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Phylogenetic studies of the core Alismatales inferred from morphology and *rbc*L sequences

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Abstract

The phylogeny of Alismatales remains an area of deep uncertainty, with different arrangements being found in studies that examined various subsets of genes and taxa. Herein we conducted separate and combined analyses of 103 morphological characters and 52 *rbc*L sequences to explore the controversial phylogenies of the families. Congruence between the two data sets was explored by computing several indices. Morphological data sets contain poor phylogenetic signals. The homology of morphological characters was tested based on the total evidence of phylogeny. The incongruence between DNA and morphological results; the hypothesis of the 'Cymodoceaceae complex'; the relationships between Najadaceae and Hydrocharitaceae; the intergeneric relationships of Hydrocharitaceae; and the evolutionary convergence of morphological characters were analyzed and discussed.
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Keywords: Alismatales; Morphological phylogeny; *rbc*L sequences; Cymodoceaceae complex; Najadaceae; Hydrocharitaceae

1. Introduction

All known marine angiosperms (12 genera) and all hydrophiles angiosperms (17 genera) are concentrated in the Alismatales with only a few species found in the Ceratophyllaceae [1] and Callitrichaceae [2]. All the members of this order are plant rhizomatous; mycorrhizae absent; starch grains pteridophyte-type, amylophilic; a extrorse, tapetum amoeboid, cells uninucleate, carpels with completely unfused canals, stylodia+, stigma dry (common); endosperm helobial; embryo large, (chlorophyllous); seedling with hypocotyl and root well developed (data from Angiosperm Phylogeny Website http://www.mobot.org/MOBOT/research/APweb/orders/alismatalesweb.htm#Alismatales).

Many of the phylogenies of the Alismatales have been published, some based on morphological data and others based on molecular data [3–13]. However, there is no evidence that these hypotheses of the relationship are converging on a single viewpoint. Relationships within the order Alismatales are still less certain. Many early studies [14,15] did not use explicit methods of phylogenetic analysis, therefore their conclusions are difficult to evaluate. Furthermore, most of these were less than comprehensive, with phylogenetic relationships suggested for only subsets of taxa, or for larger numbers of taxa but based on a small number of characters; their results should be less persuasive. The only existing studies of relationships in the Alismatales that incorporate explicit phylogenetic methods, contain a representative sample of taxa, and use a large number of characters are the analyses of non-molecular data by Dahlgren and Rasmussen [16] and the analysis of DNA sequence data by Les and Haynes [17] and Les et al. [3,18]. The phylogenetic relationships of Alismatales have been discussed by Les et al. [3] in detail. However, cladograms of the Alismatales generated from the molecular data differ in many respects from those obtained using non-molecular data. Donoghue

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and Sanderson [19] argued strongly in favor of combined analyses of such data sets. Here, we attempt to evaluate the outcome of combined analyses for the first time based on 52 *rbc*L sequences and 103 morphological characters from 52 genera; representing all currently recognized families and 81% of the genera in the order Alismatales. Delimitation of families and genera are according to APG II [20], Cook [21], Cronquist [22], and Tomlinson [23]. In this paper, incongruence between the two different data sets is examined by use of several indices. Despite the fact that the *rbc*L data set is incompatible with the morphological data set, we argue that the best solution for handling the two data sets is to combine them all into a single matrix.

The goals of this study are: (1) to compare results obtained from morphology and *rbc*L sequences to assess whether there is significant incongruence between the two and whether there is greater homoplasy in the morphological data; (2) to determine whether analysis of the combined data provides additional phylogenetic information beyond that obtained from either morphology or molecules alone; (3) to present a phylogenetic analysis of the intrafamilial relationships within Alismatales based on two different data sets: morphology and *rbc*L sequences; and (4) to analyze the specific characters which might contribute to the conflict between morphological and *rbc*L data sets. This study adds to our understanding of relationships of the Alismatales and is an important step towards understanding the evolutionary processes that have led to those complicated patterns of the Alismatales.

2. Materials and methods

2.1. Taxa sampling

The morphological characters of 52 taxa of Alismatales were compiled from the literature resources and from herbarium specimens from KIB of China [1,3,7,16,24–34]. In total, 103 characters were analyzed, covering internal anatomy, external morphology, and ecological characters (Appendix A). In this analysis, we increased the number of taxa from the 15 families used by Les and Haynes [17] to 52 genera and the number of morphological characters from 59 to 103. The *rbc*L sequences for 52 species of Alismatales in GenBank were obtained (Table 1). All phylogenetic trees were rooted by Lemnaceae and Araceae according to Les et al. [3], Tamura et al. [9], Janssen and Bremer [8], Givnish et al. [11], and Chase et al. [10] (all place Araceae as a sister to the rest of the order).

2.2. Data analysis

Morphological characters were equally weighted. All characters were considered unordered. Molecular and morphological data sets were combined into a single matrix for a parsimony analysis. No weighting was applied to any character.

All phylogenetic analyses were performed using maximum parsimony as the optimization criterion, as implemented in PAUP* 4.0b4a [35]. Three analyses were carried out on the morphological data for 53 taxa; *rbc*L sequence data for 52 taxa; morphological and *rbc*L data combined for 48 taxa. For the separate phylogenetic analyses of the morphological data and *rbc*L sequence data, all changes among character states were weighted equally. To simultaneously accommodate taxa with multiple character states resulting from uncertainty or polymorphy, the variable option in PAUP* 4.0b4a was used.

Phylogenetic analysis was done using heuristic search options with 1000 random sequence additions, tree bisection–reconnection (TBR) branch swapping, MULPARS on, steepest descent off and saving all most parsimonious trees without any restrictions. Branch support was estimated by 1000 bootstrap replicates using PAUP* 4.0b4a with TBR and MULPARS options in a heuristic search with a random addition of sequence of taxa per bootstrap replicate.

2.3. Congruence among data sets

Measures of character congruence examined were the Mickevich and Farris original measures (I_{MF}) [36]. The I_{MF} is the proportion of between data set homoplasy (the difference between the extra number of steps required for a most parsimonious tree from the combined data set and the extra number of steps required by each data set on its most parsimonious trees) relative to the extra homoplasy required by the combined data set; data sets with no conflict will yield an I_{MF} value of 0.0.

A global test for homogeneity was accomplished by the incongruence length difference (ILD) test [37,38], implemented in PAUP* 4.0b4a as the partition homogeneity test. The data sets are combined into a single data matrix with two partitions: the test compares the sum of the shortest tree lengths based on the original partitions (the two separate 48-sample data sets) to a distribution of sums of lengths of trees generated by random repartitioning of all the data. When 99% or more of those random partitions show an I_{MF} smaller than the original, we reject the null hypothesis and conclude that the data sets are significantly heterogeneous. For this test, invariant characters were excluded [39], and heuristic searches were conducted with 100 replicates, each with 10 random addition replicates using TBR branch swapping, and the MULTREES option on.

2.4. Reconstruction of character evolution

To reconstruct historical shifts in morphology, we overlaid characters onto a representative tree from the combined data set by using WinClada [40] and NONA [41].

3. Results

3.1. Phylogenetic analysis of morphological data

Of the 103 characters considered, 12 were variable parsimony-uninformative characters, and 79 were parsi-

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Table 1
List of taxa and the accession numbers in GenBank for *rbc*L sequences.

Family	Species	GenBank Accession No.
Alismataceae	*Alisma plantago aquatica* L.	L08759
	Baldellia ranunculoides (L.) Parlatore	U80677
	Caldesia oligococca (F. Muell.) Buchenau	AY277799
	Damasonium alisma Mill.	U80678
	Echinodorus grandiflorus (Cham. and Schltdl.) Micheli	U80679
	Luronium natans (L.) Raf.	U80680
	Ranalisma humile (Kuntze) Hutch.	U80681
	Sagittaria latifolia Willd.	L08767
	Wiesneria triandra Micheli	U80682
Aponogetonaceae	*Aponogeton fenestralis* (Pers.) Hook. f.	AB088808
Butomataceae	*Butomus umbellatus* L.	AY149345
Cymodoceaceae	*Amphibolis antarctica* (Labill.) Asch.	U80686
	Cymodocea serrulata (R. Br.) Asch. and Magnus	U80687
	Halodule uninervis Boiss.	AY952436
	Syringodium isoetifolium (Asch.) Dandy	U80691
	Thalassodendron pachyrhizum Hartog	U80692
Hydrocharitaceae	*Apalanthe granatensis* (Bonpl.) Planch.	U80693
	Blyxa japonica Miq.	AB004886
	Egeria densa Planch.	AB004887
	Elodea nuttallii (Planch.) St. John	AB004888
	Enhalus acoroides L.C. Rich. ex Steud.	AB004889
	Halophila ovalis (R.Br.) Hook. f.	AB004890
	Hydrilla verticillata (L.f.) Royle	AB004891
	Hydrocharis dubia (Bl.) Backer	AB004892
	Lagarosiphon madagascariensis Casp.	AB004893
	Limnobium laevigatum (Humb. and Bonpl. ex Willd.) Heine	AB004894
	Nechamandra alternifolia (Roxb.) Thwaites	U80706
	Ottelia alismoides (L.) Pers.	AB004895
	Stratiotes aloides L.	AB004896
	Thalassia hemprichii (Ehrenberg) Ascherson	AB004897
	Vallisneria asiatica Miki	AB004898
Juncaginaceae	*Cycnogeton procerum* Buchenau	U80713
	Triglochin maritimum L.	AB088811
Lilaeaceae	*Lilaea scilloides* (Poir.) Hauman	U80715
Limnocharitaceae	*Hydrocleys nymphoides* (Willd.) Buchenau	AB004900
	Limnocharis flava (L.) Buchenau	AB088807
Najadaceae	*Najas flexilis* (Willd.) Rostk amd W.L.E. Schmidt	U03731
Potamogetonaceae	*Groenlandia densa* (L.) Fourr.	U80720
	Potamogeton distinctus A. Benn.	AB004901
Posidoniaceae	*Posidonia australis* Hook. f.	U80718
Ruppiaceae	*Ruppia megacarpa* L.	U80728
Scheuchzeriaceae	*Scheuchzeria palustris* L.	U03728
Zannicheliaceae	*Zannichellia palustris* L.	U03725
Zosteraceae	*Heterozostera tasmanica* (Martens ex Ascherson) den Hartog	U80730
	Phyllospadix torreyi S. Watson	U80731
	Zostera japonica L.	AB125353
Araceae	*Gymnostachys anceps* R. Br.	AB088806
	Lasia spinosa (L.) Thwaites	L10250
	Orontium aquaticum L.	AJ005632
	Symplocarpus foetidus (L.) Salisb. ex Nutt.	L10247
	Xanthosoma sagittifolium (L.) Schott	L10246
Lemnaceae	*Lemna trisulca* L.	AY034237

mony-informative characters. The percentage of phylogenetically informative characters was 87.38%. Parsimony analysis of the morphological matrix produced 9348 parsimonious trees of 284 steps with $CI = 0.4572$, $RI = 0.8518$, $HI = 0.5428$, and $RC = 0.4139$.

The 50% majority rule consensus tree of 9348 most parsimonious trees based on the morphological data was weakly supported without resolution. The Alismatales was resolved as monophyletic with a low bootstrap value (<50%). However, the morphological data still identified several strongly supported monophyletic groups, e.g., the clade of Hydrocharitaceae with a bootstrap support of 94%, the Alismataceae subclade (100%), the Limnocharitaceae subclade (94%), the Cymodoceaceae subclade (89%), the Zosteraceae subclade (95%), and the Potamogetonaceae subclade (80%). The 50% majority rule consensus tree of these trees showed that Hydrocharitaceae might be the sister to the remainders that were divided into two clades with no internal supports. The first one included Alismataceae, Limnocharitaceae, Butomaceae, Aponogetonaceae, Scheuzeriaceae, Lilaeaceae, and Juncaginaceae with 62% bootstrap support, which was subdivided into two subclades with low internal supports, i.e., a weakly supported Alismataceae, Limnocharitaceae, Butomataceae, and Aponogetonaceae subclade; and a Lilaeaceae, Juncaginaceae, and Scheuzeriaceae subclade with no support (<50%). The other clade included the remaining seven families within Alismatales, where Potamogetonaceae and Ruppiaceae are basal with 96% bootstrap support, followed by Zannichelliaceae and Najadaceae in turn; Posidoniaceae and Zosteraceae together are a sister group of Cymodoceaceae with 55% bootstrap support. The topology indicated that Lilaeaceae and Juncaginaceae were unresolved polychotomies. The low resolution of interrelationships among the main clades is likely to be due to characters used in the

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analysis that were not chosen to study internal Alismatales phylogeny.

3.2. Phylogenetic analysis of molecular data

Sequence alignment of the *rbc*L sequences yielded 1274 bp, of which 801 were variable sites, 94 were variable characters that are parsimony-uninformative, and 287 are parsimony-informative variable sites. The percentage of phylogenetically informative sites was 24.28%. Parsimony analysis of the data yielded 21 most parsimonious trees of 1267 steps with $CI = 0.3783$, $RI = 0.573$, $HI = 0.6217$, and $RC = 0.2950$.

The strict consensus of these trees indicates two major lineages in Alismatales: One contains five families arranged in two subclades, consisting of (1) Alismataceae and Limnocharitaceae, and (2) Butomaceae, Hydrocharitaceae, and Najadaceae. The bootstrap value of this lineage is 80%. The other lineage includes 10 families, in which: (1) Aponogetonaceae and Scheuchzeriaceae are basal; (2) one clade constitutes the families Lilaeaceae and Juncaginaceae; (3) one clade includes Cymodoceaceae, Posidoniaceae, and (4) another clade comprises Ruppiaceae, Zosteraceae, Potamogetonaceae, and Zannichelliaceae where Ruppiaceae is basal. The bootstrap value of this lineage is 82%. The monophyly of the Alismatales is strongly supported with a bootstrap value of 97%. The strict consensus tree also indicates some unresolved polytomies: (1) Hydrocharitaceae and Najadaceae; (2) Alismataceae and Limnocharitaceae; (3) Lilaeaceae and Juncaginaceae, and (4) Cymodoceaceae and Posidoniaceae.

3.3. Combined data sets: Congruence indices and phylogenetic analyses

The I_{MF} index (0.0612) indicated that the incongruence that was less then 10% was attributable to differences between data sets. This is a relatively low level of incongruence [42,43]. However, the partition homogeneity test indicated that the molecular and morphological data sets have significantly different phylogenetic structures. The null hypothesis that the two data sets are homogeneous was rejected ($P = 0.01$).

The combination of morphological and *rbc*L data sets produced four trees, and the strict consensus tree, of 1801 steps with $CI = 0.4000$, $RI = 0.7087$, $HI = 0.6000$, and $RC = 0.3664$, was well resolved (Fig. 1). The only difference among the four trees was the relative placement of genera *Ottellia*, *Blyxa*, and *Lagarosiphon* of the Hydrocharitaceae. This data set included 1181 base pairs and 103 morphological characters (687 variable characters; 363 informative characters). The percentage of phylogenetically informative sites was 28.27%.

With 287 informative character state changes possible in *rbc*L and 79 possible in the morphological data set, the phylogenetic trees from the combined data set (Fig. 1) show more shifts to molecular trees than to the morphology trees. The monophyly of the Alismatales, again, is strongly supported with a bootstrap value of 99%. The strict consensus of these trees indicates two major lineages in the Alismatales, as seen in the molecular data. One clade includes four families, where Hydrocharitaceae and Butomataceae are basal in turn, a subclade of Limnocharitaceae and Alismataceae, and their sister relationships with Butomataceae with 100% bootstrap support. The other clade contains eleven families, where Aponogetonaceae is a sister group to the other ten families in this clade, which received 50% bootstrap value in the combined analysis. Scheuchzeriaceae diverged as the next branch and are a sister to the remainders with 52% bootstrap support. This is followed by a subclade consisting of Juncaginaceae and Lilaeaceae, which are placed as a sister group of the subclade comprising the other seven families. However, the placement of Najadaceae, Posidoniaceae, Ruppiaceae, Scheuchzeriaceae, and Aponogetonaceae is still uncertain in the current analyses. Lilaeaceae and Juncaginaceae are still realized as unresolved polychotomies.

3.4. Analysis of morphological characters

After analyzing the distribution of each character and its states on the phylogenetic tree, characters 2, 3, 9, 17, 21, 37, 38, 42–45, 47, 50, 55, 60, 63, 64, 76, 82, 87–89, 93, and 95 were recovered as homologous characters, with the other characters homoplasious in the Alismatales (Fig. 2). A 'homoplasious character' means that its diverse states are due to convergent, parallel, or reverse evolution and not due to inheritance from a common ancestor. Such a character still contributes to constructing the phylogenetic tree in a cladistic analysis (Fig. 2), but it is prone to override the exact characters that may imply the real evolutionary history if over-weighted in building a phylogeny.

4. Discussion

4.1. Incongruence between morphological and rbcL analyses

Partition homogeneity tests between morphological data and *rbc*L sequence data showed incongruence ($P = 0.01$). The results demonstrate that morphological data possess different phylogenetic information compared with *rbc*L data, and indicate that there are some heterogeneity among the data partitions. The phylogeny produced by the original (non-randomized) data sets is significantly shorter than the trees produced when the two data sets were randomly recombined. In other words, a given character from one data set tends to support other characters from the same data set more strongly than it supports characters from the other data set. Thus, partition homogeneity tests indicated substantial incongruence between the morphology and *rbc*L data sets. However, the original Mickevich–Farris measures of percent variation indicated little variation between morphology and *rbc*L data sets ($I_{MF} = 0.0612$).

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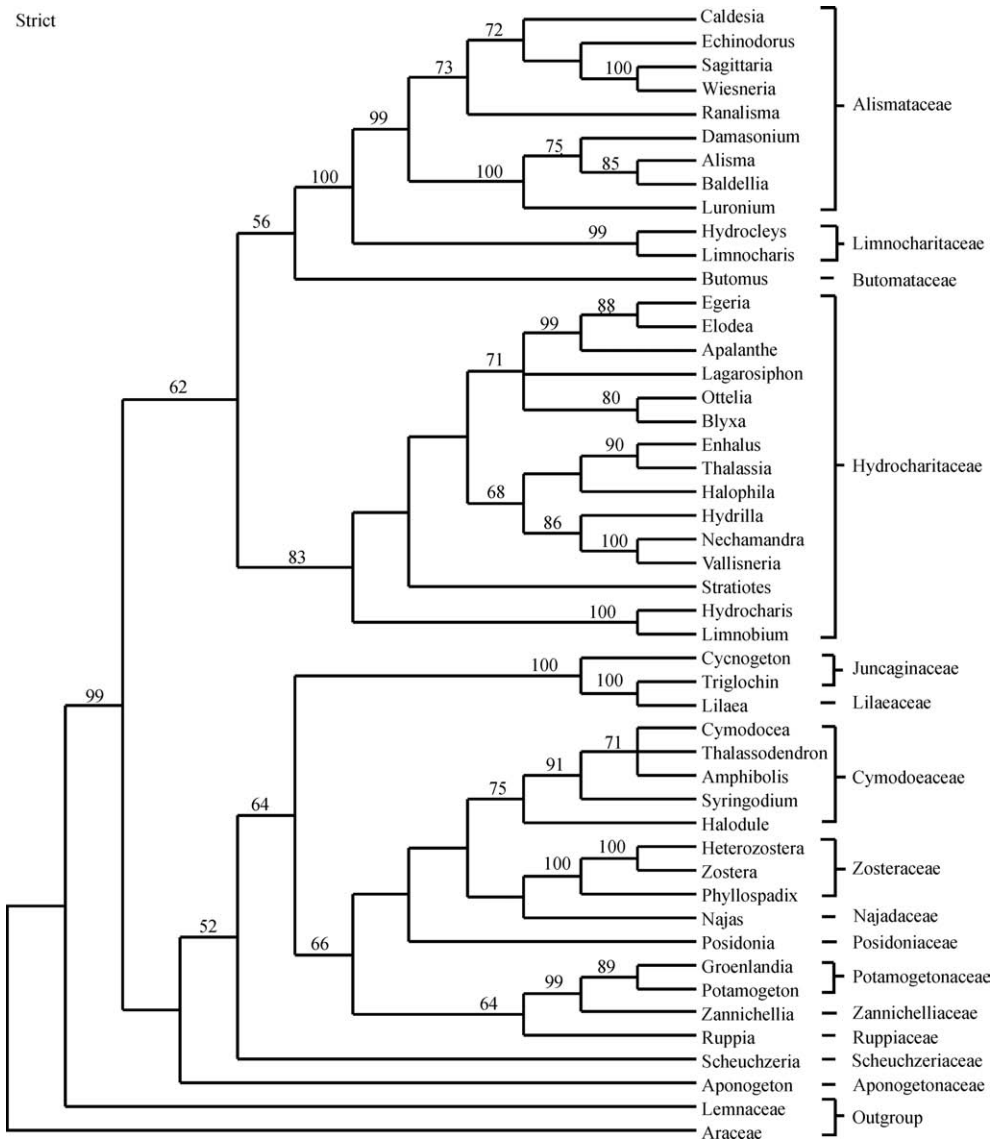

Fig. 1. Strict consensus tree of four most parsimonious trees resulting from a combined analysis of morphology and *rbc*L data. Tree length = 1801 steps, *CI* (excluding uninformative characters) = 0.4000, *HI* = 0.6000, *RI* = 0.7087. Bootstrap values greater than 50% are shown as percentages on the branches.

In addition, the summary statistics resulting from the parsimony analysis of the morphological data set are clearly worse than those from the combined data set (Table 2), and the bootstrap support for most groups is lower in the morphological trees, as is the resolution. In contrast, the number of most parsimonious trees is much higher in the morphological analyses (9348 vs. 12). Therefore, although morphological characters are few, they contain a considerable amount of incongruence among themselves, probably the result of errors in homology assessment [44]. These morphological characters seem to contain poor recoverable signal for the reconstruction of species phylogeny. The fact that the topology of the cladogram from the morphological matrix is incompatible with that from the *rbc*L matrix seems to further indicate that our interpretation of the morphological data set contains an excess of incorrect homology assessments.

The appropriate treatment of multiple independent data sets is an area of debate in systematics [19,35,45–49]. A total evidence approach advocates that combined analysis improves the opportunity to detect phylogenetic signals amid background noise, by increasing the number of characters [50]. In addition, some authors have indicated that the partition homogeneity test is extremely conservative [39,51]. Furthermore, such incongruence is biologically more interesting since they may be the indicators of previously unsuspected biological processes [52–55].

Based on the above argument, and given the results of both Partition homogeneity tests and Mickevich–Farris measures, we merged the data together into a single matrix (total evidence) because we believe that the combined analysis of the two data sets constitutes a homology test for the morphological characters against the molecular characters [56]. The results of such a test can be read on the resulting

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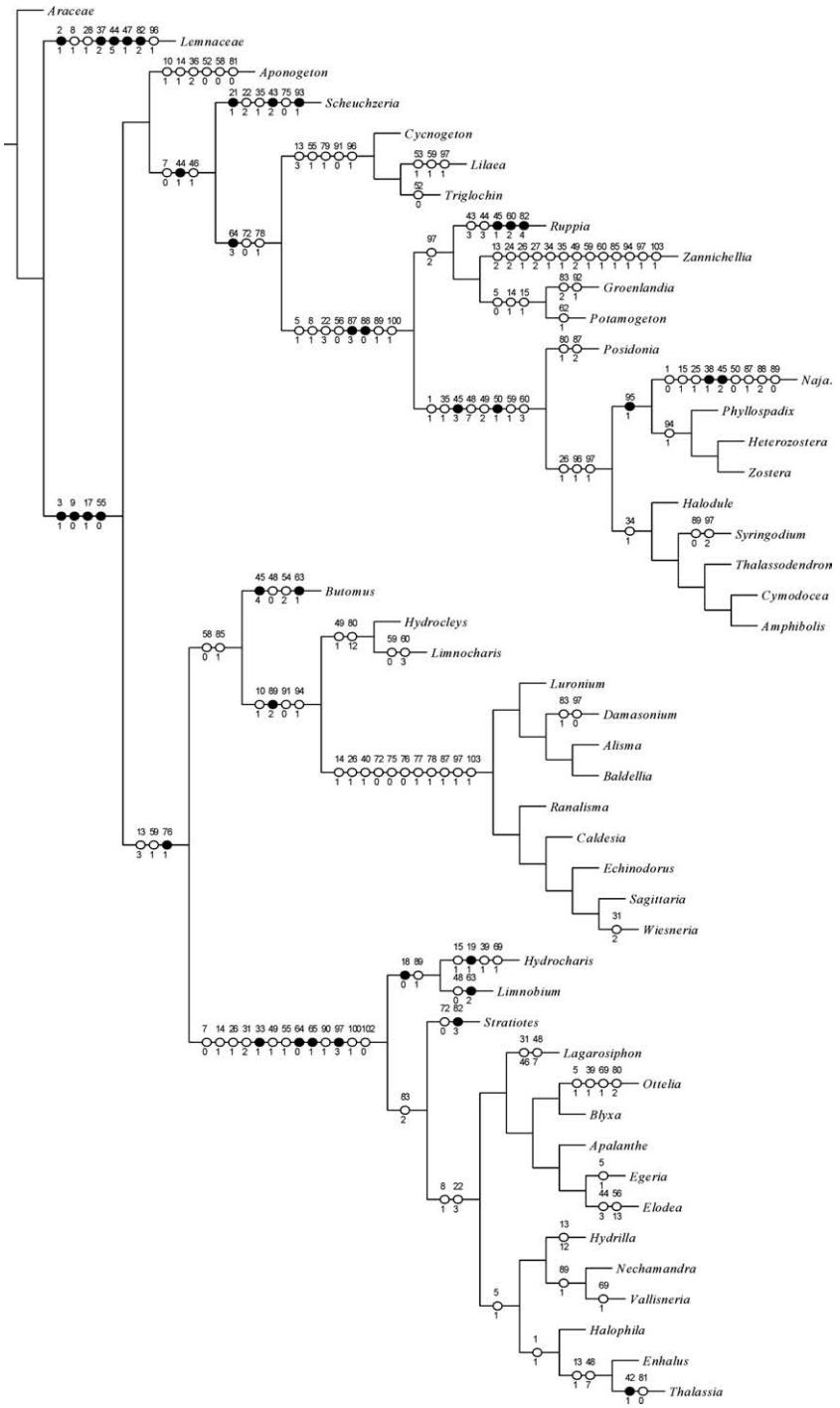

Fig. 2. One of the four most parsimonious trees from the combined analysis, with morphological characters mapped at the nodes. The numbers above branches indicate characters; the numbers below branches refer to corresponding character states. Solid black circles represent homologous characters with empty circles representing homoplasious characters.

cladograms generated from the parsimony analysis of the combined matrix, which provide justification for letting molecular characters decide which of the morphological characters contain more noise in reconstructing the intrageneric phylogeny of the Alismatales (Fig. 2). Given that the molecular data set appears to convey more phylogenetic signal for reconstructing the intrageneric phylogeny, if we were to merge the molecular data together with a high number of morphological characters containing much homoplasy, the true phylogenetic signal might be partially overridden. To better understand the conflicts between the morphological and molecular data sets, specific morphological characters contributing to this incongruence are examined later.

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Table 2
Comparison of metrics for the various data sets.

Data set	Total length	No. of variable char.	No. of informative char.	No. of trees	Length of trees	*CI*	*RI*	*RC*
Morphological	103	12	79	9348	284	0.4572	0.8518	0.4139
*rbc*L	1182	94	287	21	1272	0.3783	0.6898	0.2950
Combined	1284	687	363	4	1801	0.400	0.7087	0.3664

4.2. Combined and independent analysis

Preliminary summaries of interfamilial phylogenetic relationships in the Alismatales have been discussed by Les and Haynes [17], and Les et al. [3,18], and the references therein. The analysis given here presents a more comprehensive estimation of relationships, with analyses of molecular and morphological data sets based on the study of the majority of the genera in the order. The outcome from the *rbc*L sequence-based data set in this study is similar to that of Les et al. [3], showing the presence of the same two major clades. However, they differ in details such as the topology of the families in the complex clade (Cymodoceaceae, Posidoniaceae, and Ruppiaceae), and the placement of two fresh-water families (Aponogetonaceae and Scheuchzeriaceae). The low bootstrap value of this clade in the earlier study and the unresolved polytomies in the present study indicated that the *rbc*L gene is not capable of resolving the position of Cymodoceaceae, Posidoniaceae, and Ruppiaceae and that additional gene sequences should be included in further studies.

The molecular data provided a rather different picture from the morphology-based phylogeny herein. The combined data topology shows several shifts in the direction of the molecular results, indicating that including morphological data may give better phylogenetic results. However, maximum parsimony analysis of the combined data produced a topology (Fig. 1) that was only slightly more resolved than that based on *rbc*L alone.

The consensus of evidence from molecular and morphological studies argues strongly for the monophyly of the Alismatales, characterized by the following synaptomorphies: root-hair cells shorter than other epidermal cells (character 3), sieve-tube plastids with starch grains absent (character 9), intravaginal squamules present (character 17), and apocarpy carpel (character 55). Cladograms of the Alismatales generated from molecular data and morphological data differ mainly by the placement of Najadaceae and Ruppiaceae.

The topology in the combined cladogram for Hydrocharitaceae, Butomataceae, Alismataceae, and Limnocharitaceae is consistent with the cladogram presented by Dahlgren et al. [57], Les and Haynes [17], and Les et al. [3,18]. However, the relationships among the remaining representatives are different from the results from Les et al. [3], especially for the 'Cymodoceaceae complex', the relationships between Najadaceae and Hydrocharitaceae, as well as the intergeneric relationships of Hydrocharitaceae.

Based on *rbc*L data, in spite of the great differences in the morphology and the anatomy of their reproductive structures, as well as their modes of pollination, Les et al. [3] treated the families Cymodoceaceae, Posidoniaceae, and Ruppiaceae together as one phylogenetic unit, the 'Cymodoceaceae complex', to distinguish it from the other seagrass groups such as the Zosteraceae and the marine Hydrocharitaceae. However, the degree of internal supported (bootstrap values) for the monophyly of the 'Cymodoceaceae complex' is not particularly high (40%). Cox and Humphries [58] have suggested that Zosteraceae, Posidoniaceae, and Cymodoceaceae represent a monophyletic clade, but their analysis was limited by the small number of taxa considered. Our combined analysis shows a close relationship between the Ruppiaceae and the clade Potamogetonaceae–Zannichelliaceae, with a 64% bootstrap value, and between the Posidoniaceae and the clade Cymodoceaceae–Zosteraceae–Najadaceae, which is mainly supported morphologically by filiform pollen, exine absent or greatly reduced of pollen sculpturing, pollen wall ultrastructure atectate, and filiform stigma.

Within the Alismatales, contemporary taxonomists have often assigned the families Hydrocharitaceae and Najadaceae to different suborders Hydrocharitales. 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. Les et al. [3,18] supported the hypothesis that the Najadaceae are implicated either as the sister group to, or an integral member of, the Hydrocharitaceae. Analyses of morphological and molecular combined data sets (Figs. 1 and 2), however, indicated that Najadaceae have a much closer phylogenetic relationship to families of the 'Najadales' (Cymodoceaceae, Potamogetonaceae, Ruppiaceae, Scheuchzeriaceae, Zannichelliaceae, Zosteraceae) than to the Hydrocharitaceae.

Hydrocharitaceae occupy a wide spectrum of habitats, from freshwater to marine, and exhibit remarkable diversity in vegetative and reproductive morphology and pollination mechanisms. The family illustrates stages in the evolution of angiospermous plants inimitably adapted to life in water. Yet, intergeneric relationships have not been resolved confidently enough to facilitate critical evaluation of evolutionary trends. The *rbc*L and combined results showed that *Hydrocharis* and *Limnobium* occupy a basal position in the family and are resolved in both the molecular and combined analyses, whereas highly specialized taxa are derived phylogenetically. Marine genera (*Enhalus*, *Halophila*, *Thalassia*) are monophyletic. Morphologically similar submersed freshwater genera are polyphyletic. Morphologically, Hydrocharitaceae appear to be supported mainly by the presence of staminodia, intracarpel-

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lary fusion only by the secretion, inferior ovary, berry or berrylike fruit.

4.3. Evolutionary convergence of morphological characters

Morphological characters perceived as adaptive for aquatic life have arisen repeatedly among unrelated groups of the Alismatales, which includes all known marine angiosperms (12 genera) and all water-pollination angiosperms (17 genera) excluding the two dicotyledons genera (*Ceratophyllum* and *Callitriche*) [59]. The results of the combined analysis of morphological and *rbc*L data sets show that at least 29 of the 103 morphological characters require high amounts of homoplasy to be optimized in the representative most parsimonious tree (Fig. 2). Therefore, their coding as the same character is not confirmed by the parsimony analysis and instead seems to result from incorrect homology assessment. As Les et al. [3] stated, several characters used to characterize the Alismatales appear to be the consequence of parallel and convergent evolution associated with aquatic adaptations.

Members of the Alismatales are found in fresh, brackish and marine environments or in marsh, based on the habitat of the majority of species in a genus. The phenomenon that similar suites of characters evolved in different lineages in response to similar ecology is well demonstrated in the Alismatales, which developed convergent features as adaptations to the marine environment. For example, floral reduction, embryo type and embryo-sac formation, which are considered an outcome of abandoning insect pollination, seem to follow a general trend of more specialized habitats, in going from fresh, to brackish and finally marine environments.

Les and Haynes [17] re-examined hydrophily and unisexuality in light of the *rbc*L phylogeny and found both characters have evolved many times. The high likelihood of parallelism for unisexuality and hydrophily is also acknowledged by Dahlgren and Rasmussen [16]. Chen et al. [7] examined the evolution of carpy based on the topology from *rbc*L phylogeny. Two independent origins of apocarpy in the Alismatales are explored in this study. Three separate origins of a single carpel and two separate origins of syncarpy in the order are also proposed. We re-explored the evolution of several characters that are of great evolutionary interest in themselves and that probably contribute to the conflict between the morphological and the molecular phylogeny herein.

Reductions and losses of perianth occur within several alismatid families. An undifferentiated perianth appears to have been the basal condition among the Hydrocharitaceae and was then lost at least twice in three genera of Zosteraceae, Posidoniaceae, Zannichelliaceae, and Aponogetonaceae (Fig. 3). The presence of an undifferentiated perianth, or losses of perianth, occurred through the evolution of showy, petaloid sepals, associated with highly specialized mechanisms of entomophilous pollination. It is interesting to note that *Aponogeton* genera with both an undifferentiated perianth and a differentiated perianth, is indeed animal pollinated. In contrast, the Butomataceae, Hydrocharitaceae, Alismataceae, and Hydrocharitaceae families with a differentiated perianth are all animal pollinated or hydrophily. A homologous loss of perianth in Cymodoceaceae, Najadaceae, Posidoniaceae, Potamogetonaceae, Zannichelliaceae and Zosteraceae as suggested by Dahlgren and Rasmussen [16] is not a conclusion that can be accepted with confidence; nor is the absence of

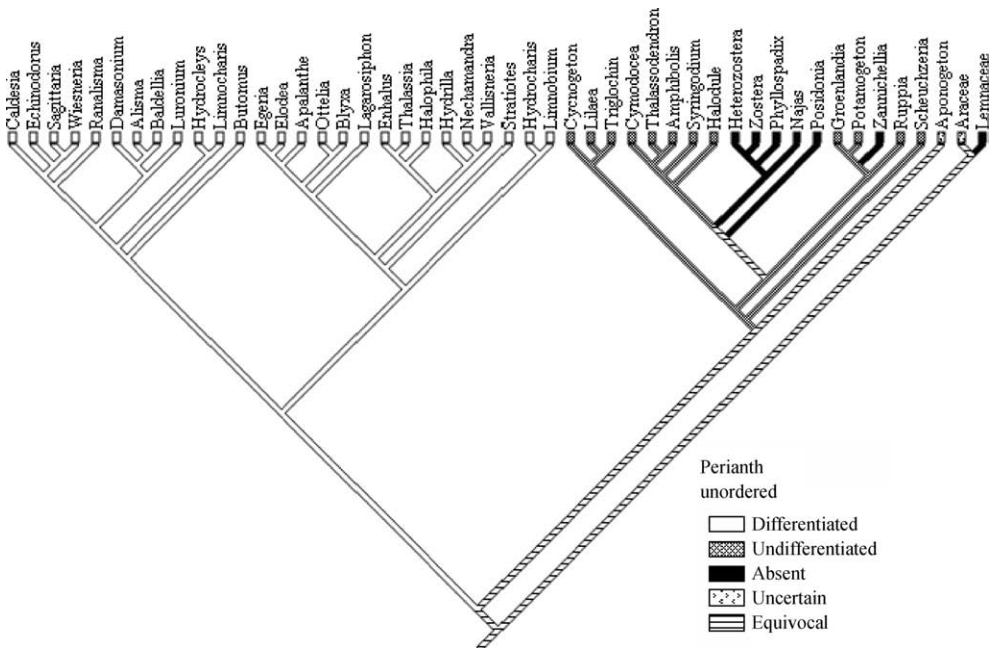

Fig. 3. Reconstruction of perianth (character 27) for selected most parsimonious trees based on the combined data sets.

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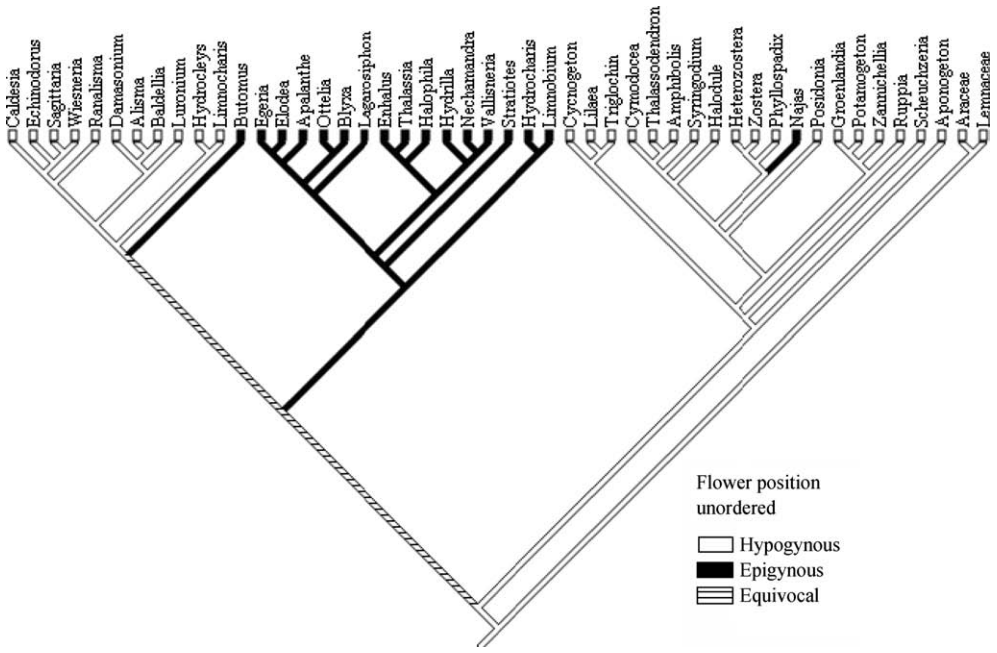

Fig. 4. Reconstruction of flower position (character 25) for selected most parsimonious trees based on the combinined data sets.

perianth in the Alismatales as a homology suggested by Dahlgren et al. [57].

Hypogynous flowers appear to have re-evolved at least twice in the families within Alismatales, in Alismataceae, Limnocharitaceae, and in other alismatid families except for Hydrocharitaceae, Butomataceae and Najadaceae (Fig. 4). Dahlgren et al. [57] considered epigyny as restricted to (and autapomorphic for) Hydrocharitaceae. However, in its later developmental stages, the flower of both Butomataceae and Najadaceae tends to become epigynous [60]. The floral outer envelope of Najadaceae has been interpreted as a bract similar in origin, structure, and vascularization to the spathe found in Hydrocharitaceae [61]. Some have regarded the structure as an abnormal feature resulting from a morphogenetic shift [62]. However, at present there is no convincing evidence to resolve whether the flower of Najadaceae is hypogynous and apocarpous or epigynous and syncarpous. Thus, there is still doubt about its use as one of the morphological synapomorphies defining the Najadaceae, Hydrocharitaceae and Butomataceae.

Several other morphological characters also appear to be highly homoplasious within the alismatid families.

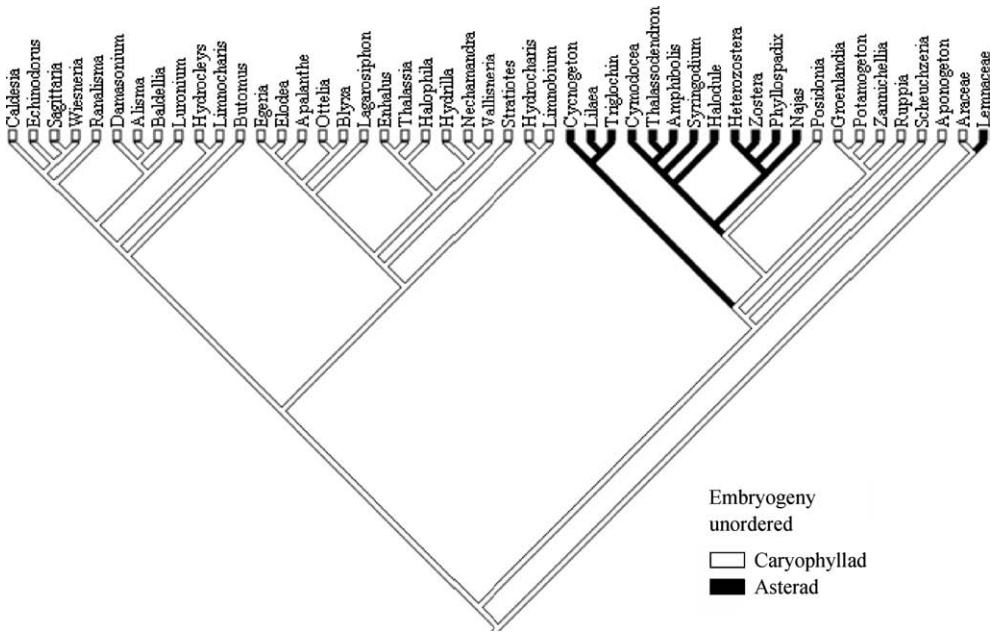

Fig. 5. Reconstruction of embryogeny (character 96) for selected most parsimonious trees based on combined data sets.

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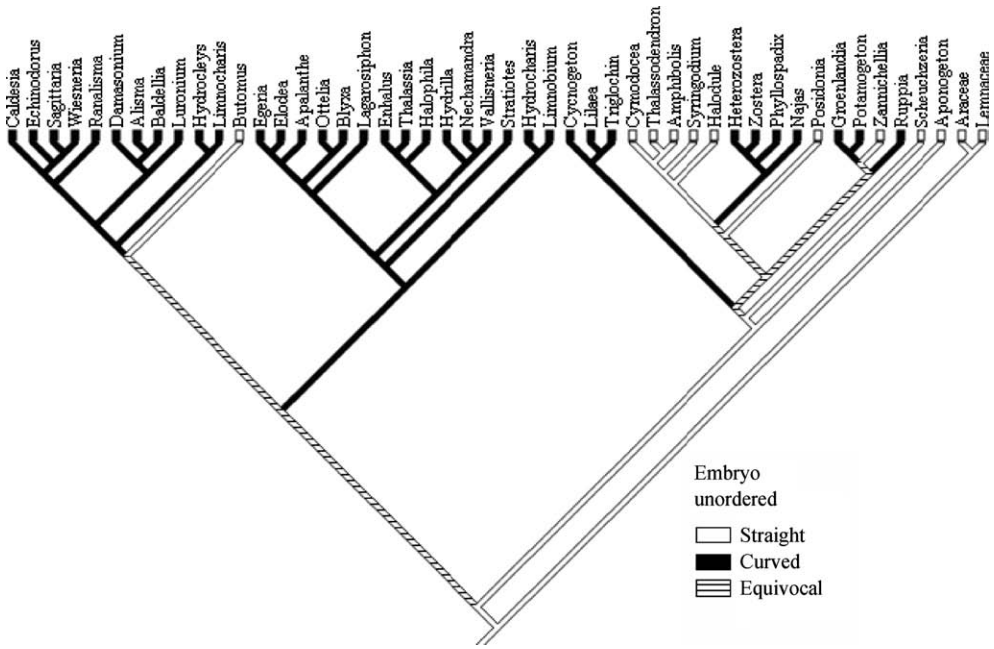

Fig. 6. Reconstruction of embryo (character 101) for selected most parsimonious trees based on the combined data sets.

For example, asterad embryogeny appears to have arisen at least twice (Fig. 5). Embryo straight has arisen at least five times, in Aponogetonaceae, Scheuchzeriaceae, Zannichelliaceae, Posidoniaceae, Cymodoceaceae, and Butomataceae (Fig. 6). *Polygonum* embryo-sac formation evolved twice independently in Zannichelliaceae and Alismataceae. The adaptive significance, if any, of embryo features is unknown. The highly labile nature of embryo characteristics in the alismatid families under study raise doubts on the suitability of these characters for phylogenetic analyses.

Acknowledgments

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Appendix A. Character states used in cladistic analysis

1 Habitat: hydrophytic, fresh or brackish (0), hydrophytic, marine (1), helophytic (2)
2 Shoot type: shoot differentiation into stem and leaves (0), shoot reduced to platelike bodies (Lemnaceae) (1)
3 Root-hair cells: equal to other epidermal cells (0), shorter than other epidermal cells (1)
4 Velamen: absent (0), present (1)
5 Root vessels: present (0), absent (1)
6 Stem vessels: present (0), absent (1)
7 Secretory ducts in stem: absent (0), present (1)
8 Stele: atactostely (0), protostele or strand, reduced (1)
9 Sieve-tube plastids with starch grains: absent (0), present (1)
10 Laticifers in stem: absent (0), present (1)
11 Leaf dissection: simple (0), lobed or compound (1)
12 Ptyxis: aduplicate (0), supervolute (1), conduplicate (2), involute (3)
13 Phyllotaxy: alternate or clustered on branches (0), opposite (1), spiral (2), basal concentrations or ranked (3)
14 Petiole: absent (0), present (1)
15 Stipules: absent (0), present (1)
16 Ligules: absent (0), present (1)
17 Intravaginal squamules: absent (0), present (1)
18 Primary venation: palmate (actinodromous or acrodromous) or crowded (pinnate with crowded basal secondaries, upward decreasing angle) (0), pinnate with secondaries at more or less constant angle (1), parallel to midvein from base of blade to apex (2), absent (3)

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Appendix A (*continued*)

19 Reticulate venation: absent (0), present (1)
20 Pubescence: absent (0), present (1)
21 Leaf/frond axil hairs: absent (0), present (1)
22 Stomata: anomocytic (0), paracytic (1), tetracytic (2), absent (3)
23 Leaf/frond air canals: absent (0), random (1)
24 Flowers clustering: racemes or panicles (0), spikes or spikelike inflorescences (1), small, dense clusters in leaf axils (2), spadix (with a single spathe) (3)
25 Flower position: hypogynous (0), epigynous (1)
26 Sexuality of flowers: bisexual (0), unisexual (1)
27 Perianth: differentiated (0), undifferentiated (1), absent (2)
28 Tepals: present (0), absent (1)
29 Perianth connation: absent (0), present (1)
30 Perigonal nectarines: absent (0), present (1)
31 Stamens: one (0), two (1), three (2), four (3), six (4), nine (5), numerous (more than ten) (6)
32 Stamens with flat petaloid appendage: absent (0), present (1)
33 Staminodia: absent (0), present (1)
34 Connate stamen filaments: absent (0), present (1)
35 Connective protrusion: absent (0), present (1)
36 Anther dehiscence orientation: introse (0), extrose (1), latrose (2)
37 Anther dehiscence: longitudinal (0), poricidal (1), transverse slit (2)
38 Anther wall formation: monocotyledonous type (0), reduced type (1)
39 Sporangia per anther: tetrasporangiate (0), bisporangiate (1)
40 Endothecial wall thickenings: spiral (0), 'girdle-like' (1)
41 Tapetum types: 'glandular-secretory' (0), 'plasmodial-amoeboid' (1)
42 Microsporogenesis: successive (0), simultaneous (1)
43 Pollen unit: monads (0), tetrads (1), dyads (2), moniliform chains (3)
44 Pollination mechanisms: entomophily (0), anemophily (1), male flower-epihydrophily (2), pollen-epihydrophily (3), hypohydrophily (4), 'contact' pollination (5), autogamy (6)
45 Pollen shape: spheroidal (0), ovoid (1), ellipsoidal (2), filiform (3), boat-shaped (4)
46 Pollen aperture types: monosulcate (including monoulcerate) (0), inaperturate (1), bisulcate (2), two to many foraminate (3), polyaperturate (4), 1–4 porate (5)
47 Pollen aperate margin: non-annulate (0), annulate (1)
48 Pollen sculpturing: reticulate (0), echinate (1), microreticulate (2), granular (3), psilate (4), scabrate (5), striate (6), exine absent or greatly reduced (7)
49 Pollen wall ultrastructure: columellate (0), granular (1), atectate (2)
50 Pollen disperse: pollen grains dispersed in their original form (0), pollen grains germinating in water, dispersed as pollen tubes (1)
51 Endexine: absent (0), present (1)
52 Pollen nuclei: two (0), three (1)
53 Carpel number per flower: more than one (0), one (1)
54 Carpel size: small (1–3 mm long) (0), medium (4–9 mm) long (1), large (10–12 mm long) (2), very large (more than 20 mm long) (3)
55 Carpel fusion: apocarpy (0), syncarpy (1), solitary carpel (2)
56 Carpel form: ascidiate (0), plicate (1), symplicate-synascidiate (2), symplicate (3), synascidiate (4)
57 Carpel apex extending into two lateral tips: absent (0), present (1)
58 Nectar production on carpel sides: present (0), lacking (1)
59 Style: absent (stigma sessile) (0), present (elongated apical portion of carpel distinctly constricted relative to the ovary, including cases in which the apical portion is mostly or entirely stigmatic) (1)
60 Stigma shape: capitate (0), wide, discoid (1), peltate (2), filiform (3)
61 Stigma: extended (all around the ventral slit or extending half or more of the way down the style-stigma zone) (0), restricted (above slit or around its upper part) (1)
62 Stigmatic surface: dry (0), wet (1)
63 Stigma papillate: unicellular only (or stigma smooth) (0), some or all pluricellular-uniseriate (1), some or all multicellular-multiseriate (2)

(*continued on next page*)

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Appendix A (*continued*)

64 Intracarpellary fusion: angiospermy type 1 (0), angiospermy type 2 (1), angiospermy type 3 (2), angiospermy type 4 (3)
65 Ovary position: superior (0), inferior (1)
66 Ovary locule filled with secretion: absent (0), present (1)
67 Pollen tube transmitting tissue: one-layered and well differentiated (0), one-layered and only weakly differentiated (1)
68 Compitum: absent (0), present (1)
69 Tanniferous cells in the Carpel Wall: absent (0), present (1)
70 Ethereal oil cells in the Carpel Wall: absent (0), present (1)
71 Mucilage cells in the Carpel Wall: absent (0), present (1)
72 Cells with oxalate crystals in the Carpel Wall: absent (0), present (1)
73 Cells with oxalate druses in the Carpel Wall: absent (0), present (1)
74 Cells with oxalate raphides in the Carpel Wall: absent (0), present (1)
75 Intercellular cavities: absent (0), present (1)
76 Ovules per locule: few (<5) (0), numerous (>=5) (1)
77 Ovules filling the locules: absent (0), present (1)
78 Ovules in close contact with the ovary wall: absent (0), present (1)
79 Ovules size: medium (0.3–0.5 mm long) (0), large (0.6–1.0 mm long) (1), very large (more than 1.0 mm long) (2)
80 Ovules type: crassinucellar (0), pseudocrassinucellar to tenuinucellar (1), tenuinucellar (2)
81 Ovules: unitegmic (0), bitegmic (1)
82 Micropyle formation: the single integument (0), the inner integument (1), the outer integuments (2), the inner and outer integuments (3), lacking (4)
83 Nucellus size: narrow (less than 0.1 mm broad) (0), medium (0.1–0.2 mm broad) (1), broad (more than 0.2 mm broad) (2)
84 Nucellus with meiocyte: a single meiocyte (0), 1–3 meiocyte (1)
85 Nucellar cap: absent (0), present (1)
86 Cells with oxalate crystals in the ovules: absent (0), present (1)
87 Placentae: laminar (0), basal (1), marginal (2), apical (3), axile (4)
88 Ovule direction: pendent (0), horizontal (1), ascendant (2), more or less along the length of the ovary (3), irregularly directed (4)
89 Ovule curvature: anatropous (including hemianatropous) (0), orthotropous (including hemitropous) (1), campylotropous (2)
90 Shape of outer integument: semiannular (0), annular (1)
91 Lobation of inner integument: unlobed (0), lobed (1)
92 Thickness of outer integument: two cells (0), two and three to four (1), four and five, or more (2)
93 Thickness of inner integument: two or three cells (0), four cells (1), five and more cells (2)
94 Parietal cell in ovule: present (0), absent (1)
95 Endosperm formation: helobial (0), nuclear (1), cellular (2)
96 Embryogeny: caryophyllad (0), asterad (1)
97 Fruit types: follicle (0), achene (1), drupe (2), berry or berrylike (3), capsule (4), nutlet (5)
98 Seed storage: endosperm absent (0), endosperm (1)
99 Starchy endosperm: none or little (0), abundant (1)
100 Macropodous embryos: absent (0), present (1)
101 Embryo: straight (0), curved (1)
102 Embryo type: *Trillium* type (0), *Urginea* type (1)
103 Embryo-sac formation: *Allium* type (0), *Polygonum* type (1)

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Appendix B. Data matrix showing character states for all taxa included in this analysis

Taxon/Node	1111111111222222222233333333334444444445555555556666666666777777777788888888899999999990000 12345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123
Alisma	B0100110010331001200011100A0000400001000110000403000100010010010000000000000110E1100101220000100100011 1
Baldellia	B0100110010331001200011001000040000100011000040300010001001001000000000000011001100101220000100100011 1
Burnatia	B0100110010331001200011001000050000100011000040100010001001001000000000000011001100101220000100100011 1
Caldesia	B0100110010331001200011001000T0000100011000040F0001000100101001000000000000011001100101220000100100011 1
Damasonium	B0100110010331001200011001000040000100011000040F0001000100101001000000000000A1101111010122000010000001 11
Echinodorus	B0100110010331001200011001000M0000100011000040F0001000100101001000000000000011011100101220000100100011 1
Luronium	B0100110010331001200011001000040000100011000040100010001001001000000000000011001100101220000100100011 1
Limnophyton	B0100110010331001200011001000040000100011000040100010001001001000000000000011011100101220000100100011 1
Ranalisma	B0100110010331001200011001000040000100011000040100010001001001000000000000011001100101220000100100011 1
Sagittaria	B010011001A3310012000110 0A0000T0000100011000040100010001001001000000000000011011100101220000100100011 1
Wiesneria	B0100110010331001200011001000020000100011000040100010001001001000000000000011001100101220000100100011 1
Aponogeton	0010011001030100120001100 0E000L00A0200001A0000020000000100001101 0A000001001A00000A100A22001200000000010
Butomus	0010?11000003000120001101000004000020000100040000001020110101011000000010011000011001002001100000000010
Amphibolis	1010110100000000012000312011000100111000010043107210102 0??113?0??0??0?1?????0?????????1?301????0011001010
Cymodocea	1010110100000000012000312011000100111000010043107210102 0??113?0?30??0?1?????0????11??1?301????0011001010
Halodule	1010110100000000012000312011000100111000010043107210102 0??113?0??0??0?1?????0?????????1?301????0011001010
Syringodium	1010110100000000012000312011000100111000010043107210102 0??113?0??0??0?1?????0???011??1?300????0012001010
Thalassodendron	1010110100000000012000312011000100111000010043107210102 0??113?0??0??0?1?????0?????????1?301????0011001010
Apalanthe	0010010100?2100120103101100002010010000100001011001 0?10?110?0001101 00??0011???01???000?????0003001100
Blyxa	0010010100?B1001201031011000I0100100001000010110010?10?110?0001101 00??001100?01F?0000?0??2000030011 00
Egeria	0010110100?2100120103101100005010010000100001011001 0?10?110?0001101 00??001100?01???000?1????0003001100
Elodea	0010010100?2100120103101100 0?0100100001 0A30101100103 1F?1100000110100010011001011 2000A2111200003001100
Enhalus	1010110100?1100120103101100002010010010100201071001 0?10?110?0?0?1101 00??001100?011??000?0????0003001100
Halophila	1010110100?31001E0103101100002010010 0A0103401011001 0?10?110?0?0?1101 00??001100?01??0000?0??0000003001100
Hydrilla	0010110100?E1001201031011000020100100A0100301011001 0?10?110?0001101 00??001100?011?A000?0????0003001100
Hydrocharis	0010010000?3110101101101100002010010010100001011001011D?1100000110110010011000 01?0?000H111000003001100
Lagarosiphon	0010010100?0100120103101100 0L0100100001002010710010?10?110?0001101 00??001100?01???000?1????0003001100
Limnobium	0010010000?3100100101101100002010010000100A0100100 10?1D?110?0201101 00??001100?01??0000?1????0003001100
Nechamandra	0010110100??1001201031011000E0100100001002010110010?10?110?0001101 00??001100?011?0000?1????0003001100
Ottelia	0010110100?3100120103101100 0J0100100101000010110010?10?110?0001101 10??001100?21??0000?0????0003001100
Stratiotes	0010010000?3100120101101100002010010000100000011001 0310?1101000110100000011002 0132?000H0A1200003001100
Thalassia	1010110100?1100120103101100 0S0100100A0113401071001 0?10?110?0?0?1101 00??001100?00???000?????0003001100
Vallisneria	0010110100?3100120103101100002010010000100201011001 0310?1100000110110110011001 01F100A0K1110000003001100
Cycnogeton	B0100100000030011200011000100040000100001001010000010?1101000003000000000010011011100012000E00010000100
Maundia	B0100100000030011200011000100060000100001001010000010?1101000003000000000010011011100030100E00010000100
Tetroncium	B0100100000030011200011000100040000100001001010000010?1101000003000000000010011011100012000E00010000100
Triglochin	B0100100000030011200011000100 0Q0000100001001010000000001G0100000300000000001001101110001 2000E00010000100
Lilaea	B0100100000030011200011 00A1000000001000010010100000011?1101100003000000000010011011100012000E00011000100
Butomopsis	2010011001033000120001100000005000020000100 0?3011001010110101001000000010011000E1100100220000100000011 0
Hydrocleys	0010011001033000120001100000000L0000200001000?3011001010110101001000000010011000E1100100220000100000011 0
Limnocharis	0010011001033000120001100000006000020000100003011001010?1003100100000000100110 00E1100100220000100000011 0
Najas	0010110100000010120003121120000000011 01A0100421072001 1?20?113????0100??1????0???011?00?120??100111001110
Groenlandia	0010010100 0?A11012000F1A0011003100010001100601000001A0001100003001010000010110011 2000H0E1110000020011 10
Potamogeton	0010010100 0?A11012000F1A0011003100010001100101000001A0001100010300101000001011001 12000H0E1100000200111 0
Posidonia	1010110100 00C0011200031100210 0?1001100001?0431072101 1?2??113????0??0????????0???11???0?201????0000001010
Ruppia	A0101101000?A000120?031A0011003100010001103311000001000011020 00?0?????????0???014?00?301??0000020011 10
Scheuchzeria	0010010000000C001?2011210001000400011000010210100000100011100000100000001000000000111?001E001210000000000
Zannichellia	0010110100002000120003120120000000111000010040100200100?0?111????0??0??1????0???0?11?01?301??0010010010111
Heterozostera	1010110100000000012000310012100010011000010043107210110 2??113????0??0??1????0????1???1?301????1111001110
Phyllospadix	1010110100000000012000310012100010011000010043107210110 20?113????0??0??1????0????1???1?301????1111001110
Zostera	1010110100000000012000310012100010011000010043107210110 20?113????0??0??1????0????111??1?F01??101111001110
Araceae	B00A0A101AA10A0A0EAA0EAC00E0AAP00A01A0A010A0DW0YA0AAA0EB010AAAA10101A001A1AAAAA0ARA0A0RBNAAE?0B0XAAA010
Lemnaceae	0100011110 0??0000H?1?2?C002100A0000120A010050011A0?1102B0101?1?1010??00????00?1012????4?A??A00B1DA1?010

Note: For certain characters, some taxa were assigned multiple character states because they were highly polymorphic and were analyzed as such: $A = 0$ and 1; $B = 0$ and 2; $C = 0$ and 3; $D = 0$ and 4; $E = 1$ and 2; $F = 1$ and 3; $G = 1$ and 4; $H = 2$ and 3; $I = 2$ and 5; $J = 2$ and 6; $K = 3$ and 4; $L = 4$ and 6; $M = 5$ and 6; $N = 0$ and 1 and 2; $O = 0$ and 1 and 3; $P = 0$ and 1 and 4; $Q = 0$ and 2 and 4; $R = 1$ and 3 and 4; $S = 2$ and 4 and 6; $T = 4$ and 5 and 6; $W = 0$ and 1 and 2 and 5; $X = 0$ and 2 and 3 and 4 and 5; $Y = 0$ and 1 and 2 and 4 and 5 and 6. ? indicates unknown or missing data.

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