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PHYLOGENETIC ANALYSES OF THE BRYOPSIDALES (ULVOPHYCEAE, CHLOROPHYTA) BASED ON RUBISCO LARGE SUBUNIT GENE SEQUENCES[1]

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Current taxonomy of the Bryopsidales recognizes eight families; most of which are further categorized into two suborders, the Bryopsidineae and Halimedineae. This concept was supported by early molecular phylogenetic analyses based on rRNA sequence data, but subsequent cladistic analyses of morphological characters inferred monophyly in only the Halimedineae. These conflicting results prompted the current analysis of 32 taxa from this diverse group of green algae based on plastid-encoded RUBISCO large subunit (*rbc*L) gene sequences. Results of these analyses suggested that the Halimedineae and Bryopsidineae are distinct monophyletic lineages. The families Bryopsidaceae, Caulerpaceae, Codiaceae, Derbesiaceae, and Halimediaceae were inferred as monophyletic, however the Udoteaceae was inferred as non-monophyletic. The phylogenetic position of two taxa with uncertain subordinal affinity, *Dichotomosiphon tuberosus* Lawson and *Pseudocodium floridanum* Dawes & Mathieson, were also inferred. *Pseudocodium* was consistently placed within the halimedinean clade suggesting its inclusion into this suborder, however familial affinity was not resolved. *D. tuberosus* was the inferred sister taxon of the Halimedineae based on analyses of *rbc*L sequence data and thus a possible member of this suborder.

Key index words: **Bryopsidales; Caulerpales; Chlorophyta; Phlyogeny; *rbc*L; RUBISCO; taxonomy; Ulvophyceae**

Abbreviations: **BI, Bayesian inference; ML, maximum likelihood; MP, maximum parsimony; MPT, most parsimonious tree; TLD, tree length distribution.**

The order Bryopsidales (also referred to as the Caulerpales, Codiales, and Siphonales) is comprised of green, mostly macroscopic, siphonous algae with multicellularity arising only in some taxa during sexual reproduction (Silva 1982). The Bryopsidales exhibit a cosmopolitan distribution; however, some groups are restricted to tropical marine environments. One genus, *Dichotomosiphon*, is found in freshwater habitats. Similarly, the Bryopsidales exhibit extremely broad morphological diversity (from the simple uniaxial siphonous construction found in *Bryopsis*, *Derbesia*, and *Caulerpa* to the complex interwoven multiaxial siphon patterns found in *Codium, Halimeda*, and *Penicillus*). Some genera are heavily calcified as in the genus *Udotea*, while the family Caulerpaceae and the majority of the suborder Bryopsidineae exhibit no calcification. Some bryopsidalean taxa are invasive and ecologically problematic and are known to flourish in temperate marine waters (e.g. *Caulerpa taxifolia* and *Codium fragile* subsp. *tomentosoides*; Bouk and Morgan 1957, Trowbridge 1995, Jousson et al. 1998).

Smith (1955) separated the Bryopsidales (his Siphonales) into the families Halicystidaceae (including the algae currently classified under the genus *Derbesia*), Bryopsidaceae (*Bryopsis*), Caulerpaceae (*Caulerpa*), Codiaceae (*Codium*) and Dichotomosiphonaceae (*Dichotomosiphon* and *Boodleopsis*). Hillis-Colinvaux (1984) subdivided the order into two suborders on the basis of thallus morphology, reproduction, plastid types and geographic distributions. She defined the Bryopsidineae (including *Bryopsis, Codium*, and *Derbesia*) based on non-holocarpic reproduction and the Halimedineae (e.g. *Caulerpa*, *Halimeda*, and *Udotea*) on holocarpic reproduction. Furthermore, Hillis-Colinvaux (1984) noted that the Halimedineae exhibit heteroplasty (containing both chloroplasts and amyloplasts) while amyloplasts are absent in bryopsidinean taxa. Global distribution patterns vary as well. That is, bryopsidinean taxa generally inhabit temperate, tropical, and subtropical marine waters, while halimedinean taxa are generally restricted to tropical and subtropical habitats. However, exceptions to these general patterns occur, including the occurrence of some *Caulerpa* species in temperate waters (e.g. *Caulerpa taxifolia*, Jousson et al. 1998, 2000) and non-holocarpy in *Caulerpella* (Prud'homme van Reine and Lokhorst 1992).

Pseudocodium and *Dichotomosiphon* are two bryopsidalean genera with uncertain affinity at the subordinal level. Hillis-Colinvaux (1984) tentatively placed the genus *Pseudocodium* into the Bryopsidineae because of a reported common mannan cell wall component with the genus *Codium* (Dawes and Mathieson 1972). Likewise, cladistic analyses of morphological traits grouped *Pseudocodium* with *Codium* (Vroom et al. 1998). However, morphological features, including heteroplasty (Feldman 1946), suggest that the alga has more in common with the Halimedineae. In addition, Weber

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van Bosse (1896), Levring (1938), and Womersley (1955) noted that the formation of utricles in *Pseudocodium* is closer in affinity to *Halimeda* than to *Codium*. The freshwater alga, *Dichotomosiphon tuberosus*, possesses plastid features of the Halimedineae (i.e. heteroplasty, Moestrup and Hoffman 1973), however, sexual reproduction is non-holocarpic (Ernst 1902, Smith 1955). Previous results (Vroom et al. 1998, Hanyuda et al. 2000) inferred a sister relationship between *Dichotomosiphon* and a clade containing halimedinean taxa.

Previous morphology-based phylogenetic analyses of the order (Vroom et al. 1998) suggested monophyly of the Halimedineae. However, bryopsidinean taxa formed a non-monophyletic assemblage. In contrast, molecular phylogenies (Zechman et al. 1990, Hanyuda et al. 2000) supported the monophyly of both suborders; however these data sets contained limited numbers of bryopsidalean taxa. Recent molecular phylogenetic analyses of bryopsidalean taxa (Hillis et al. 1998, Woolcott et al. 2000, Fama et al. 2002, Kooistra 2002, Kooistra et al. 2002) were aimed at resolving more specific family, genus and species level relationships. The current study seeks to elucidate the phylogenetic position of major evolutionary lineages within the Bryopsidales. This was accomplished by phylogenetic analyses of the RUBISCO large subunit (*rbc*L) sequences for 32 bryopsidalean taxa. Sequencing and amplification of additional taxa was attempted but not successful with the methods described. To the extent possible with current taxon and character sampling, phylogenetic analyses sought to: (1) infer phylogenetic relationships among the Bryopsidales at the subordinal and familial levels; (2) compare *rbc*L sequence-based phylogenies to results obtained from previous data sets; and (3) determine the phylogenetic affinity of the genera *Pseudocodium* and *Dichotomosiphon*.

MATERIALS AND METHODS

Taxon sampling and outgroup selection. The *rbc*L data set contained 20 new sequences and twelve published sequences obtained from GenBank (Table 1). The final ingroup data set included representatives from two suborders, eight families, and 14 genera. Variable *rbc*L introns that were present in some taxa (e.g. the 1813 base pair intron in *Codium fragile*) were excluded from the data set. Outgroup selection was based on previous phylogenetic analyses of the Ulvophyceae. Relationships based on rRNA (Zechman et al. 1990) suggested the Dasycladales as a possible sister group to the Bryopsidales, but included an unresolved trichotomy with the Cladophorales. Because of these results, and because no published cladophoralean *rbc*L sequences are currently available, four published dasycladalean *rbc*L sequences (Zechman 2003) were used as outgroups to root phylogenetic trees.

DNA extraction, PCR, and sequencing template purification. Field collected samples were frozen in liquid nitrogen and stored at –80° C or preserved in silica gel desiccant (Fisher Scientific, Fair Lawn, NJ, USA) before DNA extraction. The DNA was extracted following Lee and Taylor (1990) with a modified lysis buffer (55 mM cetyltrimethylammonium bromide, 1.4 M sodium chloride, 20 mM EDTA (pH 8.0), 20 mM Tris, 2.5 mM polyvinylpyrrolidone, and 2 μL/ · mL 2-mercaptoethanol) DNA extractions were also performed using the Dneasy Plant Mini Kit (Qiagen Inc. Valencia, CA, USA) following the protocols found therein.

The *rbc*L gene was amplified in two overlapping fragments from total genomic DNA with oligonucleotide primers (Table 2). Each 50 μL PCR reaction consisted of 10 μL diluted DNA, 5 μL 10 × Buffer with 1.25 U of Taq polymerase (Fisher Scientific Okasis), 2.0 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, 0.2 μM each primer, and 0.024% non-acetylated BSA (Sigma Chemicals, St. Louis, MO, USA). Thermocycling was accomplished on a GeneAmp 2700 PCR system (Applied Biosystems, Foster City, CA, USA). PCR parameters included an initial denaturation at 94° C for 5 min followed by 40 repeated cycles of 94° C for 45 s, 41.5° C for 45 s, and 72° C for 90 s. A final incubation at 72° C for 10 min was used to insure complete polymerization of DNA strands. Resulting PCR products were electrophoresed on 1% Tris Borate EDTA agarose gels. Products were purified for sequencing using Qiaquick Spin Columns (Qiagen Inc.) based on protocols contained therein. The purified PCR product was quantified for cycle sequencing reactions by comparison of band intensities against known concentrations of unmethylated λ virus genomic DNA (Fisher Scientific) on 1% TBE agarose gels.

DNA sequencing. Approximately 40–50 ng of purified PCR product were used as a template for cycle sequencing reactions. Sequencing reactions were performed with Big Dye Terminator version 3.1 (Applied Biosystems), purified with G-50 fine Sephadex (Amersham Biosciences, Uppsala, Sweden) columns, dried and dissolved in template suppression reagent (Applied Biosystems). Automated sequencing was performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Two opposing strands were sequenced for each fragment using the aforementioned PCR primers. The RUBISCO large subunit sequence fragments were edited and assembled into contigs using Sequencher version 3.1.1 (Gencodes Corp., Ann Arbor, MI, USA).

Sequence alignment and molecular phylogenetic analyses. Sequences were aligned with ClustalX (Thompson et al. 1997). Introns were removed from the data set and the data set was imported into MacClade version 4.03 (Maddison and Maddison 2001). Unweighted MP analyses performed with PAUP* 4.0b10 (Swofford 2002) utilized the heuristic search option with 10 replicates of random taxon addition and tree-bisection-reconnection (TBR) branch swapping. Characters state changes were treated as unordered (Fitch 1971) with equal weights. Bootstrap support (*bs*) values (Felsenstein 1985) were calculated based on 10,000 replicates of heuristic tree searching. Phylogenetic signal was estimated by measuring the skewness (g_1 score) for the distribution of 1,000,000 random tree lengths (Hillis 1991).

Model-based phylogenetic analyses were also conducted on DNA sequences. Models of nucleotide substitution were estimated from Modeltest 3.07 (Posada and Crandall 1998). The hierarchical likelihood ratio test, Akaike information criterion, and Bayesian information criterion implemented through Modeltest 3.07 all selected the same model (GTR + I + Γ, General Time Reversible model of nucleotide substitution with a portion of invariable sites, and gamma distributed rate variation among sites) and identical model parameter settings. Maximum likelihood (ML) analysis was conducted using the heuristic search algorithm and TBR branch swapping using PAUP*. Bayesian inference (BI) was performed with MrBayes version 3.1.1 (Huelsenbeck and Ronquist 2001) with the GTR + I + Γ model (number of substitution types = 6, rates = invgamma, number of generations = 10,000,000, print frequency = 500, sample frequency = 100, and number of Markov chains = 4). The BI results yielded 100,000 trees of which 65 were required for the burn-in phase. The burn-in

TABLE 1. Taxon sampling for phylogenetic analysis.

Taxon	Collection location	Source of data	Accession
Ingroup Taxa			
Dichotomosiphonaceae			
Dichotomosiphon tuberosus G.W. Lawson		Hanyuda et al. (2000)	AB038487
Pseudocodiaceae			
Pseudocodium floridanum C.J. Dawes & A.C. Mathieson	Florida USA	This study	AY942178
Bryopsidineae			
Bryopsidaceae			
Bryopsis corticulans Setchell	Bodega Bay, CA, USA	This study	AY942163
Bryopsis hypnoides J.V. Lamouroux	Bodega Bay, CA, USA	This study	AY942169
Bryopsis maxima Okamura		Kono et al. (1991)	X55877
Bryopsis pennatula J. Agardh	Laguna Beach, CA, USA	This study	AY942165
Bryopsis plumosa (Hudson) C. Agardh		Hanyuda et al. (2000)	AB038480
Trichosolen myura (J. Agardh) W.R. Taylor		Woolcott et al. (2000)	AF212144
Codiaceae			
Codium fragile (Suringar) Hariot		Manhart and VonderHaar (1991)	M67453
Codium fragile ssp. *tomentosoides* (van Goor) P.C. Silva	Appledore Island, ME, USA	This study	AY942179
Codium lucasii Setchell		Hanyuda et al. (2000)	AB038481
Derbesiaceae			
Derbesia marina (Lyngbye) Solier		Woolcott et al. (2000)	AF212142
Pedobesia ryukyuensis (Yamada & T. Tanaka) Kobara & Chihara		Woolcott et al. (2000)	AF212143
Halimedineae			
Caulerpaceae			
Caulerpa brachypus Harvey		Hanyuda et al. (2000)	AB038483
Caulerpa okamurae Weber-van Bosse		Hanyuda et al. (2000)	AB038484
Caulerpa paspaloides (Bory de Saint-Vincent) Greville	Florida Keys, USA	This study	AY942171
Caulerpa prolifera (Forsskål) J.V. Lamouroux	Florida Keys, USA	This study	AY942173
Caulerpa racemosa (Forsskål) J. Agardh		Hanyuda et al. (2000)	AB038486
Caulerpa sertulariodes (S.G. Gmelin) M. Howe	Florida Keys, USA	This study	AY942170
Halimedaceae			
Halimeda discoidea Decaisne		Hanyuda et al. (2000)	AB038488
Halimeda incrassata (J. Ellis) J.V. Lamouroux	Florida Keys, USA	This study	AY942167
Halimeda opuntia (Linnaeus) J.V. Lamouroux	Florida Keys, USA	This study	AY942174
Halimeda tuna (J. Ellis & Solander) J.V. Lamouroux	Florida Keys, USA	This study	AY942177
Udoteaceae			
Penicillus dumetosus (J.V. Lamouroux) Blainville	Florida Keys, USA	This study	AY942175
Penicillus pyriformis A. Gepp & E.S. Gepp	Florida Keys, USA	This study	AY942162
Rhipilia tomentosa Kützing	Banco Chinchorro Quintana Roo, Mexico	This study	AY942164
Rhipocephalus phoenix (J. Ellis & Solander) Kützing	Florida Keys, USA	This study	AY942176
Rhipocephalus phoenix f. *brevifolius* A. Gepp & E. Gepp	Florida Keys, USA	This study	AY942172
**Tydemania expeditionis* Weber-van Bosse	Finger's Reef, Apra Harbor, Guam	This study	AY942161
Udotea conglutinata (J. Ellis & Solander) J.V. Lamouroux	Banco Chinchorro Quintana Roo, Mexico	This study	AY942168
Udotea flabellum (J. Ellis & Solander) M.A. Howe	Banco Chinchorro Quintana Roo, Mexico	This study	AY942166
Udotea spinulosa M.A. Howe	Florida Keys, USA	This study	AY942160
Outgroup Taxa			
Acetabularia acetabulum (Linnaeus) P.C. Silva	Naples, Italy	Zechman (2003)	AY177738
Batophora occidentalis Howe	Florida, USA	Zechman (2003)	AY177747
Chlorocladus australasicus Sonder	Cape York, Australia	Zechman (2003)	AY177750
Polyphysa parvula Berger & Kaever	Caribbean	Zechman (2003)	AY177741

Collection locations (when known), source of the data, and GenBank accession numbers are provided.

TABLE 2. Oligonucleotides primers for PCR amplification and cycle sequencing.

*rbc*L binding site	Sequence	Direction	Source
bp 7–26	5′-CCAMAAACWGAAACWAAAGC-3′	Forward	Hanyuda et al. (2000)
bp 6–29	5′-TCCAAAAACTGAAACTAAAGCAGG-3′	Forward	Hanyuda et al. (2000)
bp 689–667	5′-GCTTGWGMTTTRTARATWGCTTC-3′	Reverse	Hanyuda et al. (2000)
bp 905–886	5′-TCAATAACCGCATGCATTGC-3′	Reverse	Hanyuda et al. (2000)
bp 427–449	5′-GCTTATGCWAAAACATTYCAAGG-3′	Forward	This study
bp 1396–1372	5′-AATTTCTTTCCAAACTTCACAAGC-3′	Reverse	This study

phase was determined by plotting the number of generations versus −ln likelihood scores. Of the remaining 99,935 trees in the stationarity phase, the final 10,000 trees were used to determine posterior probabilities (pp) of clades through a 50% majority rule consensus tree created by PAUP*. Wherever appropriate, pp values were used as nodal support values in the ML tree topology. Shimodaira-Hasegawa (SH) tests (1999) were performed in PAUP* (full optimization and 1000 bootstrap replicates) to determine whether there was a significant difference between tree topologies obtained in different analyses and to compare the ML results with alternative hypotheses based on topological constraints enforced in PAUP*. In all cases where topological constraints were used, only the specified nodes were constrained allowing for other relationships to assume optimal phylogenetic patterns.

Results of MP and ML analyses were considered in the context of morphological traits reported as relevant to bryopsidalean taxonomy (e.g. plastid type, reproduction, thallus construction, calcification, and utricles). State changes for these characters were optimized via parsimony on the MP and ML trees to assess their congruence with the inferred phylogenies (see Fig. 1 legend for character coding).

RESULTS

Parsimony analysis. Summary statistics for the aligned bryopsidalean and dasycladalean RUBISCO large subunit gene sequence data are given in Table 3. Maximum parsimony (MP) resulted in nine equally most parsimonious trees (MPTs) with a length of 2223 steps. A strict consensus tree was generated and is displayed in Figure 1. MP analysis indicates that the Bryopsidineae and the Halimedineae are separate monophyletic suborders, but with relatively weak nodal support, (*bs* 61% and 65%, respectively). The freshwater alga *D. tuberosus* was inferred as a sister to the marine Halimedineae (*bs* = 90%). *Pseudocodium* was inferred to be the sister to the family Halimedaceae. The families Halimedaceae (weakly supported with *bs* <50%), Caulerpaceae (*bs* = 100%), Bryopsidaceae (*bs* = 80%), Derbesiaceae (*bs* = 81%), and Codiaceae (*bs* = 100%) were also inferred as separate monophyletic lineages. Within the Bryopsidineae, the family Codiaceae was a sister group to the lineage containing the members of the Bryopsidaceae and Derbesiaceae, although without any *bs* support. Within the Bryopsidaceae, *Trichosolen myura* (*Pseudobryopsis myura*) was sister to a clade of taxa classified in the genus *Bryopsis*.

Within the halimedinean family Caulerpaceae, *Caulerpa okamurae* and *C. paspaloides* formed a grade below an unresolved clade (*bs* = 78%) comprised of *C. sertularioides*, *C. prolifera* and a clade formed by *C. brachypus* and *C. racemosa*. Taxa assigned to the Udoteaceae, were not monophyletic. A clade formed by *Tydemania expeditionis* and *Rhipilia tomentosa* was sister to a clade formed by the remaining Udoteaceae, Halimedaceae, and Pseudocodiaceae, but this relationship was not supported. The genera, *Penicillus, Rhipocephalus*, and *Udotea* formed a clade (*bs* = 100%) sister to a *Pseudocodium* and *Halimeda* clade (*bs* <50%). Within a monophyletic but unsupported *Halimeda* clade, *H. incrassata*, and *H. opuntia* (*bs* = 57%) were sister to *H. discoidea* and *H. tuna* (*bs* = 57%). The genus *Udotea* was not monophyletic, a result with strong *bs* support. *Udotea flabellum* was inferred as sister to the clade (*bs* = 100%) formed by the remaining udoteacean taxa (i.e. two species each of *Udotea*, *Rhipocephalus*, and *Penicillus)*. The genera *Penicillus (P. pyriformis* and *P. dumentosus*, *bs* = 97%), and *Rhipocephalus (R. phoenix* and *R. phoenix* f. *brevifolius, bs* = 99%) were each resolved as monophyletic.

ML and BI. The ML tree topology is presented in Figure 2. BI analysis (not shown) resulted in a tree topology nearly identical to that of ML analysis with one exception: that *T. myura* is sister to the genus *Bryopsis* in the ML tree, while BI suggested that *T. myura* is sister to *Bryopsis* plus *Codium*. However, the ML tree and BI consensus tree were not significantly different based on SH test results ($P = 0.483$). There was also no significant difference between the ML tree and all MPTs (SH test, P values ranged from 0.212 to 0.325). The ML tree topology was seven steps longer than the MPTs (2230 steps, a 0.31% difference).

ML analysis suggested that the Halimedineae and Bryopsidineae formed separate monophyletic groups with strong support (pp = 0.99 and 1.00, respectively). As in the MP analysis, the freshwater alga *D. tuberosus* was sister to the Halimedineae clade (pp = 1.00). Analysis of a tree topology constraining *D. tuberosus* within the Bryopsidineae was significantly different (SH test, $P = 0.000$) from the ML tree topology. Within the Bryopsidineae, the families Bryopsidaceae (in ML analysis only), Codiaceae (pp = 1.00), and Derbesiaceae (pp = 1.00) formed separate monophyletic groups. The primary difference between the ML and MP analyses was that ML placed the Derbesiaceae as sister to the Bryopsidaceae and Codiaceae (compared with MP that placed the Codiaceae as sister to the Bryopsidaceae and Derbesiaceae).

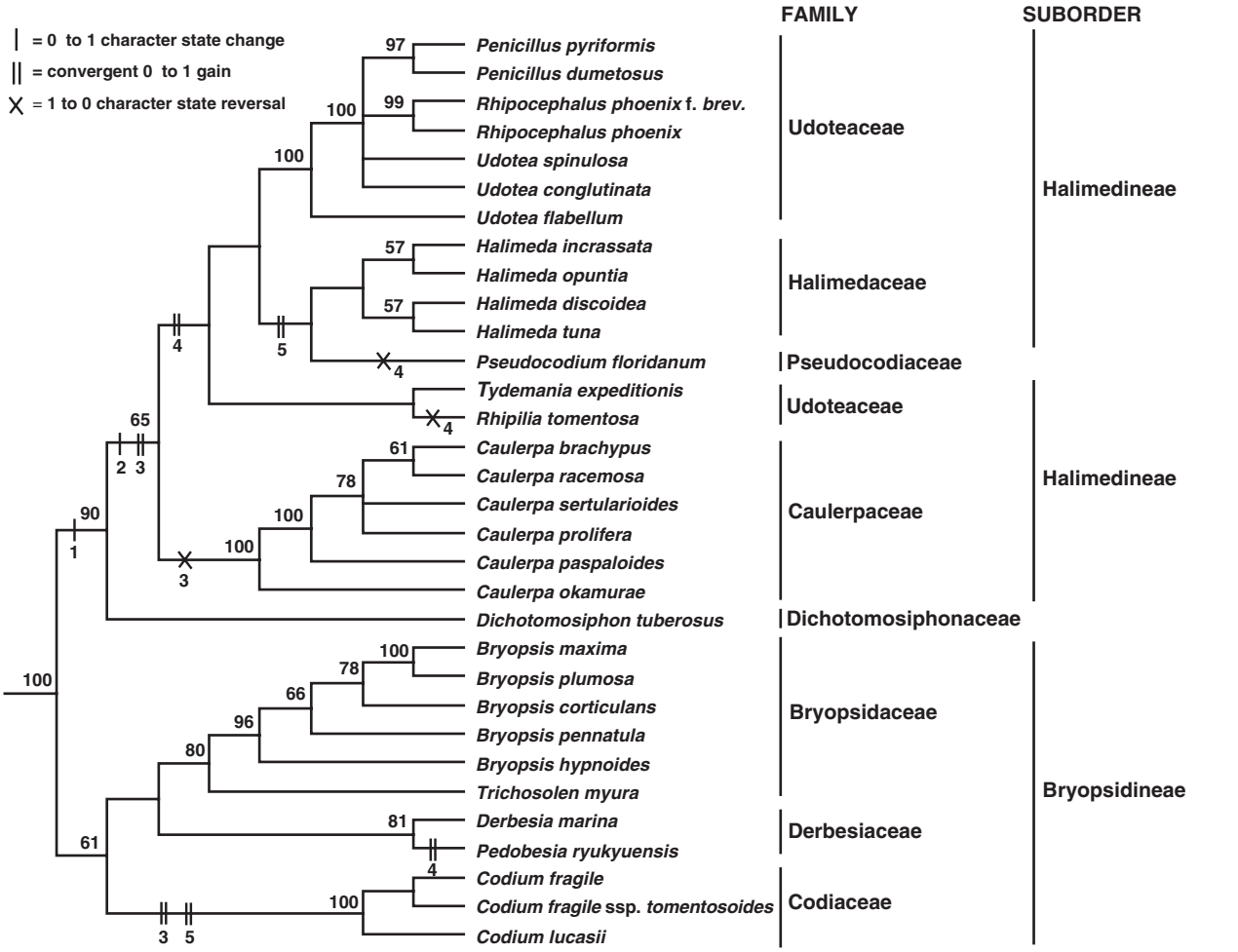

FIG. 1. A strict consensus of nine equally most parsimonious trees based on the *rbc*L nucleotide data set and rooted with dasycladalean outgroups (pruned from the tree). Bootstrap values were obtained from 10,000 replicates; values above 50% marked at respective nodes. Morphological traits mapped onto the maximum parsimony tree are shown below the branches on which they are optimized. Characters: (1) plastid types, homoplasty = 0 heteroplasty = 1 (Hillis-Colinvaux 1984, Moestrup and Hoffman 1973, Bold and Wynne 1985); (2) reproduction, nonholocarpic = 0 holocarpic = 1 (Ernst 1902, Bold and Wynne 1985); (3) thallus structure, uniaxial = 0 multiaxial = 1 (Fritsch 1945); (4) calcification, absent = 0 present = 1 (Hillis-Colinvaux 1984); (5) utricles, absent = 0 present = 1 (Weber van Bosse 1896, Levring 1938, Womersley 1955, Dawes and Mathieson 1972).

Within the Halimedineae, the families Caulerpaceae and Halimedaceae (pp = 1.00 and 0.98, respectively) were inferred as monophyletic. However, the family Udoteaceae was not. Instead, *R. tomentosa* was sister to the assemblage formed by the Caulerpaceae, Halimedaceae, and the remaining udoteacean taxa. Constraining the Udoteaceae as monophyletic resulted in a tree topology that was significantly different from the ML tree (SH test, $P = 0.000$). The genus *Halimeda* was monophyletic (pp = 0.98) and sister (pp = 0.99) to an unsupported clade formed by *Penicillus*, *Udotea*, *Rhipocephalus*, *Tydemania*, and *Pseudocodium*. Within this group, *T. expeditionis* and *Pseudocodium floridanum* were sister to the assemblage comprised of *Udotea*, *Penicillus*, and *Rhipocephalus* (pp = 1.00). *Penicillus* and *Rhipocephalus* formed separate monophyletic groups (pp = 1.00 each). However, the genus *Udotea* was strongly inferred to be non-monophyletic (pp = 1.00). *Udotea flabellum* was sister to a clade comprised of *Penicillus*, *Rhipocephalus*, and two other *Udotea* species. An analysis constraining the genus *Udotea* as monophyletic resulted in a tree topology that was significantly different from the ML tree (SH test, $P = 0.000$).

DISCUSSION

Phylogenetic analyses of *rbc*L sequences of the Bryopsidales have helped to clarify a number of long standing issues related to the phylogeny and evolution of these diverse green algae. These issues include (1) hypotheses of monophyly for the bryopsidalean suborders and families, (2) the phylogeny of taxa with uncertain subordinal affinity (e.g. *Pseudocodium* and *Dichotomosiphon*), and (3) the patterns of morphological character evolution relevant to the taxonomy of the Bryopsidales.

TABLE 3. Summary of alignment and tree statistics for analyzed data set.

Alignment length	1428
Invariable positions	790
Variable positions	638
Parsimony informative positions	507
MPT's	9
TLD skewness, g_1	−0.55
TLD skewness, g_1(outgroups excluded)	−0.61

MPT, most parsimonious tree; TLD inferred from 1,000,000 random trees.

Monophyly of the suborders Halimedineae and Bryopsidineae. Although levels of statistical support may vary, the results of this study imply the monophyly of the suborders, Halimedineae and Bryopsidineae. Monophyly of the Halimedineae is dependent, however, on the subordinal classification of the genus *Pseudocodium*, previously of uncertain affinity (Hillis-Colinvaux 1984). Results of MP and ML analyses unequivocally indicate that this genus should be classified in the Halimedineae (discussed in more detail below). Similar results supporting monophyly of the two bryopsidalean suborders (sensu Hillis-Colinvaux 1984) were found in a ML analysis of *rbc*L introns for 12 bryopsidalean taxa (Hanyuda et al. 2000). In previous phylogenetic analyses based on morphological data Vroom et al. (1998) inferred monophyly of only the Halimedineae, while the Bryopsidineae formed a non-monophyletic grade. Increased taxon sampling may result in a robust and richly resolved phylogeny (Zwickl and Hillis 2002), and thus future molecular phylogenetic studies of the group should include additional bryopsidalean taxa, including *Ostreobium*, *Boodleopsis*, *Caulerpella*, *Rhipidosiphon*, *Chlorodesmis*, *Avrainvillea*, and *Rhipiliopsis*.

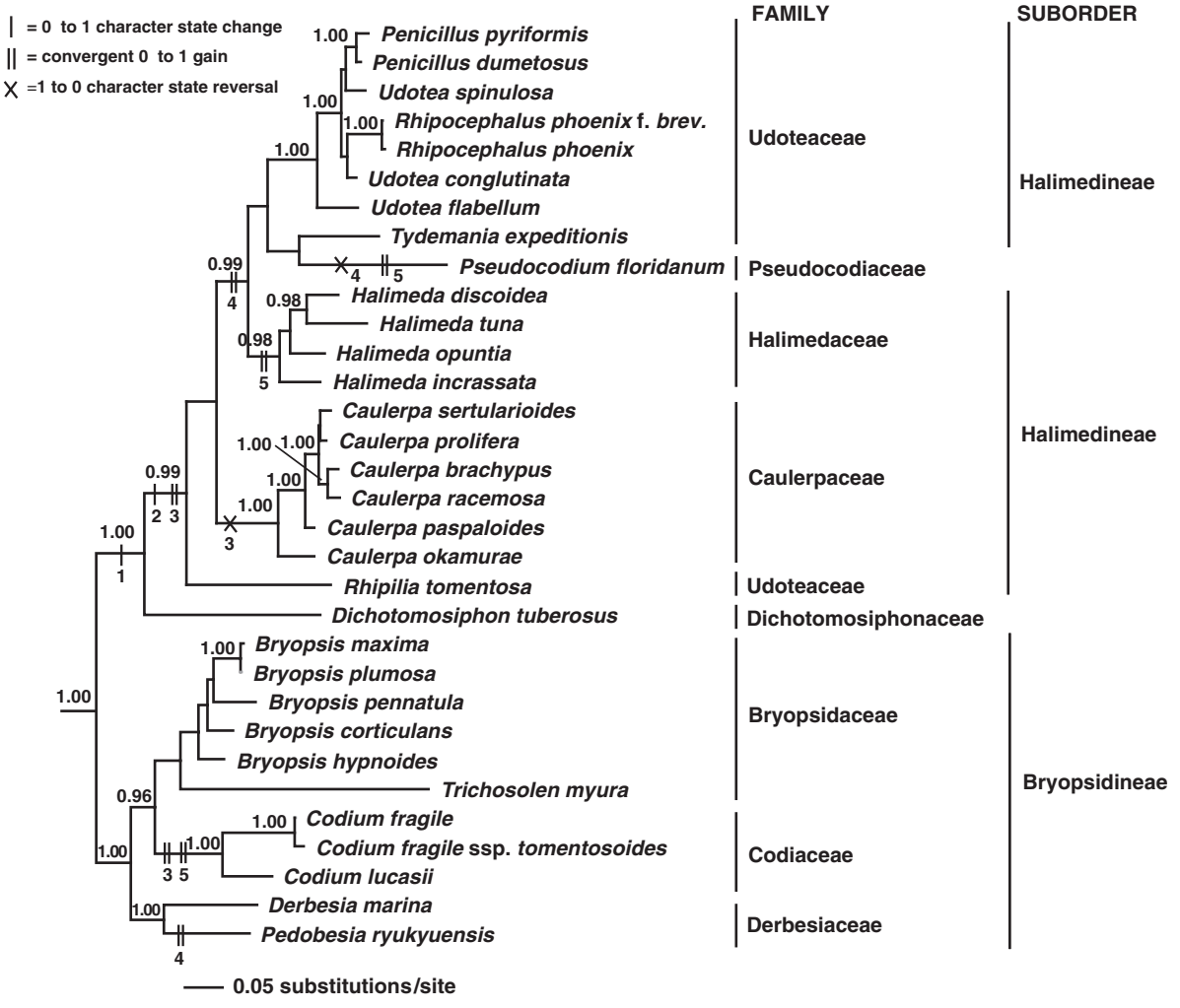

FIG. 2. Optimal tree topology of maximum likelihood analysis (–ln 11918.46125). Posterior probabilities above 0.95 obtained from Bayesian inference are given at their respective nodes. Parameters for GTR + I + Γ model inferred from ModelTest for use in PAUP* were: base = 0.3331 0.1056 0.1933; nst = 6; rmat = 3.1589 2.3951 1.7331 2.1811 8.7434; Rates = γ, shape = 0.6915; Pinvar = 0.3851. Morphological character coding as in Fig. 1.

It would be most parsimonious to suggest that the ancestral bryopsidalean alga exhibited homoplasty and non-holocarpic reproduction (see Figs. 1 and 2 for relevant morphological characters mapped on the MP and ML tree topologies). Despite differences in inferred relationships, the study by Vroom et al. (1998) is consistent with this interpretation. These ancestral states are inferred to have persisted in the Bryopsidineae, but evolved into the derived states of heteroplasty and holocarpic reproduction in the Halimedineae. The absence of holocarpy in *Caulerpella* (Prud'homme van Reine and Lokhorst 1992) is, therefore, a reversal.

Bryopsidinean families. Although current results imply that the Bryopsidineae is monophyletic, family-level relationships remain in question. Indeed, both MP and ML phylogenies imply that the families Bryopsidaceae, Codiaceae, and Derbesiaceae are distinct clades, but the relationships among these clades differed between the two analyses. In fact, when our analysis and those of previous studies (discussed below) are compared, all possible relationships among bryopsidinean families have been inferred, and is possibly an artifact of limited taxon sampling of these families. Current MP analysis suggested a sister relationship between the Bryopsidaceae and Derbesiaceae. In contrast, ML suggested a sister relationship between the Bryopsidaceae and the Codiaceae, and ML analysis of *rbc*L introns (Hanyuda et al. 2000) implied that the Codiaceae is sister to the Derbesiaceae. A sister relationship between the Derbesiaceae and Bryopsidaceae has been suggested (Silva 1982, Vroom et al. 1998) defined by the shared occurrence of uniaxial construction and sporic meiosis. Although these features separate these taxa from the Codiaceae, these are not synapomorphic traits, but are instead sympleisomorphic when more global comparisons are made within the green algae. Silva (1982) suggested that *Derbesia* and *Bryopsis* should be classified in the same family on the basis of similar life history characters. The occurrence of a heteromorphic life history is shared by these two genera, as well as the formation of stephanokont zoospores (van den Hoek 1981). Although stephanokont zoospores are also found in the chlorophycean taxon, *Oedogonium*, structural details differ from those in *Derbesia* and *Bryopsis* (Roberts et al. 1980, 1981). These observations imply that stephanokont zoospores are homologous within the Bryopsidales, but are convergent in *Oedogonium*. It should be noted, however, that relationships among the bryopsidinean families in current and previous phylogenetic analyses based on morphological and molecular data lack robust nodal support. Thus, more data and additional taxa are necessary to resolve relationships among the bryopsidinean families. MP results are consistent with morphological characters such as thallus construction and site of meiosis. That is, both the Derbesiaceae and the Bryopsidaceae possess uniaxial thallus construction and sporic meiosis, however, these characters are likely to be pleisiomorphic and not diagnostic of relationships.

Within the Bryopsidaceae, the species *T. myura* was inferred as sister to a clade of *Bryopsis* species in MP and ML analyses (a result consistent with Woolcott et al. 2000). *Trichosolen* and *Bryopsis* differ in reproductive traits, with gametes formed in unmodified pinnules on *Bryopsis*, and in specialized gametocysts that are arranged laterally on the pinnules of *Trichosolen* (Feldman 1969). Within the Derbesiaceae, both *Pedobesia* and *Derbesia* form erect filamentous thalli. The latter is distinguished from other bryopsidinean species by the presence of a calcified basal disc (MacRaild and Womersley 1974, Littler and Littler 2000). The results of the current MP and ML analyses suggest that calcification of bryopsidalean algae was gained in two independent evolutionary events, having originated once in the suborder Halimedineae (Halimedaceae and Udoteaceae, but subsequently lost in *Pseudocodium*) and again in the Bryopsidineae (*Pedobesia*).

Halimedinean families. The family Udoteaceae was not monophyletic based on MP and ML analyses. This result is primarily because of the ambiguous resolution of *Rhipilia*, *Tydemania*, and *Pseudocodium*, and was perhaps caused by long branch or taxon sampling effects. The genera *Penicillus* and *Rhipocephalus* each formed separate monophyletic groups, but the genus *Udotea* was not monophyletic. These results are consistent with a nuclear rDNA-based phylogeny of the Udoteaceae (Kooistra 2002). Therefore, both nuclear- and plastid-based molecular phylogenies suggest the genus is not monophyletic. The inclusion of other udoteacean genera not included in our study, such as *Rhipidosiphon*, *Avrainvillea*, *and Cladocephalus* may facilitate elucidation of family-level relationships and stabilize relationships within genera such as *Udotea*.

The Halimedaceae and Caulerpaceae were monophyletic in MP and ML analyses. Historically, the Halimedaceae has been classified (along with other multiaxial taxa) in the Codiaceae (e.g. Fritsch 1945), and later considered to be in the Udoteaceae (e.g. Bold and Wynne 1985). The relationships among the Halimedaceae, Pseudocodiaceae, and Udoteaceae are unresolved because of the ambiguous placement of *Pseudocodium*, *Tydemania*, and *Rhipilia* in different analyses. However, the close affinity of the multiaxial halimedinean taxa (except *Rhipilia* in ML) is apparent (a result inferred in MP and well supported in ML).

The Caulerpaceae, represented by six species of the genus *Caulerpa* was a well-supported monophyletic group in all analyses. The addition of the genus *Caulerpella* to our analyses could have improved the resolution of this group with respect to other families, and may have helped to elucidate the evolution of holocarpy. The sister relationship of *C. paspaloides* to most other *Caulerpa* species inferred in this study agrees with previous family-level analyses (Fama et al. 2002).

Genera of uncertain taxonomic affinity. The taxonomic affinity of the genus *Pseudocodium* has long been

debated. The cylindrical spongy thallus, with multiaxial construction, a cortex comprised of utricles and presence of β, 1–4 mannan in cell walls have led some authors to suggest that *Pseudocodium* is closely related to *Codium* (Gepp and Gepp 1911, Dawes and Mathieson 1972). However, utricle branching patterns (Weber van Bosse 1896, Levring 1938, Womersley 1955) and the polygonal facets of utricles in surface view (Fritsch 1945) have led others to suggest a closer affinity to the genus *Halimeda*. Our results support the latter of these two views. The results of all analyses in the current study placed *Pseudocodium* solidly within the Halimedineae; however specific placement within the suborder is still in question because of ambiguity and lack of character support. If this hypothesis is correct, the prediction of halimedinean characteristics, such as holocarpy and heteroplasty would be warranted. To date, reproduction has not been confirmed. Dawes and Mathieson (1972) reported putative ''gametangia,'' but observed no gametes or division products. Their illustrations, however, of the structures reported as ''gametangia'' did not possess basal cross-walls. Similarly, reports of heteroplasty in *Pseudocodium* are conflicting and somewhat confused. Dawes and Mathieson (1972) did not mention whether amyloplasts were present in *P. floridanum*. Their omission may have been mistaken by Hillis-Colinvaux (1984) as ''implied'' homoplasty. In her description of the genus, Weber van Bosse (1896) likewise did not report the presence of amyloplasts, but noted the occurrence of ''grains of amylum'' in the cytoplasm. More recently, Coppejans et al. (2001) reported heteroplasty for *P. floridanum* from Papua New Guinea.

Hillis-Colinvaux (1984) tentatively assigned *Pseudocodium* to the Bryopsidineae, but conceded that it did not fit the characteristics of any of the families within the suborder and thus erected a separate family, the Pseudocodiaceae, to accommodate the genus. Current MP results placed *Pseudocodium* as sister to *Halimeda*, a result consistent with utricle structure and arrangement, and could indicate a familial affinity between these two genera. However, ML placed *Pseudocodium* as sister to *Tydemania*, indicating an affinity to the Udoteaceae. Thus, although the genus *Pseudocodium* is robustly resolved within the suborder Halimedineae, it was not reliably resolved in any family, and Hillis-Colinvaux's (1984) tentative assignment to the Pseudocodiaceae is retained.

The taxonomy of the genus *Dichotomosiphon* has long been problematic. Fritsch (1945) classified this genus in the family Vaucheriaceae of the green algal order Siphonales (which included the Dasycladales and Bryopsidales). Smith (1955) placed the genus in its own family, Dichotomosiphonaceae, within the Siphonales. Hillis-Colinvaux (1984) declined to assign subordinal affinity of the genus because of character conflicts in her classification scheme. This confusion has been largely because of conflicting reproductive and plastid characters observed in this alga. Both MP and ML analyses suggested that *D. tuberosus* is the sister taxon to the Halimedineae. Like the Halimedineae, *Dichotomosophon* is heteroplastic (Moestrup and Hoffman 1973). However, the occurrence of septations during sexual reproduction suggests that the alga is non-holocarpic (Lee 1999) and possibly allied with the Bryopsidineae. There are other taxa currently classified in the Halimedineae that have also been reported to be non-holocarpic (e.g. *Caulerpella*, Prud'homme van Reine and Lokhorst 1992). Hanyuda et al. (2000) also reported that *Dichotomosiphon* is sister to the Halimedineae in their analysis of *rbc*L introns and cladistic analysis of morphology (Vroom et al. 1998) placed *Dichotomosiphon* within a clade of halimedinean taxa. These molecular and morphological data, as well as our own well-supported hypotheses that *Dichotomosiphon* is sister to the Halimedineae, provide evidence for the inclusion of this genus in the Halimedineae.

One could argue against the classification of *Dichotomosiphon* in the Halimedineae because of numerous morphological differences between these taxa. Besides the aforementioned differences in reproductive modes these traits include differences in habitat (i.e. *D. tuberosus* is found in freshwater habitats versus marine habitats for the remaining halimedinean taxa) and the unique occurrence of striated tubules found in *Dichotomosiphon* chloroplasts (Moestrup and Hoffman 1973). These differences, however, are unique to *Dichotomosiphon* (autapomorphic) and not useful for inference of phylogeny.

One can conclude from our results and those of others (e.g. Prud'homme van Reine and Lokhorst 1992) that holocarpic reproduction is not a universal feature of the Halimedineae. In contrast, however, these algae seem to share the presence of both chloroplasts and amyloplast and are thus heteroplastic. Although heteroplasty should perhaps be further confirmed in *P. floridanum*, this sole feature appears to be a synapomorphy to define the Halimedineae. Unfortunately, corresponding synapomorphic morphological feature cannot be identified for the Bryopsidineae, and is perhaps why Vroom et al. (1998) did not infer monophyly of the group in their cladistic analysis of morphological features. A review of traits defining the Bryopsidineae in Hillis-Colinvaux (1984) (e.g. homoplasty, absence of concentric lamellae, non-holocarpy, broad geographic distribution, absence of allelochemicals and presence of cell wall components mannan, xylan, and cellulose) reveals that these traits are either plesiomorphic or homoplastic, and thus not suitable to define clades. It may be that despite best intentions to identify morphological traits that define bryopsidalean clades, the antiquity of these algae has likely provided many opportunities for convergence and reversal of these traits.

Fossil record. The bryopsidalean algae possess a relatively rich fossil history that estimates their origin to be 350 million years old. However, the possible sister orders, Dasycladales and Cladophorales, are estimated to be about 550 and 700 million years old,

respectively (Tappan and Loeblich 1971, Butterfield et al. 1988). The differences in fossil ages among these orders are possibly attributable to missing fossil data for the most ancient dasycladalean and bryopsidalean taxa. Our phylogenetic trees suggest that the ancestral bryopsidalean algae were non-calcified, and that heavy calcification exhibited in some udoteacean taxa is a more derived feature. Thus, the non-calcified cell walls of primitive bryopsidalean algae would have limited their fossilization, led to missing fossil data and perhaps resulted in underestimates of Bryopsidales fossil ages.

CONCLUSIONS

Phylogenetic analyses of RUBISCO large subunit sequence data support the hypothesis of monophyly in both the Bryopsidineae and Halimedineae proposed by Hillis-Colinvaux (1984). These data also support family-level monophyly, with various degrees of support, for the Bryopsidaceae, Derbesiaceae, Codiaceae, Caulerpaceae, and Halimedaceae, but not the Udoteaceae. Although multiaxial construction and thallus habit of *P. floridanum* appears superficially similar to the genus *Codium* in the Codiaceae and Bryopsidineae, our results suggest this alga should be classified in the Halimedineae, and that the utricles in *Pseudocodium* are homologous with those in *Halimeda* but not *Codium*. Similarly, *D. tuberosus*, a freshwater alga of uncertain subordinal affinity, was sister to the marine Halimedineae, supporting its inclusion in that suborder. Consideration of the morphological features used to define subordinal and family-level lineages within the Bryopsidales leads one to infer that most are either plesiomorphic or homoplastic. One exception is the heteroplastic condition, which, if confirmed in *Pseudocodium*, is a diagnostic feature for the Halimedineae. Increased taxon sampling and additional genetic markers can result in a more robust and richly resolved phylogeny for this important group of algae.

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