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SYSTEMATICS OF THE GRACILARIACEAE (GRACILARIALES, RHODOPHYTA): A CRITICAL ASSESSMENT BASED ON *rbc*L SEQUENCE ANALYSES[1]

Carlos Frederico D. Gurgel[2] *and Suzanne Fredericq*

University of Louisiana at Lafayette, Department of Biology, Lafayette, Louisiana 70504-2451, USA

Generic concepts in the economically important agarophyte red algal family Gracilariaceae were evaluated based on maximum parsimony, Bayesian likelihood, and minimum evolution analyses of the chloroplast-encoded *rbc*L gene from 67 specimens worldwide. The results confirm the monophyly of the family and identify three large clades, one of which corresponds to the ancestral antiboreal genera *Curdiea* and *Melanthalia*, one to *Gracilariopsis*, and one to *Gracilaria sensu lato*, which contains nine distinct independent evolutionary lineages, including *Hydropuntia*. The species currently attributed to *Hydropuntia* comprise a single well-supported clade composed of two distinct lineages. The two most basal clades within *Gracilaria sensu lato* deserve generic rank: a new genus centered around *G. chilensis* Bird, McLachlan et Oliveira and *G. aff. tenuistipitata* Chang et Xia and a resurrected *Hydropuntia* encompassing primarily Indo-Pacific (*G. urvillei* [Montagne] Abbott, *G. edulis* [S. Gmelin] P. Silva, *G. eucheumatoides* Harvey, *G. preissiana* [Sonder] Womersley, and *G. rangiferina* [Kützing] Piccone) and western Atlantic species (*G. cornea* J. Agardh, *G. crassissima* P. et H. Crouan in Mazé et Schramm, *G. usneoides* [C. Agardh] J. Agardh, *G. caudata* J. Agardh, and *G. secunda* P. et H. Crouan in Mazé et Schramm). Cystocarpic features within the *Gracilaria sensu lato* clades appear to be more phylogenetically informative than male characters. The *textorii*-type spermatangial configuration is represented in two distinct clusters of *Gracilaria*. The *rbc*L genetic divergence among the Gracilariaceae genera ranged between 8.46% and 16.41%, providing at least 2.5 times more genetic variation than does the 18S nuclear rDNA. *rbc*L also resolves intrageneric relationships, especially within *Gracilaria sensu lato*. The current number of gracilariacean species is underestimated in the western Atlantic because of convergence in habit and apparent homoplasy in vegetative and reproductive anatomy.

Key index words: **Bayesian; *Gracilaria*; Gracilariaceae; *Hydropuntia*; maximum parsimony; phylogeny; *rbcL*; Rhodophyta; systematics**

Abbreviations: **BP, bootstrap proportions; *G.*, *Gracilaria*; *Gp.*, *Gracilariopsis*; ITS, internal transcribed spacer; ME, minimum evolution; MP, maximum parsimony; PP, Bayesian posterior probabilities; SSU, small subunit**

The Gracilariales comprises a recently described order of marine red algae (Fredericq and Hommersand 1989a) based on the Gracilariaceae Nägeli (1847), a family previously placed in the Gigartinales (Kylin 1932, 1956). Members are characterized by the following: 1) a female reproductive apparatus composed of a two-celled carpogonium branch, 2) a supporting cell producing other two to three two-celled sterile filaments, 3) the fertilized carpogonium fusing with its supporting cell to form the fusion cell, 4) the cells of the sterile filaments fusing and transfer their cellular-rich contents into the fusion cell, and 5) the gonimoblasts developing directly and primarily outward from the fusion cell (Fredericq and Hommersand 1989a, 1990b, Hommersand and Fredericq 1990). Studies targeting the higher evolutionary relationships among red algae based on molecular analyses confirm the monophyly of the Gracilariales (Freshwater et al. 1994, Fredericq et al. 1996).

The order is composed of a single family, Gracilariaceae, because the status of the parasitic Pterocladiophilaceae is still unresolved by molecular methods. Generic concepts within the Gracilariaceae have been based on anatomical details in cystocarp ontogeny that reflect strategies for the provision of nutrients by the gametophyte to the developing carposporophyte (Fredericq and Hommersand 1990b). Currently, the Gracilariaceae is composed of up to seven genera (Fredericq and Hommersand 1990b), namely *Gracilaria* Greville (1830), *Hydropuntia* Montagne (1842), *Melanthalia* Montagne (1843), *Curdiea* Harvey (1855), *Gracilariophila* Setchell et Wilson in Wilson (1910), *Gracilariopsis* Dawson (1949), and *Congracilaria* Yamamoto (1986). Identification keys and short diagnoses for each genus are provided in Fredericq and Hommersand (1990b). *Curdiea* and *Melanthalia* are restricted to the temperate regions around southern Australia, Tasmania, and New Zealand; the former is also found

[1]Received 23 August 2002. Accepted 18 September 2003.
[2]Author for correspondence and present address: Universidade Federal do Rio de Janeiro, CCS, Instituto de Biologia, Departamento de Botânica, Ilha do Fundão, Rio de Janeiro, RJ, Brasil, 21941-900. e-mail f-gurgel@biologia.ufrj.br.

in Antarctica. *Curdiea* is characterized by a thick foliose habit and a high degree of morphological plasticity.

Considered synonymous with *Gracilaria* are *Corallopsis* Greville (1830) and *Tyleiophora* J. G. Agardh (1890). *Polycavernosa* Chang et Xia (1963) has been subsumed into *Hydropuntia* (Wynne 1989). Morphologically and genetically *Curdiea* and *Melanthalia* are considered distinct genera from each other and from the remaining Gracilariaceae (Fredericq and Hommersand 1989a,b,c, 1990a,b, Bird et al. 1992). Although some authors consider *Gracilaria* (hereafter *G.*), *Gracilariopsis* (hereafter *Gp.*), and *Hydropuntia* distinct genera (Wynne 1989, 1998), others consider *Hydropuntia* a synonym of *Gracilaria* (Abbott et al. 1991, Bellorin et al. 2002), and still others place all three genera in synonymy with *Gracilaria* (Gargiulo et al. 1992, Abbott 1995, 1999). The proposed synonymy between *Gracilaria* and *Gracilariopsis* is based on practical taxonomic considerations (Abbott 1999) instead of true phylogenetic uncertainties, because there has been overwhelming morphological and genetic evidence supporting the separation of these two genera (Fredericq and Hommersand 1989a,b, Bird et al. 1992, Bellorin et al. 2002). Nonetheless, the precise taxonomic identification of sterile cylindrical specimens remains a difficult task, especially in cases of morphological modification due to the influence of particular biotic (e.g. herbivory) and abiotic (e.g. drifting habit, wave exposure, sand scours) factors. In these cases, vegetative characters of cylindrical species from both genera often converge.

Subgenera within *Gracilaria* are based on the shape of the mature spermatangial conceptacle: the *textorii* type, the *verrucosa* type, and the *Hydropuntia* type (*sensu* Yamamoto, 1978). However, distinguishing subgenera on such a basis is said to be unreliable because several species display features characteristic of more than one subgenus (Abbott et al. 1991, Schneider and Searles 1991, Gargiulo et al. 1992).

Whereas all genera but *Gracilaria* are relatively small, comprising fewer than 20 species each, *Gracilaria* has nearly 300 described species, of which 110 are currently recognized worldwide (Oliveira and Plastino 1994). At present, the genus *Gracilaria* is the major source of agar and the third largest farmed seaweed worldwide (Zemke-White and Ohno 1999). Propelled by an economic interest in phycocolloids, the study of *Gracilaria* has resulted in numerous proposals for taxonomic and nomenclatural changes (Silva et al. 1996). After a very confused and dynamic lectotypification history (nomenclatural reviews in Fredericq and Hommersand 1989a, Steentoft et al. 1991, Silva 1994, Bird 1995, Irvine and Steentoft 1995, Silva et al. 1996), *Gracilaria* was officially typified with *G. compressa* (C. Agardh) Greville 1830 (Greuter et al. 2000:168); this name is a synonym of *G. bursa-pastoris* (S. Gmelin) Silva (1952:265).

In addition to morphological features, molecular and caryological studies have also been applied to solve taxonomic and systematic problems within the Gracilariaceae. Chromosome counts, reported so far only for some species of *Gracilaria* and *Gracilariopsis*, reveal that these two genera have distinct chromosome numbers (24 and 32, respectively), corroborating that these two genera are indeed distinct taxonomic entities (Kapraun 1993, Kapraun et al. 1993). However, this technique has no resolution below the generic level and has not helped to solve systematic questions below the level of genus. Chromosome counts for the remaining Gracilariaceae genera did not provide reliable results (*Hydropuntia*, Kapraun et al. 1993) or have not been assessed (*Curdiea* and *Melanthalia*).

Allozyme profiles have been used only to infer genetic variation at population level within a single species, *Gracilaria chilensis*, from New Zealand (Intasuwan et al. 1993). The authors found population structure despite the fact that intrapopulation variation (heterozygosity) and genetic distance among New Zealand *G. chilensis* populations are low. Allozyme profiles have not been applied to resolve broad-scale systematic questions in red algae because this technique has limited applications and is more suitable for studies at, and especially below, the species level.

Restriction maps (RFLP) and primer-DNA similarity random amplified polymorphic DNA (RAPD) has been more widely used to address systematic questions in the Gracilariaceae. RAPD has been successfully applied to infer intraclonal genetic variation within and among populations, strains, and coalesced versus noncoalesced thalli in *G. chilensis* (González et al. 1996, Santelices et al. 1996, Meneses and Santelices 1999). RFLPs of isolated plastid DNA have also been successfully applied to infer the potential usefulness of this technique (Goff and Coleman 1988) in red algal systematics. The RFLP pattern generated upon the electrophoretic separation of digestion fragments showed that within *Gracilariopsis andersonii* (as *Gracilariopsis lemaneiformis*), patterns are identical among populations spread 2000 miles along the western North America coast but not between higher taxa. Even though Goff and Coleman (1988) showed that this molecular technique can distinguish different genera and species, only one *Gracilariopsis* and two *Gracilaria* species were used in their study. RFLPs of specifically chosen PCR-amplified markers were applied to infer population differences between Chilean and New Zealand populations of *Gracilaria chilensis* (internal transcribed spacer [ITS], Candia et al. 1999) and to assess species limits within *Gracilaria* and *Gracilariopsis* (18S rDNA, Scholfield et al. 1991). Both studies included small numbers of species, the former target a single species, and the latter, two species (*Gp. longissima* and *G. gracilis* [as "*G. verrucosa*"]) from different parts of the world. Microsatellite primers, a codominant marker, have been developed for Gracilariaceae, but this technique, besides being developed for a single species, *G. gracilis*, focuses on inferring population level questions and proved to be able to identify individuals within a population (Wattier et al. 1998, Luo et al. 1999).

DNA sequence analysis has been the most reliably and widely used molecular technique to infer phylo-

genic relationships at the species level within the Gracilariaceae. These studies have used regions of the nuclear ribosome cistron (Bird et al. 1992, 1994, Goff et al. 1994, Bird 1995), the chloroplast encoded *rbc*L gene (Gurgel et al. 2003a,b), and the *rbc*L-*rbc*S spacer region (Goff et al. 1994). In the Gracilariaceae these studies have 1) identified new *Gracilariopsis* species (Gurgel et al. 2003a,b), 2) showed that worldwide distributed species are indeed artificial assemblages of distinct taxa (e.g. the "*G. verrucosa*" and *Gp. lemaneiformis* species complexes) (Bird et al. 1994, Goff et al. 1994), and 3) provided new insights about correlations between molecular and morphological phylogenetic relationships (Bird et al. 1992, 1994, Bird 1995, Bellorin et al. 2002). All these DNA sequence studies produced strong evidence supporting the taxonomic distinctiveness of the genera *Curdiea*, *Gracilaria*, *Gracilariopsis*, and *Melanthalia*, but the position of the genus *Hydropuntia* remains controversial. Despite these advances in the systematics of the Gracilariaceae, all molecular techniques applied so far have focused on only a few species (e.g. *G. chilensis*, *G. gracilis*, *G. tenuistipitata*, *G. tikvahiae*, *Gp. longissima*, *Gp. lemaneiformis*) and were geographically restricted (mostly North America, Atlantic Europe, Chinese, and Chilean species). The most extensive phylogenetic surveys published to date are provided by Bird et al. (1992) and Bellorin et al. (2002) in which 19 and 39 Gracilariaceae 18S rDNA sequences were analyzed, respectively. Unfortunately, the 18S rDNA provides insufficient resolution at the species level in the *Gracilaria*/*Hydropuntia* complex (Bird 1995:263, Bellorin et al. 2002, fig. 1B). The two ITS of the ribosomal cistron have proven useful in distinguishing between some closely related congeners. However, the level of genetic variation is too great to allow unambiguous alignment of sequences among most species within the genera *Gracilaria* or *Gracilariopsis*, let alone between genera (Bird et al. 1994, Bellorin et al. 2002). Therefore ITS cannot be used for determining phylogenetic relationships in the family as a whole (Bird 1995).

In this study, the *rbc*L is considered to provide optimal resolution for inferring species level phylogenetic relationships within the Gracilariaceae, relative to other commonly used genetic markers (18S, ITS1, 5.8S, ITS2, and 28S rDNA regions). The main goal of this study is to use *rbc*L sequence data to provide a critical assessment of generic and subgeneric concepts and to resolve species-level phylogenetic and biogeographic questions pertaining to the Gracilariaceae. This study is the most extensive systematic survey and phylogenetic analysis of the Gracilariaceae with molecular data performed to date.

MATERIAL AND METHODS

Silica-gel dried specimens and extracted DNA samples were deposited in the Seaweed Laboratory at the University of Louisiana at Lafayette and stored at $-20°$ C. DNA samples were prepared using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) or were submitted to a CTAB-cesium chloride DNA procedure (Freshwater and Rueness 1994). Plastid-encoded *rbc*L was selected to infer a phylogeny for the Gracilariaceae. PCR (FrbcLstart-R753, F57-R753, F577-R1381, F993-RrbcSstart) and sequencing primers (FrbcLstart, F7, F57, F492, F577, F753, F993, R753, R1105, R1381, RrbcSstart) used in this study are listed in Freshwater and Rueness (1994) and Gavio and Fredericq (2002). Protocols for gene amplification, automated sequencing, and multiple sequence alignment are identical to those given in Lin et al. (2001). Voucher specimens and materials for morphological studies were fixed and stored in 5% formalin/seawater and/or pressed as herbarium sheets and deposited in the Herbarium of the University of Louisiana at Lafayette. Herbarium abbreviations follow those of Holmgren et al. (1990). Species identifications were based on the original descriptions, critical analysis of published literature, and on the type method (Silva 1952). An extensive photographic collection of type species of Gracilariaceae housed in Herbarium of the University of Louisiana at Lafayette was used to match recently collected specimens.

Partial and complete *rbc*L sequences were produced for a total of 67 specimens of Gracilariaceae (Table 1) comprising 3 *Curdiea*, 4 *Melanthalia*, 13 *Gracilariopsis*, and 47 *Gracilaria sensu lato* specimens (*Gracilaria sensu lato*, as defined by Abbott et al. [1991], includes all species once placed in *Hydropuntia*). DNA sequences have been deposited in GenBank (Benson et al. 1994). GenBank accession numbers, species identification and authors, and information concerning origin, date, and collector are listed in Table 1. The generated sequence data were compiled and aligned with Sequencher (Gene Codes Corp., Ann Arbor, MI, USA) and MacClade 4.0 (Maddison and Maddison 2000) and exported for phylogenetic analysis. Because some sequence data were incomplete at the 5′ terminus of the coding region in many taxa, the data set was restricted to the last 1368 base pairs (bp) of the 1467-bp *rbc*L.

Phylogenetic analyses were conducted with maximum parsimony (MP) and minimum evolution (ME) as implemented in PAUP* v.4.0 beta 10 (Swofford 2002), and the Bayesian likelihood as implemented in MrBayes 1.11 (Huelsenbeck and Ronquist 2001). Parsimony trees obtained under the Fitch criterion of equal weights for all substitutions (Fitch 1971) were inferred in a two-part heuristic search scheme, excluding uninformative characters. Initial searches designed to increase the likelihood of swapping within the "island" of trees leading to the most parsimonious solution (Maddison 1991) consisted of 5000 random sequence additions holding 25 trees at each step, MULPARS, and tree-bisection-reconnection algorithms with MULTREES (saving multiple trees) and STEEPEST DESCENT options. All most parsimonious trees found in this initial search were then swapped to completion using the tree-bisection-reconnection algorithm. Consistency and retention indices were calculated (Farris 1989, Kluge and Farris 1989).

The optimal model of sequence evolution to fit the data alignment estimated by hierarchical likelihood ratio tests performed by Modeltest v.3.04 (Posada and Crandall 1998) was the GTR $I+\Gamma$ (general time reversible model with invariable sites and gamma distribution). The parameters used were as follows: assumed nucleotide frequencies A = 0.3475; C = 0.1202; G = 0.1556; T = 0.3767; substitution rate matrix A–C substitutions = 1.0663, A–G = 6.4763, A–T = 0.7696, C–G = 1.7148, C–T = 11.5115, G–T = 1.0; proportion of sites assumed to be invariable = 0.5445; and rates for variable sites assumed to follow a gamma distribution with shape parameter = 1.2152. These likelihood parameters were applied in Bayesian [Lset Nst = 6 revmat = (1.0663, 6.4763, 0.7696, 1.7148, 11.5115, 1.0) rates = invgamma shape = 1.2152 ncat = 4 basefreq = estimate;] and ME analyses.

For the Bayesian analysis, we ran four chains of the Markov Chain Monte Carlo, sampling 1 tree every 10 generations for

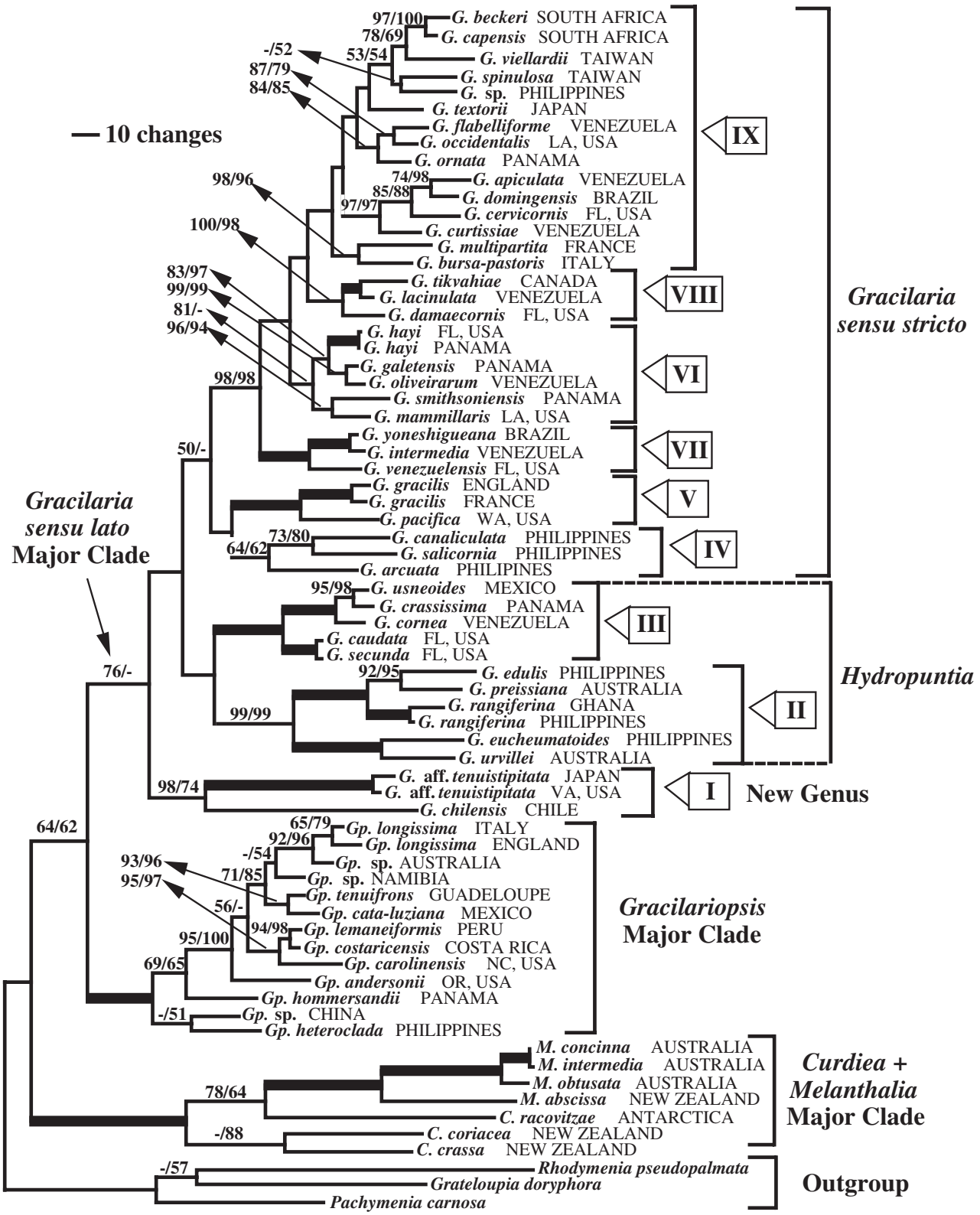

FIG. 1. One of 36 most parsimonious trees from analysis of the *rbc*L gene sequence data of the family Gracilariaceae (tree length = 2805 steps, consistency index = 0.293, retention index = 0.6421, number of phylogenetically informative characters = 490). Numbers above the branches correspond to bootstrap proportion values (%) from maximum parsimony and minimum evolution analyses, respectively (= MP/ME, both based on 1000 resamplings) and thick bold branches correspond to 100% bootstrap proportion values obtained in both phylogenetic methods (= 100/100). Roman numerals correspond to (nine) distinct evolutionary lineages (= subgroups) within the genus *Gracilaria sensu lato*, as also seen in Figure 2A.

TABLE 1. List of species identification, collection information, and the *rbc*L GenBank accession numbers followed by *rbc*L fraction sequenced (in %).

Entity	Collection data	GenBank accession number and percent sequenced
Outgroups (non-Gracilariales)		
Rhodymenia pseudopalmata (Lamouroux) Silva	Port Aransas Jetty, TX, USA; coll. C.F. Gurgel, 17 May 1998	AY1686565
Grateloupia doryphora (Montagne) Howe	Playa de San Francisco, Bahia de Ancon, Peru; coll. P. Carbájal, 15 September 2001	AF488817
Pachymenia carnosa (J. Ag.) J. Agardh	Kommetjie, Cape Penisula, South Africa; coll. O. De Clerck, 9 November 1999	AF385640
Ingroup (Gracilariales)		
Curdiea coriacea (Hook. et Harv.) J. Agardh	Doubtless Bay, New Zealand; coll. W. Nelson, 1 December 1993	AY049425, 66.5%
Curdiea crassa Millar	Bongin Bay, North of Sydney, NSW Australia; coll. A. Millar & P. Richards; 18 February 1994	AY049427, 98.1%
Gracilaria apiculata P. et H. Crouan in Schramm et Mazé	Playa Barranquita, Puerto Cumarebo area, Falcón State, Venezuela; coll. C. F. Gurgel, 14 July 1999	AY049341, 98.8%
Gracilaria arcuata Zanardini	Hilutangdu, Cebu, Philippines; coll. S. M. Lin, 19 April 1998	AY049383, 98.6%
Gracilaria beckeri (J. Agardh) Papenfuss	Sharks Bay, Port Alfred, South Africa; coll. M. H. Hommersand, 19 July 1993	AY049377, 96.3%
Gracilaria bursa-pastoris (Gmelin) Silva	Italy; coll. E. Cecere, 25 July 1994	AY049376, 91.6%
Gracilaria canaliculata Sonder	Philippines; coll.: S. M. Lin, April 1998	AY049390, 87.9%
Gracilaria capensis Schmitz ex Mazza	Sharks Bay, Port Alfred, South Africa; coll. M. H. Hommersand, 19 July 1993	AY049378, 96.5%
Gracilaria caudata J. Agardh	Walton Rocks, St. Lucie Co., FL, USA; coll. C. F. Gurgel, J. N. Norris & S. Fredericq, 11 April 1998	AY049358, 76.4%
Gracilaria cervicornis (Turner) J. Agardh	Higgis Beach, Key West, FL, USA; coll. C. F. Gurgel, July 1998	AY049365, 95.6%
Gracilaria chilensis Bird, McLachlan et Oliveira	Playa Charia, Coquimbo, Chile; coll. S. Fredericq, 19 January 1995	AY049396, 98.2%
Gracilaria cornea J. Agardh	Puerto Escondido, Peninsula Paraguana, Falcon State, Venezuela; coll. C. F. Gurgel, 13 July 1999	AY049338, 98.8%
Gracilaria crassissima P. et H. Crouan in Mazé et Schramm	Fort Randolph, Colon City, Panama; coll. B. Wysor, 6 March 1999	AY049351, 98.0%
Gracilaria hayi Gurgel, Fredericq et J.N. Norris	Hutchinson Is. beach close to Fort Pierce jetty, Fort Pierce, FL, USA; coll. C. F. Gurgel, October 1998	AY049319, 95.6%
Gracilaria hayi Gurgel, Fredericq et J.N. Norris	Galeta Point, Colon City, Panama; coll. B. Wysor, 21 September 1999	AY049315, 98.1%
Gracilaria curtissiae J. Agardh	Mague Lloroso, Peninsula Paraguana, Falcon State, Venezuela; coll. C. F. Gurgel, 13 July 1999	AY049327, 98.4%
Gracilaria damaecornis J. Agardh	Beach behind the Harbor Branch Oceanographic Institution jetty, Fort Pierce, FL, USA; coll. C. F. Gurgel, 13 July 1998	AY049326, 100%
Gracilaria domingensis Sonder ex Kützing	Praia Rasa, Búzios city, Rio de Janeiro State, Brazil; coll. C. F. Gurgel, 12 December 1998	AY049371, 98.6%
Gracilaria edulis (Gmelin) Silva	Little Santa Cruz, Philippines, coll. L. M. Liao, 28 April 1998	AY049387, 98.6%
Gracilaria eucheumatoides Harvey	Tambuli, Cebu, Philippines; coll.: S. M. Lin; 18 April 1998	AY049389, 93.3%
Gracilaria flabelliformis comb. nov. (P. et H. Crouan in Mazé et Schramm) Gurgel et Fredericq	Playa Barranquita, Puerto Cumarebo area, Falcon State, Venezuela; coll. C.F. Gurgel, 14 July 1999	AY049343, 98.8%
Gracilaria galetensis Gurgel, Fredericq et J. N. Norris	Galeta Point, Atlantic Panama; coll. B. Wysor, 20 June 1999	AY049320, 97.3%
Gracilaria gracilis (Stackhouse) Steentoft, Irvine et Farnham	W. Angle Bay, Wales, England; coll. M. H. & F. Hommersand, 22 July 1997	AY0494000, 98.0%
Gracilaria gracilis (Stackhouse) Steentoft, Irvine et Farnham	Ile Verte, Roscoff, Brittany, France; coll. J. Cabioch, 22 June 1993	AY0493399, 98.0%
Gracilaria intermedia J. Agardh	Puerto Escondido, Peninsula Paraguana, Venezuela; coll. C. F. Gurgel, 13 July 1999	AY049336, 97.6%
Gracilaria lacinulata (Vahl) Howe	La Encrucijada, Peninsula Paraguana, Falcon State, Venezuela; coll. C. F. Gurgel; 13 July 1999	AY049344, 97.1%
Gracilaria mammillaris (Montagne) Howe *in* Britton	Offshore LA, USA; coll. C. F. Gurgel & S. Frdericq, 26 May 2000	AY049323, 97.1%
Gracilaria multipartita (Clement) Harvey	Carantec, Brittany, France; coll. J. Cabioch, 22 May 2000	AY049322, 98.6%
Gracilaria occidentalis (Børgensen) Bodard	Offshore LA, USA; coll. C. F. Gurgel & S. Fredericq, 26 May 2000	AY049322, 98.6%
Gracilaria ornata Areschoug	Fort Randolph, Colon city, Panama; coll. B. Wysor, 26 February 1999	AY049318, 92.9%
Gracilaria pacifica Abbott	Indian Island, WA, USA; coll. M. H. Hommersand	AY049397, 97.7%
Gracilaria preissiana (Sonder) Womersley in Min-Thein et Womersley	Cervantes, Australia; coll. M. H. & F. H. Hommersand, 20 September 1995	AY049403, 93.7%

TABLE 1. (*continued*)

Entity	Collection data	GenBank accession number and percent sequenced
Gracilaria rangiferina (Kützing) Piccone	Terna, Ghana; coll. G. Ameka, leg. M. H. Hommersand, March 2001	AY049379, 86.0%
Gracilaria rangiferina (Kützing) Piccone	La Vista del Mar, upper Calarian, Zamboanga City, Philippines; coll. S.M. Lin; 27 April 1998	AY049380, 95.5%
Gracilaria salicornia (C. Agardh) Dawson	Sulpa, Cebu, Philippines; coll. S. M. Lin, 19 April 1998	AY049385, 98.0%
Gracilaria secunda P. et H. Croaun in Schramm et Mazé	Tampa Bay, FL, USA; coll. C. Dawes, 26 October 1999	AY049360, 97.8%
Gracilaria sp.	Bulusan, South Luzon, Philippines; coll. S. M. Lin, 21 April 1998	AY049384, 93.2%
Gracilaria smithsoniensis Gurgel, Fredericq et J. N. Norris	Galeta Point, Atlantic Panama; coll. B. Wysor, BW#737, 20 June 1999	AY049321, 97.3%
Gracilaria spinulosa (Okamura) Chang et Xia	Taiwan; coll. S. M. Lin, 11 May 1998	AY049395, 93.3%
Gracilaria aff. tenuistipitata Chang et Xia	Tokawa, Japan; coll. M. Yoshizaka, 7 June 1993	AY049324, 97.3%
Gracilaria aff. tenuistipitata Chang et Xia	Hog Island Bay, Eastern Shore, VA, USA; coll. C. Tyler, 10 February 1999, leg. T. Frankovich	AY049312, 98.0%
Gracilaria textorii (Suringar) De Toni	Gobogahana, Japan; 10 July 1994, leg. M. Hommersand	AY049325, 97.5%
Gracilaria tikvahiae McLachlan	Morret pond, Pomquet harbor, Antigonish Co., Nova Scotia, Canada; coll. C. J. Bird, 3 July 1999	AY049434, 97.0%
Gracilaria urvillei (Montagne) Abbott in Abbott, Zhang et Xia	Lee Point, Darwin, Australia; coll. M. H. Hommersand, 22 November 1995	AY049402, 97.4%
Gracilaria usneoides (C. Agardh) J. Agardh	Santa Rosalia bridge, Campeche Bay, Mexico; coll. C. F. Gurgel, 14 February 1999	AY049346, 98.0%
Gracilaria venezuelensis Taylor	Indian River, Fort Pierce, FL, USA; coll. C. F. Gurgel, October 1998	AF539603, 95.4%
Gracilaria viellardii Silva	Taiwan; coll. S. M. Lin, 22 April 1998	AY049394, 95.5%
Gracilaria oliveirarum Gurgel, Fredericq et J. N. Norris	La Vela de Coro, Falcon State, Venezuela; coll. C. F. Gurgel, 13 July 1999	AY049330, 91.8%
Gracilaria yoneshigueana Gurgel, Fredericq et J. N. Norris	Prainha beach, Arraial do Cabo City, Rio de Janeiro State, Brazil; coll. A. Taouil, 13 March 1998	AY049372, 93.4%
Gracilariopsis andersonii (Grunow) Dawson	Seal Rock, Lincoln Co., Oregon, USA; coll. S. Fredericq, 15 May 1999	AY049414, 96.4%
Gracilariopsis carolinensis Liao & Hommersand in Gurgel, Liao, Fredericq & Hommersand	Kure Beach, Fort Fisher, NC, USA; coll. D. W. Freshwater, 14 April 1991	AY049412, 96.7%
Gracilariopsis cata-luziana Gurgel, Fredericq & Norris	19° 03.31' N × 96° 0.44' W, Vera Cruz area, Mexico; coll. C. F. Gurgel; 10 February 1999	AY049406, 80.2%
Gracilariopsis costaricensis Dawson	South end, Playa Tamarindo, Nicoya Penninsula, Guanacaste, Costa Rica; coll. D. T. Talbot & D. W. Freshwater, 17 March 1999	AY049423, 98.4%
Gracilariopsis heteroclada (Zhang et Xia) Zhang et Xia in Abbott	Dapdap, Bulusan, Luzon, Philippines; coll. S. M. Lin, 22 April 1998	AY049411, 91.1%
Gracilariopsis hommersandii Gurgel, Fredericq & Norris	Fort Randolph, Colon City, Panama; coll. B. Wysor, 26 March 1998	AY049405, 97.1%
Gracilariopsis lemaneiformis (Bory) Dawson, Acleto et Foldvik	Yacilla, Paita, Piura, Peru; coll. C. Acleto & R. Zuniga, 3 March 1994	AY049415, 97.6%
Gracilariopsis longissima (Stackhouse) Irvine, Steentoft et Farnham	Venetian lagoon, Adriatic Sea, Italy; coll. K. S. Cole, 7 September 1998	AF527881, 97.5%
Gracilariopsis longissima (Stackhouse) Irvine, Steentoft et Farnham	Off Sandfoot Castle, Portland Harbour, Dorset, England; coll. Wm. Farnham & M. Steentoft, 30 August 1992, leg. C. J. Bird;	AY049420, 97.3%
Gracilariopsis sp.	Lake Butler, Robe, Australia; coll. H. B. S. Womersley, 3 March 1995	AY049422, 97.8%
Gracilariopsis sp.	Swakopsmund, Namibia; coll. M. H. Hommersand, 6 July 1993	AY049410, 98.2%
Gracilariopsis sp.	Qingdao, Shandong Prov., China; coll. M. H. Hommersand, 23 April 1994	AY049421, 65%
Gracilariopsis tenuifrons (Bird et Oliveira) Fredericq et Hommersand	Ilet Caret, Guadeloupe, French West Indies; coll. A. Renoux, 2 December 1993	AY049418, 97.8%
Melanthalia abscissa (Turner) Hooker & Harvey	New Zealand; coll. W. Nelson, 25 April 1994	AY049428, 97.9%
Melanthalia concinna J. Agardh	Warrnambool, Australia; coll. M. H. Hommersand, 13 July 1995	AY049429, 96.1%
Melanthalia intermedia Harvey	Warrnambool, Australia; coll. M. H. Hommersand, 13 July 1995	AY049430, 97.9%
Melanthalia obtusata (Labillardière) J. Agardh	Warrnambool, Victoria, Australia; coll. M. H. Hommersand, 13 July 1995	AY049431, 99%

TABLE 2. Comparisons of Gracilariaceae genetic diversity between *rbc*L and 18S rDNA (SSU) sequences.

	*rbc*L (this study)	SSU (Bellorin et al. 2002)
Intergeneric		
Curdiea vs. *Melanthalia*	14.13–15.40	1.47
Gracilaria sensu lato vs. *Gracilariopsis*	8.45–11.84	2.24–4.65
Curdiea + *Melanthalia* vs. *Gracilariopsis*	14.44–16.41	3.65–6.35
Proposed new intergeneric divisions		
Subgroup I vs. *Gracilaria sensu stricto*	10.01–13.05 ($n = 3$)	—
Subgroup II vs. *Gracilaria sensu stricto*	12.31–8.46 ($n = 6$)	—
Subgroup III vs. *Gracilaria sensu stricto*	9.91–6.71 ($n = 5$)	—
Interspecific		
Curdiea	11.79–16.20 ($n = 3$)	—
Melanthalia	0.14–8.70 ($n = 4$)	—
Gracilariopsis	2.37–7.47 ($n = 13$)	0.47–2.88
Gracilaria sensu lato	2.00–13.61 ($n = 47$)	0.00–1.29
Gracilaria subgroup I	12.12–12.67 ($n = 3$)	—
Gracilaria subgroup II	4.22–10.08 ($n = 6$)	—
Gracilaria subgroup III	0.40–4.18 ($n = 5$)	—
Gracilaria sensu stricto (= subgroups IV–IX)	2.10–9.05 ($n = 33$)	—
Intraspecific[a]		
Gracilariopsis	0.00–0.07	0.18
Gracilaria sensu stricto	0.00–1.89	0.00–0.41

Genetic divergence expressed as uncorrected percentages (%, "p" distances). *Gracilaria sensu lato* as defined by Abbott et al. (1991) to include *Hydropuntia*. *Gracilaria sensu lato* subgroups composed of subgroup I, *G. chilensis* + *G. aff. tenuistipitata*; subgroup II, *G. urvillei*, *G. eucheumatoides*, *G. edulis*, *G. preissiana* and *G. rangiferina*; subgroup III, *G. crassissima*, *G. cornea*, *G. caudata*, *G. secunda*, *G. usneoides*.

[a] *rbc*L data used to compute intraspecific genetic distances from Gurgel et al. (2001).

1,000,000 generations starting with a random tree. Stationarity was reached at above generation 20,200. Thus, the first 20,200 generations were the "burn in" of the chain, and inferences about the phylogeny were based on those trees sampled after the burn in point. A 50% consensus tree (majority rule as implemented by PAUP*) was computed from the 98,981 trees saved after the burn in point. Reliability of the Bayesian consensus tree is given by the frequency at which each node appears among all saved trees after the burn in generation. This frequency corresponds to the true probability of the clades (Hall 2001).

ME was performed using a heuristic search of 1000 replications, holding 10 trees each stepwise addition step, with a tree-bisection-reconnection swapping algorithm, MULTREES and STEEPEST DESCENT options. Starting trees were obtained via stepwise addition with a random sequence addition and a random nine digits starting seed (= 86366). A maximum likelihood distance correction was used and set with the GTR substitution rate matrix parameters listed above, excluding invariable sites and the gamma distribution. The ME method only recovered the most probable hypothesis similar to those given by the MP and Bayesian methods when simpler models of sequence evolution were applied (data not shown). Support for nodes in the MP and ME analyses were assessed by calculating bootstrap proportion (BP) values (Felsenstein 1985) based on 1000 resamplings.

Mutational saturation of third codon positions in *rbc*L was examined by plotting all pairwise genetic distances uncorrected for multiple substitutions ("p" distance) against those corrected for multiple substitutions with Kimura-2 parameter (Kimura 1980), according to the procedure in Daugbjerg and Andersen (1997) in which for all pairwise combinations, corrected and uncorrected values for multiple substitutions were calculated for first/second positions only and third codon positions only.

Sequences of Rhodymeniaceae (*Rhodymenia pseudopalmata*) and Halymeniaceae (*Grateloupia doryphora* and *Pachymenia carnosa*) were selected as the outgroup based on phylogenetic hypotheses derived from earlier global analyses of the Florideophycidae as a whole (Fredericq et al. 1996). Pairwise genetic distances were calculated based on uncorrected percentages, "p" distances (Table 2).

RESULTS

No insertion or deletion mutations were found in the *rbc*L sequences, permitting unambiguous alignment of all sequences. Tree lengths of 100,000 randomly generated trees had a skewed distribution ($g_1 = -0.5708$, $P < 0.01$), indicating the presence of nonrandom structure (Hillis and Huelsenbeck 1992, Hillis et al. 1993).

FIG. 2. Gracilariaceae phylogeny based on *rbc*L DNA sequences. (A) Majority rule consensus of 97,991 trees sampled according to a Bayesian MCMC tree sampling procedure (number of generations = 10^6, burning point = 20,200, evolutionary model = GTR + I + Γ). Numbers above the branches correspond to Bayesian posterior probability support, and thick bold branches correspond to 100% Bayesian posterior probability. *Curdiea* and *Melanthalia* monophyletic clade is in yellow, the *Gracilariopsis* clade in pink, and species from the genus *Hydropuntia* complex in gray. Roman numerals correspond to distinct evolutionary lineages (= subgroups) within the genus *Gracilaria sensu lato*. (B–J) transverse sections of *Gracilaria sensu lato* reproductive structures. (B–E) Reproductive morphological features characteristic of species from *Gracilaria sensu stricto* only (subgroups IV–IX). (F–H) Reproductive morphological features characteristic of the genus *Hydropuntia* (subgroups II and III). (I and J) Reproductive morphological features characteristic of a new genus (subgroup I). (B, C, F, I) Spermatangial conceptacles. (B) *Textorii* type (*G.* "*blodgettii*", reproduced from Terada and Yamamoto 2000). (C) *Verrucosa* type (from *G. gracilis*). (F) *Hydropuntia* type (from *G. crassissima*). (I) *chilensis* type (*G. chilensis*, reproduced from Bird et al. 1990). (D, E, G, H, J) Cystocarps with different carposporophyte designs. (D) *G. flabelliforme*, (E) *G. tikvahiae*, (G) *G. crassissima*, (H) *G. edulis*, (J) *G. aff. tenuistipitata* from USA.

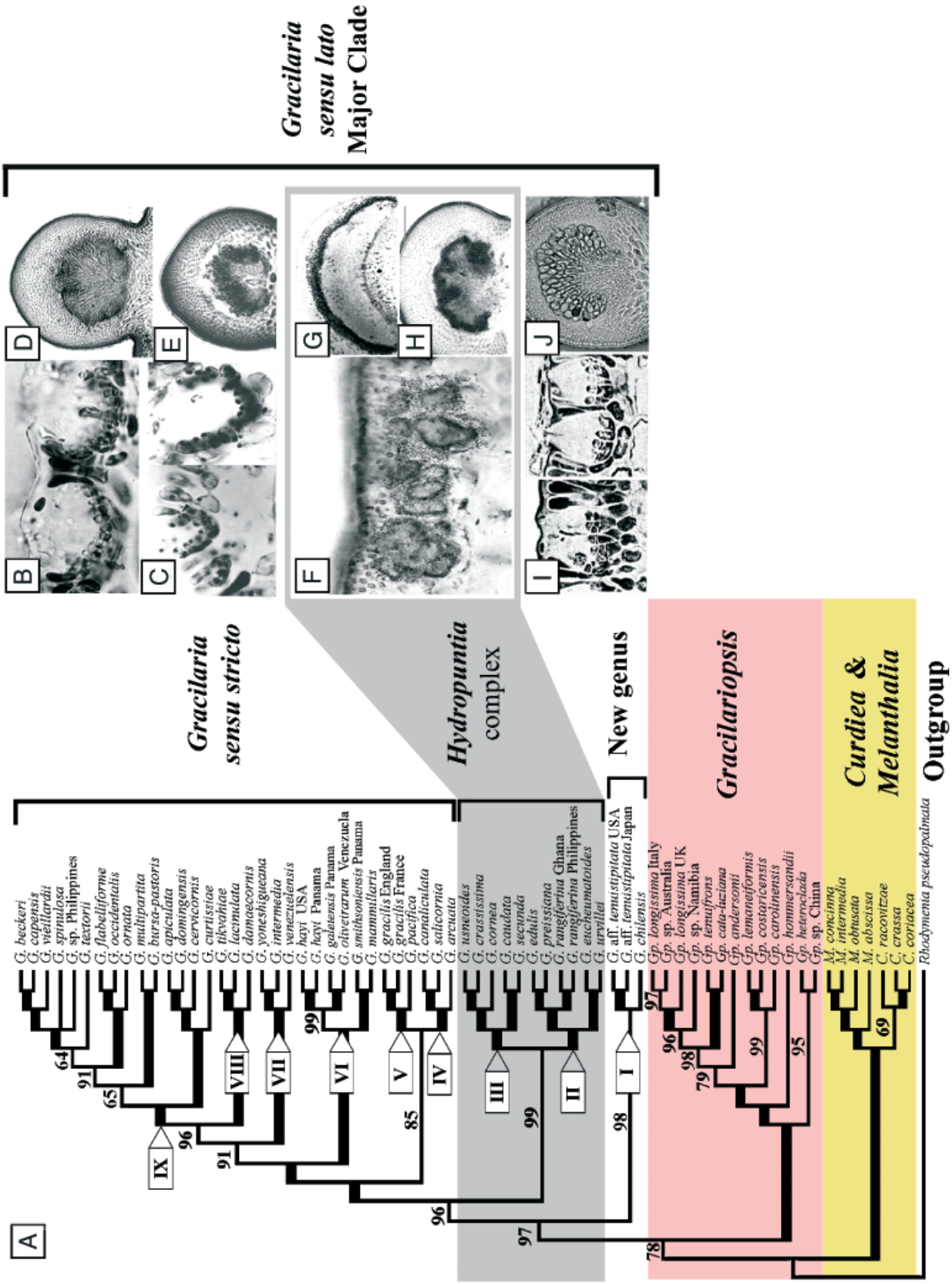
Gracilaria sensu lato Major Clade
Gracilaria sensu stricto
Hydropuntia complex
New genus
Gracilariopsis
Curdiea & Melanthalia
Outgroup
A
B
C
D
E
F
G
H
I
J
G. beckeri
G. capensis
G. vieillardii
G. spinulosa
sp. Philippines
G. textorii
G. flabelliforme
G. occidentalis
G. ornata
G. multipartita
G. bursa-pastoris
G. apiculata
G. domingensis
G. cervicornis
G. curtissiae
G. tikvahiae
G. lacinulata
G. damaecornis
G. yoneshigueana
G. intermedia
G. venezuelensis
G. hayi USA
G. hayi Panama
G. galetensis Panama
G. oliveiranum Venezuela
G. smithsonensis Panama
G. mammillaris
G. gracilis England
G. gracilis France
G. pacifica
G. canaliculata
G. salicornia
G. arcuata
G. uncoides
G. crassissima
G. cornea
G. caudata
G. vecunda
G. edulis
G. preissiana
G. rangiferina Ghana
G. rangiferina Philippines
G. encheumatoides
G. urvillei
G. aff. tenuistipitata USA
G. aff. tenuistipitata Japan
G. chilensis
Gp. longissima Italy
Gp. sp. Australia
Gp. longissima UK
Gp. sp. Namibia
Gp. tenuifrons
Gp. cata-laziana
Gp. andersonii
Gp. lemaneiformis
Gp. costaricensis
Gp. carolinensis
Gp. hommersandii
Gp. heterociada
Gp. sp. China
M. concinna
M. intermedia
M. obtusata
C. racovitzae
C. crassa
C. coriacea
Rhodymenia pseudopalmata
I
II
III
IV
V
VI
VII
VIII
IX
97
78
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TABLE 3. Comparative morphological differences of *textorii*-type spermatangial conceptacle between the *Gracilaria chilensis*/*G. aff. tenuistipitata* and *G. bursa-pastoris*/*G. textorii* lineages.

Spermatangial characters	Subgroup I *G. chilensis* lineage	Subgroup IX *G. textorii* lineage
Cortical cells	Elongated, as in *Gracilariopsis*	Variable: isodiametric, rounded, squarish, or elongated
Cortical cells flanking spermatangia	Club shaped, wide	Linear to obovoid, thin
Conceptacle in transverse section	Squarish	Concave
Spermatangial parent cell layer	Restricted to floor of conceptacle only	Surrounding entire internal surface
Three-dimensional shape of noncoalesced conceptacles	Well shaped	Bow shaped

Among the 1368 bp in the data set (excluding outgroup sequences), 561 are constant and 807 vary (of which 490 are phylogenetically informative). Informative and noninformative variable sites, regardless of codon position, were evenly distributed throughout the gene (data not shown) with an overall average transition/transversion rate of 2.289. (A larger than 1.0 transition/transversion rate is expected because among single-step substitutions in the universal genetic code at a third codon position, only 3% of the transitions cause amino acid replacements compared with 41% for transversions [Wakeley 1996]). The translated amino acid data set (not shown) is less variable than the DNA sequence data set and does not provide phylogenetic resolution. No evidence of widespread saturation is present among the first and second codon positions (data not shown), but saturation was observed in nucleotide sites at the third codon position that differ in pairwise distance comparisons between evolutionarily distant taxa (data not shown). Even though some nucleotides at third codon position are saturated, they contributed to the biggest number of informative sites in the data set (79%) compared with the first (17%) and second (about 4%) codon positions.

Comparisons between published 18S rDNA (small subunit [SSU]) sequences (Bird et al. 1992) and the *rbc*L sequences used in this study reveal a greater phylogenetic signal in *rbc*L (35.81% = 490/1368 bp, n = 67 taxa) than in SSU rDNA (6.86% = 119/1734 bp, n = 19 taxa) in the Gracilariaceae. The overall genetic variation displayed by these two markers also shows that *rbc*L has more variation than SSU, and the lowest genetic values of intergeneric distances for *rbc*L are twice as large as those for SSU rDNA (Table 2). Among *Gracilaria sensu lato* species, the smallest distance is larger than the largest value in SSU rDNA (Table 2).

MP analyses resulted in 36 equally most parsimonious trees of 2805 steps (tree length), consistency index = 0.293 and retention index = 0.6421. The MP phylogram presented (Fig. 1) is the one among all 36 most parsimonious trees that presents the highest likelihood value (–ln = 15362.054) under the best evolutionary model found by the hierarchical likelihood ratio test. ME analysis resulted in 23 minimum trees with an ME score = 2.085450 (data not shown). The overall majority rule consensus topology given by the Bayesian analysis is fully resolved and well supported, with the exception of *G. textorii* and *G.* sp. from the Philippines (Fig. 2A).

All three phylogenetic analyses identified three major assemblages (Figs. 1 and 2A): a *Curdiea*/*Melanthalia* clade, a *Gracilariopsis* clade, and a *Gracilaria sensu lato* clade (including *Hydropuntia*). In the *Gracilaria sensu lato* clade, nine distinct evolutionary lineages (subgroups) were also identified (Figs. 1 and 2A): a *G. chilensis*/*G. aff. tenuistipitata* clade (subgroup I); a *G. urvillei* clade (subgroup II); a *G. caudata*/*G. crassissima* clade (subgroup III); a *G. gracilis* /*G. pacifica* clade (subgroup IV); a *G. arcuata*/*G. salicornia* clade (subgroup V); a *G. venezuelensis*/*G. intermedia* clade (subgroup VI); a *G. mammillaris* clade (subgroup VII); a *G. tikvahiae*/*G. damaecornis* clade (subgroup VIII); and, the most derived lineage, the *G. bursa-pastoris*/*G. textorii* clade (subgroup IX) (Figs. 1 and 2A).

A strict consensus of the 36 most parsimonious trees resulted in a fully resolved tree except for the presence of two polytomies: one at the deeper nodes of the three most derived subgroups within *Gracilaria sensu lato* clade (subgroups VI, VIII, and IX), for which bootstrap support is low (BP = 75%) to lacking (Fig. 1), and the other at the position of *Gp*. sp. from Namibia within *Gracilariopsis*. The strict consensus of the 23 ME trees resulted in a less resolved phylogram when compared with the MP consensus, with polychotomous nodes at the three most basal species in *Gracilariopsis*, *Gp*. sp. from Namibia, *Gracilaria* subgroup I (*G. chilensis*), and at the basal nodes among *Gracilaria* subgroups III to V and among subgroups VI to IX (data not shown). Major inconsistencies among the MP and ME phylograms pertain to the position of *G. textorii*, *G. multipartita*, and *G. bursa-pastoris* within *Gracilaria sensu lato* subgroup IX; the position of *Gracilaria sensu lato* subgroup VIII; the position of *Gp*. sp. from Namibia; and the two most basal species in *Gracilariopsis*. In all 36 most parsimonious trees, *Gracilaria sensu lato* subgroups II and III form a monophyletic clade without bootstrap support (Fig. 1); however, high support for the same topology was obtained in the Bayesian tree (PP = 99%, Fig. 2A). Only ME did not cluster these subgroups together (data not shown). The Bayesian tree (Fig. 2A) has the same topology as the MP tree (Fig. 1) except for the position of subgroups VI and VII that in the MP tree (Fig. 1) appears inverted: subgroup VI at a more derived position than subgroup VII. In both trees

(Figs. 1 and 2A), however, the internal node leading to subgroup VII received high support (BP = 98%, PP = 91%), making this phylogenetic difference the most striking one between the results from these two phylogenetic methods. Detailed characterization of more derived relationships will be treated in the discussion of each *Gracilaria sensu lato* subgroup.

Comparisons between *rbc*L molecular phylogenies (Figs. 1 and 2A) and morphological characters of male reproductive structures within the *Gracilaria sensu lato* clade revealed two independent origins for the *textorii* type of spermatangial conceptacles (Yamamoto 1978): one at *Gracilaria sensu lato* subgroup I and the other at *Gracilaria sensu lato* subgroups VI–IX. A reexamination of the spermatangial conceptacles from these two distinct monophyletic groups revealed stable morphological differences (Table 3).

DISCUSSION

Although it was previously shown that the Gracilariales is a monophyletic order as inferred from chloroplast-encoded *rbc*L and nuclear-encoded 18S SSU rDNA and 28S large subunit rDNA sequence analyses (Fredericq et al. 1996, Harper and Saunders 2001), phylogenetic information is published that clarifies the generic concepts within the order, especially within *Gracilaria*. The most recent and comprehensive phylogenetic study based on DNA sequence analysis of the Gracilariaceae is given by Bellorin et al. (2002) using 36 SSU rDNA sequences. Of SSU rDNA, large subunit rDNA, and *rbc*L, *rbc*L sequences show the highest genetic variation (Freshwater et al. 1999) in the Gelidiales. ITS rDNA sequences in the Gracilariaceae are alignable only among closely related species, whereas alignments among distant species are limited only to specific regions presumed constrained by secondary structures (Bird 1995, Bellorin et al. 2002). SSU rDNA sequences among distinct species are sometimes identical and not sufficiently variable to differentiate between closely related species, producing low bootstrap support and equivocal relationships (Bellorin et al. 2002). In contrast, *rbc*L DNA sequences in the Gracilariaceae revealed higher levels of interspecific genetic diversity (Table 2), especially within *Gracilaria sensu lato*, enabling us to detect not only interspecific relationships among closely related species but also population structure among a sample of haplotypes within a species (Gurgel and Fredericq 2000, Gurgel et al. 2001). Major clades and subgroups within the Gracilariaceae are discussed below along with phylogeographic and morphological remarks.

Curdiea *and* Melanthalia *major clade*. The genera *Curdiea* and *Melanthalia* form a monophyletic group endemic to the antiboreal regions, well supported in MP and ME analyses (BP = 100 in both) and moderately supported in the Bayesian analysis (PP = 75%) (Figs. 1 and 2A). These two genera share morphological features supportive of their alliance, including a cystocarp cavity completely filled with gonimoblasts and carposporangia formed in long straight chains (Fredericq and Hommersand 1989c, 1990a,b). *Melanthalia* differs from *Curdiea* by having dark, linear, narrow thalli with distinct subdichotomous branches, a prominent apical zone composed of a thick multicellular meristematic cortex, and sterile thick-walled gonimoblasts in the cystocarp (Fredericq and Hommersand 1990a,b, Womersley 1996). In all MP (Fig. 1) and ME (not shown) results, *C. racovitzae*, a species endemic to the Antarctic Peninsula, appears as sister to the genus *Melanthalia*, rendering *Curdiea* paraphyletic in excluding the former genus. However, in the Bayesian tree (Fig. 2A), *C. racovitzae* is placed back in *Curdiea*, rendering both genera monophyletic and sister to each other. Recent vegetative and reproductive morphological analyses of extensive collection of *C. racovitzae*, made by Dr. R. Moe and deposited in UC (e.g. UC#1557573, UC#1557575), reveal that this species corresponds better to the genus *Curdiea*.

Three of four *Melanthalia* species included in this study, *M. abscissa*, *M. concinna*, and *M. obtusata*, are reported from southern Australia. The first was originally described from New Zealand and the latter two from Australia. Womersley (1996), although recognizing all three species, pointed out that morphological similarities among them might warrant their treatment as varieties of the same species. The phylogenetic results herein indicate that despite vegetative and reproductive similarities, *M. abscissa* is genetically distinct from the other two species (Figs. 1 and 2A).

The *rbc*L data set presented in this study indicates that uncorrected pairwise genetic distances ("p" distance) between two haplotypes from the same species range between zero and 1.5%. Morphologically distinct and well-defined species usually have values equal to or greater than 2%. Species whose "p" distances lie between 1.5% and 2.0% often require further systematic analysis before a final taxonomic conclusion can be made. Between *M. concinna* and *M. obtusata* there is a 1.65% pairwise "p" distance, and our phylogenetic results (tree topologies) support the recognition of two distinct species. *Melanthalia abscissa* is reported to have distinctly compressed branches, mostly 0.7–1.3 mm broad, whereas *M. concinna* has terete to slightly compressed mostly 0.3–0.8 mm broad branches (Womersley 1996, p. 31). A fourth species, *M. intermedia*, was originally described as *M. obtusata* var. *intermedia* Harvey (1858), but Womersley (1996) merged it with *M. abscissa*. The *rbc*L sequence of *M. intermedia* was nearly identical to that of *M. concinna* (0.14%), suggesting that these two species are conspecific. *Melanthalia abscissa* is the only species of the genus reported from New Zealand (Adams 1994), and the inclusion of new *rbc*L haplotypes of *M. abscissa* from Australia could reveal geographic isolation among New Zealand and Australian populations.

Gracilariopsis *major clade*. The genus *Gracilariopsis* has been well characterized morphologically (Fredericq and Hommersand 1989b, Steentoft et al. 1995, Gurgel et al. 2003b) and genetically (Goff and Cole-

man 1988, Kapraun 1993, Kapraun et al. 1993, Bird et al. 1994, Goff et al. 1994, Gurgel et al. 2003a,c). The present phylogenetic analysis also confirms the monophyly of the genus (Figs. 1 and 2A).

Gurgel et al. (2003c) provided an *rbc*L phylogeny of *Gracilariopsis* and reinstated the generitype, *Gp. sjoestedii* (Kylin) Dawson, to include plants distributed from Vancouver, British Columbia to Pacific California, Mexico, and the name was corrected to *Gp. andersonii*. *Gracilariopsis lemaneiformis* was shown not to have a worldwide distribution but to be restricted to the vicinity of Peru in South America, with *Gp. costaricensis* from Costa Rica most likely being the same species. *Gracilariopsis carolinensis*, a new species from North Carolina (Gurgel et al. 2003c), is related to *Gp. lemaneiformis* and *Gp. costaricensis*. Entities that have been referred to as *Gp. lemaneiformis* from China and Japan constitute an undescribed species that occupies a basal position in association with *Gp. heteroclada* from the Philippines (Figs. 1 and 2A). *Gracilariopsis tenuifrons* from the Caribbean sea is identified as a distinct sister species to *Gp. cata-luziana*, a species so far endemic to the southwestern Gulf of Mexico. *Gracilariopsis longissima* is recognized from Western Europe. An undescribed species from Namibia and an unidentified invasive species from the Gulf of California, Mexico, and South Australia are represented in a clade that includes *Gp. longissima* from Europe. Three new species for the northwestern Atlantic Ocean, *Gp. silvana* Gurgel, Fredericq et Norris from Venezuela, *Gp. hommersandii* Gurgel, Fredericq et Norris from Venezuela and Panama, and *Gp. cata-luziana* Gurgel, Fredericq et Norris from the Mexican Gulf of Mexico were recently described (Gurgel et al. 2003a). *Gracilariopsis silvana* is the first confirmed flat *Gracilariopsis* species, a genus currently characterized by only cylindrical species.

Gracilaria sensu lato *major clade*. *Gracilaria*, the most species-rich genus in the Gracilariaceae and one of the most taxonomically difficult genera in the Rhodophyta, comprises at least nine distinct evolutionary lineages (Figs. 1 and 2A). The phylogenetic relationships among deeper nodes within the most derived *Gracilaria* lineage (subgroup IX, Figs. 1 and 2A) are not resolved in MP phylograms (Fig. 1) and received the lowest values of phylogenetic support in the Bayesian tree (Fig. 2A). This lineage is composed of smaller clades with variable degrees of phylogenetic support that include the generitype *G. bursa-pastoris*. This subgroup is characterized by flat-foliose species with *textorii*-type spermatangial conceptacles.

There is a stronger correlation between thallus shape and type of spermatangial conceptacle than between female and male reproductive features (Yamamoto 1984), with a tendency for cylindrical species to either display a *verrucosa*- (deep pits) or *chorda*-type (superficial) spermatangial arrangement and for flat, compressed, and foliose species to be of the *textorii* type (shallow pits). Major evolutionary trends among the 43 distinct *Gracilaria* species recognized in the data set emerge. The five most basal *Gracilaria sensu lato* subgroups (I–V) are characterized mainly by cylindrical species; exceptions such as *G. crassissima* and *G. eucheumatoides* possess a range of phenotypic variation that extends from totally cylindrical to compressed habits. Only *G. rangiferina* and *G. preissiana* among the 17 species in this assemblage are characterized by exclusively flat thalli. In contrast, the four most derived subgroups (VI–IX) are composed mainly of flat species. Twenty-seven of the 31 species in these four subgroups are flat, one (*G. tikvahiae*) displays both phenotypes, and three have cylindrical to slightly compressed thalli (*G. damaecornis*, *G. venezuelensis*, and *G. bursa-pastoris*). These results suggest that the cylindrical habit is the plesiomorphic condition in *Gracilariopsis* and *Gracilaria sensu lato*.

Subgroup I—the Gracilaria chilensis complex: The first divergent clade within *Gracilaria sensu lato* is composed of *G. chilensis* from Chile and *G. aff. tenuistipitata* from Japan and Virginia, USA. This assemblage shares several cystocarp characters with *Gracilariopsis*, such as the lack of multinucleated tubular nutritive cells linking gonimoblasts to the pericarp and a gradual morphological transition between gonimoblasts and mature carposporangia (Fig. 2J) (Bird et al. 1986, 1990, Nelson and Ryan 1991).

Both species typically inhabit protected estuarine environments. Specimens newly identified as *G. aff. tenuistipitata* collected from the east coast of the United States possess cystocarp features that are remarkably similar to those found in *G. chilensis* and *G. aff. tenuistipitata* from Tokawa, Japan. The low pairwise genetic distance between the Japanese and U.S. specimens (0.88% bp) suggests that the *G. aff. tenuistipitata* is a non-native introduction in the northwest Atlantic from Japan. The species seems to be spreading in northern Europe (J. Rueness, University of Oslo, personal communication), consistent with the general trend of numerous Asiatic invaders in European and North American Atlantic waters as a result of aquaculture introductions (Ribera and Boudouresque 1995, Maggs and Stegenga 1999, Gavio and Fredericq 2002).

Subgroup I, so far, is characterized exclusively by cylindrical and irregularly branched Pacific and Indo-Pacific species with a *textorii*-type spermatangial conceptacle (Fig. 2I). The non-Pacific terete counterpart, *G. bursa-pastoris*, has proved to be part of a separate evolutionary lineage (subgroup IX). Our results suggest that the *textorii* type of spermatangial conceptacle arose at least twice in the evolutionary history of *Gracilaria sensu lato* (Table 3, Fig. 2, B and I). It is likely that additional Asian cylindrical species with *textorii*-type spermatangial pits are part of this complex and that future inclusion of DNA sequences from those taxa (e.g. *G. chouae* Zhang et Xia, *G. minuta* Lewmanomont, and *G. parvispora* Abbott) should confirm this hypothesis. Future critical examination and comparative analysis of spermatangial development between *G. chilensis* and *G. bursa-pastoris*/*G. textorii* may reveal further developmental differences not discernible in mature stages.

The reproductive characters of *G. chilensis* and *G. aff. tenuistipitata* (Fig. 2, I and J) are sufficiently different from the type of *Gracilaria* for these species to be elevated to generic rank. This observation was formulated previously by Nelson and Ryan (1991).

Subgroup II—Pacific Hydropuntia*:* This clade includes only Indo-Pacific species to date. The type species of *Hydropuntia* (*G. urvillei* [Montagne] Abbott in Abbott et al. [1991]) is found in this clade (Figs. 1 and 2A). The name *Hydropuntia* has priority over *Polycavernosa* Chang et Xia (*G. edulis* [S. Gmelin] P. Silva 1952) (Wynne 1989). All members of this clade possess diagnostic features that correspond to the generic concept of *Hydropuntia*, such as the development of deeply embedded, often confluent, spermatangial conceptacles of subcortical-medullary origin that produce spermatangia in clusters (Chang and Xia 1963, Xia and Abbott 1985, 1987). Xia and Abbott (1987) considered the distinct cystocarp ontogeny described for *G. crassissima* (Fredericq and Norris 1985, Fredericq and Hommersand 1990b) as the *Hydropuntia* type. Subgroup II does not contain *G. cornea* and *G. crassissima*, species previously grouped and currently placed in *Hydropuntia* (Wynne 1998). The latter two species are part of a distinct clade (subgroup III). However, both MP (Fig. 1) and the Bayesian analysis (Fig. 2A) places species with *Hydropuntia*-like reproductive characters (Fig. 2, F–H) within a monophyletic clade of the genus *Gracilaria*, with varying support (MP, no support; Bayesian, PP = 99%). The reproductive features that characterize this clade are different enough from those found in the type of *Gracilaria* for the species in this assemblage to be elevated together with subgroup III to generic rank, as a *Hydropuntia*.

Subgroup III—Atlantic Hydropuntia*:* This subgroup thus far includes only western Atlantic species. All species are typically cylindrical, with *G. crassissima* sometimes displaying a compressed prostrate thallus. *Gracilaria secunda* is a validly published species that has been considered a synonym of *G. cornea* (Taylor 1960, as *G. debilis*). Our phylogenetic results support the recognition of this species distinct from *G. cornea* and sister to *G. caudata*. There is a striking morphological similarity between *G. caudata* and *G. secunda*. The morphological plasticity within each of these two species is huge and overlaps, making their taxonomic delineation problematic. Nevertheless, two distinct genetic entities do exist, and the type of *G. secunda* is the best match to the specimens used in this study.

Gracilaria usneoides is phylogenetically closer to *G. crassissima* than to *G. cornea* (Figs. 1 and 2A), but morphologically *G. cornea* and *G. usneoides* are much alike. Fredericq and Norris (1985) provided an account of the development of reproductive features of *G. crassissima* from Belize and demonstrated its distinctness in terms of an elaborate reticulate postfertilization fusion cell and origin of male reproductive parent cells. A reinterpretation of the photographs of *G. caudata* in Plastino and Oliveira (1997) reveals that carposporophyte development is of the same type as that of *G. crassissima*, even though a few upper tubular nutritive cells are depicted. The sexual reproductive structures of *G. secunda* are still unknown. It is clear from the current study that there are two distinct evolutionary lineages encompassing the concept of *Hydropuntia*.

Because subgroups II and III form a well-supported monophyletic group (Fig. 2A) and the type species of *Hydropuntia* is found in this clade (*H. urvillei*), all the species pertaining to these two subgroups are, in this study, transferred to *Hydropuntia* (see below for new combinations).

Subgroup IV—the G. salicornia *complex: Gracilaria canaliculata*, *G. crassa* Harvey ex J. Agardh (1876), and *Corallopsis opuntia* J. Agardh (1872) were considered conspecific by Newton (1953). The first two species were subsumed into *G. salicornia* by Xia (1986) and Meneses and Abbott (1987), who independently came to the same conclusion (Abbott 1988). However, Silva et al. (1996) recognize both *G. canaliculata* and *G. salicornia* as distinct species.

In this lineage, the morphological variation displayed by species with cylindrical and constricted thalli corresponds to a phenotypic continuum from one species description to the next, which makes species delineation impossible based solely on morphological grounds. Abbott (1988) mentioned that plants of *G. canaliculata* or *G. crassa* could be placed in any of those proposed names. The molecular results obtained in this study, however, do not support the conspecificity of *G. salicornia* and *G. canaliculata*. *Rbc*L-based phylogenetic trees show that these two species are related but taxonomically distinct (Figs. 1 and 2A) (genetic distance based on *rbc*L is 5.35%). The future inclusion of sequences from *G. cacalia* (J. Agardh) Dawson (1954, p. 2), *G. crassa* Harvey ex J. Agardh (1876, p. 417), and other morphologically similar species is necessary to resolve the taxonomic status of this species complex, which is characterized by some degree of constrictions in their terete thalli and *verrucosa* type of spermatangia.

The presence of distinct constrictions at the nodal and internodal regions were once used to create the genus *Corallopsis* J. Agardh 1876, which included *G. urvillei* (as *Hydropuntia urvillei*) and the 11 names currently in synonym with *G. salicornia* (Xia 1986, Abbott 1988). Thallus constriction grades from almost nonexistent (e.g. *G. canaliculata*) to very pronounced (e.g. *G. salicornia*). When comparing plants with divergent morphologies (e.g. with or without constrictions), the identification is clear but in many cases there is a phenotypic continuum between distinct phenotypes among specimens in the same population. Dawson (1954) concluded that nodal constrictions may not define genera and placed *Corallopsis* in synonymy with *Gracilaria*. The presence of some degree of thalli constrictions and the presence of only *verrucosa* type of spermatangial conceptacles characterize this highly polymorphic lineage. Although characteristic of this species complex, the pattern of thallus constriction

is not shared by all members of this clade, which includes *G. arcuata*, with strong bootstrap support. The placement of *G. urvillei* in a distinct separate clade (*Gracilaria* subgroup II) is evidence that sharp constrictions at the base of branches arose more than once in *Gracilaria*, increasing the degree of morphological homoplasy. However, the thallus constriction pattern and habit of *G. urvillei* are different from that found in species of this *G. salicornia* lineage. All members of this clade for which reproductive characters are known have cystocarp structures typical of *Gracilaria sensu stricto* (*sensu* Fredericq and Hommersand 1990b) (Fig. 2, D and E). *Corallopsis* is not supported by *rbc*L sequence analysis as generically distinct from *Gracilaria* Greville. All members of this subgroup are characterized by having *verrucosa* type of spermatangial conceptacles (Fig. 2C) that do not develop into the *Hydropuntia* type (Fig. 2F).

Subgroup V: Gracilaria gracilis and *G. pacifica* form a well-supported clade based on *rbc*L sequence analysis (BP = 100%, in both MP and ME results; PP = 100%), and their genetic distance is 4.47%. Currently, *G. gracilis* is the only terete species of *Gracilaria* described for the flora of the northeastern Atlantic (excluding the Mediterranean). The genetic distance between the southern U.K. *G. gracilis* haplotype and the northern France specimen included in the data set is not large enough (0.95%) to suggest that these two populations are distinct species. The ontogeny of reproductive structures of *G. gracilis* (as *G. verrucosa* [Hudson] Papenfuss) was used as the reference to define the order Gracilariales (Fredericq and Hommersand 1989a). All members of this subgroup are characterized by having *verrucosa* type of spermatangial conceptacles (Fig. 2C) that do not develop into the *Hydropuntia* type (Fig 2F).

Subgroup VI—the Gracilaria mammillaris complex: Morphologically, this is one of the most problematic lineages in the genus *Gracilaria*. The overall habit of members of this subgroup show remarkable similarities, especially when dealing with atypical phenotypes and small specimens. In other parts of the world, phenotypic plasticity and striking morphological similarities among flat *Gracilaria* species also produce similar taxonomic problems (e.g. *G. textorii*) (Yamamoto 1984). This subgroup is composed entirely of foliose dichotomously branched species restricted to the western Atlantic that are often misidentified as *G. mammillaris*. The current concept of *G. mammillaris* corresponds indeed to a species complex. Morphological comparisons among phylogenetic distinct populations (based on *rbc*L phylogenies) and type specimens revealed that at least four species were new and have been recently described: *G. galetensis*, *G. hayi*, *G. oliveirarum*, and *G. smithsoniensis,* (Gurgel et al. 2003b). Sterile specimens of *G. galetensis* may have commonly been misidentified as *Rhodymenia pseudopalmata*. The expansion of systematic surveys of flat *Gracilaria* species in the Caribbean may reveal an even greater number of new species currently passing under the name of *G. mammillaris*.

SubgroupVII: This is a well-supported group composed of only poorly known western Atlantic species. All specimens examined have a distinct habit: *G. intermedia* (Venezuela) and *G. yoneshigueana* (Brazil) are flat, whereas *G. venezuelensis* has terete to slightly compressed thalli (Taylor 1942). More extensive collections of fertile members of this clade are necessary before a full characterization of their reproductive structures can be accomplished. Since its description, *G. venezuelensis* has been cited only twice in floristic surveys: one record from the Mexican Gulf of Mexico (Dreckmann and Perez-Hernandez 1994) and the other from the Philippines (Westernhagen 1973, 1974). Reports of this species outside the western Atlantic should be considered tentative, because it is unlikely that southeastern Asia and the Caribbean share *Gracilaria* species that were not artificially introduced. In the western Atlantic, *G. venezuelensis* seems to be common but not recognized and often presents as thin cylindrical and very branched phenotypes when growing as drifting mats at protected environments such as bays and the U.S. intracoastal waterways (e.g. Tampa Bay, western Florida, and the Indian River, eastern Florida; C. F. Gurgel, personal observation). When growing attached to subtidal rocky substrata (e.g. Capron Shoal, Fort Pierce Co., FL, USA), *G. venezuelensis* develops a more robust, thicker, and regular dichotomous branched phenotype. *Gracilaria yoneshigueana* is a delicate 7-cm-long flat endemic species from Brazil, so far collected only in the Rio de Janeiro state, occurring in exposed as well as protected intertidal rocky shores (Gurgel et al. 2003b).

Subgroup VIII—The Gracilaria *tikvahiae clade:* This clade is composed of the western Atlantic species *G. damaecornis* and *G. tikvahiae* and the poorly defined *G. lacinulata*. All species are restricted to the western Atlantic with the exception of *G. tikvahiae*, which has been introduced in Hawaii (Abbott 1999, p. 216). All species in this subgroup display a persistent large postfertilization fusion cell (Fig. 2E). *Gracilaria tikvahiae*, a dichotomously branched species, displays a wide range of habit morphologies ranging from thin to thick, entirely flat to terete phenotypes. Morphologically different specimens of *G. tikvahiae* are often found growing on the same rock. Branching pattern in *G. damaecornis* is still more constant even among specimens from distinct geographic regions, and the degree of thallus compression also vary greatly. *Gracilaria lacinulata* shows a broad range in blade width and branching pattern but is characterized by a distinctly flattened green thallus.

Previous *rbc*L analysis (unpublished data) revealed that the green *Gracilaria* sp. variety farmed at Harbor Branch Oceanographic Institute is part of this *Gracilaria* subgroup. This taxon corresponds to an unidentified species originally collected on the eastern U.S. coast that has never been found in the field again. All three

Harbor Branch Oceanographic Institution *Gracilaria* varieties (brown, green, and red) have been kept in culture since 1977, 1982 and 1982, respectively, (D. Hanisak, HBOI, personal communication) and do not develop reproductive structures in the cultures. The brown variety is a true *G. tikvahiae* and part of subgroup VIII, but the red variety, producing the highest quality agar, is part of subgroup IV (unpublished data). The *rbc*L DNA sequence analysis of the three *Gracilaria* varieties mentioned above have also been deposited in GenBank (brown = AY049362, red = AY049310, green = AY049311).

Subgroup IX—the G. bursa-pastoris *clade:* Deeper nodes within *Gracilaria* subgroup IX, composed of the 15 most derived species, have low bootstrap support in the MP (Fig. 1) analysis and even less in the ME trees (data not shown). In the Bayesian tree these nodes have higher support, with the exception of *G. spinulosa* and *Gracilaria* sp. from the Philippines. In this subgroup, four distinct clades are resolved with high support. Two of these clades are composed of flat and compressed species from the western Atlantic: *G. occidentalis*, *G. ornata*, and *Plocaria flabelliforme*, P. et H. Crouan in Schramm & Mazé (1865, p. 21) with 100/84/85% support (Bayesian/MP/ME, respectively) and *G. apiculata*, *G. cervicornis*, *G. domingensis* with 100/97/97% support (Bayesian/MP/ME, respectively). The phylogenetic position of *G. curtissiae* in the latter clade had 100% Bayesian support but was not resolved in either MP or ME analyses (no BP support). The third clade is composed of the eastern Atlantic species *G. bursa-pastoris* and *G. multipartita* with 100/98/96% support (Bayesian/MP/ME, respectively). The fourth and most derived clade is the least supported cluster (64% Bayesian support and no BP support for neither MP nor ME) composed of the remaining six species in this subgroup, all with an east South African and Indo-Pacific distribution.

Thirteen of the fifteen species in subgroup IX have both a foliose habit and *textorii*-type spermatangial conceptacles. The exceptions are the compressed species *G. bursa-pastoris* and *G. cervicornis*; and *G. domingensis* bearing *verrucosa*-type spermatangial conceptacles. This lineage is composed of well-known species of *Gracilaria* and one unidentified species from the Philippines that is morphologically similar to, but genetically distinct from, *G. textorii*.

The most derived lineage in subgroup IX holds the type of the South African endemic genus *Tyleiophora* J. Agardh, *G. beckeri* (J. Agardh) Papenfuss. This species is sister to another South African-Madagascar endemic (Silva et al. 1996), *G. capensis*. Jacob Agardh (1890) established the genus *Tyleiophora* to contain *Gracilaria* species with tetrasporangia aggregated in nemathecia. Dawson (1949) reported that a tetrasporangial nemathecium is not a stable character and demonstrated that many flat northeastern Pacific species display a variable degree of nemathecal development. Papenfuss (1952) subsequently concluded that this character should not be used to recognize genera, merging *Tyleiophora* back into *Gracilaria*. *Gracilaria spinulosa*, *Gracilaria* sp. from the Philippines, and *G. textorii* received low bootstrap support (Figs. 1 and 2A), and their phylogenetic relationship within subgroup IX is still unresolved.

Three frequently confused and morphologically similar species, *G. cervicornis*, *G. domingensis*, and *G. ferox* J. Agardh (1852), were reassessed by Oliveira et al. (1983). They concluded based on morphological grounds that *G. cervicornis* and *G. ferox* are conspecific and that *G. domingensis* can only be reliably distinguished from *G. cervicornis* based on spermatangial features. The molecular study herein confirms that *G. cervicornis* is a distinct sister taxon to what is known as *G. domingensis* from Brazil. Specimens of *Gracilaria* recently collected from Venezuela and Mexico, resembling atypical phenotypes of *G. cervicornis*, turned out to belong to a distinct species, herein identified as *G. apiculata* P. et H. Crouan in Schramm and Mazé (1865, p. 19) based on examination and comparison of photographs of type material housed in BM! and PC!. Another resurrected name, previously recognized in Kapraun (1993), is based on *Plocaria flabelliforme*. A new combination is made in this study for this common, flat, western Atlantic tropical *Gracilaria* species. Several specimens of *G. apiculata* and *G. flabelliforme* were sequenced (data not shown); these taxa represent common species for the Caribbean and the southern Gulf of Mexico passing under the names *G. cervicornis* and *G. mammillaris*, respectively.

Male reproductive structures. Dawson (1949) was the first to stress the importance of the shape and origin of spermatangial conceptacles in the taxonomy of the Gracilariaceae. Ohmi (1958) pointed out that they were the most important character to distinguish among species. Yamamoto (1975, 1978) divided the genus *Gracilaria sensu lato* (including *Gracilariopsis*) into three subgenera based on three different types of spermatangial conceptacles previously recognized by Thuret and Bornet (1878) and Dawson (1949, 1961): a subgenus *Gracilariella* (spermatangia flush with surface: *chorda* type, as in *Gracilaria chorda* [Holmes] Ohmi [1958, p. 50]), *Textoriella* (spermatangia organized in shallow pits: *textorii* type, as in *G. textorii*, Fig. 2B), and *Gracilaria* (spermatangia organized in deep pits: *verrucosa* type as in *Gracilaria verrucosa* = *G. gracilis*, Steentoft et al. 1995, Fig. 2C). Later, Yamamoto (1984) included other kinds of spermatangial types in his classification, such as the *symmetrica* type and the *henriquesiana* type (Bird 1995), for which he did not established any new subgenera.

Tseng and Xia (1999) formally described the new subgenus *Hydropuntia* to include species with spermatangial conceptacles in multiple cavities (Fig. 2F) in which the spermatangia cover the entire surface of the conceptacles (encompassing the *Polycavernosa* and *henriquesiana* types). The superficial spermatangia of the *chorda* type have been considered ancestral with the deeper and complex conceptacles considered derived (Yamamoto 1975, 1978, 1984, Tseng and Xia 1999). Yamamoto (1984) presented a schematic diagram

depicting a sequence that had the *chorda*-type configuration giving rise to the other more complex types: *chorda* type (type 1, spermatangia continuously and homogeneously distributed along the thallus surface), to the *symmetrica* type (type 2, spermatangia superficial but discontinued by large scattered cortical cells), to the *textorii* type (type 3, shallow cavity, Fig. 2B), to the *verrucosa* type (type 4, deep cup-shaped cavity, Fig. 2C), to the *henriquesina* type (type 5, aggregation of *verrucosa*-type conceptacles), and finally to the *Polycavernosa* type (= *Hydropuntia* type, Fig. 2F) with deep confluent and branched conceptacles.

The *chorda* type is now known to be characteristic of and restricted to the genus *Gracilariopsis*. With the exception of *Melanthalia* for which there is still no reported spermatangial description, the two major basal lineages in the *rbc*L tree (i.e. *Gracilariopsis* and *Curdiea*) have superficial spermatangia with small colorless spermatia and do not form conceptacles (Fredericq and Hommersand 1989b, 1990b, Nelson and Knight 1997). Male structures in *Gracilariopsis* differ from those of *Curdiea* in having the spermatangium cut off singly by transverse division of the spermatangial parent cell and by not having spermatangia organized in nemathecia. The ancestral condition for the Gracilariales appears to be one in which undifferentiated cortical cells produced spermatangia by oblique longitudinal divisions, following the same division pattern as the surface cells.

Our results agree with Yamamoto (1984) and Bellorin et al. (2002) in which the most plesiomorphic spermatangial state in the Gracilariaceae seems to be the *chorda* type. However, this study does not support the phylogenesis hypothesis provided by Yamamoto (1984). In light of the new molecular evidence produced in this study, the evolutionary history of spermatangial types in the Gracilariaceae is more variable and complex than currently appreciated. Based on literature accounts for the species included in this study, the *Hydropuntia* type of spermatangial conceptacle (hereafter treated as synonymous to the *henriquesiana* and the *Polycavernosa* type) is present in four *Gracilaria* subgroups: in all taxa placed in subgroup II (*G. edulis*, Abbott et al. 1991) and subgroup III (*G. caudata*, Plastino and Oliveira 1997; *G. crassissima*, Fredericq and Norris 1985), in *G. domingensis* from subgroup IX (Guimarães et al. 1999), and in *G. damaecornis* (Ganesan 1989) from subgroup VIII. However, the independent acquisition of the *Hydropuntia* type by *G. damaecornis* and *G. domingensis* may not correspond to a true homoplasy but to misidentifications. It is possible that more than one species may be passing under the names *G. damaecornis* and *G. domingensis* and that these taxa may indeed correspond to two distinct species complexes in need of a careful systematical revision.

This study reveals that a "*chilensis*" type of spermatangial conceptacle is possibly the ancestral spermatangial configuration within *Gracilaria sensu lato*. The *textorii* type, considered the most primitive, is in fact present in the most derived lineages (subgroup IX). These results suggest that the *textorii* type as currently defined arose independently at least twice in *Gracilaria sensu lato*, once in the *G. chilensis* lineage (proposed here as the *chilensis* type) (Table 3, Fig. 2I), and once in the most derived subgroups characterized by *G. bursa-pastoris*, *G. cervicornis*, and *G. textorii* (proposed here as the true *textorii* type) (Table 3, Fig. 2B).

There are reports that the *verrucosa* and *Hydropuntia* type of spermatangia have been found on the same thallus in several Indo-Pacific (Abbott et al. 1991) and Atlantic (Ganesan 1989, Plastino and Oliveira 1997) species and that both the *textorii* and *verrucosa* type of spermatangial conceptacles have been reported from the same thallus in "*G. blodgettii*" Harvey (Zhang and Xia 1985, Reading and Schneider 1986, Abbott 1988). Those reports suggest that the *verrucosa*-type conceptacle may develop into a *Hydropuntia* type when intercalary spermatangial parent cells fuse back to vegetative cells (see fig. 14 in Fredericq and Hommersand 1990b for *H. crassissima*) or that a *textorii* type may develop into a *verrucosa* type if the conceptacle deepens into the thallus. However, our observations do not point in this direction. So far, only subgroups IV and V seem to have spermatangia restricted to the *verrucosa* type, which are morphologically distinct from the *verrucosa* type reported for species placed in other subgroups in our *rbc*L phylogenies. Comparisons between *verrucosa* type of spermatangia between species from subgroups IV and V with those found on species from subgroups II and III reveal a remarkable distinction. The spermatangial parent cells from species from subgroups IV and V never interact with medullary cells, and the *verrucosa* type of spermatangia in these species never develop into the *Hydropuntia* type. On the other hand, spermatangial parent cells from species from subgroups II and III always interact (via secondary pit-connections) with medullary cells, and it is on these groups that reports of plants carrying both kinds of spermatangial conceptacles have been made (Abbott et al. 1991). Therefore, the way the spermatangial parent cells interact with vegetative cells is the key character to distinguish these two groups of *Gracilaria sensu lato* species and helps to recognize *Hydropuntia* as a distinct, stable, and reliable genus from *Gracilaria sensu stricto*. In species of *Gracilaria sensu stricto* with the true *verrucosa* type of spermatangia (the one that does not interact with medullary cells) (Fig. 2C), as the thallus age the cortical cells multiply after the spermatangial conceptacle growth. This development pattern is very evident in mature spermatangia from *G. salicornia* (Abbott 2000, p. 215) and *G. shimodensis* (Tereada and Yamamoto 2000, p. 192), species characterized by having only *verrucosa* type of spermatangial conceptacles that do not ever develop into the *Hydropuntia* type.

The observation of more than one kind of spermatangial conceptacle for a particular *Gracilaria* species, especially when the *textorii* type and the *verrucosa* type are said to co-occur on the same species, should be considered with caution because more than one species may have been considered in those studies. Also, the

possibility of hybridization between two distinct taxa should be considered. Hybridization tests *in vitro* have been applied to infer limits among species of *Gracilaria* (Bird and McLachlan 1982, Plastino and Oliveira 1988), but nothing is known about how this corresponds *in situ*.

The integration of newly generated *rbc*L phylogenies (Figs. 1 and 2A) and new morphological interpretations of male reproductive structures support the separation of *Gracilaria sensu lato* into four distinct groups based on kind of spermatangial conceptacle: the *chilensis* type (Fig. 2I), composed of only *Gracilaria* subgroup I; the *Hydropuntia* type (Fig. 2F), composed of subgroups II and III; the *verrucosa* type (Fig. 2C), composed of subgroups IV and V; and the *textorii* type (Fig. 2B), composed of the most derived subgroups VI–IX. A more detailed comparative analysis of spermatangial ontogeny and anatomy among these four groups may reveal news insights about how to interpret them in a taxonomically and evolutionary framework. Ontogenetic observations show that the degree of interactions between spermatangial mother cells with cortical or medullary vegetative cells is a more important morphological character than the shape of mature conceptacles (Fredericq and Norris 1985).

Female reproductive structures. The *rbc*L phylogenies presented in this study (Figs. 1 and 2A) corroborate Fredericq and Hommersand's (1990b) synthesis of cystocarp types for the family Gracilariaceae. These authors recognized four distinct cystocarps for the nonparasitic genera:

1. The *Curdiea/Melanthalia* type: Cystocarp cavity completely filled by gonimoblasts, multinucleated tubular cells absent, carposporangia formed in distinct, elongate, narrow files of similarly sized cells, gametophytic cells in the floor of cystocarp becoming cytologically transformed, incorporation of gametophytic vegetative cells into the fusion cell restricted to cells of the sterile filaments of the supporting cell.

2. The *Gracilariopsis* type: Cystocarp cavity not completely filled by gonimoblasts; multinucleated tubular cells absent; carposporangia aligned in comparatively straight chains with smaller immature carposporangia at the base and larger mature carposporangia at distal portion of chains; gametophytic cells in floor of cystocarp cytologically transformed, incorporation of gametophytic vegetative cells into the fusion cell restricted to cells of the sterile filaments of the supporting cell.

3. The *Hydropuntia* type: Cystocarp cavity not completely filled by gonimoblasts, multinucleated basal and lateral tubular cells often present, multinucleated upper tubular cells often absent, carposporangia often aligned in straight chains with sharp transition between gonimoblast mass and a mature narrow carposporangial layer, cytologically transformed gametophytic cells in floor of the cystocarp often present and composed of small cells arranged in layers, incorporation of gametophytic vegetative cells into the fusion cell not restricted to sterile filaments of the supporting cell, fusion cell becomes highly reticulate and inconspicuous, gonimoblast mass with regular development, gonimoblasts with variable cell wall thickness (Fig. 2G).

4. The *Gracilaria* type: Cystocarp cavity not completely filled by gonimoblasts, multinucleated basal and upper tubular cells often present, carposporangia organized in clusters of cells of variable sizes, gametophytic cells in floor of cystocarp not transformed cytologically, incorporation of gametophytic vegetative cells into the fusion cell not restricted to sterile filaments of the supporting cell, mature fusion cell globose, variedly ramified or indistinct from other large gonimoblasts, inner gonimoblasts with conspicuous thick cell walls.

In light of our molecular results, at least four distinct cystocarp types can now be distinguished within the genus *Gracilaria sensu lato* alone (Fig. 2, D and E, G and H, and J):

1. The *chilensis* type. This cystocarp is morphologically similar to the *Gracilariopsis* type but with a less dissected organization of the inner gonimoblasts and with carposporangia in unbranched chains formed by gonimoblasts that gradually transform into large terminal carposporangia. This cystocarp type (Fig. 2J) is characteristic and so far only found among members of *Gracilaria sensu lato* subgroup I. Morphological similarities and differences between the cystocarps of *G. chilensis* and *Gp. lemaneiformis* have been well documented (Bird et al. 1986, Ryan and Nelson 1991). Among *Gracilaria sensu lato* species, this cystocarp type lacks multinucleated tubular cells connecting outer pericarp and gonimoblasts, has an extensive cystocarp cavity, a regular pattern of gonimoblast development, large external gonimoblast cells, and orderly arrays of carposporangia (Fig. 2J).

2. The *Hydropuntia* type. This cystocarp type is present so far only in Indo-Pacific species once placed in *Hydropuntia*, members of *Gracilaria sensu lato* subgroup II (Fig. 2A). Distinct features include an irregularly shaped fusion cell enclosed within the pericarp before gonimoblast initiation, with gonimoblast filaments developing in nearly complete straight files with the inner derivatives linking rapidly with one another by means of secondary pit-connections, mature mass of gonimoblasts often branched or lobed, carposporangia organized in short chains with sharp transition with gonimoblast mass (Fig. 2H).

3. The *crassissima* type. This type of cystocarp is characteristic of *Gracilaria sensu lato* subgroup III. Distinct features include gonimoblast filaments developing in complete straight files producing a regular, not lobed, centralized, and broad-based mature gonimoblast mass with carposporangia in usually short chains (Fig. 2G). Morphological observations from species included in this study agree with those of Abbott et al. (1991), who noted that gonimoblast organization in the Caribbean *Hydropuntia* species (subgroup III) is centralized, whereas that of Pacific *Hydropuntia* (subgroup II) species is diffuse. This type

of cystocarp was illustrated by Fredericq and Norris (1985), Fredericq and Hommersand (1990b), and Plastino and Oliveira (1997).

4. The *Gracilaria sensu stricto* type. This type of cystocarp is characteristic of the most derived subgroups within *Gracilaria sensu lato* (subgroups IV–IX). Its distinctive features include a regularly shaped, often persistent, fusion cell that can be quite conspicuous throughout gonimoblast development, gonimoblast filaments developing at regular or irregularly rates but often producing a gonimoblast mass composed of large cells with conspicuous cell walls, and with carposporangia organized in dichotomously branched chains (Fig. 2, D and E). Usually, when the fusion cell is persistent, the rate of gonimoblast development is irregular, thus producing an irregularly shaped gonimoblast mass composed of variably sized cells (e.g. *G. gracilis*, *G. tikvahiae*). Cystocarps with a more regular rate of gonimoblast development typically produce a rounded gonimoblast mass composed of large longitudinally elongated cells with conspicuous cell walls (e.g. *G. flabelliforme*).

The presence or absence and location of tubular multinucleated cells connecting the pericarp with the gonimoblasts alone is not a stable taxonomic characters to define *Gracilaria sensu lato* subgroups, with the exception of subgroup I that completely lacks these cells. However, despite the many homoplasies, a pattern can be observed where subgroups II and III are often characterized by having them restricted to the base of the cystocarp cavity, whereas the most derived subgroups (IV–IX) are often characterized by typical *Gracilaria sensu stricto* cystocarps with tubular cells distributed along the entire cystocarp cavity.

Tetrasporangia in the Gracilariales are cruciately divided or decussate. The development of nemathecia observed in some species (e.g. *G. beckeri*) was corroborated by this molecular study as being indistinct at the generic level (Dawson 1949, Papenfuss 1952).

CONCLUSIONS

Morphological characters found in the Gracilariaceae that do not take into account development often resemble one another in the mature state and are noninformative taxonomically, even among distant taxa (e.g. shape and size of vegetative cells frequently overlap among species). Also, ontogenetic features should be assessed with care because abortive pre- and postfertilization stages are common (Fredericq and Hommersand 1990b).

Delineation of new and previously defined subgeneric groups within *Gracilaria sensu lato* needs to be reassessed and requires the combination of male and female reproductive characters coupled with molecular phylogenies inferred from informative genetic markers. Distinct spermatangial conceptacle types within *Gracilaria sensu lato* are represented by the *chilensis* type, the *Hydropuntia* type, the *verrucosa* type, and the *textorii* type. Distinct cystocarps types within *Gracilaria sensu lato* are likewise represented by the *G. chilensis* type, the *Hydropuntia*-*G. crassissima* types, and the *G. bursa-pastoris* type. These four kinds of spermatangial and cystocarp organizations plus the phylogenetic relationships inferred from *rbc*L sequence analyses produced in this study define stable clades at the generic rank. Also in the literature there is already a plethora of genetic (Bird et al. 1992, 1994, Bird 1995, Bellorin et al. 2002) and morphological (Nelson and Ryan 1991, Ryan and Nelson 1991) evidence supporting the uniqueness of the subgroup I and its recognition as a distinct genus within the Gracilariaceae. The description and delineation of a new genus to accommodate the *G. chilensis* clade will be done elsewhere. The genus *Hydropuntia*, defined by the generitype *G. urvillei*, is herein reinstated encompassing species from subgroups II and III. The remaining terminal taxa (subgroups IV–IX) are defined by the generitype *G. bursa-pastoris* and considered the true genus *Gracilaria sensu stricto*.

At the species level, distinct evolutionary lineages within the *Gracilaria sensu lato* clade (= subgroups) present strong biogeography patterns of distribution. Subgroups I, II, and IV have to date a restricted Indo-Pacific distribution, whereas subgroups III, VI, VII, and VIII have a western Atlantic restricted distribution. In subgroup IX, the most recent clade in the *Gracilaria sensu lato* phylogeny, certain lineages are restricted to the western Atlantic (e.g. the *G. ornata* and the *G. cervicornis* lineages), whereas others have a Indo-Pacific-South African distribution (e.g. *G. capensis* lineage). These patterns suggest that ecological radiation and local speciation are a common phenomenon in the genus *Gracilaria sensu lato*. On the other hand, the biogeography patterns observed in the genus *Gracilariopsis* are different from the ones found in *Gracilaria* (Gurgel et al. 2003b). *Curdiea* and *Melanthalia* clade may present interesting biogeographic patterns, but more data are needed before they can emerge.

The *rbc*L genetic distances among the four distinct *Gracilaria sensu lato* generic groups mentioned above are consistent with the generic rank differences found among accepted Gracilariaceae genera. Placing more than 100 species within a single genus underestimates our capacity to recognize different evolutionary histories among genetically distinct lineages of Gracilariaceae. However, when morphological characters, informative molecular data, and sound phylogenetic hypotheses are combined, the recognition of three distinct genera currently placed within *Gracilaria sensu lato* corresponds to a more natural, phylogenetically informative, and information-rich taxonomy than the one currently in use.

Our results suggest that several independent dispersal events took place in *Gracilaria*, *Gracilariopsis*, and *Hydropuntia* but not in *Curdiea* and *Melanthalia*. In this study, the first divergent node in all three Gracilariaceae major clades are typically Indo-Pacific in distribution, lending support to the biogeographic hypothesis that the order's ancestor originated in eastern Gondwana before the opening of the Tethyan

Ocean (Hommersand 1990). The austral Tethyan group is composed of *Melanthalia* and *Curdiea* with extant taxa currently found in South Australia, Tasmania, New Zealand, South Africa, and the Antarctic Peninsula. Representatives of *Gracilaria*, *Gracilariopsis*, and *Hydropuntia* are present in both the Southern and Northern hemispheres, ranging from temperate to tropical environments. Hommersand (1990) suggested three major dispersal scenarios: 1) *Gracilariopsis* and some taxonomic sections of *Gracilaria* were distributed from the northwest Pacific to North America by way of the Alaskan Peninsula, either in late Cretaceous or in the Paleocene, ultimately reaching the Caribbean Sea, and possibly also Europe and the Mediterranean Sea; 2) species clusters in *Gracilaria* may have a Tethyan distribution; and 3) others appear to have extended their range westward from the Indian Ocean to the western Atlantic and Caribbean Sea by way of South Africa. The inclusion in the *rbc*L data set of more species worldwide is needed to fully assess such scenarios of ancient dispersal patterns. SSU rDNA data also places a *Curdiea* and *Melanthalia* clades at the root of Gracilariaceae trees (Bellorin et al. 2002).

The three major clades of Gracilariaceae may have diverged early in the evolution of the family. The low bootstrap support for nodes determining the relationships among these three clades and of the *Gracilaria sensu lato* subgroups in the MP analysis may be interpreted as the lack of sufficient phylogenetic signal in *rbc*L to resolve these relationships (genetic conservation) or a high level of mutation accumulation and a faster rate of gene evolution (gene saturation). The latter hypothesis is more likely due to detected saturation at the third codon position. Similar low bootstrap results for particular clades are indicative of adaptive radiation, lineage sorting, or of a fast origin for major evolutionary clades that originated within a short period of time from one another, as has been suggested for other organisms (Mardulyn and Whitfield 1999, Hampl et al. 2001). Phylogenetic analysis of other red algal families and orders based on different genetic markers often present similar bootstrap results supporting the latter interpretation (Saunders and Kraft 1996, Freshwater and Bailey 1998, Freshwater et al. 1999). The Bayesian analysis has a maximum likelihood correction for heterogeneity in the mutation rates and provided high probability support for the same nodes generated by MP.

The inclusion of more *Gracilaria* taxa in the data set may better resolve the evolutionary relationships of the clades already observed, especially those with low bootstrap support. Nevertheless, the *rbc*L gene showed signs of significant saturation at the third codon position, suggesting that another nonsaturated genetic marker should be used to confirm the phylogenetic relationships among deeper nodes in the Gracilariaceae. As important as sequencing distinct species or using the proper genetic marker is sequencing multiple specimens belonging to a same species. By doing so, the full range of the phenotypic plasticity for a particular species can be assessed, different forms and varieties attributed to a particular species confirmed, transferred to another species or recognized as a distinct taxon, and the true taxonomy for certain species complexes, cryptic species, and superspecies evaluated.

NEW COMBINATIONS

Gracilaria flabelliforme (P. et H. Crouan in Schramm et Mazé) Fredericq et Gurgel comb. nov.

Basionym: Plocaria flabelliforme P. et H. Crouan in Schramm et Mazé 1865, Essai Class.Alg. Guadeloupe, p. 21, #131

Homonym: Plocaria flabelliforme P. et H. Crouan in Schramm et Mazé 1866, p. 48

Lectotype: BM!, largest specimen on sheet, Algae Guadeloupensis, H. Mazé, Purchased 1890, in the *Gracilaria foliifera* folder, 57th 1st series, Monle Vieux Bourg.

Type locality: Guadeloupe, collected on submerged rocks in April.

The clade composed of *Gracilaria sensu lato* subgroups II and III (Figs. 1 and 2A) is herein recognized and confirmed as the genus *Hydropuntia*, distinct from the remaining *Gracilaria* species. Some of the species names that are part of this clade were previously placed in *Hydropuntia* before; thus, the remaining and following ones are transferred to *Hydropuntia* for the first time:

Hydropuntia caudata (J. Agardh) Gurgel et Fredericq comb. nov.

Basionym: Gracilaria caudata J. Agardh 1852, Sp. Gen. et Ordines Alg., 2(2), p. 598

Taxonomic synonyms: See Plastino and Oliveira (1997, p. 229).

Hydropuntia edulis (Gmelin) Gurgel et Fredericq comb. nov.

Basionym: Fucus edulis Gmelin, 1768. Hist. Fuc., p. 133.

Synonyms: Polycavernosa fastigiata Chang et Xia, 1963, p. 15, p. 120, pl. I: figs. 1–12, pl. II: figs. 1–6; *Gracilaria edulis* (Gmelin) Silva, 1952, p. 293; *Hydropuntia fastigiata* (Chang & Xia) Wynne, 1989, p. 477; *Sphaerococcus lichenoides* C. Agardh 1822, p. 310; *Sphaerococcus lichenoides* var. *tenuis* C. Agardh 1824, p. 234.

Taxonomic synonyms: See Silva et al. (1996, p. 175).

Hydropuntia eucheumatoides (Harvey) Gurgel et Fredericq comb. nov.

Basionym: Gracilaria eucheumoides Harvey, 1860, Proceedings Amer. Acad. Arts Sci. 4:331 (as "*eucheumioides*").

Taxonomic synonyms: See Silva et al. (1996, p. 168) as *Gracilaria eucheumatoides* Harvey.

Hydropuntia preissiana (Sonder) Gurgel et Fredericq comb. nov.

Basionym: Rhodymenia preissiana Sonder, 1845, Botanische Zeitung 3, p. 56 (as "*Rhodomenia*")

Synonyms: Rhodophyllis preissiana (Sonder) Kützing, 1849, pp. 786–7; *Calliblepharis preissiana* (Sonder) Harvey, 1859, pl. cvi.; *Gracilaria preissiana* (Sonder)

Womersley in Min-Thein et Womersley, 1976, p. 109; Withell, Millar et Kraft, 1994, pp. 294–7, figs. 11–13.

Taxonomic synonyms: See Silva et al. (1996, p. 174).

Hydropuntia rangiferina (Kützing) Gurgel et Fredericq comb. nov.

Basionym: Sphaerococcus rangiferinus Kützing, 1849, Sp. Alg., p. 779 (= *Sphaerococcus cervicornis* Kützing, 1843, Phyc. Gen. Pl. 62, fig. II; see Abbott et al. 1991)

Synonyms: Gracilaria dentata J. Agardh 1852, p. 603; *Polycavernosa dentata* (J. Agardh) Lawson & John 1982, p. 228; *Hydropuntia dentata* (J. Agardh) Wynne 1989, p. 477; *Gracilaria henriquesiana* Hariot 1908, p. 162; *Polycavernosa henriquesiana* (Hariot) Chang et Xia 1968, pl. 2, fig. 6; *Hydropuntia henriquesiana* (Chang et Xia) Wynne 1989, p. 477.

Hydropuntia secunda (P. et H. Crouan) Gurgel et Fredericq comb. nov.

Basionym: Gracilaria secunda P. et H. Crouan in Schramm et Mazé 1865, Essai Class. Alg. Guadeloupe, p. 19. Not *Gracilaria secunda* (Ag.) Zanardini 1840: Biblioth. Ital. (Milano) 99, p. 214 [*Sphaerococcus secundus* Ag.]

Hydropuntia usneoides (C. Agardh) Gurgel et Fredericq comb. nov.

Basionym: Sphaerococcus usneoides C. Ag. 1822, Sp. Alg., p. 333.

Synonym: Gracilaria usneoides (C. Ag.) J. Agardh 1852, Sp. Alg., 2(2), p. 595.

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