of

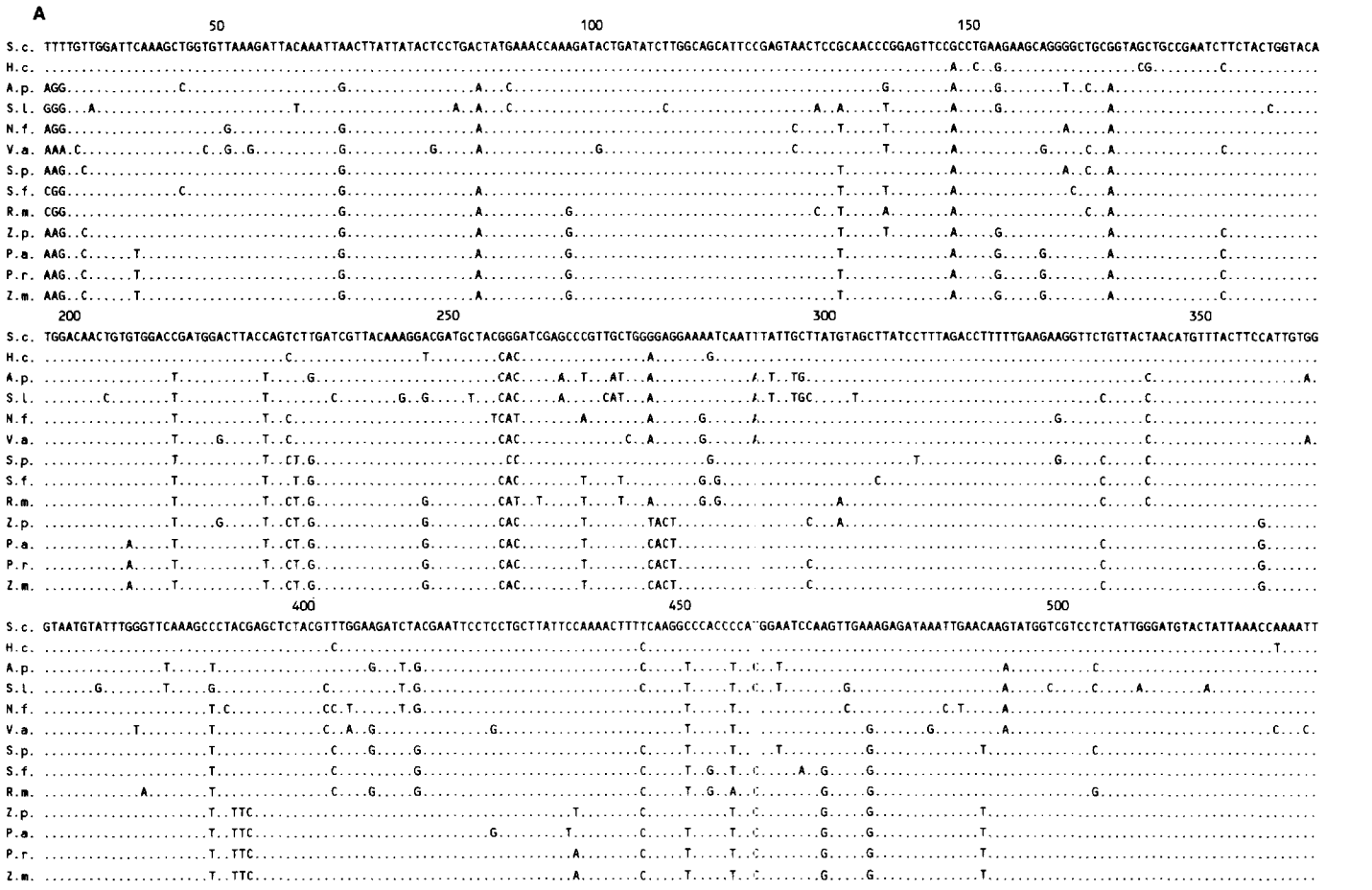

FIG. 2. An 1183-bp sequence of *rbc*L compared in *Saururus cernuus* (S.c.), *Houttuynia cordata* (H.c.), *Alisma plantago-aquatica* (A.p.), *Sagittaria latifolia* (S.l.), *Najas flexilis* (N.f.), *Vallisneria americana* (V.a.), *Scheuchzeria palustris* (S.p.), *Syringodium filiforme* (S.f.), *Ruppia maritima* (R.m.), *Zannichellia palustris* (Z.p.), *Potamogeton amplifolius* (P.a.), *Potamogeton richardsonii* (P.r.), and *Zostera marina* (Z.m.). Dots indicate identity with the *Saururus cernuus* sequence.

dinucleotide probabilities was performed for all species using the program DINUC written by S. Duncan, Indiana University.

A comprehensive phylogenetic analysis of 499 taxa using *rbc*L sequence data (Chase *et al.*, 1993) has facilitated our selection of an appropriate outgroup for the Alismatidae. This analysis, which included three alismatid genera, depicted the "paleoherbs" (predominantly the orders Nymphaeales and Piperales) as the closest dicot sister group to the Alismatidae. Taxa from the Piperales, Nymphaeales, and Ceratophyllales (the basal angiosperm lineage identified by the above analysis), used singly or in combination as outgroups, all generated the same topology for our ingroup taxa. We selected two taxa from the order Piperales (*Saururus, Houttuynia*) to serve as a functional outgroup in our analyses (Watrous and Wheeler, 1981).

The phylogenetic significance of the sequence data was assessed by maximum parsimony and maximum likelihood methods. Parsimony analyses were conducted using PAUP, v. 3.1 (Swofford, 1993). *Saururus* and *Houttuynia* were specified as the outgroup for rooting. The "branch and bound" algorithm was used to search for minimum-length trees with all substitutions weighted equally. After a single minimum-length tree was obtained, we compared the result to those of four "forced topologies" which differed from the branch and bound solution (user tree 1 below) by requiring the pairing of the two *Potamogeton* species (user tree 2) and the association of Najadaceae with other submersed "Najadales" rather than with Hydrocharitaceae (user tree 3), and by forcing the entire topology to comply with the cladogram published by Dahlgren and Rasmussen (1983) (user tree 4).

The format of these user trees was as follows (abbreviations of taxa are explained in the legend to Fig. 2):

1. ((S.c.,H.c.),(((N.f.,V.a.),(A.p.,S.l.)),(((((P.r.,Z.m.),P.a.),Z.p.),(R.m.,S.f.)),S.p.)))
2. ((S.c.,H.c.),(((N.f.,V.a.),(A.p.,S.l.)),(((((P.r.,P.a.),Z.m.),Z.p.),(R.m.,S.f.)),S.p.)))
3. ((S.c.,H.c.),((V.a.,(A.p.,S.l.)),(((((P.r.,Z.m.),P.a.),Z.p.),(R.m.,S.f.)),N.f.),S.p.)))
4. ((S.c.,H.c.),((V.a.,(A.p.,S.l.)),(((((P.a.,P.r.),R.m.),Z.m.),((S.f.,Z.p.),N.f.)),S.p.))).

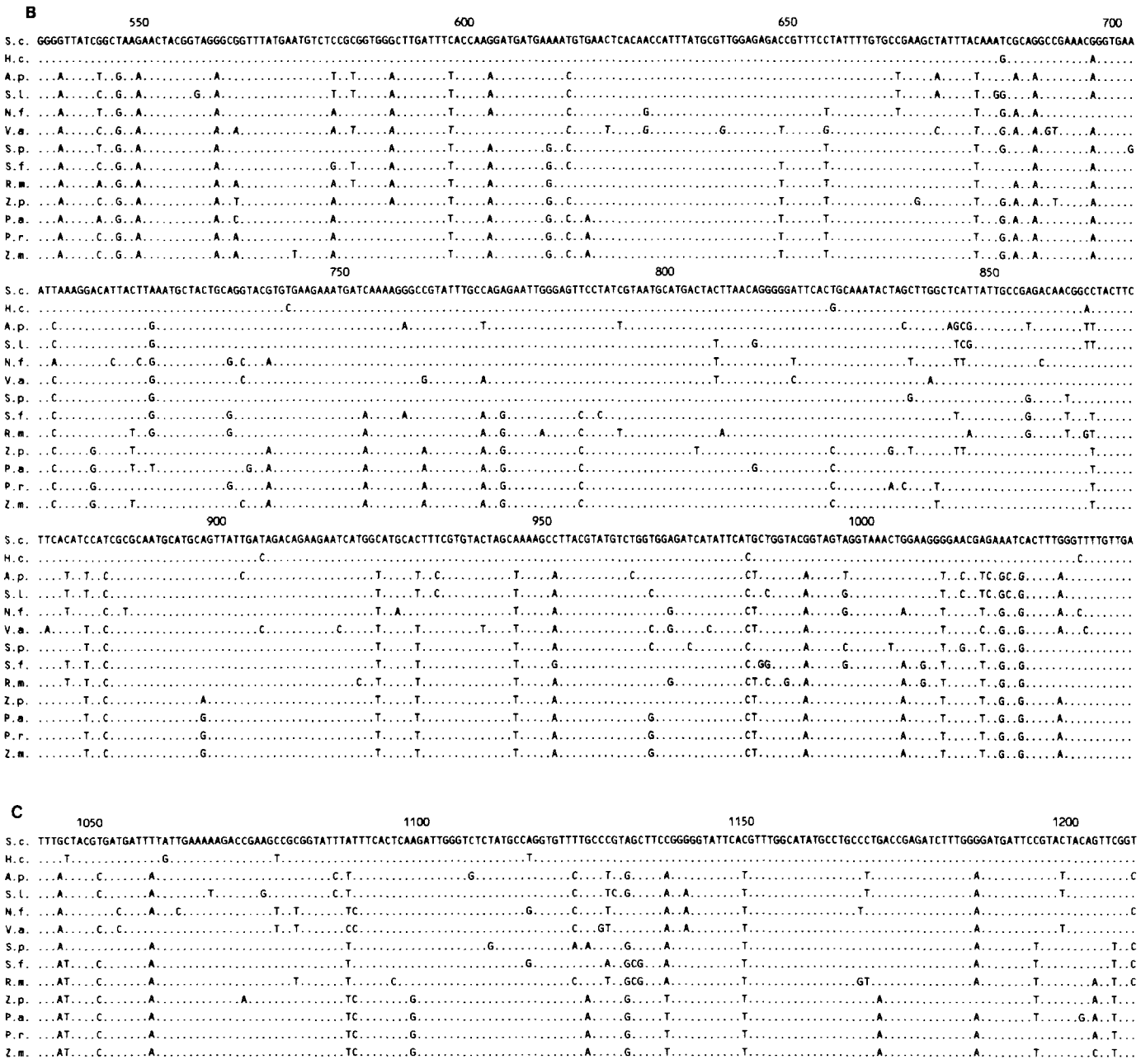

FIG. 2—*Continued*

The length of each tree and its consistency and retention index were noted. A "bootstrap" analysis with 500 replications was used to provide an estimate of confidence intervals for branches of the minimum-length solution. To further assess the stability of our topology, we performed a "decay analysis" (following the methods of Qiu *et al.*, 1993) to indicate the number of additional steps required to collapse ingroup branches that supported monophyletic groups in the maximum parsimony solution. For this we examined all trees up to five steps longer than the most parsimonious solution.

We used the program PHYLIP, v. 3.5/c (Felsenstein, 1993) to conduct maximum likelihood estimations (MLE) of phylogeny from the sequence data. Results of the parsimony analysis were used to determine the initial input order of taxa for MLE ("closest" taxa were added sequentially). The MLE analysis of this input order was run with the "global" option, empirically determined base frequencies, and the default transition:

transversion ratio of 2.0 (close to the average ratio of all pairwise comparisons). We were unable to improve the log-likelihood value of this result using alternate input orders. We then compared the log-likelihood of this "best" topology to those of the same user trees indicated above.

RESULTS

Within an 1183-bp sequence of *rbc*L, we detected 275 substitutions (23.2% of sites) among the 13 species sampled (Table 1). 173 sites (14.6% of total; 62.9% of all substitutions) were phylogenetically informative (contained substitutions shared by at least two taxa).

First, second, and third codon positions accounted for 44 (16.0%), 26 (9.5%), and 205 (74.5%) of all site substitutions, respectively. The relative rates of substitution among codon positions agreed closely with results from a similar analysis of aquatic dicot *rbc*L sequences (Table 2).

Among all taxa studied, the percentage sequence divergence for this segment of *rbc*L ranged from 0.7 to 10.8 (Table 3). In pairwise comparisons, transition: transversion ratios ranged from 0.27 to 3.25 with a mean of 2.1 (Table 3). Relative to the outgroup taxon *Saururus, rbc*L sequence divergence ranged from 2.4 to 10.3%; transition:transversion ratios relative to *Saururus* ranged from 1.8 to 2.8 with a mean of 2.3. Transition:transversion ratios did not appear to decrease inversely to sequence divergence in this sample; the majority of ratios was close to the mean pairwise value of 2.1.

Six of the thirteen species examined yielded a total of nine dinucleotide probabilities that differed significantly from values predicted from random associations among nucleotides based upon their empirical frequencies in the *rbc*L sequence (Table 4). Deviation (underrepresentation) of the GT dinucleotide occurred in five species. *Najas flexilis* showed a significant deficiency of the CG dinucleotide, and an excess of CT and TG dinucleotides.

A single minimal length tree (length = 481; consistency index = 0.707; retention index = 0.677) was obtained as a result of parsimony analysis (Fig. 3). The topology of this tree agreed with that obtained from a maximum-likelihood analysis which had the lowest log-likelihood ratio (−4298.15) of any tree that we could find through various input order manipulations.

Forced topology analyses increased tree lengths from two to 99 additional steps and incrementally lowered the consistency and retention indices (Table 5). Two additional steps were added when the pairing of *Potamogeton* species was forced, but the log-likelihood value did not differ significantly from the best solution. Placing Najadaceae among submersed families that have traditionally comprised the order "Najadales" increased the tree length by 22 steps and yielded a log-likelihood value that differed significantly. Forcing the topology to comply exactly with the cladogram published by Dahlgren and Rasmussen (1983) increased the tree length by 99 additional steps and generated a log-likelihood value that differed significantly (Table 5).

The single topology representing the "best" parsi-

TABLE 1

Classification of Taxa Examined in This Study (Following Cronquist, 1990) and Voucher Specimens of Material Examined

Outgroup: Class Magnoliopsida (dicots); Subclass Magnoliidae;
- Order Piperales
  - Family Saururaceae
    - *Houttuynia cordata* (Les s.n., CONN)
    - *Saururus cernuus* (Tucker 4835, NYS)

Class Liliopsida (monocots); Subclass Alismatidae

- Order Alismatales
  - Family Alismataceae
    - *Alisma plantago-aquatica* (Les s.n., CONN)
    - *Sagittaria latifolia* (Les s.n. CONN)
- Order Hydrocharitales
  - Family Hydrocharitaceae
    - *Vallisneria americana* (Les s.n., CONN)
- Order Najadales
  - Family Cymodoceaceae
    - *Syringodium filiforme* (Koch s.n., CONN)
  - Family Najadaceae
    - *Najas flexilis* (Les s.n., CONN)
  - Family Potamogetonaceae
    - *Potamogeton amplifolius* (Les 483, CONN)
    - *Potamogeton richardsonii* (Les s.n., CONN)
  - Family Ruppiaceae
    - *Ruppia maritima* (Koch s.n., CONN)
  - Family Scheuchzeriaceae
    - *Scheuchzeria palustris* (Les s.n., CONN)
  - Family Zannichelliaceae
    - *Zannichellia palustris* (Haynes s.n., UNA)
  - Family Zosteraceae
    - *Zostera marina* (Miller s.n., CONN)

TABLE 2

Uniformity of *rbc*L Codon Position Substitution Compared for 23 Monocot and Dicot Aquatic Plant Species

	Alismatid species[a]	Nymphaeles species[b]
No. substituted sites:	275 (23.2)	149 (12.6)
No. first position:	44 (16.0)	23 (15.4)
No. second position:	26 (9.5)	15 (10.1)
No. third position:	205 (74.5)	111 (74.5)

Note. Percentages are given in parentheses
[a] This study
[b] Data from Les *et al.* (1991).

TABLE 3

Matrix of Transition:Transversion Ratios (Upper) and Percentage Sequence Divergence (Lower) for *rbc*L in Pairwise Comparisons of Taxa Studied

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 *Alisma plantago-aquatica*	—	1.68	1.97	1.67	1.89	1.70	1.87	1.81	1.96	1.38	1.64	2.00	1.74
2 *Houttuynia cordata*	10.0	—	2.14	1.95	1.89	2.54	1.80	1.98	2.21	2.26	2.13	1.97	1.85
3 *Najas flexilis*	7.8	9.3	—	2.83	3.00	2.57	2.35	2.28	2.86	2.68	3.25	3.23	3.10
4 *Potamogeton amplifolius*	9.0	9.5	9.3	—	0.60	2.00	2.26	1.86	1.89	2.04	2.52	1.60	0.27
5 *Potamogeton richardsonii*	8.8	9.3	9.1	1.4	—	2.04	2.20	1.95	2.16	2.21	2.48	1.77	0.60
6 *Ruppia maritima*	7.4	10.4	8.4	6.8	6.9	—	2.84	1.87	2.04	2.20	2.29	2.00	1.93
7 *Saururus cernuus*	9.2	2.4	8.8	9.4	9.2	9.9	—	2.13	2.65	2.64	2.23	2.29	2.14
8 *Sagittaria latifolia*	5.0	10.8	8.9	10.4	10.2	9.6	10.3	—	2.37	1.73	1.97	2.17	1.90
9 *Scheuchzeria palustris*	6.8	7.9	7.2	6.6	6.7	6.6	7.0	8.5	—	2.05	2.33	2.35	2.00
10 *Syringodium filiforme*	6.8	9.2	7.6	6.3	6.4	4.1	8.5	8.5	5.4	—	2.61	2.15	2.12
11 *Vallisneria americana*	8.7	9.8	7.2	9.2	9.1	9.5	9.6	10.0	8.5	9.3	—	2.38	2.57
12 *Zannichellia palustris*	9.0	9.5	9.0	2.7	2.5	7.1	9.4	10.4	7.0	6.6	9.3	—	1.87
13 *Zostera marina*	9.0	9.4	8.8	1.2	0.7	6.9	9.3	10.3	6.5	6.5	8.9	2.4	—

mony and MLE solutions for the *rbc*L data agrees with Miki's concept of a phylogenetic relationship between Hydrocharitaceae and Najadaceae, but does not support the often proposed alliance of Najadaceae and Zannichelliaceae (Fig. 1). Bootstrap and decay values strongly supported (100%; $d > 5$) clades including Zannichelliaceae/Potamogetonaceae/Zosteraceae and the Alismataceae (*Alisma* and *Sagittaria*). The association of Najadaceae and Hydrocharitaceae was moderately supported (83%; $d = 5$) as was the association of *Potamogeton richardsonii* and *Zostera marina* (90%; $d = 2$). The association of Ruppiaceae (*Ruppia*) and Cymodoceaceae (*Syringodium*) was fairly well supported (92%; $d = 5$).

The *rbc*L sequence of *Zostera marina* is most similar to that of *Potamogeton richardsonii* in our sample. The compared region of *rbc*L was only 0.7% divergent for these species, but 1.4% divergent for the two *Potamogeton* species sequenced (Table 3).

DISCUSSION

The evolutionary transition to an aquatic existence has occurred rarely in higher plants with estimates that fewer than 3.5% of vascular plant genera contain aquatic species (Cook, 1990). In flowering plants, only approximately 17% of all families contain aquatic species (Cook, 1990). The monocot subclass Alismatidae

TABLE 4

Dinucleotide Bias in *rbc*L for Taxa Studied

	A.p.	H.c.	N.f.	P.a.	P.r.	R.m.	S.c.	S.f.	S.l.	S.p.	V.a.	Z.m.	Z.p
AA	1.052	1.116	1.030	1.047	1.061	1.003	1.145	1.060	1.049	1.003	0.976	1.041	1.043
AC	1.129	1.108	1.104	1.082	1.091	1.079	1.063	1.101	1.070	1.080	1.080	1.086	1.096
AG	0.872	0.826	0.921	0.865	0.847	0.877	0.795	0.851	0.867	0.916	0.959	0.882	0.874
AT	0.962	0.953	0.960	1.006	0.998	1.044	0.987	1.001	1.008	1.007	1.000	0.992	0.995
CA	1.018	0.954	0.962	0.967	0.964	1.062	0.936	0.967	1.023	0.983	1.049	0.975	1.013
CC	1.120	1.149	0.986	1.010	1.005	1.150	1.165	1.147	1.175	1.126	0.965	1.012	1.004
CG	0.813	0.859	0.727[a]	0.795	0.808	0.752	0.850	0.836	0.872	0.793	0.818	0.775	0.710[a]
CT	1.029	1.048	1.235[a]	1.178	1.145	1.030	1.065	1.053	0.958	1.082	1.108	1.181	1.203
GA	1.109	1.063	1.158	1.072	1.055	1.115	1.056	1.130	1.087	1.161	1.150	1.066	1.070
GC	0.930	0.945	0.995	1.086	1.019	1.003	0.992	0.985	0.981	1.010	1.073	1.066	1.065
GG	1.045	1.171	1.038	1.192	1.204	1.105	1.207	1.075	1.108	1.040	0.979	1.181	1.179
GT	0.903	0.832	0.822	0.726[a]	0.772[a]	0.801	0.778[a]	0.824	0.837	0.807	0.819	0.754[a]	0.757[a]
TA	0.842	0.866	0.859	0.905	0.907	0.862	0.859	0.855	0.861	0.859	0.853	0.912	0.885
TC	0.853	0.832	0.911	0.846	0.862	0.812	0.831	0.808	0.825	0.830	0.883	0.857	0.857
TG	1.196	1.113	1.214[a]	1.095	1.103	1.178	1.114	1.173	1.107	1.175	1.166	1.107	1.147
TT	1.084	1.134	1.018	1.093	1.080	1.097	1.134	1.106	1.148	1.092	1.073	1.075	1.064

Note. Abbreviations for taxa are the same as in Fig. 2. Values indicate the ratio of observed to expected dinucleotide occurrences.
[a] Z score significant at $P < 0.05$.

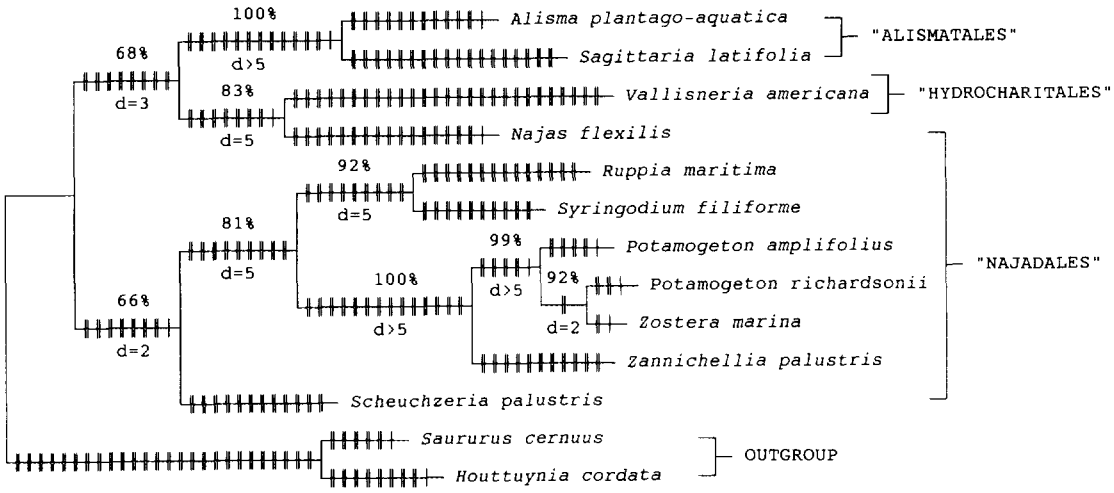

FIG. 3. Phylogenetic relationships among 13 aquatic species inferred from *rbc*L sequence data. The same tree topology was obtained from both parsimony and maximum likelihood analyses. All branch lengths were significantly positive in the maximum likelihood analysis. Substitutions indicated by parsimony analysis are represented by short vertical bars; percentages above ingroup branches provide estimates of confidence intervals from 500 bootstrap replications; numbers below branches indicate the "decay" index. Traditional concepts of alismatid orders (represented by brackets) are inconsistent with the position of Najadaceae (*Najas flexilis*) resulting from this analysis.

is a striking exception to the usual rarity of aquatic species in the angiosperms. Excluding the parasitic, terrestrial order Triuridales, whose inclusion in the group is doubtful (Tomlinson, 1982), the remaining 15 alismatid families and 56 genera all contain aquatics (Cook, 1990).

Commonplace reduction and convergent evolution among hydrophytes present systematists with difficult problems to consider in phylogenetic studies of aquatic angiosperms. Although the aquatic habitat itself is often perceived as "ecological" evidence of systematic relationships, recent studies have demonstrated the unrelatedness of several aquatic families once assumed to represent a single monophyletic lineage (Les *et al.*, 1991). Accordingly, it is difficult to assess the extent to which the prevalence of aquatic species represents a unifying feature of subclass Alismatidae (Tomlinson, 1982) as proposed by several authors (Cronquist, 1981; Dahlgren *et al.*, 1985).

Nevertheless, most systematic studies of the group and recent phylogenetic studies of monocots have indicated that the Alismatidae are probably monophyletic. However, problems associated with reduction, parallelism, and convergence have led to hypotheses of relationships within the subclass that are extremely tentative (Dahlgren and Rasmussen, 1983; Dahlgren *et al.*, 1985). Because this group represents the largest putatively natural group of aquatic plants, these problems come as no surprise. A cladogram of the Alismatidae produced by Dahlgren and Rasmussen (1983) represents, perhaps, the best comprehensive study of phylogenetic relationships for the Alismatidae, yet these authors emphasized the high probability of convergences within the data set used in their analysis.

Although the cladogram of Alismatidae by Dahlgren and Rasmussen (1985) was evaluated simply as ". . . one model expressing their possible relationships . . ." (Dahlgren *et al.*, 1985), the relationships depicted by the cladogram are congruent overall with most taxonomic classifications of the Alismatidae. In particular, the segregation of the Najadaceae and Hydrocharitaceae in different orders is a taxonomic consensus that represents more than a century of tradition (see summary in Shaffer-Fehre, 1991). Taxonomists have widely accepted the concept of the order Najadales which includes Najadaceae among many other

TABLE 5

Comparison of Tree Lengths, Consistency Indices, and Retention Indices (from Parsimony Analysis) and Significant Differences for Log-Likelihoods (from Maximum Likelihood Analysis) among Four "User Trees"

User tree	Length	Consistency index	Retention index	Log-likelihood value
1	481	0.707	0.677	−4298.15 (best)
2	483	0.704	0.673	−4305.51[a]
3	503	0.676	0.627	−4350.08[b]
4	580	0.586	0.457	−4639.42[b]

Note. Tree 1 represents topology of single most parsimonious tree and "best" obtainable maximum likelihood value; tree 2 is identical but forces monophyly of two *Potamogeton* species; tree 3 forces association of Najadaceae into "Najadales" clade; tree 4 forces entire topology to comply with the cladogram proposed by Dahlgren and Rasmussen (1983).

[a] Not significantly worse than best tree.

[b] Significantly worse ($P < 0.01$) than best tree.

submersed aquatic Alismatidae such as Potamogetonaceae, Zannichelliaceae, and Zosteraceae.

The confident acceptance of this classification for the Alismatidae, however, belies certain inconsistencies. Chrysler (1907) emphasized that the extent of morphological reduction in *Najas* had greatly obscured its relationships, and suggested that the genus may be quite isolated from other Potamogetonaceae. Dahlgren *et al.* (1985) remarked that "strongly supported alternatives" exist to their model of family relationships within the Najadales, and emphasized that features such as the basal anatropous ovule of Najadaceae are inconsistent with character states found in advanced members of the Najadales. Anomalous karyotypic evolutionary trends led Sharma and Chatterjee (1967) to conclude that the Najadaceae are a distinct lineage "... entirely independent of Aponogetonales and Potamogetonales." Several taxonomic concepts emphasizing the isolation of the Najadales (comprising only the family Najadaceae) have been suggested (Dahlgren, 1975; Thorne, 1983).

The close alliance of Najadaceae and Potamogetonaceae and their isolation from Hydrocharitaceae was emphatically contested more than 50 years ago by Miki (1937) who argued that details of floral structure clearly linked Najadaceae with Hydrocharitaceae in a position isolated from Potamogetonaceae (Fig. 1). Other evidence of a potential relationshp between Hydrocharitaceae and Najadaceae can be found in their shared traits of nonprecocious bud loss and consistent leaf number between consecutive bifurcations of the vegetative axis (Wilder, 1975). Miki's hypothesis has been revived most recently by anatomical evidence of shared specialized seed coat characters that occur in Najadaceae and Hydrocharitaceae but are not found in other Alismatidae (Shaffer-Fehre, 1991).

Our sample included all strictly aquatic freshwater alismatid families and thereby represented every group that can reasonably be considered to have a close relationship with the Najadaceae. We have yet been unable to procure material of the Australian seagrass family Posidoniaceae; however, this marine family is an unlikely ancestor to the freshwater Najadaceae.

The *rbc*L molecular phylogeny (Fig. 3) supports the relationship of Hydrocharitaceae and Najadaceae postulated by Miki (1937) and Shaffer-Fehre (1991). When the *rbc*L cladogram topology is "forced" to comply with relationships indicated by Dahlgren and Rasmussen's (1983) phylogenetic hypothesis (Fig. 1B), an additional 22 steps are added to the parsimony tree and the maximum likelihood result is significantly different (worse) (Table 5). Using *rbc*L data, efforts to place the Najadaceae into the clade containing the aquatic Najadales resulted in elongation of trees beyond a reasonable length (Table 5).

Although molecular characters such as *rbc*L sequence data are also subject to homoplasy, they are at least independent of adaptive homoplasies related to the aquatic environment. The *rbc*L data set analyzed here also appears to satisfy a number of assumptions pertinent to molecular phylogenetic data analysis. The pattern of substitution among codon positions is consistent with that found in another similar study (Table 2), an indication that substitution rates among codon positions are fairly constant. The average transition: transversion ratio approximated 2.1 for all pairwise comparisons and did not decrease inversely with divergence. This indicated that sites with transitions were not highly saturated and lowers the likelihood that the data represent numerous mutiple hits within sites. The maximum level of sequence divergence in this study was 10.8%, close to the optimal value suggested by Ritland and Eckenwalder (1990).

We detected only a few instances of significant dinucleotide bias in the *rbc*L sequences analyzed (Table 4). An underrepresentation of CG dinucleotides was characteristic of all sequences but was significant only for *Najas flexilis* and *Zannichellia palustris*. Being a methylation site, CG is also commonly underrepresented in many vertebrate sequences (Weir, 1990). An underrepresentation of GT occurred in five species (Table 4) and is also characteristic of vertebrate sequences (Weir, 1990). TG and CT display the highest overrepresentation in vertebrate sequences (Weir, 1990) and also showed high incidences of occurrence in the *rbc*L sequences. Significant deviations of these dinucleotides, however, were observed only in *Najas flexilis*. Overall, the low incidence of significant deviant dinucleotide occurrences indicated that dinucleotide bias did not contribute substantially to the overall pattern of substitution present in our data set.

Accordingly, we believe that the phylogenetic analysis of *rbc*L data is more likely to portray an accurate phylogenetic perspective of relationships in the Alismatidae than previous analyses that relied on the evaluation of highly adaptive morphological characters. Convergence of morphological characters undoubtedly contributed to the association of Najadaceae and Potamogetonaceae rendered by the Dahlgren and Rasmussen (1983) cladistic analysis. Twelve of the 56 characters used by Dahlgren and Rasmussen (1983) in their cladistic analysis have a high probability of convergence. Dahlgren and Rasmussen (1983) themselves suggest that eight of these characters are likely to represent convergences linked to adaptive aquatic traits. It is reasonable to expect that a more reliable analysis of alismatid relationships should be obtained upon exclusion of these characters.

A reanalysis of Dahlgren and Rasmussen's morphological data has been conducted following several modifications (Les and Haynes, unpublished). The revised matrix reflected the deletion of four characters for which homologies could not be reasonably assessed, the addition of three new anatomical characters, and

the correction or different interpretation of some state distributions for nine of the characters. The results of this modified analysis are more likely to be accurate than the original study because the probability of convergences is reduced.

A maximum parsimony analysis of the revised data yielded seven minimum length trees. The topology of one tree was essentially equivalent to that of the original Dahlgren and Rasmussen cladogram. In the six other trees, however, the Hydrocharitaceae and Najadaceae were paired as sister groups (Les and Haynes, unpublished). We interpret these results to indicate that morphological data provide a similar level of support for a close relationship of Najadaceae and Hydrocharitaceae as they do for an isolated position of Najadaceae among other families of Najadales. We emphasize that the majority of topologies represented by the modified analysis of morphological data were congruent with relationships suggested by the present *rbc*L analysis as well as character distributions emphasized by Miki (1937) and Shaffer-Fehre (1991).

The close association of *Zostera* and *Potamogeton richardsonii* in the *rbc*L cladogram is not entirely unexpected. An affinity between *Zostera* and *Potamogeton* has been recognized by most taxonomists as evidenced by the many classifications which place both genera within a single family (referred to as either Najadaceae, Potamogetonaceae, or Zosteraceae) or in different (but assumed closely related) families of these same names (Chrysler, 1907; Fernald, 1950; Gleason and Cronquist, 1963; Hartog, 1970; Muenscher, 1944; Voss, 1972).

More recent classifications (e.g., Cronquist, 1988; Takhtajan, 1980), however, have abandoned such a broad concept of Potamogetonaceae in favor of recognizing as many as seven distinct families (including Zosteraceae) derived from former taxonomic "subdivisions" of the Potamogetonaceae sensu lato. The high *rbc*L sequence similarity of *Zostera* and *Potamogeton* is somewhat surprising in light of the relatively high level of morphological divergence distinguishing these genera. Features contrasting in *Zostera* and *Potamogeton* include their sexual condition (unisexual vs bisexual flowers), habitat (freshwater vs marine), pollination (water vs wind), pollen shape (filiform vs globose), stamen and style number, and endosperm type (Cook, 1990; Dahlgren *et al.*, 1985). There is some question, however, concerning the actual number of stamens and styles in *Zostera* and even whether the flowers are truly unisexual (Dahlgren *et al.*, 1985).

Several traits linking Zosteraceae and Potamogetonaceae are also evident. Tepal-like "retinacula" (probably connective appendages) of some Zosteraceae are similar to floral appendages of *Potamogeton* and *Posidonia* (Dahlgren *et al.*, 1985). Spicate inflorescences characterize Posidoniaceae, Potamogetonaceae, and Zosteraceae (Dahlgren *et al.*, 1985). Chrysler (1907) considered it conceivable that the vascular anatomy of *Zostera* was derived from *Potamogeton*, although he remarked (p. 173) that "The process of evolution has apparently separated *Zostera* far from its relatives."

In light of all evidence, Dahlgren *et al.* (1985) placed the Zosteraceae closest to Potamogetonaceae and Posidoniaceae. Many years earlier, Chrysler (1907) had concluded that the relationship of *Zostera* was closer to *Potamogeton* than to other genera of Potamogetonaceae.

Although Cretaceous fossils ("*Archeozostera*") have been allied to modern zosteroids, there remains much uncertainty regarding the actual phylogenetic position of these fossils (Hartog, 1970). The *rbc*L data indicate that the extant genus *Zostera* is not at all ancient, but probably has evolved quite recently from an ancestor common to freshwater *Potamogeton*. If this is true, then the numerous morphological differences between *Zostera* and *Potamogeton* are indicative of rapid diversification. Although slow evolutionary rates often characterize hydrophilous species, high rates of diversification probably characterize their early evolutionary stages (Les, 1988). Similar patterns of accelerated morphological divergence and low molecular divergence have been reported for terrestrial species (e.g., *Heterogaura*) suspected of undergoing rapid adaptive radiation (Sytsma and Gottlieb, 1986). A rapid diversification of most monocot groups has been proposed by Stebbins (1974) and Takhtajan (1969).

The *rbc*L analysis justifies the independent recognition and taxonomic maintenance of the family Ruppiaceae. Although it has become conventional to merge the genus *Ruppia* within the family Potamogetonaceae (e.g., Dahlgren *et al.*, 1985; Tomlinson, 1982), the *rbc*L data do not support this merger but indicate that the Ruppiaceae are most closely allied to the Cymodoceaceae, a family of marine seagrasses. Dahlgren *et al.* (1985, p. 315) noted that features of Potamogetonaceae and *Ruppia* agreed well "In spite of some conspicuous diagnostic character states . . ." In opposition to the wind-pollinated freshwater Potamogetonaceae, both the Ruppiaceae and the Cymodoceaceae represent marine, water-pollinated plants with elongate pollen (Tomlinson, 1982).

Relationships of the family Zannichelliaceae are consistent in both the *rbc*L and morphological studies. The Zannichelliaceae occupy an isolated position at the base of a clade leading to Potamogetonaceae and Zosteraceae. The *rbc*L analysis does not support the concept of Miki (1937) and others who have suggested that the families Najadaceae and Zannichelliaceae are closely related.

Our molecular phylogenetic study of relationships in subclass Alismatidae provides evidence supporting the transfer of Najadaceae into the order Hydrocharitales as advocated recently by Shaffer-Fehre (1991). The *rbc*L data also justify the removal of *Ruppia* from the

Potamogetonaceae and the reestablishment of the family Ruppiaceae. Our results indicate the origin of Ruppiaceae from a common ancestor shared with the marine Cymodoceaceae. The Zannichelliaceae represent an isolated group related to Potamogetonaceae and Zosteraceae.

Even though alternate *rbc*L topologies that group *Potamogeton* species apart from *Zostera* are not significantly different (Table 5), the extremely close relationship of *Zostera* and *Potamogeton* evidenced by their *rbc*L similarity (only 0.7% divergence in an 1183-base pair sequence) supports classification schemes that merge these genera within a single family rather than those that segregate them as separate families. This disposition is particularly recommended given that the level of divergence between *Potamogeton* and *Zostera* is less than that observed between two *Potamogeton* species studied.

The results of these analyses indicate that further exploration of alismatid relationships using both molecular and morphological data should provide exciting new insights and greatly facilitate our efforts to improve the classification of this unusual aquatic subclass.

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REFERENCES

Arber, A. (1920). "Water Plants: A Study of Aquatic Angiosperms," University Press, Cambridge.

Chase, M. W., Soltis, D. E., Olmstead, R. G., Morgan, D., Les, D. H., Mishler, B. D., Duvall, M. R., Price, R. A., Hills, H. G., Qiu, Y. L., Kron, K. A., Rettig, J. H., Conti, E., Palmer, J. D., Manhart, J. R., Sytsma, K. J., Michaels, H. J., Kress, W. J., Karol, K. G., Clark, W. D., Hedrén, M., Gaut, B. S., Jansen, R. K., Kim, K., Wimpee, C. F., Smith, J. F., Furnier, G. R., Straus, S. H., Xiang, Q., Plunkett, G. M., Soltis, P. S., Swensen, S. M., Williams, S. E., Gadek, P. A., Quinn, C. J., Eguiarte, L. E., Golenberg, E., Learn, Jr., G. H., Graham, S. W., Barrett, S. C. H., Dayanandan, S., and Albert, V. A. (1993). Phylogenetics of seed plants: An analysis of nucleotide sequences from the plastid gene *rbc*L. *Ann. Missouri Bot. Gard.* **80:** 528–580.

Chrysler, M. A. (1907). The structure and relationships of the Potamogetonaceae and allied families. *Bot. Gaz.* **44:** 161–193.

Cook, C. D. K. (1990). "Aquatic Plant Book," SPB Academic Publishing, The Hague, The Netherlands.

Cox, P. A., and Knox, B. (1989). Two-dimensional pollination in hydrophilous plants: Convergent evolution in the genera *Halodule* (Cymodoceaceae), *Halophila* (Hydrocharitaceae), *Ruppia* (Ruppiaceae), and *Lepilaena* (Zannichelliaceae). *Am. J. Bot.* **76:** 164–175.

Cronquist, A. (1981). "An Integrated System of Classification of Flowering Plants," Columbia Univ. Press, New York.

Cronquist, A. (1988). "The Evolution and Classification of Flowering Plants," New York Botanical Garden, Bronx.

Dahlgren, R. (1975). A system of classification of the angiosperms to be used to demonstrate the distribution of characters. *Bot. Not.* **128:** 119–147.

Dahlgren, R., and Rasmussen, F. (1983). Monocotyledon evolution: Characters and phylogenetic estimation. *Evol. Biol.* **16:** 255–395.

Dahlgren, R., Clifford, H. T., and Yeo, P. F. (1985). "The Families of Monocotyledons. Structure, Evolution, and Taxonomy," Springer-Verlag, Berlin.

Doyle, J. J., and Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19:** 11–15.

Felsenstein, J. (1993). "PHYLIP Manual Version 3.5." University Herbarium, University of California, Berkeley, CA.

Fernald, M. L. (1950). "Gray's Manual of Botany," 8th ed., Van Nostrand, New York, NY.

Gleason, H. A., and Cronquist, A. (1963). "Manual of Vascular Plants of Northeastern United States and Adjacent Canada," Van Nostrand, New York, NY.

Hartog, C. Den. (1970). "The Sea-Grasses of the World." North-Holland Publishing Company, Amsterdam, The Netherlands.

Les, D. H. (1988). Breeding systems, population structure, and evolution in hydrophilous angiosperms. *Ann. Missouri Bot. Gard.* **75:** 819–835.

Les, D. H., Garvin, D. K., and Wimpee, C. F. (1991). Molecular evolutionary history of ancient aquatic angiosperms. *Proc. Natl. Acad. Sci. USA* **88:** 10119–10123.

Les, D. H., and Philbrick, C. T. (1993). Studies of hybridization and chromosome number variation in aquatic angiosperms: Evolutionary implications. *Aquat. Bot.* **44:** 181–228.

Les, D. H., and Sheridan, D. J. (1990). Biochemical heterophylly and flavonoid evolution in North American *Potamogeton* (Potamogetonaceae). *Am. J. Bot.* **77:** 453–465.

Miki, S. (1937). The origin of *Najas* and *Potamogeton*. *Bot. Mag. (Tokyo)* **51:** 472–480.

Muenscher, W. C. (1944). "Aquatic Plants of the United States," Cornell University Press, Ithaca, NY.

Qiu, Y., Chase, M. W., Les, D. H., and Parks, C. R. (1993). Molecular phylogenetics of the Magnoliidae: Cladistic analysis of nucleotide sequences of the plastid gene *rbc*L. *Ann. Missouri Bot. Gard.* **80:** 587–606.

Ritland, K., and Eckenwalder, J. E. (1992). Polymorphism, hybridization, and variable evolutionary rate in molecular phylogenies. *In* "Molecular Systematics of Plants" (P. S. Soltis and D. E. Soltis, Eds.), pp. 404–428, Chapman and Hall, New York.

Saiki, R. K., Gelfund, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487–491.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463–5467.

Sculthorpe, C. D. (1967). "The biology of Aquatic Vascular Plants," Edward Arnold, London.

Shaffer-Fehre, M. (1991). The position of *Najas* within the subclass Alismatidae (Monocotyledones) in the light of new evidence from seed coat structures in the Hydrocharitoideae (Hydrocharitales). *Bot. J. Linn. Soc.* **107:** 189–209.

Sharma, A. K., and Chatterjee, T. (1967). Cytotaxonomy of Helobiae with special reference to the mode of evolution. *Cytologia* **32:** 286–307.

Stebbins, G. L. (1974). "Flowering Plants. Evolution above the Species Level," Belknap Press of Harvard, Cambridge, MA.

Swofford, D. L. (1993). "PAUP: Phylogenetic Analysis Using Parsimony," Version 3.1. Computer program distributed by the Illinois Natural History Survery, Champaign, IL.

Sytsma, K. J., and Gottlieb, L. D. (1986). Chloroplast DNA evolution and phylogenetic relationships in *Clarkia* sect. *Peripetasma* (Onagraceae). *Evolution* **40:** 1248–1261.

Takhtajan, A. (1969). "Flowering Plants. Origin and Dispersal," Oliver and Boyd, Edinburgh.

Takhtajan, A. (1980). Outline of the classification of flowering plants. *Bot. Rev.* **46:** 225–359.

Thorne, R. F. (1983). Proposed new realignments in the angiosperms. *Nord. J. Bot.* **3:** 85–117.

Tomlinson, P. B. (1982). VII. Helobiae (Alismatidae). *In* "Anatomy of the Monocotyledons" (C. R. Metcalfe, Ed.), 559 pp., Clarendon Press, Oxford.

Voss, E. G. (1972). "Michigan Flora. Part 1: Gymnosperms and Monocots." Cranbrook Institute of Science, Cranbrook, MI.

Watrous, L., and Wheeler, Q. (1981). The out-group comparison method of character analysis. *Syst. Zool.* **30:** 1–11.

Weir, B. S. (1990). "Genetic Data Analysis: Methods for Discrete Population Genetic Data." Sinauer Associates, Sunderland, MA.

Wilder, G. J. (1975). Phylogenetic trends in the Alismatidae (Monocotyledoneae). *Bot. Gaz.* **136:** 159–170.