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Phylogenetic Studies in Alismatidae, II: Evolution of Marine Angiosperms (Seagrasses) and Hydrophily

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ABSTRACT. Aquatic species represent fewer than two percent of all flowering plants, and only 18 aquatic genera have acquired true hydrophily (water-pollination) which is associated with an unusually high incidence of unisexual flowers. From the subset of submersed, hydrophilous angiosperms, only 13 genera have colonized marine habitats. The evolution of hydrophily, unisexuality, and marine habit in angiosperms was explored using estimates of phylogeny obtained by phylogenetic analyses of chloroplast (*rbcl*) gene sequence data. Despite what might appear to be difficult evolutionary transitions, hydrophiles are highly polyphyletic with independent origins in the monocotyledon subclass Alismatidae in addition to two derivations in the dicotyledon families Ceratophyllaceae and Callitrichaceae. Yet, even in alismatids, hydrophily has evolved many times. Unisexuality has also evolved repeatedly in the Alismatidae, and is ancestral to the evolution of hydrophiles and marine plants in the Hydrocharitaceae. Marine angiosperms (known only from Alismatidae) have evolved in three separate lineages. The multiple origins of hydrophilous, marine plants offer an extraordinary example of convergent evolution in angiosperms.

Angiosperms are an important component of saltwater ecosystems (Dawson 1966; McRoy and Helfferich 1980; Rasmussen 1977; Kikuchi 1980). Although the ecology of marine flowering plants (seagrasses) has been studied extensively, their systematic relationships have not. The scarcity of evolutionary studies of seagrasses is due in part to their few extant species and past neglect by collectors (Hartog 1970, 1980). Seagrasses are often viewed as relatively unimportant in the general picture of angiosperm evolution. This impression underestimates the colonization of marine habitats by angiosperms, a remarkable achievement for a group whose evolutionary history essentially reflects adaptation to terrestrial conditions. A better understanding of seagrass evolution may provide valuable insight into understanding adaptive evolution in flowering plants.

Aquatic angiosperms are relatively uncommon and occur only in approximately 17% of the families, 1.5% of the genera and represent fewer than two percent of all flowering plant species (Cook 1990; Les and Philbrick 1993; Les and Schneider 1995). Despite their rarity, present estimates propose as many as 100 independent origins of aquatic angiosperms (Cook 1990). Fewer than 130 aquatic angiosperm species are hydrophilous (water-pollinated) and only about 60 species are

marine (Cook 1990; Kuo and McComb 1989; Les 1988; Philbrick 1991). Water-pollinated angiosperms (hydrophiles) are concentrated in the subclass Alismatidae (Table 1) with only a few species found elsewhere in the Ceratophyllaceae (Les 1988) and Callitrichaceae (Philbrick 1993). Marine angiosperms are confined taxonomically to five monocotyledon families (Cook 1990) within the single subclass Alismatidae, a group of approximately 450 aquatic and helophytic (wetland) species (Table 1). Because hydrophiles represent only 0.04% and seagrasses only 0.02% of an estimated 300,000 angiosperm species, both water pollination and colonization of marine habitats must represent difficult evolutionary transitions for flowering plants. Furthermore, these evolutionary events are apparently correlated given that all but one marine angiosperm species (*Enhalus acoroides*) are hydrophilous.

Hartog's (1970) phylogenetic hypotheses are the basis of most contemporary discussions of seagrass evolution. Although subsequent reviews of seagrasses have been made (e.g., Hartog 1980; Phillips and Meñez 1988; Pollard et al. 1993) the coverage of specific evolutionary questions has been minimal with a primary focus on biogeography (e.g. Brasier 1975). More specific information on the origin and phylogeny of seagrasses could enhance the "basic

TABLE 1. Families and genera of subclass Alismatidae with indications of: I. habitat (B = brackish; F = freshwater; M = marine); II. floral condition (H = hermaphroditic; U = unisexual); III. pollination (A = anemophilous; AUT = autogamous; B = “B” type of Cook (1981); E = entomophilous; H = hydrophilous). Estimated number of species in parentheses. Species surveyed in present study and voucher data are indicated in brackets. Adapted from Cook (1981; 1990), Cronquist (1981), Tomlinson (1982).

	I	II	III
A. Alismataceae Vent.			
1. <i>Alisma</i> L. (9)	F	H	E
[<i>A. plantago-aquatica</i> L., Les s.n. (CONN)]			
2. <i>Baldellia</i> Parl. (2)	F	H	E
[<i>B. ranunculoides</i> (L.) Parlatore, Charlton s.n. (MANCH)]			
3. <i>Burnatia</i> Micheli (1)	F	U	E
4. <i>Caldesia</i> Parl. (4)	F	H	E
5. <i>Damasonium</i> Mill. (4)	F	H	E
[<i>D. alisma</i> Mill., Charlton s.n. (MANCH)]			
6. <i>Echinodorus</i> Rich. ex Engelm. (45)	F	H	E
[<i>E. grandiflorus</i> (Cham. & Schltdl.) Micheli, Les s.n. (CONN)]			
7. <i>Limnophyton</i> Miq. (4)	F	H, U	E
8. <i>Luronium</i> Raf. (1)	F	H	E
[<i>L. natans</i> (L.) Raf., Charlton s.n. (MANCH)]			
9. <i>Ranalisma</i> Stapf (2)	F	H	E
[<i>R. humile</i> (Kuntze) Hutch., Charlton s.n. (MANCH)]			
10. <i>Sagittaria</i> L. (20)	F	U	E
[<i>S. latifolia</i> Willd., Les s.n. (CONN); <i>S. graminea</i> Michx., Chase 210 (NCU)]			
11. <i>Wiesneria</i> Micheli (3)	F	U	E
[<i>W. triandra</i> Micheli, Cook s.n. (Z)]			
B. Aponogetonaceae J. Agardh			
1. <i>Aponogeton</i> L.f. (43)	E, B	H, U	E
[<i>A. elongatus</i> F. Muell. ex Benth., Les s.n. (CONN); <i>A. distachyos</i> L.f., Les s.n. (CONN)]			
C. Butomaceae Rich.			
1. <i>Butomus</i> L. (1)	F	H	E
[<i>B. umbellatus</i> L., Les 499 (CONN)]			
D. Cymodoceaceae N. Taylor			
1. <i>Amphibolis</i> C. Agardh (2)	M	U	H
[<i>A. antarctica</i> (Labill.) Asch., Waycott 94003 (UWA)]			
2. <i>Cymodocea</i> K. Koenig (4)	M	U	H
[<i>C. serrulata</i> (R. Br.) Asch. & Magnus, O'Donohue 21395 (BRN); <i>C. nodosa</i> (Ucria) Aschers., Procaccini s.n. (CONN)]			
3. <i>Halodule</i> Endl. (6)	M	U	H
[<i>H. beaudettei</i> (Hartog) Hartog, Wimpee s.n. (CONN); <i>H. pinifolia</i> (Miki) Hartog, Walker 1611941 (UWA)]			
4. <i>Syringodium</i> Kütz. (2)	M	U	H
[<i>S. filiformis</i> Kütz., Wimpee s.n. (CONN); <i>S. isoetifolium</i> (Asch.) Dandy, Waycott 94008 (UWA)]			
5. <i>Thalassodendron</i> Hartog (2)	M	U	H
[<i>T. pachyrhizum</i> Hartog, Waycott 95002 (UWA)]			
E. Hydrocharitaceae Juss. (incl. Najadaceae Juss.)			
1. <i>Apalanthe</i> Planch. (1)	F	H	E
[<i>A. granatensis</i> (Humb. & Bonpl.) C. D. K. Cook & Urmi-König, Cook s.n. (Z)]			
2. <i>Appertiella</i> C. D. K. Cook & Triest (1)	F	U	B
3. <i>Blyxa</i> Noronha ex Thouars (9)	F	H, U	E
[<i>B. aubertii</i> L. C. Richard, Charlton s.n. (MANCH)]			
4. <i>Egeria</i> Planch. (2)	F	U	E
[<i>E. densa</i> Planch., Les s.n. (CONN)]			
5. <i>Elodea</i> Michx. (5)	F	U	H
[<i>E. nuttallii</i> (Planch.) H. St. John, Les s.n. (CONN)]			
6. <i>Enhalus</i> Rich. (1)	M	U	B
[<i>E. acoroides</i> (L.f.) Rich. ex Steud., Walker 1611942 (UWA)]			

TABLE 1. Continued.

	I	II	III
E. Hydrocharitaceae Juss. (incl. Najadaceae Juss.) Continued			
7. <i>Halophila</i> Thouars (10) [<i>H. decipiens</i> Ostenf., Walker 1811941 (UWA); <i>H. engelmannii</i> Asch., Wimpee s.n. (CONN)]	M	U	H
8. <i>Hydrilla</i> Rich. (1) [<i>H. verticillata</i> (L.f.) Casp., Cook s.n. (Z)]	F	U	A
9. <i>Hydrocharis</i> L. (3) [<i>H. morsus-ranae</i> L., Les & Waycott s.n. (CONN)]	F	U	E
10. <i>Lagarosiphon</i> Harv. (9) [<i>L. muscoides</i> Harv., Cook s.n. (Z); <i>L. major</i> (Ridley) Moss, Cook s.n. (Z)]	F	U	B
11. <i>Limnobia</i> Rich. (2) [<i>L. spongia</i> (Bosc.) Steud., Cook s.n. (Z)]	F	U	A, E
12. <i>Maidenia</i> Rendle (1)	F	U	B
13. <i>Najas</i> L. (45) [<i>N. flexilis</i> (Willd.) Rostk. & W. L. E. Schmidt, Les s.n. (CONN); <i>N. marina</i> L., Wakeman s.n. (CONN)]	F	U	H
14. <i>Nechamandra</i> Planch. (1) [<i>N. alternifolia</i> (Roxburgh ex Wight) Thwaites, Cook s.n. (Z)]	F	U	B
15. <i>Ottelia</i> Pers. (21) [<i>O. alismoides</i> (L.) Pers., Bogner s.n. (M); <i>O. ulvifolia</i> Walp., Bogner s.n. (M)]	F	H, U	E
16. <i>Stratiotes</i> L. (1) [<i>S. aloides</i> L. Les s.n. (CONN)]	F	U	E
17. <i>Thalassia</i> K. Koenig (2) [<i>T. hemprichii</i> (Ehrenb.) Asch., Walker 1611943 (UWA); <i>T. testudinum</i> Banks ex K. Koenig, Wimpee s.n. (CONN)]	M	U	H
18. <i>Vallisneria</i> L. (4) [<i>V. americana</i> Michx., Les s.n. (CONN); <i>V. spiralis</i> L., Cook s.n. (Z)]	F	U	B
F. Juncaginaceae Rich.			
1. <i>Cyanogeton</i> Endl. (1) [<i>C. procerum</i> Buchenau, Beesley 449 (CBG)]	F	H	A
2. <i>Maundia</i> F. Muell. (1)	F	H	A
3. <i>Tetroncium</i> Willd. (1)	F	U	A
4. <i>Triglochin</i> L. (15) [<i>T. maritimum</i> L., Les s.n. (CONN)]	F, B	H	A
G. Lilaeaceae Dumort.			
1. <i>Lilaea</i> Humb. & Bonpl. (1) [<i>L. scilloides</i> (Poir.) Hauman., Philbrick 3031 (WCSU)]	F	H, U	A?
H. Limnocharitaceae Takht. ex Cronquist			
1. <i>Butomopsis</i> Kunth (1)	F	H	E
2. <i>Hydrocleys</i> Rich. (5) [<i>H. nymphoides</i> (Willd.) Buchenau, Les s.n. (CONN)]	F	H	E
3. <i>Limnocharis</i> Humb. & Bonpl. (2) [<i>L. flava</i> (L.) Buchenau, Les s.n. (CONN)]	F	H	E
I. Posidoniaceae Lotsy			
1. <i>Posidonia</i> K. Koenig (6) [<i>P. australis</i> Hook.f., Gadek s.n. (CONN); <i>P. oceanica</i> (L.) Delile, Waycott 94021 (UWA)]	M	H	H
J. Potamogetonaceae Dumort.			
1. <i>Groenlandia</i> J. Gay (1) [<i>G. densa</i> (L.) Fourr., Philbrick 4585 (WCSU)]	F	H	AUT
2. <i>Potamogeton</i> L. (100) [<i>P. amplifolius</i> Tuck., Les 483 (CONN); <i>P. confervoides</i> Rchb., Philbrick 4633 (WCSU), <i>P. crispus</i> L., Les s.n. (CONN); <i>P. gramineus</i> L., Philbrick 4608 (WCSU); <i>P. perfoliatus</i> L., Les 505 (CONN); <i>P. richardsonii</i> (A. Benn.) Rydb., Les s.n. (CONN); <i>P. robbinsii</i> Oakes, Les 504 (CONN); <i>P. zosteriformis</i> Fernald, Les 494 (CONN)]	F, B	H	A

TABLE 1. Continued.

	I	II	III
J. Potamogetonaceae Dumort. Continued			
3. <i>Coleogeton</i> Les and Haynes (3) [<i>C. pectinatus</i> (L.) Les and Haynes, Philbrick 4609 (WCSU)]	F, B	H	AUT
K. Ruppiaceae (Kunth) Hutch.			
1. <i>Ruppia</i> L. (10) [<i>R. maritima</i> L., Koch s.n. (CONN); <i>R. megacarpa</i> R. Mason, Carruthers 95034 (UWA)]	M	H	H
L. Scheuchzeriaceae F. Rudolphi			
1. <i>Scheuchzeria</i> L. (1) [<i>S. palustris</i> L., Les s.n. (CONN)]	F	H	A
M. Zannichelliaceae Dumort.			
1. <i>Althenia</i> F. Petit. (1)	B	U	H
2. <i>Lepilaena</i> J. L. Drumm ex Harv. (5) [<i>L. australis</i> J. L. Drumm ex Harv., Waycott 950110 (UWA)]	F, B	U	H
3. <i>Pseudalthenia</i> (Graebn.) Nakai (1)	F	U	H
4. <i>Zannichellia</i> L. (6) [<i>Z. palustris</i> L., Haynes s.n. (UNA)]	F, B	U	H
N. Zosteraceae Dumort.			
1. <i>Heterozostera</i> (Setch.) Hartog (1) [<i>H. tasmanica</i> (Martens ex Asch.) Hartog, Waycott 94007 (UWA)]	M	U	H
2. <i>Phyllospadix</i> Hook. (5) [<i>P. torreyi</i> S. Watson, Philbrick 2274 (WCSU)]	M	U	H
3. <i>Zostera</i> L. (12) [<i>Z. marina</i> L., Yarish s.n. (CONN); <i>Z. mucronata</i> Hartog, Waycott 94018 (UWA); <i>Z. noltii</i> Hornemann, Procaccini s.n. (CONN)]	M	U	H

understanding” of their communities (Hartog 1980), yet these issues remain inadequately investigated. In a review of seagrass taxonomy, Kuo and McComb (1989) concluded that even though seagrass genera and families have been clearly circumscribed, their classification and evolutionary relationships at higher levels remain “obscure.” A major obstacle has been the widespread occurrence of ‘extra-simple’ (i.e., highly reduced) floral organization (Poluszny and Charlton 1993) in seagrasses, which has complicated efforts to evaluate their systematic relationships.

The distribution of unisexual reproductive systems (dicliny) is another issue relevant to the evolution of hydrophiles and marine angiosperms. More than 90% of hydrophilous species possess unisexual flowers; dioecy and monoecy occur respectively in approximately 72% and 44% of hydrophile genera (Les 1988). The preponderance of unisexual flowers in hydrophiles has suggested to some authors that water pollination is closely associated with outcrossing. However, recent appraisals of this question have challenged that interpretation (Les 1988; Waycott and Les 1996).

Sculthorpe (1967) observed parallel trends of hermaphroditism to unisexuality, anemophily to hydrophily, and freshwater to marine colonization

in Hydrocharitaceae. His conclusion that hydrophily evolved from anemophilous (wind-pollinated) ancestors raises the possibility that hydrophiles may be unisexual as a consequence of their diclinous, anemophilous ancestors (Les 1988). However, Philbrick (1988) hypothesized that hydrophily may have preceded the acquisition of unisexual flowers. The resolution of such debates must ultimately confront the question of whether unisexuality has preceded or succeeded the evolution of hydrophily. A satisfactory resolution of this issue will occur only when phylogenetic relationships among hydrophilous taxa and their ancestors are clarified (Barrett et al. 1993). Philbrick (1991) and Barrett et al. (1993) both emphasized that previous interpretations of evolutionary patterns for unisexuality, hydrophily and the marine habit have been complicated by the lack of reliable phylogenies.

Historically, taxonomists have recognized two or three basic groups of seagrasses. Most traditional classifications have placed the marine genera *Enhalus*, *Halophila* and *Thalassia* within the otherwise freshwater family Hydrocharitaceae, and have grouped the remainder of seagrass genera (together with a variety of freshwater genera) within or among s.l. family concepts such as the ‘Najadaceae’ of Bentham and Hooker (1880), the ‘Potamogetona-

ceae' of Engler (1904) or the 'Zosteraceae' of Lindley (1853). Details of interrelationships among these families have been specified only ambiguously. Balfour (1879) suggested that all marine angiosperms were closely related with *Halophila* 'linking' marine Hydrocharitaceae to other seagrasses. Arber (1920) believed that marine angiosperms showed a "strong affinity," but not necessarily that they were derived from a common ancestor. Arber (1920) theorized that marine angiosperms were uncommon because it was difficult to acquire specific prerequisites to marine life: a submersed habit, tolerance to salinity, hydrophily and an effective anchorage system. Arber regarded seagrasses as a "biological group" derived from freshwater ancestors that possessed what she believed to be the necessary requirements for life in the sea.

The rarity of seagrasses, their taxonomic confinement to a single subclass, and the many specialized attributes necessary for survival in marine habitats could circumstantially suggest their monophyly. However, polyphyletic origins of seagrasses have been indicated since early classifications and continue to be proposed (e.g. Hartog 1970; Tomlinson 1982). The polyphyly of families containing marine angiosperms has also been endorsed by phylogenetic approaches using morphological data (Dahlgren et al. 1985). Polyphyletic origins of seagrasses, at least with respect to marine Hydrocharitaceae and other seagrasses, have been indicated by molecular phylogenetic analyses at the family level (Les et al. 1993; Les and Haynes 1995). Although phylogenetic studies of the Alismatidae (including seagrasses) have been made by Dahlgren et al. (1985) and Les and Haynes (1995), neither study specifically evaluated seagrass relationships but focused on family rather than generic level comparisons to address broader relationships. Tomlinson (1982) included generic comparisons, but did not conduct a formal phylogenetic analysis. Cox and Humphries (1993) conducted a cladistic analysis of seagrass genera in the families Cymodoceaceae, Posidoniaceae, and Zosteraceae, but excluded critical taxa essential for a meaningful interpretation of relationships. Larkum and Hartog (1989) reconsidered the phylogeny of seagrass genera, but did not provide a character based analysis. Thus, the extent of polyphyly among marine genera remains to be determined.

Clarification of seagrass phylogeny is necessary to address related issues such as the evolution of hydrophily. Because hydrophily is considered to be

a prerequisite to marine angiospermous life (Arber 1920; Hartog 1970), it is important to evaluate evolutionary relationships between hydrophilous and marine species, particularly with reference to the suggested polyphyletic origin of both groups (Tomlinson 1982; Philbrick 1991).

Existing phylogenetic hypotheses for most water plants are tentative. Molecular studies have clarified relationships of some aquatic angiosperms where morphological reduction and convergent aquatic adaptations have complicated efforts to elucidate their affinities (e.g., Chase et al. 1993; Les et al. 1991, 1993; Les 1994; Les and Haynes 1995; Philbrick and Jansen 1991).

This study specifically explores evolutionary relationships among marine and hydrophilous angiosperms. We have expanded the scope of previous studies by presenting a phylogenetic analysis of chloroplast *rbcl* gene sequences for 69 species that represent all currently recognized families and 83% of the genera in subclass Alismatidae. Our analysis includes species from every known genus of marine angiosperms, and all but two of the hydrophilous genera. A comprehensive molecular phylogeny of Alismatidae should not only facilitate efforts to improve the classification of the subclass, but serve as a framework on which to evaluate character evolution with respect to the derivation of unisexuality, hydrophily and marine habits in the group.

MATERIALS AND METHODS

The phylogenetic analysis of Chase et al. (1993) was used to examine the distribution of selected aquatic features among angiosperms. Several sources (Arber 1920; Cook 1990; Cronquist 1981; Dahlgren and Clifford 1982; Duncan 1974; Eleuterius 1981; Les 1988; Philbrick 1991, 1993) were used to identify occurrences of aquatic, salt-tolerant, hydrophilous, and marine species within 16 major lineages depicted by the *rbcl* cladogram.

We produced *rbcl* sequences for 57 alismatid species (Table 1) to supplement 12 alismatid sequences reported previously (GenBank accessions: LO8759, LO8765, LO8767, UO3725-UO3731; Chase et al. 1993; Les et al. 1993). We re-sequenced material of *Zostera marina* given that the sequence (UO3724) reported earlier by Les et al. (1993) was incorrect, presumably an artifact of DNA contamination. We avoided similar errors by obtaining corroborating sequences (e.g. at least two species from each family) whenever possible. Our final

TABLE 2. Effect of topological constraints on tree elongation (number of additional steps from the minimal length solution obtained using a heuristic search algorithm). Constraints forced each set of groups to be monophyletic (numbers in parentheses identify each analysis as described in the methods). N/A = not applicable; CI = consistency index with value in exclusion of uninformative sites (exc) indicated in parentheses; RI = retention index.

Constrained groups (analysis #)	Original topology	Tree length	Elongation	CI (exc) %	RI%
None (unconstrained)	N/A	1545	N/A	39 (34)	76
All hydrophilous genera (2)	polyphyletic	1676	+131	36 (32)	73
Cox & Humphries topology (11)	polyphyletic	1626	+81	37 (33)	74
All marine genera (1)	polyphyletic	1585	+40	38 (34)	75
Ruppiaceae/Potamogetonaceae (5)	polyphyletic	1576	+31	38 (34)	75
Tillich's "Hydrocharitales" (10)	polyphyletic	1568	+23	38 (34)	75
Zosteraceae/Cymodoceaceae					
"complex" (3)	polyphyletic	1556	+11	39 (34)	76
Freshwater Hydrocharitaceae (6)	paraphyletic	1551	+6	39 (34)	76
<i>Zostera</i> (7)	paraphyletic	1550	+5	39 (34)	76
Hydrocharitaceae (8)	paraphyletic	1549	+4	39 (34)	76
Potamogetonaceae (4)	paraphyletic	1546	+1	39 (34)	76

data set consisted of 69 alismatid species (approximately 15% of the subclass) that represented all recognized families, 83% (47/57) of the genera, 27 species representing 89% (16/18) of the hydrophilous genera of subclass Alismatidae, and 22 species from all 13 genera of marine angiosperms. We included 10 *rbcL* sequences reported previously from genera of Acoraceae (*Acorus* L.), Araceae (*Anchomanes* Schott., *Ariopsis* J. Grah., *Gymnostachys* R. Br., *Lasia* Lour., *Montrichardia* Crueg., *Pistia* L., *Symplocarpus* Salisb., *Xanthosma* Schott.), and Lemnaceae (*Lemna* L.); (GenBank accessions: M91625, L10254, L10255, M91629, L10250, L10248, M96963, L10247, L10246, M91630) for use as outgroups.

Our procedures for DNA extraction, isolation, purification, amplification and sequencing (by direct, double-stranded methods) are described in Les et al. (1993). Due to difficulty in obtaining complete sequences for the entire coding region of *rbcL* for all taxa (see Les et al. 1993), we restricted our analysis to 1,179 nucleotides of sequence data (positions 29–1,207 of the coding region) that were complete for all taxa.

Phylogenetic analysis of the complete data set of 79 sequences was conducted using the test version of PAUP* 4.0.0d51/d52 (Swofford 1993; use of test versions by permission of D. L. Swofford) and employed COLLAPSE, MULPARS, STEEPEST DESCENT and TBR branch-swapping options. Step matrices were used to differentially weight transversions over transitions and to partition weights among codon positions. Transitions were weighted equally (1:1) at first and third positions, but were weighted higher (2:1) at second positions. Transver-

sions were assigned higher weights than transitions at all positions at a ratio of 4:3:2 (relative to transition weights) for positions 2, 1 and 3 respectively. The alismatid cladogram was rooted using the taxa from the Acoraceae, Araceae, and Lemnaceae, a group shown previously as the appropriate outgroup (Les and Schneider 1995). We searched for multiple islands of minimal length trees (Maddison 1991) using random starting points for taxon addition in 50 replicate analyses (MULPARS on; STEEPEST DESCENT on; TBR branch-swapping). Bootstrap support for the monophyly of indicated clades was obtained from 400 replicates (MULPARS off; STEEPEST DESCENT off) using PAUP.

Topological constraints (Table 2) were performed to evaluate the degree of tree elongation as taxa were forced to comply with several alternative taxonomic schemes. Trees were evaluated where 1) all marine taxa were monophyletic; 2) all hydrophiles were monophyletic; 3) two marine clades (Zosteraceae and Cymodoceaceae "complex"—see Discussion) were monophyletic; 4) the Potamogetonaceae were monophyletic; 5) Ruppiaceae and Potamogetonaceae were sister groups; 6) marine and freshwater Hydrocharitaceae were each monophyletic; 7) the genus *Zostera* was monophyletic; 8) the family Hydrocharitaceae was monophyletic, and 9) the family Alismataceae was monophyletic. We also constrained the topology to conform to 10) the "Hydrocharitales" proposed by Tillich (1995) and 11) the phylogenetic scheme proposed by Cox and Humphries (1993).

Floral condition (unisexual or hermaphroditic),

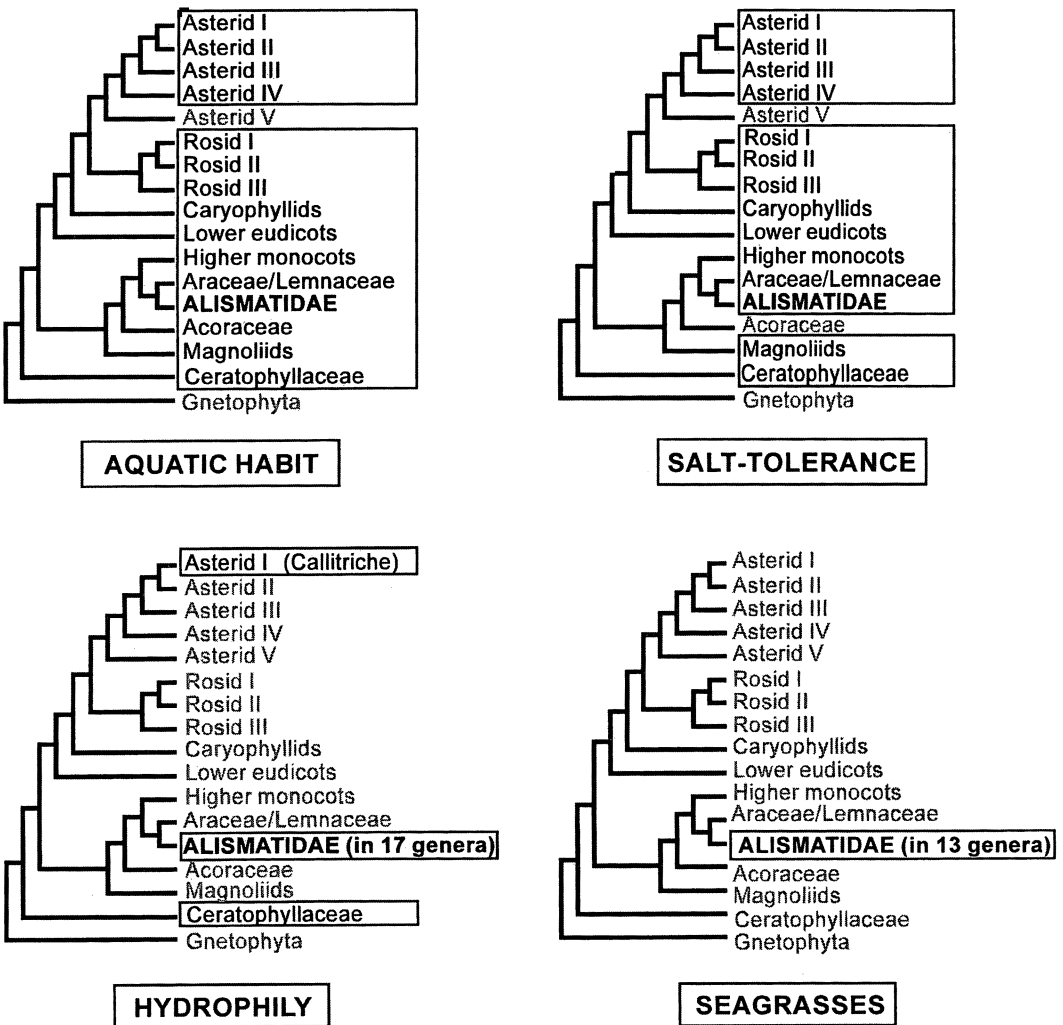


FIG. 1. Phylogenetic distribution of aquatic, salt-tolerant, hydrophilous, and marine angiosperms (in boxes) superimposed on cladogram of major angiosperm groups derived from *rbcL* sequence data (simplified from Chase et al., 1993). Hydrophilous angiosperms arose in three unrelated angiosperm clades; twice in the dicotyledons and again in the monocotyledons. Marine species have evolved only within the monocotyledonous subclass Alismatidae.

pollination system (anemophily, entomophily, hydrophily) and habitat (freshwater or marine) were scored for all taxa (Table 1) and added to the data matrix of molecular characters. These characters were excluded from all phylogenetic analyses, but their distribution on the molecular cladogram was mapped a posteriori using the ACCTRAN (which favors reversals over parallelisms) and DELTRAN (which favors parallelisms over reversals) optimization options of PAUP. Both optimizations were used to evaluate the distribution of marine taxa and

floral condition; however, the evaluation of hydrophily was limited to DELTRAN because it is unlikely that hydrophilous pollination systems revert back to terrestrial types (see Results).

RESULTS

Aquatic species occur in 94% (15/16) of the major lineages identified previously in comparative *rbcL* sequence studies (Fig. 1). Salt-tolerance (but not necessarily tolerance to immersion in saltwater) is

reported in species from 14 of these lineages (88%), but hydrophily from only three (19%) (Fig. 1). The subclass Alismatidae, which contains all seagrasses, is one of only three lineages where all three conditions occur (Fig. 1).

Of the 1,179 *rbcL* nucleotide positions compared among the alismatid taxa and outgroups, 518 sites (44%) were variable and 315 (27%) provided parsimony-informative characters. Parsimony analysis of the data yielded 4,295 equally minimal length trees of 1,545 steps with a consistency index (CI) of 39% (34% excluding uninformative characters) and a retention index (RI) of 76% (Table 2). The use of stepmatrices to account for codon position and transition bias yielded 32 equally minimal length trees of 2,329 steps. Only one island of the same minimal length trees found in the heuristic search was recovered using random sequence additions. The strict consensus of 4,295 unweighted trees differed from the strict consensus of 32 trees obtained with stepmatrix weighting only by reduced resolution at some nodes. Only the trees obtained using the stepmatrix weighting are considered further.

The strict consensus of these trees (Fig. 2) indicates two major lineages in the Alismatidae. One of these clades contains five families arranged in two subclades consisting of 1) Alismataceae and Limncharitaceae, and 2) Butomaceae, Hydrocharitaceae and Najadaceae. The other clade includes 10 families where 1) Scheuchzeriaceae and Aponogetonaceae are basal; 2) a subclade occurs with the families Lilaeaceae and Juncaginaceae; 3) a subclade occurs with the families Cymodoceaceae, Posidoniaceae, and Ruppiaceae, and 4) a subclade comprises Zosteraceae, Zannichelliaceae and Potamogetonaceae.

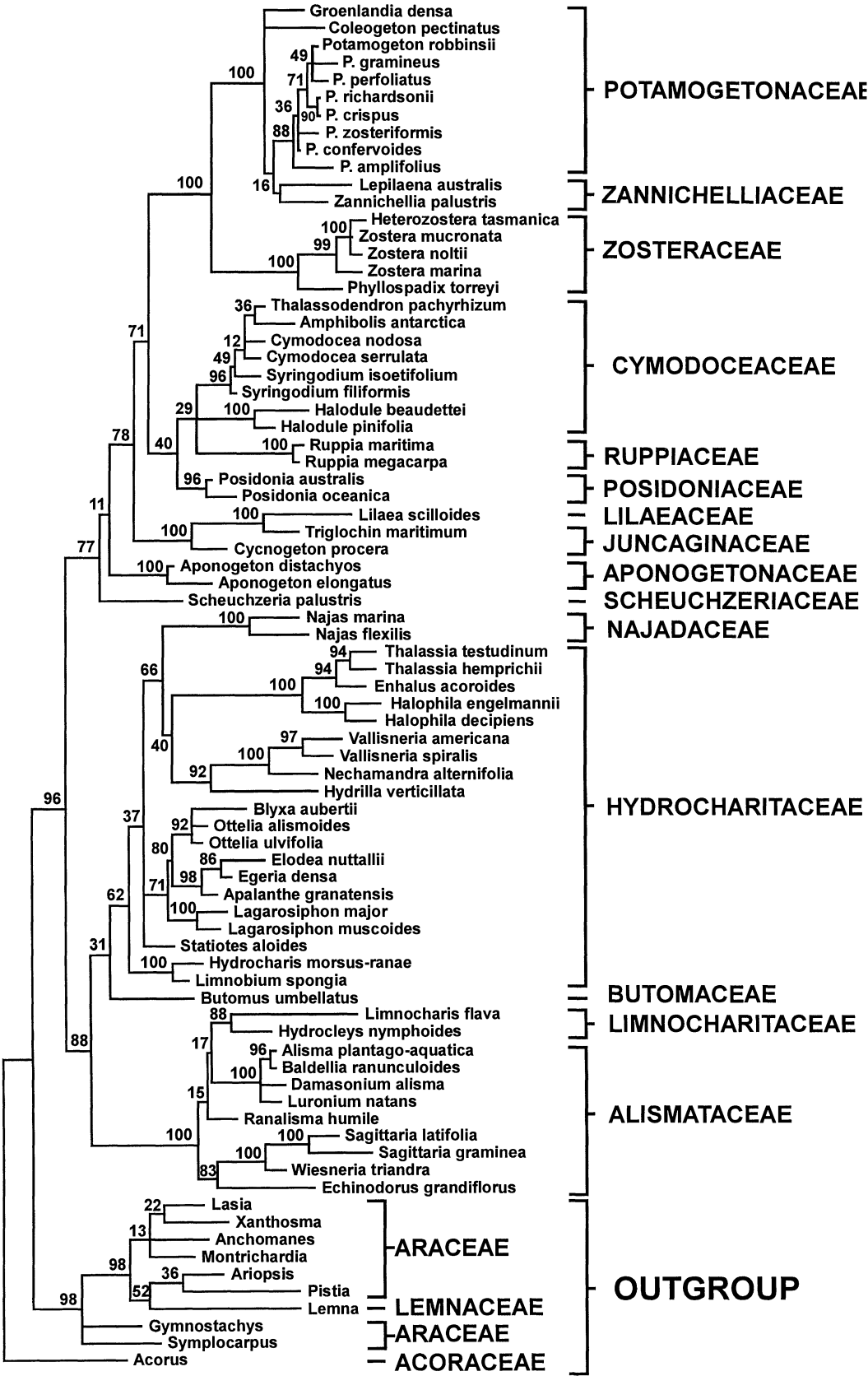
Topological constraint analyses increased minimal tree length from one to 131 additional steps (Table 2). Forcing the monophyly of paraphyletic taxa such as Alismataceae, Potamogetonaceae (+ one step) or Hydrocharitaceae (+ four steps) resulted in only slightly longer trees, whereas forcing the monophyly of polyphyletic groups such as hydrophiles (+ 131 steps), seagrasses (+ 40 steps), or Ruppiaceae/Potamogetonaceae (+ 31 steps) resulted in considerably longer trees. Unrea-

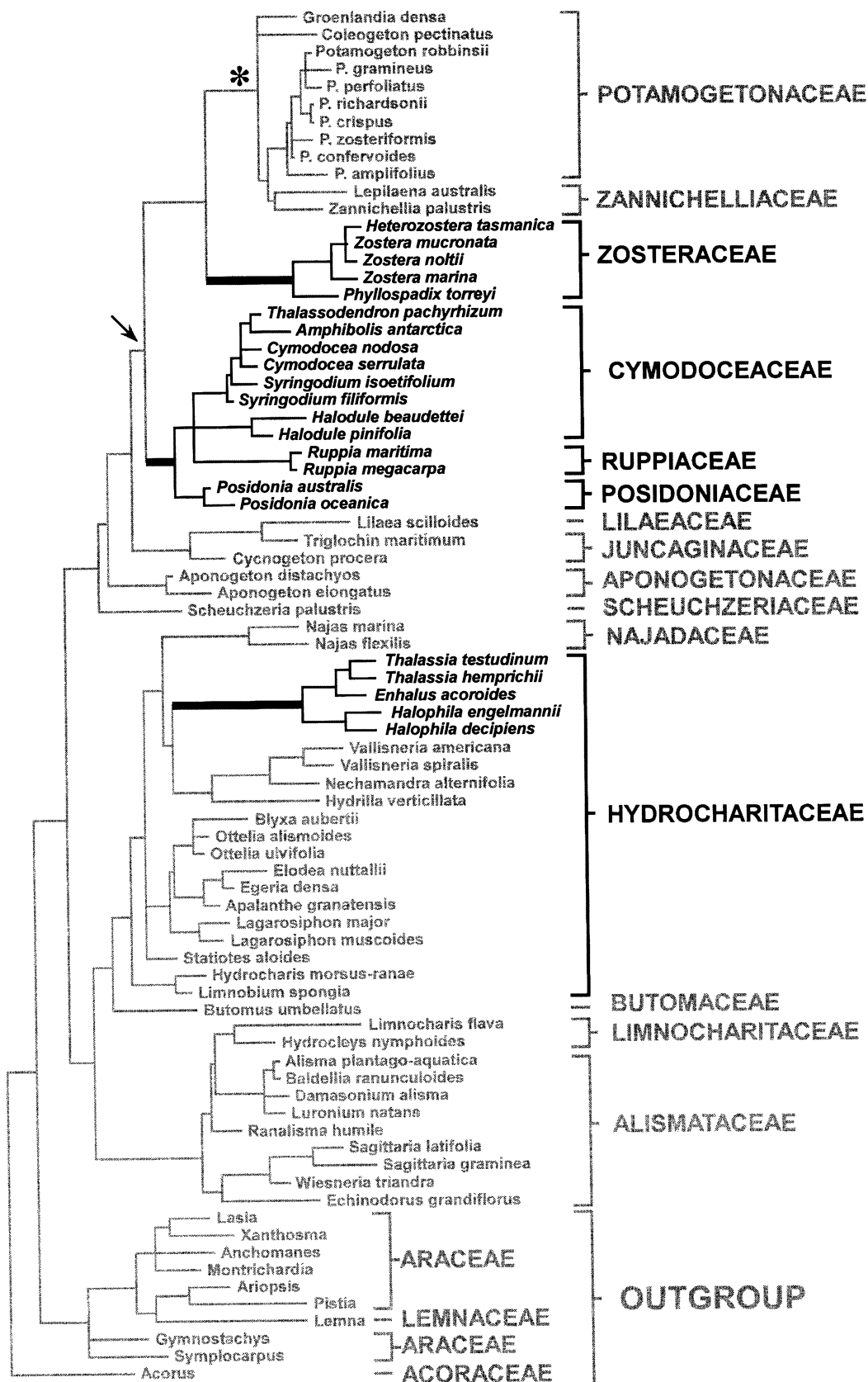
sonably long trees were also obtained by forcing the topology to comply with hypotheses rendered by Cox and Humphries (1993)(+ 81 steps) and Tillich (1995)(+ 23 steps). In all cases, topological constraints had little effect on values for consistency or retention indices, lowering the values by no more than three percent in even the most extreme case (Table 2).

Three origins of seagrasses were indicated by DELTRAN optimization of the marine habit. ACCTRAN optimization depicted two origins of marine plants with a reversal back to freshwater habit in the Potamogetonaceae/Zannichelliaceae clade (Fig. 3). The prospect that marine angiosperms have evolved in three monophyletic clades is supported by moderate to high bootstrap values (40% for Cymodoceaceae complex; 100% for Zosteraceae; 100% for marine Hydrocharitaceae). DELTRAN optimization indicated that hydrophiles are highly polyphyletic with a minimum of seven independent origins in the Alismatidae (Fig. 4) in addition to the two additional origins in the dicotyledons (Fig. 1). ACCTRAN optimization of hydrophily also indicated seven independent origins, but necessitated several reversals from highly specialized hydrophilous mechanisms to unspecialized states which seems unlikely. For this reason, the ACCTRAN mapping of hydrophily was not considered further.

Both ACCTRAN and DELTRAN gave similar results for all trees with respect to the polarity of unisexual flowers in the ingroup. ACCTRAN indicated six (and DELTRAN seven) independent origins of unisexual flowers (Fig. 5). With either optimization, the Hydrocharitaceae are unisexual primitively with one reversal to bisexuality in *Apalanthe* (the two genera of Hydrocharitaceae not surveyed in this study are unisexual). Unisexuality in Zosteraceae is universal but their immediate common ancestor with the Zannichelliaceae/Potamogetonaceae clade is bisexual. Bisexuality is primitive in the Cymodoceaceae complex; inadequate resolution indicates two origins of unisexuality in the group although a single origin of unisexuality in the Cymodoceaceae complex is likely.

FIG. 2. Maximum parsimony *rbcL* cladogram (strict consensus tree shown) of species from the 15 families typically recognized in the monocotyledon subclass Alismatidae with representatives from three outgroup families. At each node, the degree of internal support is indicated by bootstrap values. Values for nodes with < 5% bootstrap support are excluded. Monophyly of the subclass is indicated by 96% bootstrap support. Branch lengths are proportional to the number of substitutions.





DISCUSSION

What Are Seagrasses? Oceanic organisms enjoy a uniform habitat that occupies 71% of the earth's surface (Dawson 1966). Although thalassic communities support a rich variety of life, the scarcity of marine angiosperm species is conspicuous. Arber (1920) viewed marine colonization as a difficult adaptive obstacle because of the necessity to acquire tolerance to submergence and salinity as well as hydrophily and a capacity for vegetative anchorage all within a single lineage. Hartog (1970) regarded these same four features as seagrass criteria, but believed that seagrasses may have evolved from salt-tolerant, mangrove-like "terrestrial" (i.e. helophytic) species rather than from freshwater aquatic progenitors. Indeed, the monocotyledon subclass Alismatidae (which contains all seagrasses), represents one of only three angiosperm groups in which aquatic, salt-tolerant, and hydrophilous species occur (Fig. 1). Most alismatid species are rhizomatous perennials with effective anchorage systems. Arber (1920) attributed the lack of marine species in the Callitrichaceae or Ceratophyllaceae (dicotyledons with aquatic, salt-tolerant, hydrophilous species; Fig. 1) to the lack of rhizomes in the former and absence of roots in the latter.

Seagrasses are marine monocotyledons but not actually grasses (i.e. Poaceae), or their close relatives. The common name reflects their typically linear, sheathed, grasslike leaves. Historically, seagrasses have represented exclusively marine genera (12 genera are recognized by Hartog 1970) and have not included *Ruppia*, *Coleogeton* or various Zannichelliaceae which can inhabit both fresh and marine environments. This convention was first suggested by Ascherson (1867–68) and has been widely followed thereafter. *Ruppia* (Ruppiaceae) and *Lepilaena* (Zannichelliaceae) have been included among the seagrasses by other authors (e.g. Dawson 1966; McMillan 1974; Robertson 1984). Hartog (1970, p. 13) excluded these genera, despite their salinity tolerance, because they "seldom penetrate into the purely marine environment." Concluding that true seagrasses must effectively compete in marine environments, Hartog (1980)

later incorporated this concept as a fifth seagrass criterion.

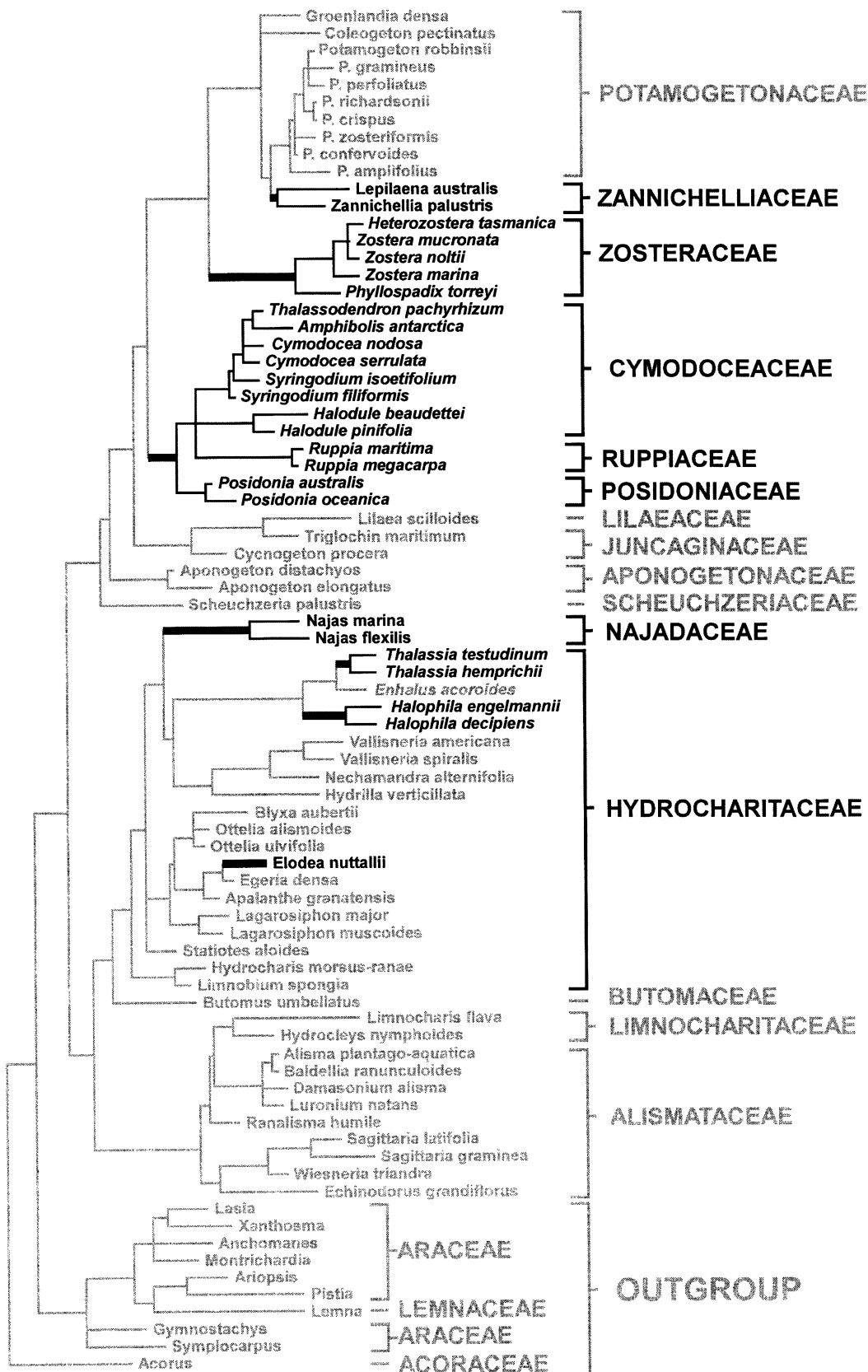
Ambiguity in Hartog's seagrass definition is illustrated by *Enhalus* (Hydrocharitaceae) which is categorized as a seagrass yet not hydrophilous (Hartog 1970). Furthermore, some seagrasses (e.g. *Halophila*) have species with broad rather than grasslike leaves. Evidently, seagrasses represent a loosely circumscribed group and can be defined in various ways. At one extreme, any aquatic angiosperm capable of surviving in marine environments could be considered a seagrass. Stricter definitions (such as that of Hartog 1980) dictate specific morphological, physiological, and ecological requirements.

Seagrasses are either temperate (*Amphibolis*, *Heterozostera*, *Phyllospadix*, *Posidonia*, *Zostera*) or tropical/subtropical (*Cymodocea*, *Enhalus*, *Halodule*, *Halophila*, *Syringodium*, *Thalassia*, *Thalassodendron*) (Hartog 1970). Marine Hydrocharitaceae do not occur where minimum temperatures fall below 20°C (Setchell 1920; Miki 1934). Nine genera span both northern and southern hemispheres; whereas, *Phyllospadix* is exclusively northern and *Amphibolis* and *Heterozostera* exclusively southern hemisphere in their distribution. *Amphibolis*, *Cymodocea*, *Enhalus*, *Heterozostera*, *Posidonia* and *Thalassodendron* are exclusively Old World. The remaining genera occur in both eastern and western hemispheres.

Tropical and subtropical genera fall into two geographical groups; a concentration in the Indo-Pacific region and a small center in the Caribbean (Dawson 1966; Sculthorpe 1967; Hartog 1970). The center of origin for tropical seagrasses is assumed to be the Indo-Pacific region where the highest diversity and most putatively primitive features occur; the Caribbean species are narrowly endemic (Sculthorpe 1967; Hartog 1970).

What Are Hydrophiles? Attempts to define hydrophiles (hydrophilous; water-pollinated species) are problematic (Philbrick and Les 1996). Because water pollination is polyphyletic in angiosperms (Fig. 1), it is reasonable to conclude that hydrophily also represents an arbitrary concept. Cook (1982) described a diversity of pollination systems (both hydrophilous and non-hydrophi-

FIG. 3. Mapping species of marine habitats onto the *rbcL* phylogeny indicates that seagrasses (italicized names) have evolved in three separate, monophyletic clades as indicated by the thick, horizontal lines (strict consensus tree; DELTRAN optimization). ACCTRAN optimization indicates only two origins of marine plants; the marine Hydrocharitaceae and a second origin indicated by the arrow. However, a reversal back to freshwater plants in the Potamogetonaceae and Zannichelliaceae (asterisk) is also required by the ACCTRAN optimization.



lous) even within one family (Hydrocharitaceae). Consequently, it is understandable that various definitions of hydrophily have been suggested. In the strictest sense (e.g. Les 1988), hydrophily is defined as a pollination system where pollen grains are actually transported on the surface (ephydrophily; epihydrophily) or below the surface (hyphydrophily; hypohydrophily) of the water. Cox (1988) advocated a relaxed definition that includes any system where water serves as a vector in pollen transport but not necessarily that pollen comes in direct contact with water. Species with free-floating stamens that carry dry pollen to dry stigmas of female flowers by direct contact (*Appertiella*, *Enhalus*, *Lagarosiphon*, *Nechamandra*, *Vallisneria*), or via aerial transport (*Hydrilla*) would be categorized as hydrophiles by the latter definition, but not by the former (see Cook 1982 for further details of these mechanisms). Philbrick (1988, 1991) recognized these variations as a distinct subtype of hydrophily but emphasized that the evolutionarily significant aspects of hydrophily are adaptations that allow for release of wet pollen and its capture by wet stigmas.

Several unusual aquatic pollination systems defy precise categorization. Proctor and Yeo (1973) suggested that certain species of *Potamogeton* and *Coleogeton* (Les and Haynes 1995) are hydrophilous. Guo and Cook (1989, 1990) presented evidence of hydrophily for *Coleogeton pectinatus* and also for *Groenlandia densa* (Potamogetonaceae). In both species, however, the pollination system resembles what Philbrick and Anderson (1987) distinguished as 'hydroautogamy.' In this system, pollen is transported on the surface of an air bubble from the anther to the stigma of the same flower but does not move from flower to flower. In such cases (e.g. *Potamogeton pusillus* L.), pollen and stigma remain functionally dry even though the flower itself is completely submersed (Philbrick and Anderson 1987). *Groenlandia* exhibits a system similar to *P. pusillus*, although both stigmas and pollen supposedly function when wet (Guo and Cook 1990). Even though the capture of released, water-borne pollen is possible (and can result in fertilization), the principal means of pollination in *Groenlandia* is autogamy (Guo and Cook 1990). *Coleogeton pectina-*

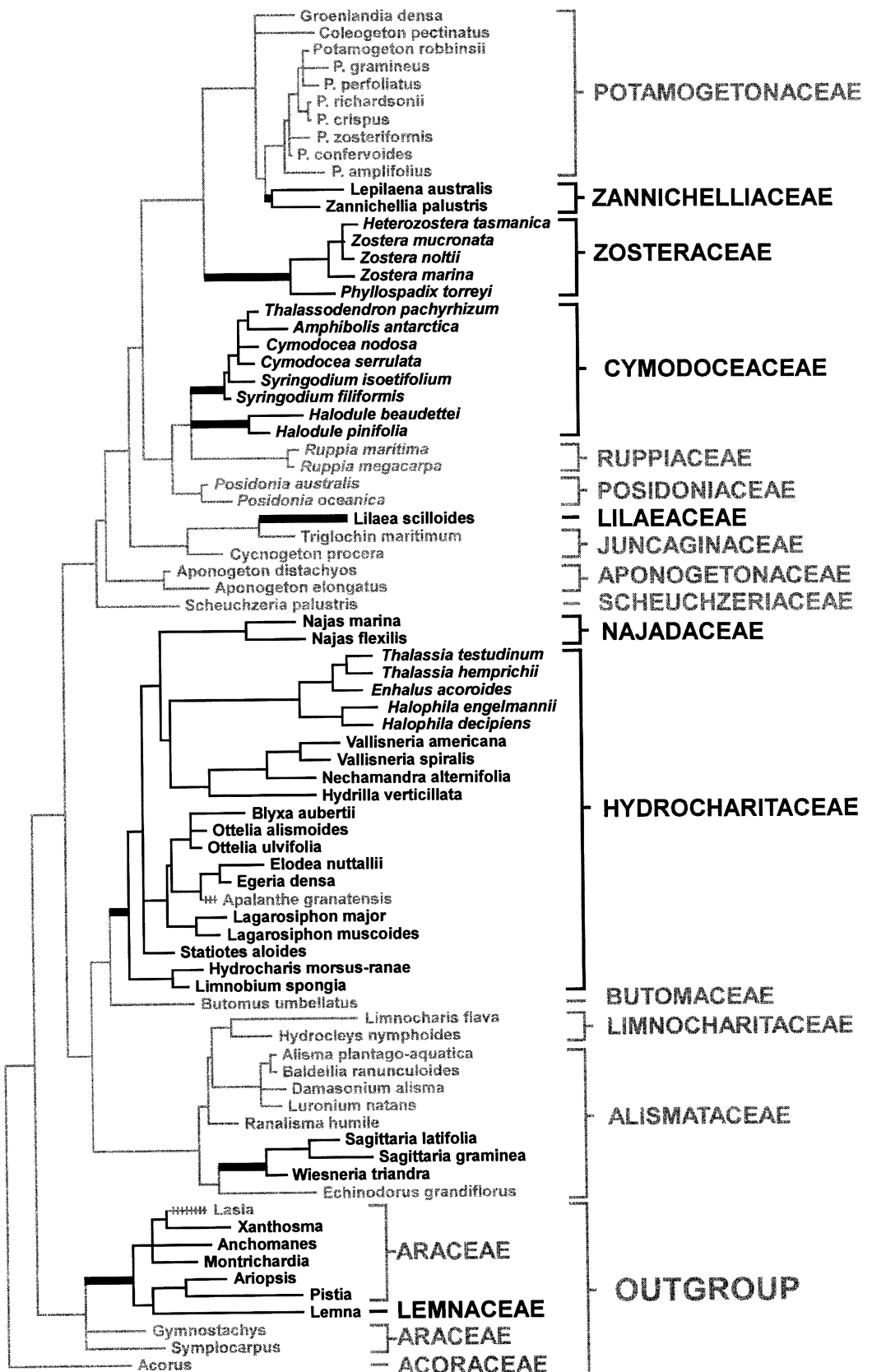
tus sets seed in completely submersed flowers (Guo and Cook 1989), but this is probably due again to autogamy rather than xenogamy (Les, pers. obs.). Hydroautogamy is theorized as an intermediate stage between aerial and true water pollination in aquatic plants (Philbrick and Anderson 1987; Philbrick 1988; Philbrick and Les 1996).

Potamogeton lucens L. presents a more complex question. Although pollination by floating pollen grains has been reported (Daumann 1963; Guo and Cook 1989), the species appears to be anemophilous in nature. Without more persuasive evidence, we have refrained from categorizing the unusual pollination systems in *Potamogeton* and *Groenlandia* as examples of hydrophily. Although the definition of hydrophily will remain subject to individual interpretations, we elect to adopt the stricter definition of hydrophily suggested by Les (1988) because it facilitates evolutionary comparisons of hydrophilous systems from the standpoint of what are perceived as major adaptive transitions; i.e. the ability to function when pollen and stigma are wet. By this definition, we categorized 18 genera as hydrophiles (Table 1).

Molecular Phylogeny Of Seagrasses. Preliminary summaries of interfamilial phylogenetic relationships in Alismatidae have been discussed by Les et al. (1993), Les and Haynes (1995) and the references therein. The present analysis provides a more comprehensive estimation of relationships based upon a study of the majority of genera in the subclass, including all marine genera and all but two hydrophilous genera. Sampling of multiple species in many genera has expanded phylogenetic insights.

The polyphyly of seagrasses has never been seriously disputed (e.g., Tomlinson 1982), but rather has remained a question of degree. A wide range of estimates on the number of seagrass origins has been suggested by various classifications rendered for the 13 marine genera. Larkum and Hartog (1989) relaxed an earlier opinion (Hartog 1970) that marine plants are best classified into two families and considered the possibility of recognizing several; however, they still maintained that Zosteraceae, Cymodoceaceae, and Posidoniaceae "evolved directly from the (same?) ancestral

FIG. 4. Mapping hydrophilous (water-pollinated) species onto the *rbcl* phylogeny (strict consensus tree; DELTRAN optimization) indicates that water pollination has evolved at least seven independent times (thick, horizontal lines) within the Alismatidae. Including the two origins of hydrophily in dicotyledons (Fig. 1), water pollination has evolved at least nine separate times in flowering plants. A close association between hydrophily and marine species (italicized names) is evident.



stock." Such statements are cryptic phylogenetically, because they would be true whether these families were monophyletic, or if they evolved independently from a more remote common ancestor. Mapping the marine habit on the *rbcl* cladogram using the DELTRAN optimization (Fig. 3) indicates three independent origins of marine plants in Alismatidae. Although the ACCTRAN optimization indicates only two seagrass origins (Fig. 3), it also requires a reversal back to freshwater habit in the Potamogetonaceae/Zannichelliaceae clade which is unlikely. Constraining the analysis to render a monophyletic clade of marine plants (Table 2; analysis #1) adds an additional 40 steps to the maximum parsimony solution. By following Hartog's hypothesis and constraining marine plants to two clades (Table 2; analysis #3), trees elongate by 11 steps (see also discussion on Cymodoceaceae below). Thus, the *rbcl* data support the polyphyly of marine angiosperms and provide a specific estimate of three independent evolutionary seagrass origins.

Although Hartog (1970) and Larkum and Hartog (1989) believed that most seagrasses evolved from "mangrove-like" saltwater plants without an immediate freshwater ancestor, we find little evidence to support this hypothesis. The ancestors of marine Hydrocharitaceae were likely to have been freshwater species because the remainder of the family comprises freshwater species. This scenario is admitted as likely even by Larkum and Hartog (1989). Our results indicate that the sister group to Zosteraceae is the principally freshwater Potamogetonaceae/Zannichelliaceae clade in which each family contains several salt-tolerant (but not oceanic) species as well. The sister group to these three families (and the seagrasses of the Cymodoceaceae complex) is the Juncaginaceae/Lilaeaceae clade which also includes plants adapted to both fresh and salt water. Basal to the whole group are the Scheuchzeriaceae and Aponogetonaceae, both freshwater families. Furthermore, none of these groups contain species that are even remotely "mangrove-like" in their habit. Our results indicate that all of the marine clades have evolved from groups that were originally either entirely freshwater, or that

included a mixture of freshwater and salt-tolerant species.

The genera *Enhalus*, *Halophila*, and *Thalassia* (marine Hydrocharitaceae) provide a striking example of uncertainty regarding the phylogenetic affinities of seagrasses, even those presumed to be closely related. These genera are usually placed within Hydrocharitaceae, although Kimura (1956) assigned them to separate families (Vallisneriaceae, Halophilaceae, Thalassiaceae, respectively). Even classifications that place these genera within one family (i.e. Hydrocharitaceae), usually separate them among three different subfamilies (e.g. Hartog 1970, 1977). Because no formal phylogenetic analysis at the generic level has been conducted for the Hydrocharitaceae, the question has remained whether these marine members represent one, two, or three independent origins within this family. Taxonomic opinion has emphasized the differences among these genera rather than similarities related to their marine existence.

The *rbcl* phylogeny depicts the marine Hydrocharitaceae as a strongly supported monophyletic group with an affinity for tropical oceanic habitats. In the strict consensus tree (Fig. 2), this seagrass clade is imbedded within freshwater genera of Hydrocharitaceae and has 100% bootstrap support. Topological constraints that force the monophyly of both freshwater Hydrocharitaceae and the marine clade (Table 2; analysis #6) add an additional six steps to the *rbcl* cladogram. Consequently, the recognition of all marine Hydrocharitaceae as a separate, monophyletic family (e.g. Thalassiaceae) is not strongly supported by *rbcl* data. Thus, results from this analysis argue to retain the genera *Enhalus*, *Halophila*, and *Thalassia* as a single taxon within the Hydrocharitaceae (e.g. subfamily) rather than as a distinct marine family.

It is also difficult to assess the continued recognition of the freshwater families Najadaceae (which render a paraphyletic Hydrocharitaceae in this analysis) and Limncharitaceae (which render a paraphyletic Alismataceae in this analysis). Constraints forcing the monophyly of either the Alismataceae (Table 2; analysis #9) or freshwater and marine Hydrocharitaceae (Table 2; analysis #8)

FIG. 5. Mapping unisexual floral conditions onto the *rbcl* phylogeny (strict consensus tree; DELTRAN optimization) indicates that unisexuality (indicated by the thick, unbroken lines) has evolved at least seven separate times in the Alismatidae; reversals to hermaphroditism are indicated by the vertical lines. The ACCTRAN optimization differed only by indicating one gain and one reversal (rather than two independent gains) at the unresolved Ruppiaceae/Cymodoceaceae node. Italicized names delimit marine taxa.

add only one to four additional steps to the length of the heuristic minimal length solution.

The relationship of Hydrocharitaceae and Najadaceae has now been well established by cladistic analyses using anatomical, morphological and molecular data (Shaffer-Fehre 1991a, 1991b; Les et al. 1993; Les and Haynes 1995). Considering that *rbcL* data indicate the origin of *Najas* from within the Hydrocharitaceae (even though not convincingly so as the constraint experiment demonstrates), we elected to support the merger of the families (Table 1) as suggested by Shaffer-Fehre (1991a, 1991b).

We also evaluated the merger of Hydrocharitaceae with Aponogetonaceae, Juncaginaceae, and Scheuchzeriaceae into the order "Hydrocharitales" as recently proposed by Tillich (1995). Constraining this topology (Table 2; analysis #10) lengthens the shortest trees by 23 steps which does not represent a viable alternative to the minimal length topology that associates Hydrocharitaceae with Alismataceae, Limncharitaceae and Butomaceae (Fig. 2). In the *rbcL* analysis, this clade has a bootstrap support of 88%. The relationship of these families has been suggested previously by morphological cladistic studies (Dahlgren et al. 1985; Les and Haynes 1995).

The marine genera *Heterozostera*, *Phyllospadix*, and *Zostera* are less problematic. Most classifications place them within either a single family (i.e., Zosteraceae; Tomlinson 1982; Kuo and McComb 1989; Cook 1990), subfamily (i.e., Potamogetonaceae subfamily Zosterioideae; Hartog 1970) or tribe (i.e., Potamogetonaceae tribe Zostereae; Ascherson and Graebner 1907). Placement of these genera in Potamogetonaceae without subfamilial segregation has been followed by some authors (e.g. Phillips and Meñez 1988). Thus, previous taxonomic treatments have all essentially indicated that *Heterozostera*, *Phyllospadix*, and *Zostera* represent a monophyletic group. The inclusion of Zosteraceae in the Potamogetonaceae seen in previous classifications might infer a close relationship between these families. Tomlinson (1982), however, noted that Zosteraceae are clearly circumscribed, but have obscure affinities to other groups. Potamogetonaceae as depicted in the *rbcL* analysis contain no marine plants. The family is paraphyletic in the analysis, but constraint experiments (Table 2; analysis #4) indicate that only one additional step is required to resolve the family as monophyletic. Notwithstanding, their monophyly is accepted given that both Potamogetonaceae and Zannichelliaceae are distinct and clearly defined families (Cook 1990).

The *rbcL* data agree with the circumscription of Zosteraceae; i.e. internal support for the monophyly of the family is high (e.g. 100% bootstrap value) in our analyses. Our results also indicate the closest extant sister group to Zosteraceae is a clade consisting of Potamogetonaceae and Zannichelliaceae. The *rbcL* data indicate a relatively high degree of molecular divergence separating the two clades (Fig. 1), which represents an argument against a taxonomic merger of these groups and further reflects the distinctness of the Zosteraceae as indicated previously by morphological phylogenetic studies (e.g. Dahlgren et al. 1985). The clade comprising all three families (Potamogetonaceae, Zannichelliaceae, Zosteraceae) has 100% bootstrap support from the *rbcL* data (Fig. 2).

One taxonomic discrepancy has been the status of *Heterozostera tasmanica* which was named originally as *Zostera tasmanica* (Ascherson 1867-68) and placed within a section that included other *Zostera* species. Setchell (1933) retained *Zostera tasmanica*, but segregated it within a monotypic section *Heterozostera*. Hartog (1970) later elevated the taxon to the monotypic genus *Heterozostera*. Problems arise when attempting to separate *Zostera* and *Heterozostera* taxonomically. Hartog (1970) and Phillips and Meñez (1988) separate the genera by rhizome characters; i.e. monopodial in the former and sympodial in the latter. Tomlinson (1982) disputed this distinction but reemphasized the distinctness of *Heterozostera* on the basis of its wiry, erect stems and numerous cortical vascular bundles. Despite anatomical differences, *Heterozostera* is similar to *Zostera*, especially to sect. *Zosterella* (which includes all of the Australasian species) whose leaf anatomy is "indistinguishable" (Tomlinson 1982). The *rbcL* data do not support the recognition of *Heterozostera* as a distinct genus. There is 100% bootstrap support for the association of *Heterozostera* with *Zostera mucronata* and *Zostera noltii* (of sect. *Zosterella*) which renders *Zostera* as a paraphyletic genus (Fig. 2). Topologically constraining the *rbcL* tree to force the monophyly of *Zostera* (Table 2; analysis #7) adds an additional five steps to the maximum parsimony solution. This is not a trivial increase in tree length given that the homoplasy is introduced in a rather confined section of the cladogram. Consequently, the *rbcL* data support the recognition of this taxon as a morphologically distinct species of *Zostera* (i.e., *Zostera tasmanica*) rather than a monotypic genus.

The *rbcL* data (Fig. 2) resolve an interesting clade consisting of three families never previously associ-

ated, comprising genera of Cymodoceaceae, Posidoniaceae, and Ruppiaceae. We refer to this assemblage as the Cymodoceaceae complex. The latter (i.e. *Ruppia*) has traditionally been classified as a subfamily (Ruppioideae) of Potamogetonaceae and has not been exclusively associated with seagrasses in past taxonomic treatments. Forcing the monophyly of Ruppiaceae and Potamogetonaceae (Table 2; analysis #5) adds an additional 31 steps to the minimal length tree obtained in our analyses and is, as a result, difficult to accept using a maximum parsimony criterion. Although the degree of internal support (expressed by bootstrap values) for the monophyly of Ruppiaceae, Cymodoceaceae, and Posidoniaceae is not particularly high (40%), the inclusion of *Ruppia* among the other representatives of this complex is certainly supported no worse than the inclusion of *Halodule* (traditionally placed in the Cymodoceaceae), and Posidoniaceae (viewed as closely related to Cymodoceaceae). The association of Ruppiaceae and Posidoniaceae is appealing because both hermaphroditic families occur in a position basal to the unisexual Cymodoceaceae.

Cox and Humphries (1993) have suggested that Zosteraceae, Posidoniaceae and Cymodoceaceae represent a monophyletic clade, but their analysis was limited by the small number of taxa considered (notably the exclusion of Ruppiaceae). The monophyletic marine clade that they obtained would be the default result of nearly any analysis of that limited sample of families simply because other essential non-marine sister groups (e.g., Potamogetonaceae, Scheuchzeriaceae, Liliaceae, Juncaginaceae, Aponogetonaceae) were not included. When the *rbcl* data are forced to comply with the Cox and Humphries (1993) topology (Table 2; analysis #11), trees elongate by 81 steps, an unreasonable alternative to the maximum parsimony solution. Forcing only the monophyly of the marine Zosteraceae and the Cymodoceaceae “complex” (Table 2; analysis #3) requires 11 additional steps. In our opinion, this degree of elongation does not represent a feasible alternative topology to the shortest *rbcl* trees which depict the separate origin of these two groups. From the *rbcl* data analysis, we conclude that marine Zosteraceae have an independent origin from the Cymodoceaceae complex. The molecular data also present the first evidence of a close relationship of the Ruppiaceae to the seagrass families Posidoniaceae and Cymodoceaceae. Association of any of these families to the Zannichelliaceae (e.g. Hutchinson 1959; Eckardt 1964) is

TABLE 3. Classification of marine angiosperms proposed by Hartog (1977) and phylogenetic classification suggested by present study.

HARTOG, 1977	PRESENT STUDY
1. Potamogetonaceae	1. Zosteraceae
a. subfamily Zosterioideae	a. <i>Heterozostera</i>
1. <i>Heterozostera</i>	b. <i>Phyllospadix</i>
2. <i>Phyllospadix</i>	c. <i>Zostera</i>
3. <i>Zostera</i>	2. Cymodoceaceae
b. subfamily Posidonioideae	a. <i>Amphibolis</i>
1. <i>Posidonia</i>	b. <i>Cymodocea</i>
c. subfamily Cymodoceoideae	c. <i>Halodule</i>
1. <i>Amphibolis</i>	d. <i>Syringodium</i>
2. <i>Cymodocea</i>	e. <i>Thalassodendron</i>
3. <i>Halodule</i>	3. Posidoniaceae
4. <i>Syringodium</i>	a. <i>Posidonia</i>
5. <i>Thalassodendron</i>	4. Ruppiaceae
2. Hydrocharitaceae	a. <i>Ruppia</i>
a. subfamily Halophiloideae	5. Hydrocharitaceae
1. <i>Halophila</i>	a. <i>Enhalus</i>
b. subfamily Hydrocharitoidae	b. <i>Halophila</i>
1. <i>Enhalus</i>	c. <i>Thalassia</i>
c. subfamily Thalassioideae	
1. <i>Thalassia</i>	

supported neither by *rbcl* data (Fig. 2) nor by anato-morphological data (Tomlinson 1982).
The distribution of soluble carbohydrates among seagrass genera suggested to Drew (1980) a “chemical phylogeny” that indicated the derivation of various Cymodoceaceae from different marine genera of Hydrocharitaceae. We find no evidence to support this unusual proposition given that *rbcl* data (and other data sets as well) strongly indicate that the marine Hydrocharitaceae are not closely related to other seagrass genera.
Phillips and Meñez (1988) retained Hartog’s (1977) classification of seagrasses (Table 3). Kuo and McComb (1989) and Cook (1990) substantially modified the system by removing marine genera from Potamogetonaceae and recognizing Cymodoceaceae, Posidoniaceae and Zosteraceae. Our molecular phylogenetic study of Alismatidae supports the modified familial classifications and necessitates an overall reconsideration of seagrass classification. We place seagrasses into five families: Cymodoceaceae, Hydrocharitaceae, Posidoniaceae, Ruppiaceae and Zosteraceae (Table 3), which correspond to three monophyletic marine clades (Fig. 3). Accepting this convention also allows for a simple, phylogenetic definition of seagrasses; i.e.,

they are species belonging to any of these monophyletic clades.

Molecular Phylogeny Of Hydrophiles. There have been few specific estimates on the number of times that hydrophily has evolved in the angiosperms. Cox (1988) suggested that cladistic methods would probably indicate "several points" of origin for hydrophily in Alismatidae, and that hydrophily may be synapomorphic for Zosteraceae, Posidoniaceae, and Cymodoceaceae. Tomlinson (1982) also believed there to be "no single pathway" to hydrophily in Alismatidae. The present study supports the conclusion that hydrophily is indeed polyphyletic in Alismatidae. If hydrophiles are constrained as one monophyletic group (Table 2; analysis #2), trees elongate by 131 steps from the shortest trees recovered. Mapping the distribution of hydrophily onto the molecular tree (Fig. 4) indicates seven separate origins of hydrophily in Alismatidae; hydrophily has also evolved twice in the dicotyledons (Fig. 1). Even adopting a less stringent definition of hydrophily such as that suggested by Cox (1988) would still require at least four independent origins of hydrophily in the subclass. Figure 4 demonstrates the association of hydrophily and marine species. This result further emphasizes the important evolutionary affinity between water pollination and the ability of angiosperms to colonize marine habitats. Unlike the distinct monophyletic groups rendered for marine species, *rbcL* data do not indicate as confined a phylogenetic distribution of hydrophily (Fig. 4). Hydrophily is universal among the Zosteraceae and Cymodoceaceae complex, but has also evolved in the freshwater Zannichelliaceae and Hydrocharitaceae (including Najadaceae). Our results indicate separate origins of hydrophily in Zosteraceae and the Cymodoceaceae complex and do not support Cox's (1988) hypothesis that the condition may represent a unifying synapomorphy for these families. True hydrophily has evolved repeatedly within the Hydrocharitaceae but does not occur in the marine genus *Enhalus*. Thus, the phylogenetic distribution of water pollination does not provide any substantial improvement in the definition of hydrophily.

Distribution Of Unisexuality. We finally consider the occurrence of unisexual floral conditions in Alismatidae, principally with respect to opinions expressed on the extent of outcrossing that occurs in water-pollinated plants such as seagrasses. Hartog (1970) believed that the high incidence of monoecy and dioecy in seagrasses indicated that

"cross fertilization is a rule." Pettitt et al. (1981) suggested that seagrasses have "continuous outbreeding." Cox (1988) also suggested that dicliny in hydrophiles results from selection for outcrossing. As Les (1988) pointed out, however, cross-fertilization is irrelevant genetically unless the population within which it occurs is variable genetically. The extensive clonal propagation of many hydrophiles led him to suggest that cross-fertilization may often occur between genetically identical, homozygous individuals. Non-existent or minuscule levels of genetic variation found in seagrasses by early allozyme studies were consistent with this expectation (Les 1988). Consequently, Les (1988) hypothesized that dicliny in at least some hydrophiles may simply reflect the sexual condition of their progenitors rather than represent an adaptation linked to water pollination itself. In many cases, the genetic structure of hydrophile populations may reflect patterns of asexual (clonal) reproduction rather than an outcome of outcrossing (Les 1991).

We have examined the phylogenetic distribution of unisexuality by mapping the derivation of the condition onto the *rbcL* cladogram (Fig. 5). Several lines of evidence indicate that unisexuality in seagrasses has not necessarily evolved subsequent to hydrophily as Philbrick (1988) hypothesized, nor are unisexual reproductive systems inevitably tied to outcrossing breeding systems. As with hydrophily, several independent origins of unisexual floral conditions are indicated. Moreover, it is apparent that unisexuality is a primitive rather than derived condition in the Hydrocharitaceae (Fig. 5). Therefore, the diclinous conditions of all hydrophilous and marine species in this family are, as Les (1988) hypothesized, a consequence of a pre-existing (primitive) condition in their non-hydrophilous, non-marine progenitors. This realization thwarts efforts to link unisexuality with either hydrophily or marine colonization in at least this clade.

However, this is not necessarily a universal conclusion. Because the Cymodoceaceae complex is hermaphroditic primitively, it would appear that here, bisexual hydrophiles have preceded unisexual hydrophiles evolutionarily, thus conforming in this case to Philbrick's (1988) hypothesis. These disparate results emphasize the complexity of adaptive evolution which, even for hydrophiles, has occurred along convergent pathways. The relative evolution of hydrophily versus unisexuality is more problematic to interpret in the Zosteraceae given that all species are unisexual, hydrophilous, and marine.

We have evaluated outcrossing in seagrasses (see Les 1988) using population genetic data to provide a direct assessment of the question. Waycott and Les (1996) demonstrated that sexual condition (unisexuality vs. hermaphroditism) is a poor estimator of genetic variation in seagrasses. In an extreme example, the hermaphroditic genus *Posidonia* is typically highly outcrossed, whereas populations of the dioecious genus *Amphibolis* are devoid of detectable genetic variation at allozyme and even hypervariable loci (Waycott et al. 1996; Waycott and Les 1996). The hermaphroditic *Ruppia*, however, is highly clonal or inbred. Furthermore, different populations of *Posidonia* are quite heterogeneous with high outcrossing rates observed in some and low rates in others. We are currently pursuing this avenue of research to furnish additional population genetic data for seagrasses. In any event, we conclude that the reservoir of genetic variation, the degree of outcrossing, and adaptation to hydrophily and marine existence in either unisexual or hermaphroditic aquatic angiosperms appear to represent adjustments to highly complex life history traits specific to each species and can not be adequately explained by any simple, universal factor.

In conclusion, molecular phylogenetic studies of the subclass Alismatidae have provided many insights into the evolution of marine and hydrophilous angiosperms. The *rbcl* data indicate there to be three independent origins of seagrasses, and seven derivations of true hydrophily within the subclass. This provides an estimate of nine separate origins of hydrophily in the angiosperms. Unisexual floral conditions have both preceded and succeeded the evolution of hydrophily in the Alismatidae and marine species have mostly evolved in hydrophilous clades. The multiple origins of both hydrophily and seagrasses in the Alismatidae provide striking examples of convergence in the angiosperms. One particularly noteworthy factor, however, is that convergence in these features has not resulted in species with similar reproductive life-history traits. Patterns of genetic variability, outcrossing rates, and other aspects of the breeding systems of marine and hydrophilous species do not appear to be constrained by the convergent evolution of either their marine existence or hydrophilous reproductive systems. Considerable variation in these life history attributes is evident not only between, but even within clades of monophyletic, hydrophilous, marine species. Additional investigations into the genetic structure of seagrass popula-

tions should provide valuable information on how these convergently similar species differ with respect to their reproduction and other evolutionarily significant life history traits.

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