A Gene Phylogeny of the Red Algae (Rhodophyta) Based on Plastid rbcL

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A gene phylogeny of the red algae (Rhodophyta) based on plastid *rbcL*

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ABSTRACT   A phylogeny for the Rhodophyta has been inferred by parsimony analysis of plastid *rbcL* sequences representing 81 species, 68 genera, 38 families, and 17 orders of red algae; *rbcL* encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Levels of sequence divergence among species, genera, and families are high in red algae, typically much greater than those reported for flowering plants. The Rhodophyta traditionally consists of one class, Rhodophyceae, and two subclasses, Bangiophycidae and Florideophycidae. The Bangiophycidae with three orders (Porphyridiales, Compsopogonales, and Bangiales) appears to be polyphyletic, and the Florideophycidae with 17 orders is monophyletic in this study. The current classification of the Florideophycidae based on ultrastructure of pit connections is supported. With the exception of the Rhodogorgonales, which appears to be misplaced, orders with one or two pit-plug cap layers (Hildenbrandiales, Corallinales, Acrochaetiales, Palmariales, Batrachospermales, and Nemaliales) terminate long branches of basal position within Florideophycidae in the most parsimonious *rbcL* tree. Orders that lack typical cap layers but possess a cap membrane are resolved as a monophyletic clade sister to the Ahnfeltiales. The large order Gigartinales, which is distributed among five *rbcL* clades, is polyphyletic. Families that possess typical carrageenan in their cell walls are resolved as a terminal clade containing two family complexes centered around the Solieriaceae and Gigartinaceae.

The red algae (phylum Rhodophyta) are a distinct group of eukaryotic organisms characterized by chloroplasts containing phycoerythrin, phycocyanin, and allophycocyanins as accessory photosynthetic pigments and by the absence of centrioles and flagella (1). Their relationship to other groups remains uncertain. Largely a marine assemblage, the red algae predominate in extensive areas of the continental shelves in tropical, temperate, and cold-water regions (2). The most current classification recognizes one class, Rhodophyceae, and two subclasses, Bangiophycidae with 3 or 4 orders and Florideophycidae with 14 orders (1). The 3 orders of Bangiophycidae [Porphyridiales, Compsopogonales (including Rhodochaetales), and Bangiales] have been redefined based on organelle ultrastructure (3) and mode of spore formation (4) and are now thought to be only distantly related (5). The Florideophycidae, in contrast, are widely regarded as being monophyletic. Members of the Florideophycidae grow by means of apical cells and consist entirely of branched filaments in which the cells are linked by pit connections. Traditionally, the Florideophycidae have been classified into orders and families based on type of life history and mode of sexual reproduction (6). The current classification emphasizes the comparative ultrastructure of pit connections. Several types of pit plugs have been identified that

differ in the number of cap layers (zero, one, or two) and the presence or absence of a cap membrane (7, 8). Nine orders [Hildenbrandiales, Corallinales, Acrochaetiales, Palmariales, Batrachospermales, Ahnfeltiales, Bonnemaisoniales, Gracilariales (5), and Rhodogorgonales (9)] have been established or reinstated recently, primarily on the basis of pit-plug characters. Cell-wall chemistry, especially the presence of agar or carrageenan, is increasing in importance as a determinant in the characterization of some red algal orders (10), but a consistent classification of the Rhodophyta based on cell-wall characters is lacking.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) is the enzyme that facilitates the primary CO_2 fixation step in photosynthesis. The quaternary structure of the enzyme consists of eight large and eight small subunits. In green algae and land plants, the large subunit is encoded by the plastid gene *rbcL*, whereas the small subunit is encoded by the nuclear gene *rbcS* (11). In other algae, including Rhodophyta (12, 13), both subunits are encoded by plastid genes.

Recent studies using gene phylogenies have been successful at inferring taxonomic relationships in several groups of red algae. Analyses of DNA encoding 18S RNA (rDNA) have been conducted in the Gracilariales (14), and the spacer region between *rbcL* and *rbcS* has been investigated in species and populations of *Gracilaria* (15) and *Gymnogongrus* (16). Only two complete *rbcL* sequences have been published previously for Rhodophyta (12, 13); however, nucleotide analyses of *rbcL* are being used to infer relationships at several taxonomic levels in the Gelidiales (17, 18) and Gigartinaceae (19). A gene phylogeny was produced in this investigation designed to assess the usefulness of *rbcL* for exploring family and ordinal relationships in Rhodophyta.‖ In addition to establishing *rbcL* as a tool for phylogenetic studies, it is hoped that this hypothesis will be used to direct further sampling and, when combined with information from other molecular and nonmolecular sources, will contribute to a clearer understanding of red algal relationships.

MATERIALS AND METHODS

Field-collected samples or cultured isolates were either dried in silica gel and stored at −20°C (20) or kept alive. Dried material was rehydrated in seawater and sorted to ensure monospecificity prior to DNA extraction. Voucher specimens were prepared by fixation in 5% formalin in seawater and stored in the Herbarium of the University of North Carolina. DNA was extracted, and *rbcL* was amplified and

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‖Sequences reported in this paper have been deposited in the GeneBank data base (accession numbers are in *Appendix*).

sequenced with synthetic primers specific for red algae (18, 19).

Previous studies have shown the length of *rbcL* in red algae to be 1467 base pairs with no insertions or deletions (12, 13, 17). Though both *rbcL* and the spacer region between *rbcL* and *rbcS* were amplified, only sequence data from *rbcL* were used in this analysis. Preliminary observations showed that disagreement in base pair designations between the forward and reverse strand are well within the estimated range of error due to methodological factors (0.35%), and only sequences of the forward strand were produced in a majority of taxa. Base pairs that could not be determined unambiguously were designated "N," corresponding to the IUPAC-IUB Biochemical Nomenclature Commission ambiguity code. Though complete *rbcL* sequences were analyzed where possible, only partial sequence data (>70%) were available for a majority of the included taxa. Preliminary analyses for this and other studies using *rbcL* data (18, 19) have shown that analyses of partial *rbcL* sequences yield topologies that do not change upon the addition of sequence data beyond 70% of the complete gene.

Sequence data were analyzed with PAUP version 3.1.1 (21) and MACCLADE version 3.0 (22). Because sequences were incomplete for the first 54 base pairs, the data set was restricted to the last 1413 of the 1467-base-pair *rbcL* coding region. All searches were done under the Fitch criterion of equal weights for all substitutions (23). Because of the large number of taxa included in this analysis and limitations of software, heuristic searches were necessary. Searches were done with 630 random sequence additions, STEEPEST DESCENT, MULPARS (but permitting only five trees be held at each step), and NNI (nearest-neighbor interchange). Trees found in these random searches were then used as starting points for further searches with MULPARS and TBR (tree bisection-reconnection) until swapping was complete.

RESULTS

Heuristic searches of *rbcL* sequence data from 81 species containing 633 phylogenetically informative characters (characters variable in two or more taxa) resulted in a single most parsimonious tree of length = 6674, consistency index (CI) = 0.194, and retention index (RI) = 0.430 (Fig. 1). In general, families that included more than one representative genus were resolved as monophyletic clades. Exceptions were Bangiaceae, Acrochaetiaceae, Ceramiaceae, and Rhodomelaceae. Ordinal groupings were also resolved in monophyletic clades; however, the Bangiales, Acrochaetiales, Gigartinales, and Rhodymeniales were polyphyletic assemblages in this tree.

Descriptive information for the sequence data used in this analysis shows that both the number of nucleotide site mutations and informative characters were evenly distributed throughout the gene (Table 1). Likewise, ensemble consistency and retention indices varied little across the length of the data set, indicating a uniform distribution of homoplasies.

DISCUSSION

In vascular plants, *rbcL* analysis has been used to evaluate relationships among genera, families, and orders (24). However, the low levels of sequence divergence between closely related taxa generally prohibit its use for examining species relationships. Levels of sequence divergence among red algae are usually higher than those found in vascular plants, with values up to 11% occurring between some species placed in the same genus (17).

In both this (Table 1) and other studies (17-19), *rbcL* data have been found to provide a large number of evenly dis-

tributed informative characters appropriate to phylogenetic analysis. Moreover, the absence of insertion or deletion mutations eliminates problems of alignment. Our current analysis recognizes genera and families that are well founded on morphological grounds. Established orders are also identified in the overall tree, although the topological position of some is intuitively unsatisfactory.

The single most-parsimonious *rbcL* tree generated in this study contains 81 species representing 68 genera, 38 families, and 17 orders. The unicellular red alga *Porphyridium aerugineum* is specified as the outgroup. Each of the three orders placed in the Bangiophycidae is represented by one (Porphyridiales and Compsopogonales) or three (Bangiales) species. Though separated by exceedingly long branch lengths, all cluster at the base of the cladogram with *Porphyridium*.

The Florideophycidae form a monophyletic clade sister to *Porphyra* of the Bangiales in Fig. 1. Orders belonging to the Florideophycidae that are recognized as distinct based on pit-plug type (7, 8) are identified in the *rbcL* tree as clades or taxa terminating long branches of basal position. Orders with pit plugs that lack cap layers but possess a cap membrane are resolved in a single clade sister to the Ahnfeltiales lineage. Exceptions are the Gelidiales, which has one cap layer (7), and the new order Rhodogorgonales (9), which possesses two cap layers and may lack a cap membrane. The latter appears to be misplaced next to the Gracilariales, and its highly variable position in tree topologies longer than minimal length suggests that its current topological position may be incorrect.

Twenty of the 38 families shown in Fig. 1 contain more than one representative genus or species. These are resolved in monophyletic clades corresponding to recognized families in all cases in which two or more genera are represented per family, except the Acrochaetiaceae, Ceramiaceae, and Rhodomelaceae. The Acrochaetiaceae are considered to be polyphyletic based on the presence of R- or B-phycoerythrin (25) and pit-plug heterogeneity (26). Too few species of Ceramiaceae and Rhodomelaceae have been sampled to permit these large families to be resolved into monophyletic clades.

Much of the confusion in the classification of the Florideophycidae has centered historically around the Cryptonemiales and Gigartinales, two orders in which development of the fruiting bodies ("gonimoblasts") after fertilization is mediated by one or more "auxiliary cells" (6). Kraft and Robins (27) proposed merging the two into a single order (Gigartinales) containing over 40 families. Neither the traditional classification nor the proposed merger is supported by the *rbcL* tree, although a significant component of the revised order (Gigartinales I, Gigartinales II) forms a monophyletic clade at the top of the tree (Fig. 1). The central family of the former order Cryptonemiales, the Halymeniaceae, forms a clade situated between *Rhodymenia* and *Gastroclonium* of the Rhodymeniales. This unnatural splitting of the Rhodymeniales is almost certainly the result of long branch lengths due in part to the inclusion of too few taxa in the analysis.

A classification based on type of hydrocolloid present in cell walls is weakly supported by the *rbcL* tree. The agarophyte orders Gelidiales and Ceramiales are resolved in a clade sister to the clade terminated by the agarophyte order Gracilariales, whose lineages are orders and families that produce intermediate-type agarocolloids (Fig. 1). The Ahnfeltiales (28) is a unique order characterized by the absence of pit-plug membranes and appears to have evolved typical agar independently. Families that have been identified by Craigie (10) as containing typical carrageenans in their cell walls cluster at the top of the *rbcL* tree in a monophyletic clade consisting of two phylogenetic lines: (*i*) Solieriaceae, Furcellariaceae, Hypneaceae, Cystocloniaceae, and Caulacanthaceae, and (*ii*) Gigartinaceae, Petrocelidaceae, and
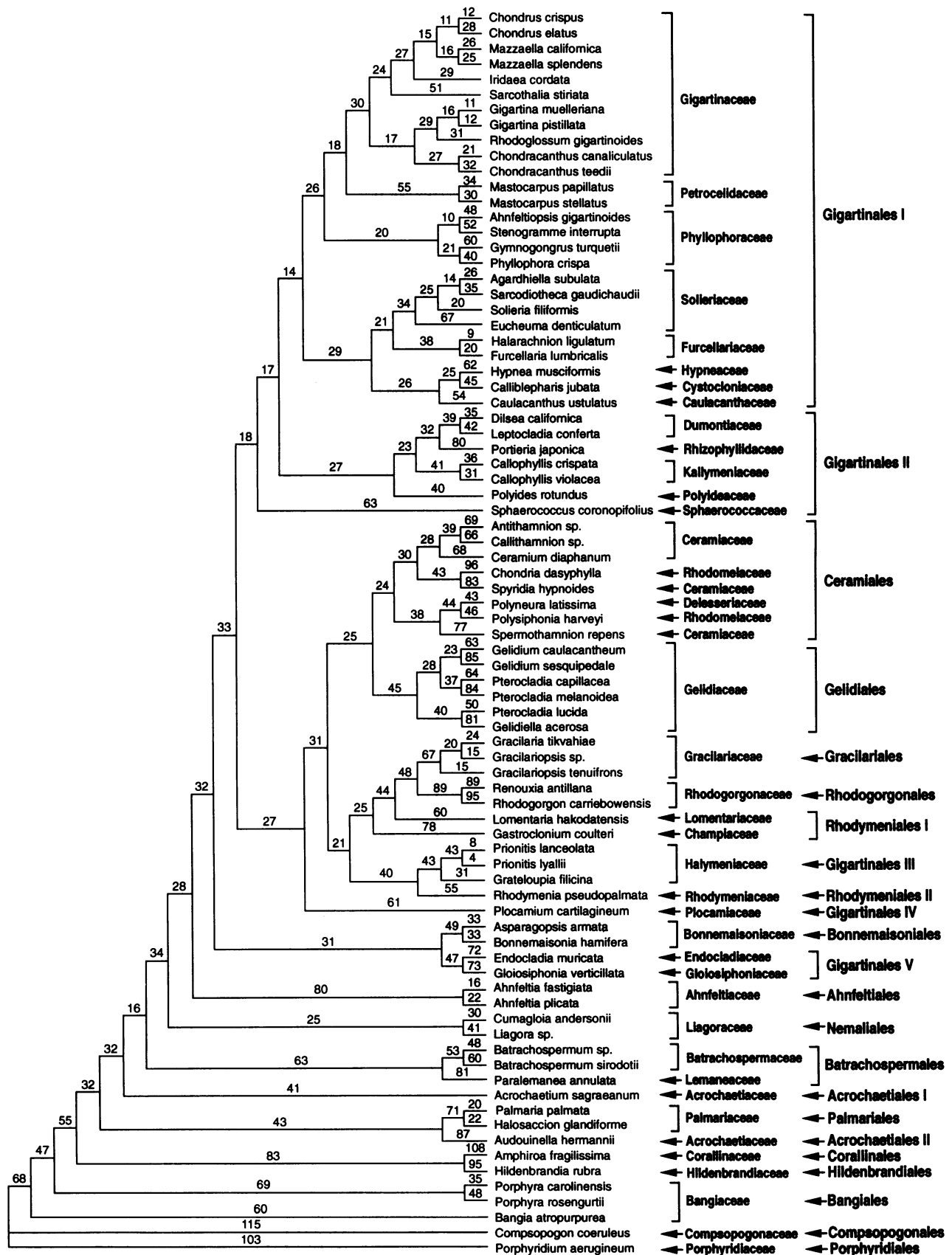
FIG. 1.　Most parsimonious tree of *rbcL* from 81 red algal taxa of length = 6674, consistency index = 0.194, and retention index = 0.430. Branch length estimates are indicated above internodal branches.

Phyllophoraceae. These two groups of families had been recognized earlier by Kylin (29), who linked them on the basis of similarities in their reproductive development. In both, the gonimoblasts develop primarily inwardly from the auxiliary cell. In the Solieriaceae complex, the auxiliary cell normally cuts off a single gonimoblast initial, connecting filaments may

Table 1. Distribution of informative characters (percent of total), steps (percent of total), consistency index (CI) and retention index (RI) across the *rbcL* sequence data set for 81 red algal species

Nucleotide sites	Informative characters, %	Steps, %	CI	RI
1–244	10.8	9.2	0.22	0.48
245–488	18.0	17.5	0.18	0.41
489–732	17.8	20.4	0.15	0.43
733–976	18.1	18.6	0.17	0.44
977–1220	18.0	19.9	0.17	0.42
1221–1467	17.3	14.4	0.22	0.42

be present or absent, and the tetrasporangia (meiosporangia) are zonately divided; in the Gigartinaceae complex, each auxiliary cell cuts off several gonimoblast initials, connecting filaments are absent, and the tetrasporangia are cruciately divided (Fig. 2).

The results of this and other studies (17–19) indicate that *rbcL* data can be useful for assessing relationships between red algal taxa at species, generic, familial, and even ordinal levels. Many of the putative anomalies seen in Fig. 1 could be due to inadequate sampling (30). Branch lengths separating taxa in the *rbcL* tree are long, indicating that evolutionary divergence is great. One remedy to the problem of large substitutional differences between taxa is the analysis of additional representative species. Based on the observations presented here we expect that, as more information is gathered, gene phylogenies founded upon *rbcL* sequence data will

contribute significantly to phylogenetic systematics of red algae at every taxonomic level.

Appendix

Species examined are listed in alphabetical order along with their localities and *rbcL* accession numbers in GenBank. Collection data are available from M.H.H.

Acrochaetium sagraeanum MA, USA (U04034); *Agardhiella subulata* NC, USA (U04176); *Ahnfeltia fastigiata* CA, USA (U04167); *A. plicata* Wales (U04168); *Ahnfeltiopsis gigartinoides* CA, USA (U04187); *Amphiroa fragilissima* FL, USA (U04039); *Antithamnion sp.* (published, ref. 13); *Asparagopsis armata* France (U04043); *Audouinella hermannii* NC, USA (U04033); *Bangia atropurpurea* Japan (U04040); *Batrachospermum sirodotii* LA, USA (U04036); *Batrachospermum sp.* NC, USA (U04035); *Bonnemaisonia hamifera* France (U04044); *Calliblepharis jubata* France (U04189); *Callithamnion sp.* NC, USA (U04020); *Callophyllis crispata* Japan (U04190); *C. violacea* CA, USA (U04191); *Caulacanthus ustulatus* France (U04188); *Ceramium diaphanum* NC, USA (U04020); *Chondracanthus canaliculatus* CA, USA (U02939); *C. teedii* France (U03024); *Chondria dasyphylla* NC, USA (U04021); *Chondrus crispus* N. Ireland (U02984); *C. elatus* Japan (U02985); *Compsopogon coeruleus* MT, USA (U04037); *Cumagloia andersonii* OR, USA (U04169); *Dilsea californica* CA, USA (U04192); *Endocladia muricata* CA, USA (U04193); *Eucheuma denticulatum* Philippines (U04177); *Furcellaria lumbricalis* France (U04194); *Gastro-*
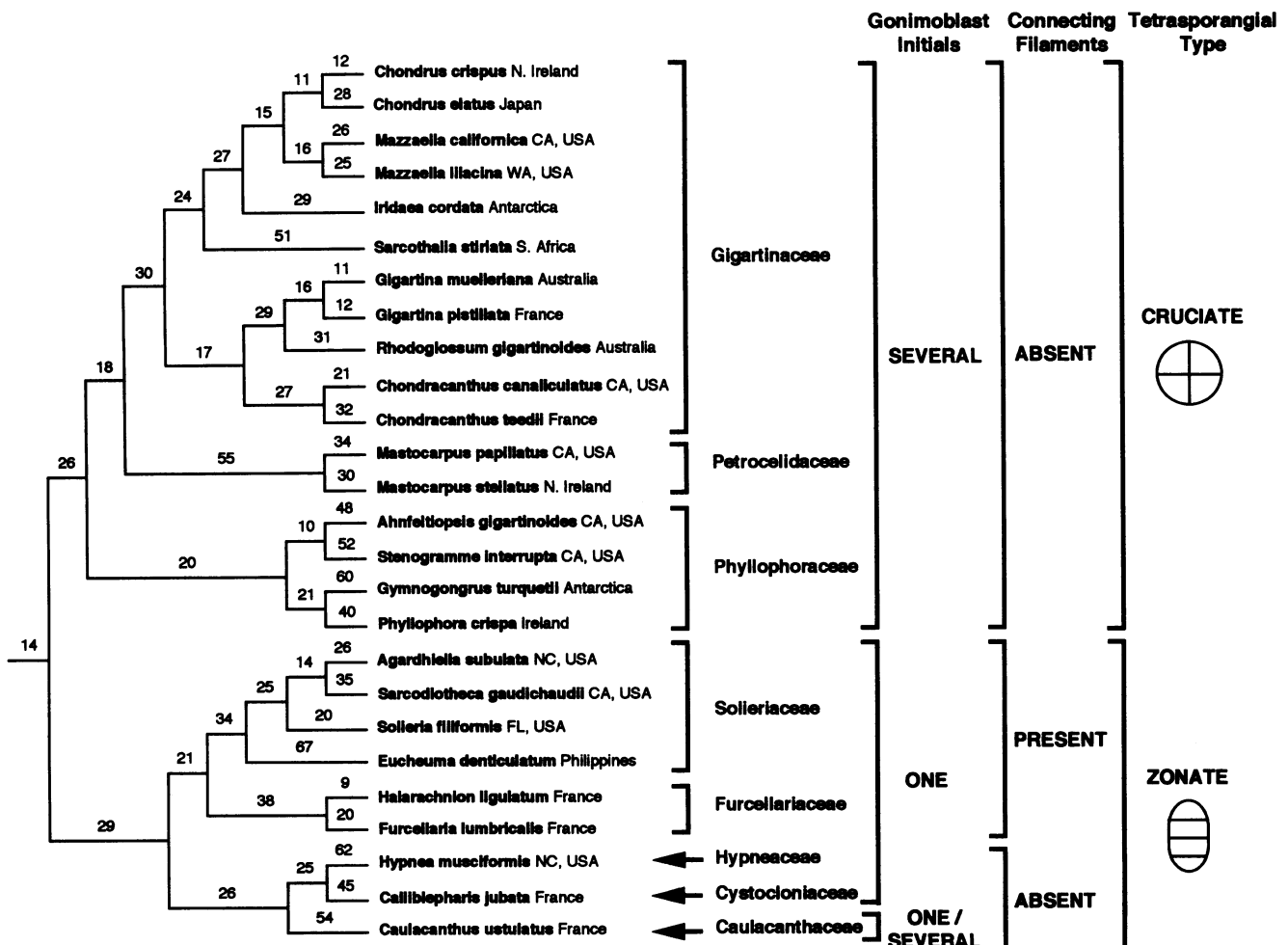

FIG. 2. Terminal clade of carrageenophyte species enlarged from Fig. 1 showing characteristics of gonimoblast initials, connecting filaments, and tetrasporangia.

clonium coulteri CA, USA (U04178); *Gelidium caulacan-theum* New Zealand (U00103); *G. sesquipedale* Spain (L22071); *Gelidiella acerosa* HI, USA (L22457); *Gigartina muelleriana* Australia (U03427); *G. pistillata* France (U03429); *Gloiosiphonia verticillata* OR, USA (U04196); *Gracilaria tikvahiae* NC, USA (U04172); *Gracilariopsis sp.* NC, USA (U04170); *Gracilariopsis tenuifrons* Venezuela (U04171); *Grateloupia filicina* FL, USA (U04212); *Gymnogongrus turquetii* Antarctica (U04196); *Halarachnion ligulatum* France (U04210); *Halosaccion glandiforme* OR, USA (U04173); *Hildenbrandia rubra* France (U04174); *Hypnea musciformis* NC, USA (U04179); *Iridaea cordata* Antarctica (U02989); *Leptocladia conferta* CA, USA (U04213); *Liagora sp.* FL, USA (U04174); *Lomentaria hakodatensis* CA, USA (U04180); *Mastocarpus papillatus* CA, USA (U04026); *M. stellatus* N. Ireland (U02992); *Mazzaella californica* CA, USA (U03082); *M. splendens* WA, USA (U03382); *Palmaria palmata* N. Ireland (U04186); *Paralemanea annulata* NC, USA (U04038); *Phyllophora crispa* Ireland (U02990); *Plocamium cartilagineum* CA, USA (U04211); *Polyides rotundus* France (U04214); *Polyneura latissima* CA, USA (U04022); *Polysiphonia harveyi* NC, USA (U04023); *Porphyra carolinensis* NC, USA (U04041); *P. rosengurtii* NC, USA (U04042); *Porphyridium aerugineum* (published, ref. 12); *Portieria japonica* Japan (U04215); *Prionitis lanceolata* CA, USA (U04216); *P. lyallii* CA, USA (U04217); *Pterocladia capillacea* Italy (U01888); *P. lucida* New Zealand (U01048); *P. melanoidea* Spain (U01046); *Renouxia antillana* Jamaica (U04181); *Rhodoglossum gigartinoides* Australia (U02991); *Rhodogorgon carriebowensis* Jamaica (U04183); *Rhodymenia pseudopalmata* NC, USA (U04182); *Sarcodiotheca gaudichaudii* CA, USA (U04184); *Sarcothalia stiriata* S. Africa (U03089); *Solieria filiformis* FL, USA (U04185); *Spermothamnion repens* MA, USA (U04024); *Sphaerococcus coronopifolius* Ireland (U04218); *Spyridia hypnoides* NC, USA (U04025); *Stenogramme interrupta* CA, USA (U07154).

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