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A multigene phylogeny of the Gelidiales including nuclear large-subunit rRNA sequence data

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Abstract

Partial sequences (1032 bp) of the nuclear-encoded large ribosomal RNA gene (LSU) were determined for 16 gelidialean species, and analyzed separately and in combination with plastid *rbc*L and nuclear SSU gene sequences. The number of informative characters and levels of sequence divergence among taxa are intermediate in LSU sequences as compared to that for *rbc*L and SSU. Analyses of the separate LSU, and a combined LSU, SSU, and *rbc*L data sets have identified early-diverging lineages within the Gelidiales including *Gelidiella*, *Pterocladia*, *Pterocladiella*, and a lineage including *Gelidium* and species classified in other genera. The relationships among most gelidialean taxa are well-resolved and well-supported by analyses of the combined data; however, the relationships of *Ptilophora* and *Capreolia* remain unclear. It is speculated that these two lineages have diverged from a common ancestor over an evolutionarily short period of time.

Introduction

Sequences for the plastid-encoded large subunit of RuBisCO (*rbc*L) and nuclear-encoded small subunit RNA (SSU) genes have been used previously to develop a phylogenetic hypothesis for the red algal order Gelidiales. Studies based on *rbc*L identify at least ten well-supported species complexes, some of which correspond to recognized genera (Freshwater & Rueness, 1994; Freshwater et al., 1995). Relationships inferred among most of the ten species complexes are robustly supported, but the *rbc*L data do not clearly resolve the order of divergence among basal lineages within the group (Freshwater et al., 1995). In an effort to improve the resolution among these early-diverging gelidialean taxa both *rbc*L and SSU sequences for 16 species were analyzed (Bailey & Freshwater, 1997). The results of the combined *rbc*L and SSU analysis also supported the recognition of ten species complexes and an early split among gelidialean lineages which corresponds to distinct developmental strategies for the female reproductive system and carposporophytes. Although the combined analysis helped to resolve relationships among the early lineages, the position of the *Capreolia* and *Ptilophora* species complexes within the Gelidiales remains uncertain. Developmental data for the female reproductive system and carposporophyte are incomplete for these taxa, but the available data seem to ally *Capreolia* and *Ptilophora* with a large *Gelidium* clade. This clade is characterized by a number of features, including: (1) carpogonia formed on both sides of the central axis, (2) nutritive filaments originating from basal cells of third-order filaments borne on opposite sides of second-order filaments, (3) gonimoblast filaments growing in an interwoven fashion among the nutritive filaments, and (4) carposporangia developing on both sides of the central plane bisecting bilocular cystocarps (Hommersand & Fredericq, 1988). Molecular data support the inclusion of *Capreolia* and *Ptilophora* as early-diverging lineages within

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the larger *Gelidium* clade, but the relative order of branching for the two taxa is unresolved (Bailey & Freshwater, 1997).

The inability to determine the affinities of *Ptilophora* and *Capreolia* in the previous studies could either be the result of inadequate taxa sampling or, perhaps, a rapid and nearly simultaneous divergence of these lineages from a common ancestor (Bailey & Freshwater, 1997). If the time between the divergence of two lineages from a third lineage is short relative to the rate of mutational change in DNA sequences, sequence data may contain little phylogenetically useful information (Lanyon, 1988). To determine if the evolutionary history of *Capreolia* and *Ptilophora* precludes resolving their relationships, or if the rates of change for *rbc*L and SSU gene sequences are inappropriate for addressing this question, data from a third gene evolving at an intermediate rate is needed.

Nuclear large-subunit ribosomal RNA (LSU) gene sequences have been used in phylogenetic studies of vascular plants (Bult et al., 1995; Hamby & Zimmer, 1988; Ro et al., 1997). To date, however, complete LSU sequences are lacking for any red alga, although a partial LSU sequence fragment is available for *Porphyridium cruentum* (Galgani et al., 1994). For this study, we developed primers to amplify and sequence a 1032 base pair fragment from the LSU gene of red algae. These primers were used to determine partial LSU sequences for 16 Gelidiales species for which *rbc*L and SSU sequences are already available. The LSU sequence data was analyzed separately and in combination with *rbc*L and SSU data in order to: (1) asses the level of variability in LSU gene sequences, (2) determine the utility of LSU sequence data in phylogenetic studies of red algae, and (3) determine if LSU sequence data would help resolve the relationships of the *Ptilophora* and *Capreolia* lineages within the Gelidiales.

Materials and methods

Species included in this study, GenBank accession numbers, and the number of base positions determined for the LSU, SSU and *rbc*L genes are listed in Table 1. Collection information for each species is provided in Freshwater et al. (1995). Total cellular DNA was extracted from samples as described in Freshwater and Rueness (1994). Primers used for amplifying and sequencing the LSU segment are shown in Table 2. Initial amplifications and sequencing reactions utilized primers described in Hamby et al. (1988). Sequences for additional primers were derived by primer walking. An approximately 1100 bp fragment of the LSU gene was amplified in PCR reactions containing 40–100 ng of DNA template, 1.25 mM $MgCl_2$, 80 μM each dNTP, 0.2 μm each flanking primer and *Tfl* DNA polymerase and reaction buffer as suggested by the manufacturer (EpiCentre Technologies, Madison, WI). Amplifications were performed in a MJ Research PT-100 thermocycler (Watertown, MA) using the following cycling protocol: initial denaturing at 94 °C for 3 min followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 50 °C for 30 s, a slow ramp of 0.2 °C per s to an elongation temperature of 72 °C for 45 s with an additional 5 min at 72 °C after the last cycle.

Amplification products were purified using either a GeneClean II Kit (Bio 101, LaJolla, CA), or gel isolating the amplification products and separating them from the agarose by spinning through an aerosol filtered pipet tip (2000 $\times$ g, 2 min). Amplification products were sequenced using the AmpliCycle sequencing method (Perkin-Elmer, Foster City, CA) with primers enzymatically end-labeled with [^{32}P]dATP using T4 polynucleotide kinase and buffer (New England BioLabs, Beverly, MA). Endlabelled primers were cleaned using the QIAquick Nucleotide Removal Kit (Qiagen, Santa Clarita, CA) before use in sequencing reactions. Sequencing reactions were separated on 6% Long Ranger gels (FMC BioProducts, Rockland, ME) and visualized by autoradiography.

The LSU sequence data was aligned using the SeqApp program (Gilbert, 1992). Manual alignment of the LSU sequences was possible since large regions of the gene are conserved and the size of gaps is small (1–3 bp). The analyzed data set included 1032 aligned sites (available upon request). The *rbc*L and SSU gene sequences included in combined analyses were taken from Bailey and Freshwater (1997).

Phylogenetic analyses of the LSU data set were performed separately and in combination with *rbc*L and SSU data sets using parsimony and maximum likelihood (ML) as implemented in PAUP (v. 3.1.1: Swofford, 1993; v. 4.0 provided by D.L. Swofford). Parsimony trees were inferred using branch and bound searches. Gaps were treated in three ways during analyses of the separate LSU data set: as missing data, as a fifth state, or coded into a presence/absence matrix that was appended to the sequence data. Consistency (CI) and retention (RI) indices (Kluge & Farris, 1969; Farris, 1989) reported for parsimony analyses were calculated excluding uninformative characters. Sup-

Table 1. Species, GenBank accession numbers, and number of base positions determined for the *rbc*L, SSU and LSU genes

Species	Accession number			Base positions		
	*rbc*L	SSU	LSU	*rbc*L	SSU	LSU
Capreolia implexa Guiry et Womersley	L22456	U60344	AF039545	1347	1595	1150
Gelidiella acerosa (Forsskål) Feldmann et Hamel	L22457	U60342	AF039551	1379	1574	1134
Gelidium americanum (Taylor) Santelices	L22459	U60347	AF039536	1467	1514	1081
Gelidium caulacantheum J. Agardh	U00103	U60343	AF039544	1411	1594	1135
Gelidium floridanum Taylor	U00107	U60351	AF039537	1467	1660	1153
Gelidium latifolium (Greville) Bornet et Thuret	U00112	U60350	AF039540	1466	1577	1113
Gelidium pusillum (Stackhouse) Le Jolis (Norway)	U00999	U60352	AF039542	1419	1633	1062
Gelidium 'pusillum' (Stackhouse) Le Jolis (NC, USA)	U00981	U60355	AF039543	1467	1478	1083
Gelidium serrulatum J. Agardh	U01042	U60340	AF039538	1465	1667	1070
Gelidium sesquipedale (Clemente) Thuret	L22071	U60354	AF039539	1439	1662	1081
Onikusa pristoides (Turner) Akatsuka	U01044	U60353	AF039541	1416	1628	1153
Pterocladia lucida (Brown et Turner) J. Agardh	U01048	U60349	AF039550	1405	1461	1158
Pterocladiella capillacea (Gmelin) Santelices et Hommersand	U01896	U60346	AF039549	1430	1474	1060
Perocladiella melanoidea (Schousboe ex Bornet) Santelices et Hommersand	U01046	U60341	AF039548	1375	1589	1081
Ptilophora pinnatifida (J. Agardh) R. Norris	U16834	U60345	AF039547	1376	1702	1150
Ptilophora subcostata (Okamura) R. Norris	U16835	U60348	AF039546	1422	1626	1155

Table 2. Oligonucleotide primers used to amplify and sequence a 1100 base pair segment of the LSU gene for 16 gelidialean species

Label	Primer sequence
Forward primers	
28C[1]	CCGAGTTTCCCTCAGGATAGC
X	GCAGGACGGTGGCCATGGAAGT
W	GAGTCGTTCCTAAGCCGAGTG
Z	CGTACCGATCACCGCATCAGG
Reverse primers	
V	CGTATCGCCAGTTCTGCTTACC
G	CACCACGTCCTCCTACTC
H	CAACTCTGCCCAGCGCCATC
28D[1]	CTTGGAGACCTGCTGCGG
Y	CGGATTCCCTTTGCCCGTTGC
28F[1]	CAGAGCACTGGGCAGAAATCAC

[1] Primer sequence from Hamby et al. (1988).

port for nodes of parsimony trees was assesed by calculating bootstrap proportion (BP) values (Felsenstein, 1985) based on 2000 resamplings of heuristic searches with simple addition (combined data) or 500 resamplings of branch and bound searches (LSU data). Decay values (Bremer, 1988), generated for trees up to 20 (combined data) or 5 (LSU data) steps longer than the minimal-length tree were also determined to provide an additional measure of support.

Likelihood analyses were performed by first determining the transition:transversion ratio (tn:tv) that maximized the log-likelihood value by plotting a range of tn:tv against the corresponding inferred log-likelihoods. The tn:tv maximizing the log-likelihoods of the combined and separate LSU analyses were de-

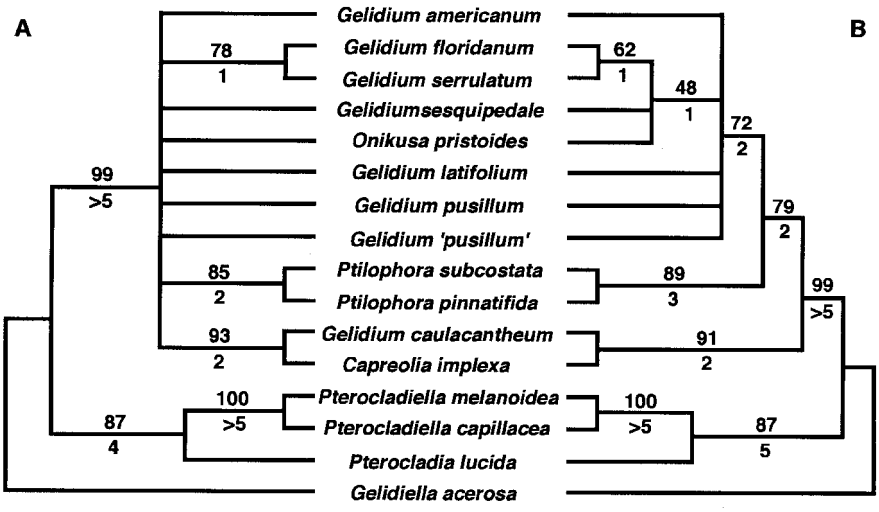

Figure 1. Trees resulting from parsimony analyses of partial LSU sequence data for 16 gelidialean species. (A) Strict consensus of 8 minimal trees of length = 187, CI = 0.648 and RI = 0.736 resulting from analyses treating gaps as missing. (B) Strict consensus of 12 minimal trees of length = 218, CI = 0.612 and RI = 0.703 resulting from analyses including gap data. Bootstrap proportion values are shown above, and decay indices below, each internode.

termined to be 2.2 and 1.9, respectively. These values were used in all subsequent replicate ML analyses of the two data sets. The ML analysis of the combined data set was replicated 10 times using empirical base frequencies, random orders of sequence addition, and tree bisection-reconnection (TBR) branch swapping. The ML tree was bootstrapped using 100 resamplings of a single random addition of taxa and TBR branch swapping. The ML analysis of the LSU data set was replicated 100 times using empirical base frequencies, random addition of sequences, and TBR branch swapping. The resulting ML tree was bootstrapped using 500 resamplings of 'Fast' stepwise addition of sequences.

The distribution of variable sites, homoplasy across sites, tree characteristics as well as distance calculations were made using PAUP and MacClade (v. 3.0: Maddison & Maddison, 1992)

Results

For each of the 16 species studied 1060 to 1160 bp of sequence from the middle region of the LSU gene was obtained. This fragment starts at approximately bp 964 of the complete LSU sequence for *Oryza sativa* (GenBank Accession No. M11585). From this data an 1032 bp alignment was compiled and analyzed. The '28C' and '28F' primer sequences from Hamby et al. (1988) have been used to amplify and sequence a variety of red algae as well as the Gelidiales species included in this study. Primers designed specifically for this study (Table 2) have also worked with a number of non-gelidialean taxa and should be useful for studies of a wide range of red algae.

Table 3. Comparison of *rbc*L, SSU, and LSU percent sequence divergence values over the analyzed gene segments from 16 gelidialean taxa at ranks of genus[a] and species. Calculations of mean distances were adjusted for missing data

	*rbc*L	SSU	LSU
Between species	3.1–11.5	0.0–0.4	0.1–2.4
Between genera	11.0–15.5	1.4–2.9	4.0–6.8

[a] Because of the uncertain status of *Capreolia* and *Ptilophora*, only comparisons between *Gelidiella*, *Gelidium*, *Pterocladia*, and *Pterocladiella* were made.

Percent LSU sequence divergence values ranged from 0.1–1.6% between species and 4.0–6.8% between genera (Table 3). These ranges are between those found for *rbc*L and SSU sequences from the same species. The distribution of variable sites, expressed as a percentage of the total number of steps required to fit a tree topology, is uneven in the studied segment (Table 4). An uneven distribution of homoplasious mutational changes is also indicated by the ensemble CI values listed for the LSU data in Table 4.

With gaps treated as missing data, the LSU sequence alignment contained 68 informative positions

Table 4. Distrubution of steps (reported as a percentage of the total) and ensemble consistency indices (CI) across 150 base pair segments of the analyzed *rbc*L, SSU and LSU sequence alignments for 16 gelidialean species. Calculation made based on the combined analyses tree topology

Nucleotide sites	*rbc*L		SSU		LSU	
	Steps (%)	CI	Steps (%)	CI	Steps (%)	CI
1–150	8.0	0.46	7.4	0.75	12.7	0.53
151–300	11.1	0.59	12.8	0.88	6.9	0.50
301–450	10.8	0.50	2.1	–[b]	18.5	0.50
451–600	12.3	0.47	27.7	0.71	3.7	0.75
601–750	9.6	0.46	10.6	1.00	15.3	0.90
751–900	10.9	0.44	5.3	0.20	11.6	0.64
901–1050	10.0	0.42	8.5	0.67	31.2[a]	0.63[a]
1051–1200	12.8	0.50	1.1	–[b]		
1201–1350	9.3	0.58	11.7	0.75		
1351–1500	4.8[a]	0.49[a]	3.2	0.50		
1501–1650			9.6[a]	0.89[a]		

[a] Less than 150 sites in these segments: *rbc*L = 116; SSU = 146; LSU = 132.
[b] No informative characters in this 150 bp segment.

and cladistic analysis resulted in eight trees of 187 steps (CI = 0.65, RI = 0.74) (Figure 1a). Analysis in which gaps (= 17 characters) were coded separately and appended to the LSU data resulted in 12 trees of 218 steps (CI = 0.61, RI = 0.70) (Figure 1b). These trees are identical to trees inferred from an analysis in which gaps were treated as a fifth character state. Coding gaps as separate characters increased the percentage of informative positions in the data set by less than 1%, or from 68 of 1032 (gaps as missing data) to 79 of 1049 characters (gaps as separate characters). The analysis in which gaps are coded as separate characters resulted in more trees; however, the consensus of these trees is more resolved than the consensus of trees inferred from the data with gaps treated as missing (cf. Figures 1a and 1b) Support for an alliance between *Pterocladia* and *Pterocladiella* was found in both parsimony analyses. However, the length of the branches separating the two *Pterocladiella* species from *Pterocladia lucida* are long (32 & 24) compared to the branch joining them (17) indicating that *Pterocladia* and *Pterocladiella* are probably only distantly related (Figure 2). A second clade resolved in these analyses is comprised of species assigned to *Gelidium*, *Capreolia*, *Onikusa*, and *Ptilophora* (Figure 1).

The maximum likelihood tree inferred from the LSU data (Figure 3) is identical to one of the most parsimonious trees. As in parsimony analyses, *Pterocladia* and *Pterocladiella* are inferred to be sister taxa. *Ptilophora* is resolved as an independent lineage that

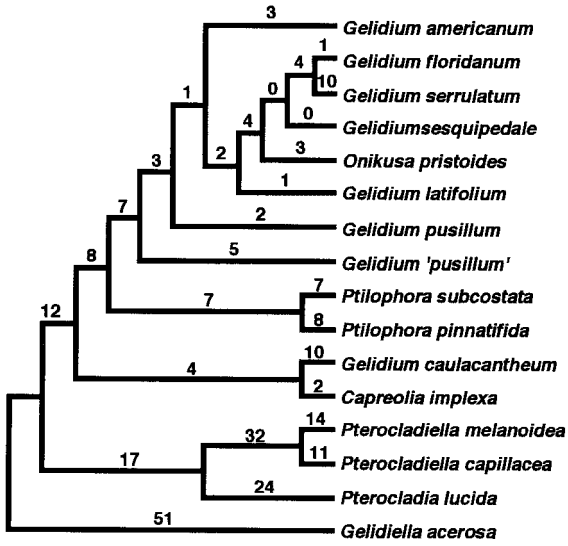

Figure 2. One of 12 minimal trees, L = 218, CI = 0.612 and RI = 0.703 resulting from parsimony searches of partial LSU sequences including gap data for 16 gelidialean species. Inferred branchlengths are shown above each internode.

is sister to a clade including species of *Gelidium* and *Onikusa*. In contrast to the parsimony analyses where there was good support for a lineage including *Capreolia* and *Gelidium caulacantheum* (BP = 93 & 92%), this lineage received only moderate bootstrap support in ML analyses (BP = 71%, Figure 3). The lack of resolution among *Gelidium* and *Onikusa* species is due to the fact that the LSU sequences for these taxa are

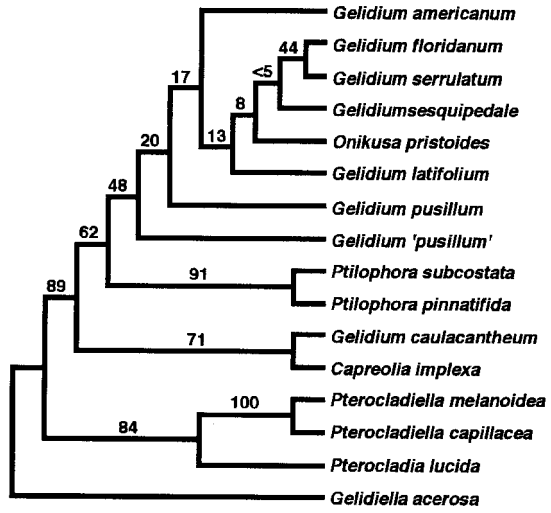

Figure 3. Maximum likelihood tree (LnLi = –2731.15916) resulting from analysis of partial LSU sequence data for 16 gelidialean species. Bootstrap proportion values are shown above each internode.

highly conserved. When these species are considered, only 21 of 1032 aligned sites are variable and only five are informative. An additional three informative characters are present when gap data are included.

The LSU data were also analyzed in combination with sequence data for the *rbc*L and SSU genes from these species (Bailey & Freshwater, 1997). The combined data set included 4145 sites of which 428 were informative. Cladistic analysis of the combined data resulted in a fully resolved tree of 1379 steps (CI = 0.52, RI = 0.56) (Figure 4) that is identical to the topology resulting from likelihood analyses of the same data (LnLi = 13922.18758). Relationships inferred among most species in the combined tree are robustly supported by bootstrap and decay values (Figure 4). The LSU and combined data sets both provide support for several independent lineages including *Pterocladiella*, *Ptilophora*, and *Capreolia*. A clade in which the *Ptilophora* and *Capreolia* lineages are positioned as sister to *Gelidium* and *Onikusa* species is also well-supported. Except for the branching order of the *Ptilophora* and *Capreolia* lineages, relationships among the taxa in this clade are well-resolved. In contrast to analyses of the separate LSU data, the combined analysis only weakly supports (BP = 63%, d3) the hypothesis that *Pterocladia* and *Pterocladiella* form a clade.

Discussion

Nuclear LSU gene sequences have been proposed as a tool for phylogenetic studies because of their longer length and greater rate of mutation as compared to sequences of the frequently used SSU gene (Larson, 1991). For example, Ro et al. (1997) found partial LSU sequences to be useful for resolving relationships in the Ranunculaceae, and that the LSU derived phylogeny was highly congruent with results of other molecular analyses. This study is the first to use nuclear LSU gene sequence data for analysis of phylogenetic relationships among red algae. The LSU gene includes variable regions termed expansion segments that alternate with conserved core regions (Clark et al., 1984; Hassouna et al., 1984). The position and number of expansion segments is conserved in angiosperms and, compared to core regions, expansion segments are characterized by a higher percentage of variable sites and more frequent and larger insertion/deletion events (Bult et al., 1995). Data for angiosperms also indicate that expansion segments may exhibit a nonrandom distribution of nucleotides, base compositional biases, and 'cryptic sequence simplicity' (Tautz et al., 1986; Bult et al., 1995).

Based on its position relative to the complete LSU sequence for *Oryza sativa*, and the conserved nature of the number and position of expansion segments in eucaryotes, the 1032 bp fragment analyzed in this study contains expansion segments D4 through D7 and the 5' end of D8 (Bult et al., 1995). As for the SSU sequences of these 16 species (Bailey & Freshwater, 1997), the distribution of variable sites in the LSU data is uneven and ranges from 3.7 to 31.2% on a nonoverlapping 150 bp scale (Table 4). Areas of increased variability in the LSU data correspond to variable areas in an alignment of six angiosperm species (Ro et al., 1997). LSU gene sequences are now being completed for these, and other, red algal species to more accurately determine the number, position, and characteristics of variable and conserved areas and to provide a secondary structural model for the LSU gene of red algae.

Levels of LSU sequence divergence among taxa are intermediate compared to that for *rbc*L and SSU genes from the same species (Table 3). This is also reflected in the percentage of informative sites for the three data sets. The percentage of informative sites in the *rbc*L and SSU data sets is 22 and 2.2% respectively (Bailey & Freshwater, 1997). When gaps are not considered, 6.6% of LSU sites were informa-

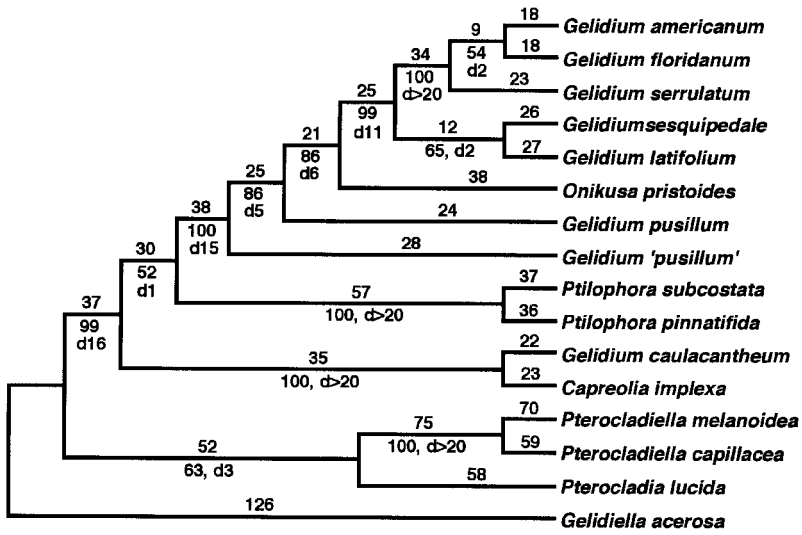

Figure 4. Single most parsimonious tree of length = 1379, CI = 0.521 and RI = 0.559 resulting from analysis of combined LSU, SSU, and *rbc*L sequence data sets for 16 gelidialean species. Inferred branch lengths are shown above, and bootstrap proportion values and decay indices below each internode.

tive. When gaps are coded separately and appended to the sequence data this adds 11 informative characters to the matrix. The informative nature of gaps in these data is demonstrated by the increased resolution observed in the consensus of trees derived from parsimony analyses when gap data were included (Figure 1). Although regions of alignments including gaps are often excluded from analyses (e.g. Lutzoni, 1997), the small size of insertion/deletion events, the conserved nature of adjacent sequence, and the relatively close relationship of the included taxa makes the probability of correctly assessing the homology of gaps in these LSU sequences high.

Both ML and parsimony analyses of the LSU data resulted in trees receiving similar levels of character support. Unresolved nodes of the parsimony consensus trees (Figure 1) receive weak (BP = 62%) to no (BP < 5%) support in the ML tree (Figure 3). Analysis of the LSU data support a *Pterocladia*/*Pterocladiella* clade that was not found in either separate or combined analyses of SSU and *rbc*L data (Bailey & Freshwater, 1997). This clade is, however, weakly supported by bootstrap (BP = 62%) and decay values (d3) in the combined analyses (Figure 4). Also, the greater number of apomorphic changes inferred along the *Pterocladia* and *Pterocladiella* branches as compared to the number of synapomorphic changes on the branch joining them indicates that the relationship between these species may not be close (Figure 2). The order of branching inferred for the *Ptilophora* and *Capreolia* lineages from combined analysis of LSU, SSU, and *rbc*L sequences (Figure 3) also differs from the earlier study (Bailey & Freshwater, 1997, Figure 4).

The results of this study corroborate the hypothesis that distinct gelidialean lineages are characterized by different types of nutritive systems as well as other carposporophytic characters (Bailey & Freshwater, 1997; Hommersand & Fredericq, 1988, 1996; Santelices & Hommersand, 1997). Nevertheless, the addition of LSU sequence data has not helped resolve the relationships of *Ptilophora* and *Capreolia* within the Gelidiales. Combined analysis of all available sequence data position *Ptilophora* as sister to a clade including species of *Gelidium* and *Onikusa* (Figure 4). In contrast, *rbc*L and SSU alone place *Capreolia* as sister to the *Gelidium/Onikusa* clade (Bailey & Freshwater, 1997). Robust support for either arrangement of these lineages is lacking (Figures 1a, 2 & 3; Bailey & Freshwater, 1997); however, *Ptilophora* is resolved as sister to the *Gelidium/Onikusa* clade with moderate support (BP = 79%) when gap data are included (Figure 1b).

The 4145 bases of sequence data included in this study do not resolve the relationships of *Ptilophora* and *Capreolia* within the Gelidiales (Bailey & Freshwater, 1997; Freshwater et al., 1995). This may be due to the high levels of sequence divergence found in *rbc*L causing branch attraction problems, and the low amount of phylogenetic signal in the conserved SSU sequence data set. The inability of LSU gene

sequences, which are less variable than *rbc*L but more variable than SSU genes, to resolve the position of *Ptilophora* and *Capreolia* within the Gelidiales suggests that this may be the result of phylogenetic history rather than an inappropriate choice of genes. If these lineages diverged rapidly and near simultaneously from a common ancestor, the sequence data may contain little phylogenetically useful information (Lanyon, 1988). Although sequence data for additional taxa from both lineages should be included to eliminate potential branch attraction problems, it is possible that the relationships of the *Ptilophora* and *Capreolia* lineages may not be resolved by molecular sequence data of any type.

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