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PHYLOGENY AND SYSTEMATICS OF THE MARINE ALGAL FAMILY GRACILARIACEAE (GRACILARIALES, RHODOPHYTA) BASED ON SMALL SUBUNIT rDNA AND ITS SEQUENCES OF ATLANTIC AND PACIFIC SPECIES[1]

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We sequenced the small subunit rDNA and internal transcribed spacer region of Gracilariaceae from the tropical Atlantic and Pacific, with emphasis on flattened or compressed species. Sequence comparisons confirmed three main lineages of Gracilariaceae: *Curdiea/Melanthalia*, *Gracilariopsis/Gracilariophila*, and *Gracilaria*. The *Curdiea/Melanthalia* diverged early in the family. *Gracilariopsis* was paraphyletic, because at least one *Gracilariophila* species evolved from it. The Atlantic *Gracilariopsis* were monophyletic and separated from the Pacific lineages. The *Gracilaria* included all species referable to its own species and to *Hydropuntia*, which was paraphyletic, formed by distantly related lineages. The new combination *Gracilaria pauciramosa* (N. Rodríguez Ríos) Bellorin, M. C. Oliveira et E. C. Oliveira is proposed for *Polycavernosa pauciramosa* N. Rodríguez Ríos. Recognition of subgenera within *Gracilaria*, based on spermatangial arrangement, was not supported. Instead, infrageneric groups were delineated by geographic origins and combinations of reproductive characters. Most Pacific species with either "*textorii*" or "*verrucosa*" type spermatangia were deeply separated from Atlantic species. Within the Atlantic *Gracilaria*, a lineage encompassing mostly tropical cylindrical species with "*henriquesiana*" type spermatangia and distinctive cystocarp anatomy was recognized. A lineage was also retrieved for cold water stringy species with *verrucosa* type spermatangia. Several species from the western Atlantic are closely related to *Gracilaria tikvahiae* McLachlan with nearly identical morphology. On the other hand, most flattened species from the tropical Atlantic were closely related despite their diverse morphologies. The interpretation of our data in addition to the literature indicates that more populations from the Indo-Pacific must be studied before a general picture of Gracilariaceae evolution can be framed.

Key index words: agarans; agarophytes; *Gracilaria*; Gracilariaceae; *Gracilariopsis*; *Hydropuntia*; ITS; phylogeny; SSU rDNA

Abbreviations: **G., *Gracilaria*; *Gl.*, *Gracilariophila*; *Gp.*, *Gracilariopsis*; GTR, general time reversible; ITS, internal transcribed spacer; ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor joining; SSU, small subunit**

The gracilarioid algae include some of the most valuable marine plants. They have been intensively investigated in the last 30 years and comprehensive information about biology (Oliveira and Plastino 1994), cultivation (Oliveira et al. 2000), and utilization (cf. Critchley and Ohno 1998) has been published. However, there is still much to be done to resolve the many remaining taxonomic problems (e.g. Bird 1995). Different approaches have been attempted to clarify the taxonomy of gracilarioid algae (*sensu* Oliveira et al. 2000), primarily the genera *Gracilaria* Greville (1830), *Gracilariopsis* E. Y. Dawson (1949), and the disputable genus *Hydropuntia* Montagne (1842; valid name for *Polycavernosa* C. F. Chang et B. M. Xia 1963, see Wynne 1989). Reproductive anatomy (e.g. Dawson 1949, Yamamoto 1978, Gargiulo et al. 1992), chemistry (Bird et al. 1987), crossability, and karyology (McLachlan et al. 1977, Bird et al. 1982, 1986, 1990a, Guiry and Freamhainn 1985, Plastino and Oliveira 1988, 1997, Yamamoto and Sasaki 1988, Godin et al. 1993, Kapraun 1993) and modern techniques, including DNA fingerprinting (Goff and Coleman 1988, Rice and Bird 1990, Wattier et al. 1997) and gene sequencing (Bird et al. 1990b, 1992, 1994, Destombe and Douglas 1991, Goff et al. 1994), have been the keystone aspects investigated. It has been concluded that species delimitation is reliable only when based on a combination of characters, preferably experimental data, because anatomical features may be equivocal and cryptic species have been reported (Bird and Rice 1990, Bird et al. 1994, Steentoft et al. 1995). Unfortunately, nonmorphological information is almost entirely restricted to the terete and economically valuable taxa, especially those from temperate waters. The large assemblage of compressed and flattened forms from tropical waters, which constitute most of the described gracilarioid algae, has been largely neglected.

Among the experimental tools for discriminating taxa within this group, the comparison of homologous gene sequences has several advantages over other ap-

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proaches. Gene sequencing is time saving compared with hybridization tests, is more informative at the species and genus level in Gracilariaceae than DNA fingerprinting or karyology, and provides testable phylogenetic and systematic hypotheses. With the techniques of PCR and automatic sequencing, this approach is also readily applicable to a large number of samples and small quantities of purified DNA.

Molecular phylogenetic studies in Gracilariaceae have been based on nucleotide sequences of nuclear-encoded small subunit (SSU) rDNA (Bhattacharya et al. 1990, Bird et al. 1990b, 1992, 1994), internal transcribed spacer (ITS) regions of ribosomal nuclear repeats (Goff et al. 1994), plastid-encoded *rbc*L, and the RUBISCO spacer region (Destombe and Douglas 1991, Freshwater et al. 1994, Goff et al. 1994, Gurgel et al. 1999). Sequence data have confirmed various aspects of the systematics and phylogeny of gracilarioid algae, for example, 1) the ordinal rank and monophyletic nature of Gracilariales, previously proposed on anatomical grounds (Fredericq and Hommersand 1989a); 2) a closer relationship of Gracilariales to the Halymeniales, Rhodymeniales, and Plocamiales (Ragan et al. 1994, Saunders and Kraft 1997) than to other primary agar-producing orders; and 3) the distinct generic status of *Gracilariopsis* (Dawson 1949, Bird 1995), which appeared as a fast-evolving clade diverging early within Gracilariaceae (Bird et al. 1992, 1994). The genera *Curdiea* Harvey (1855) and *Melanthalia* Montagne (1843), with unique morphological features (Fredericq and Hommersand 1990a) and a restricted distribution, were also supported. On the other hand, *Hydropuntia* and the subgenera of *Gracilaria* proposed by Yamamoto (1978, 1984) on the basis of spermatangial configuration were not supported as consistent groups. However, it should be taken into account that only one species referable to *Hydropuntia* has been studied so far and that the diversity of the flattened *Gracilaria* spp. was poorly represented.

Here we provide data from part of the nuclear ribosomal cistron for 28 species/populations of Gracilariales, focusing on flattened forms from the tropical Atlantic. We also include some terete forms with deep compound spermatangial conceptacles ("*henriqueziana*" type, Yamamoto 1984) that could be assigned to *Hydropuntia* and two terete species from the Pacific. To allow for broad phylogenetic resolution, we compare sequences of the slowly evolving SSU rDNA and the fast-evolving ITS.

MATERIALS AND METHODS

DNA extraction and purification. All samples were taken from natural populations (Table 1). Voucher specimens were deposited in the herbarium of the University of São Paulo, Brazil. DNA was extracted from cleaned thalli tips previously dried and stored in silica gel. Tips were ground to fine powder with liquid nitrogen, and approximately 0.1 g of ground tissue was added to 2 mL lysis buffer (1.5% CTAB, 1 M NaCl, 50 mM EDTA, 0.1 M Tris pH 8.0, 0.2% β-mercaptoethanol) and incubated for 10 min at 65° C. Lysates were cooled at room temperature, and 2 μL of RNAse (100 mg·mL^{-1}; Qiagen, Santa Clarita, CA, USA) was added, incubating 30–60 min at 37° C. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was used for extraction, followed by two washes in equal volumes of chloroform:isoamyl alcohol (24:1). DNA was precipitated with two volumes of absolute ethanol at −20° C and collected with a sterilized glass capillary or by centrifuging at ca. 15,000*g* for 20 min at 4° C. In the latter case, the supernatant was discarded and the DNA was resuspended in 0.5 mL of sterile MilliQ-filtered water (Millipore Products Division, Bedford, MA, USA). If a viscous emulsion was formed, 0.1 volumes of absolute ethanol was added, samples were centrifuged at ca. 2000*g* for 20 min at 1° C, and the supernatant recovered. DNA was precipitated by adding 0.1 volumes of 3 M NaOAC, pH 5.2, and two volumes of absolute ethanol, with subsequent incubation for 30 min at −20° C and centrifugation at ca.10,000*g* for 20 min at 4° C. After centrifugation, the DNA pellet was washed twice with 0.5 mL of 70% ethanol, and finally the DNA was dissolved in 100 μL of sterile MilliQ-filtered water.

PCR amplification. The nuclear SSU rDNA was amplified using the synthetic primers 18S5′ and 18S3′ (Table 2). Amplification of the nuclear ITS (i.e. ITS1, 5.8S rDNA, and ITS2) was accomplished with the primers 6F and 28SR (Table 2). Amplification conditions were 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer, 1.25 U of *Taq* DNA polymerase (GibcoBRL, Life Technologies, Gaithersburg, Germany), and ≈2 ng of genomic DNA per 50 μL reaction. The PCR parameters for SSU rDNA were 94° C for 5 min, 35 cycles of 94° C for 1 min, 60° C for 2 min, and 72° C for 4 min, followed by a final extension step at 72° C for 7 min in a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA, USA). The same PCR protocol was followed for the ITS, except that the times of denaturing, annealing, and extension were reduced to one half.

Sequencing. For each taxon at least three independent PCRs were pooled together (Baldwin et al. 1995). The PCR products were purified with S-300 MicroSpin HR columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA) or QUIAquick PCR Purification Kit (Qiagen). The SSU rDNA and ITS were completely sequenced in both directions, using the Sanger dideoxy chain termination method for cycle sequencing with dye-labeled terminators (Applied Biosystems) on an ABI PRISM™ 310 Genetic Analyzer or 377 DNA Sequencer (Applied Biosystems). Sequencing primers were the amplification primers, plus the internal primers listed in Table 2. Divergent positions between closely related sequences were double-checked.

Each individual sequence was assembled manually, using ESEE 3.2 (Cabot and Beckenbach 1989). In the case of ITS, which includes three component sequences, the boundaries of each component were determined as follows: (1) the SSU rDNA-ITS1 boundary was obtained by comparison with the secondary structure model of SSU rRNA for *Gracilariopsis* sp. available at R. Gutell's webpage (http://www.rna.icmb.utexas.edu); (2) the 5.8S rDNA boundaries were obtained from Hershkovitz and Lewis' (1996) ITS alignment; and (3) the ITS2-large subunit rDNA boundary was determined by comparison with the functional secondary structure model of ITS2 for yeast (van der Sande et al. 1992).

Alignment. Manual multiple alignments were made in Se-Al v1.0 (Andrew Rambaut, Department of Zoology, University of Oxford, 1996). For SSU rDNA, the secondary structure-based multiple alignment for Rhodophyta from Van de Peer et al. (2000) was used as a model. Additional SSU rDNA sequences of Gracilariaceae from GenBank (Table 3) and the sequences of *Cryptonemia undulata* Sonder (GenBank accession no. U33125), *Plocamium cartilagineum* (Linnaeus) Dixon (no. U09619), and *Sebdenia flabellata* (J. Agardh) Parkinson (no. U33138), selected as outgroups, were included in the alignment. A matrix of 39 sequences and 1700 positions was assembled for SSU rDNA, excluding positions corresponding to amplification primers, indels, and ambiguously aligned positions.

For the ITS, we predicted probable secondary structure models (see below) and used them as guides to manual alignment. Available sequences of ITS of Gracilariaceae (Table 3)

TABLE 1. Gracilariaceae representatives sequenced in this study (SPF, Institute of Biosciences Phycological Herbarium, University of São Paulo, Brazil).

Entry	Locality, date of collection, and collector	Voucher specimen
G. caudata J.Agardh Araya	Punta Escarceo, Península de Araya, Sucre, Venezuela / 26 Jan 99 / A. M. Bellorin	SPF56116
G. caudata Ceará	Flecheiras, Trairi, Ceará, Brazil / 24 Jun 99 / D. Teixeira	SPF56117
G. caudata Coro	Buchuaco, Península de Paraguaná, Falcón, Venezuela / 29 Dec 98 / A. M. Bellorin	SPF56118
G. caudata Santa Catarina	Itajaí, Santa Catarina, Brazil / 10 Mar 00 / E. C. Oliveira	SPF56119
G. cervicornis (Turner) J.Agardh	Punta Escarceo, Península de Araya, Sucre, Venezuela / 26 Jan 99 / A. M. Bellorin	SPF56121
G. cornea J.Agardh Ceará	Flecheiras, Trairi, Ceará, Brazil / 24 Jun 99 / D. Teixeira	SPF56122
G. cornea Coro	Cabo San Román, Península de Paraguaná, Falcón, Venezuela / 29 Dec 98 / A. M. Bellorin	SPF56123
G. crassissima (P.Crouan et H.Crouan in Schramm et Mazé) P.Crouan et H.Crouan in Schramm et Mazé	Arrecife, Vargas, Venezuela / 17 Mar 98 / E. C. Oliveira	SPF56124
G. cuneata Areschoug	Recife de Candeias, Jaboatão, Pernambuco, Brazil / 7 Nov 98 / E. C. Oliveira	SPF56132
G. curtissiae J.Agardh	Adícora, Península de Paraguaná, Falcón, Venezuela / 29 Dec 98 / A. M. Bellorin	SPF56125
G. domingensis (Kützing) Sonder ex Dickie Araya	Punta Arenas, Península de Araya, Sucre, Venezuela / 29 Jan 99 / A. M. Bellorin	SPF56126
G. domingensis Ceará	Flecheiras, Trairi, Ceará, Brazil / 24 Jun 99 / D. Teixeira	SPF56127
G. foliifera (Forsskål) Børgesen var. *angustissima* (Harvey) W.R.Taylor	Punta Escarceo, Península de Araya, Sucre, Venezuela / 18 Jun 98 / A. M. Bellorin	SPF56128
G. lacinulata (Vahl) M.Howe prox. Bahia	Ilhéus, Bahia, Brazil / 24 Nov 00 / A. M. Bellorin	ND
G. lacinulata prox. Cumaná	Cumaná, Sucre, Venezuela / 23 May 00 / A. M. Bellorin	SPF56129
G. mammillaris (Montagne) M.Howe Coro	Adícora, Península de Paraguaná, Falcón, Venezuela / 29 Dec 98 / A. M. Bellorin	SPF56130
G. mammillaris São Paulo	Praia Dura, Ubatuba, São Paulo, Brazil / 04 Feb 00 / E. C. Oliveira	ND
G. pauciramosa (N.Rodríguez Rios) Bellorin, M.C.Oliveira et E.C.Oliveira[a]	Punta Escarceo, Península de Araya, Sucre, Venezuela / 26 Jan 99 / A. M. Bellorin	SPF56133
G. tepocensis (E.Y.Dawson) E.Y.Dawson prox. Santa Catarina	Praia da Armação, Florianópolis, Santa Catarina, Brazil / 15 Mar 00 / E. C. Oliveira	SPF56134
G. tepocensis prox. 2B	Lagoinha, Ubatuba, São Paulo, Brazil / 16 May 00 / E. M. Plastino	SPF56135
G. tepocensis prox. 4B	Lagoinha, Ubatuba, São Paulo, Brazil / 16 May 00 / E. M. Plastino	SPF56136
G. tikvahiae McLachlan	Pomquet Harbor, Halifax, Nova Scotia, Canada / 23 Oct 00 / D. Garbary	ND
Gracilaria sp. Araya	El Rincón, Península de Araya, Sucre, Venezuela / 9 Jun 98 / A. M. Bellorin	SPF56114
Gracilaria sp. Búzios	Praia das Caravelas, Búzios, Rio de Janeiro, Brazil / 17 Jan 00 / E. C. Oliveira	SPF56115
Gracilaria sp. Ceará	Flecheiras, Trairi, Ceará, Brazil / 24 Jun 99 / D. Teixeira	SPF56120
Gracilaria sp. México	Estero de Punta Banda, Baja California, México / 7 Mar 00 / J. M. Guzmán	SPF56131
Gp. tenuifrons (C.J.Bird et E.C.Oliveira) Fredericq et Hommersand	El Rincón, Península de Araya, Sucre, Venezuela / 9 Jun 98 / A. M Bellorin	SPF56138
Gracilariopsis sp. Ecuador	Posorja, Ecuador / Jun 97 / E. C. Oliveira	SPF56137

[a] New combination from *Hydropuntia pauciramosa* (N. Rodríguez Rios) N. Rodríguez Rios proposed in this work. ND, not deposited.

were included. Because ITSs are fast-evolving sequences, unequivocal alignment among more distantly related species was possible only in regions that were probably constrained by secondary structure. Thus, two matrices were assembled for ITS excluding amplification primers, indels, and ambiguously aligned positions. ITS matrix 1 included 20 aligned sequences for material from the Pacific and Atlantic, including two *Gracilaria* species with several populations, and *Gracilariopsis lemaneiformis* as an outgroup. This matrix was formed by 64 positions of the ITS1, 138 positions of the 5.8S rDNA, and 132 positions of the ITS2. ITS matrix 2 included sequences of closely related flattened and compressed species from the Atlantic, including three species with several populations, with *Gracilaria pacifica* as an outgroup. This matrix included 14 sequences and comprised 128 positions of ITS1, 159 positions of 5.8S rDNA, and 288 positions of ITS2. All the multiple alignments and sequences were submitted to GenBank (accession nos. AF468884–AF468918, AF472416–AF472420).

Secondary structure prediction for ITS sequences. To infer secondary structure of ITS, multiple alignments on ClustalX (Thompson et al. 1997) for groups of related species were first performed to search for conservative motifs. The individual sequences were folded in the mFold web server (Mathews et al. 1999, Zuker et al. 1999; http://bioinfo.math.rpi.edu/~zukerm/) at 25° C and 20% of thermodynamic optimality, with paired complementary flanking SSU and large subunit rDNA regions as the only initial constraints, following the secondary structure model proposed for yeast ITS2 (van der Sande et al. 1992). This produced up to 15–20 possible foldings for each sequence. The helices formed by two complementary conserved motifs found in most structures were later specified as constraints in new folds, and thus the phylogenetically supported structures were progressively produced. The alignments were also manually refined in accordance with common secondary structure information, and new conserved motifs were thus revealed. As a result, most homologous positions in the ITS sequences of Gracilariaceae could be

TABLE 2. Synthetic oligonucleotide primers used for PCR and sequencing of the SSU rDNA and ITS region.

Primer	Sequence	Region and position in *G. gracilis*
18S5′	5′-dCAACCTGGTTGATCCTGCCAGT-3′	SSU rDNA, 1[a]
536R	5′-dGAATTACCGCGGCTGCTG-3′	SSU rDNA, 558[a]
530F	5′-dGAGGGCAAGTCTGGTG-3′	SSU rDNA, 524[a]
920R	5′-dCAATTCCTTTAAGTTTG-3′	SSU rDNA, 1117[a]
920F	5′-dGAAACTTAAAGGAATTG-3′	SSU rDNA, 1101[a]
1055R	5′-dCGGCCATGCACCACC-3′	SSU rDNA, 1252[a]
1055F	5′-dGGTGGTGCATGGCCG-3′	SSU rDNA, 1238[a]
6F	5′-dTGTACACACCGCCCGTCGC-3′	SSU rDNA, 1601[a]
1800F	5′-dGAGAAGTCGTAACAAGG-3′	SSU rDNA, 1723[b]
18S3′	5′-dGATCCTTCTGCAGGTTCACCTACGGAA-3′	SSU rDNA, 1767[a]
ITS3R	5′-dGCTRCGTTCTTCATCG-3′	5.8S rDNA, 216[b]
ITS3F	5′-dCGATGAAGAACGYAGC-3′	5.8S rDNA, 201[b]
58SR	5′-dGCGTTCAAARATTCGATGATTCAC-3′	5.8S rDNA, 273[b]
58SF	5′-dGTGAATCATCGAATYTTTGAACGC-3′	5.8S rDNA, 250[b]
28SR	5′-dATATGCTTAARTTCAGCGGGT-3′	LSU rDNA, 84[c]

In GenBank [a]no. L26205, [b]no. U21342, [c]no. YI1508.

unambiguously aligned. Additional RNA secondary structure predictions were made in the GeneBee server (Brodsky et al. 1992, 1995; http://www.genebee.msu.su/services/rna2_reduced.html), which may use alignments as data input, and most of the predicted conserved helices were confirmed.

Phylogenetic analyses. Mutational saturation in variable positions in SSU rDNA and ITS sequences in each multiple alignment was evaluated by plotting all pair-wise distances, uncorrected for multiple substitutions, against model corrected distances in Jukes and Cantor (1969; JC69) and Kimura (1980; K2P), estimated in PAUP* version 4 (Swofford 1998). Phylogenetic inferences were made by maximum likelihood (ML), maximum parsimony (MP), and neighbor-joining (NJ) methods for phylogenetic inferences within PAUP*. Confidence limits of individual clades were estimated as bootstrap supporting values (Felsenstein 1985) with 200–2000 replicates of heuristic searches on the 50% majority-rule consensus trees. Nodes with bootstrap values greater than 70% are significantly supported with >95% probability (Hillis and Bull 1993).

Appropriate substitution model, base frequencies, proportion of invariable sites, and rate heterogeneity among sites were estimated in Modeltest 3.04 (Posada and Crandall 1998) to establish details about the mode of evolution of sequences in each multiple alignment. For the SSU rDNA matrix, the selected model was that from Tamura and Nei (1993), with the following rate-substitution parameters: transversions A↔C, A↔T, C↔G, and G↔T = 1.0000; transition A↔G = 3.1217; and transition C↔T = 5.0463. The base frequencies were unequal (A = 0.2338, C = 0.2056, G = 0.2862, and T = 0.2543), the proportion of invariable sites was 0.6112, and the gamma distribution shape parameter for heterogeneity of rates on variable sites was 0.8912. For the ITS matrix 1, the DNA sequence evolution properties were the following: JC69 one-parameter model of substitutions, proportion of invariable sites equal to zero, and rate heterogeneity among sites with gamma distribution shape parameter = 0.2574. Finally, for ITS matrix 2, K2P was the model of substitution selected, with rate-heterogeneity correction (G = 0.2435). However, on bootstrap analyses for ML and NJ, these settings were not specified in all details because they are not necessarily appropriate for each bootstrap replicate. Because of computational constraints, bootstrapping in ML was realized with simple models (JC69). Bootstrapping in NJ for SSU rDNA (alignment with 1700 positions) was made using LogDet/paralinear distances (Lockhart et al. 1994) and general-time-reversible (GTR) model distances (Rodriguez et al. 1990), which are preferable over simple model distances when large sequences are compared (Swofford et al. 1996). For ITS (alignments with 300–600 positions), bootstrapping in NJ was made either with models specified in Modeltest and with LogDet/paralinear or GTR distances.

RESULTS

SSU rDNA. Complete SSU rDNA sequences were obtained for 23 species/populations of Gracilariaceae (Table 3). In general, the SSU rDNA gene sequenced for Gracilariaceae ranged from 1765 to 1781 base pairs (bp), and few gaps were inferred to align these sequences. The plotting of uncorrected against model-corrected distances showed that the variable sites of these sequences were not mutationally saturated (data not shown).

Pair-wise comparisons in the multiple alignment revealed that intergeneric divergences on SSU rDNA of Gracilariaceae ranged from 1.17% to 6.35% (Table 1). The interspecific divergences ranged from 0.00% within *Gracilaria* to 2.88% within *Gracilariopsis*. Intraspecific divergences ranged from 0.00% within *Gracilaria* to 0.76% within *Gracilariophila*. We found that distinct species, as revealed by ITS comparison, morphology, or hybridization, may have identical SSU rDNA sequences. Such a situation was found between *G. cornea* of Venezuela and one undescribed cylindrical species from Brazil (*Gracilaria* sp. Ceará) and between the strap shaped species *G. mammillaris* from São Paulo and *G. cuneata* from Pernambuco, both from Brazil. On the other hand, we also found that populations of the same species may have up to two substitutions on the SSU rDNA sequences. In closely related *Gracilaria* species (as the several flattened tropical species studied here), the SSU rDNA sequences have not accumulated enough differences, which explains the lower bootstrap supports for the clades, including these species in the phylogenetic inferences and the equivocal relationships (Fig. 1).

Phylogenetic inferences from SSU rDNA comparisons based on ML and MP retrieved identical topologies (Fig. 1). Distance-based inferences differed in some branch orders and details. Three main lineages were consistently recognized: 1) the *Curdiea/Melanthalia* lineage, 2) the *Gracilariopsis/Gracilariophila* lineage, and 3) the *Gracilaria* lineage, which included all of the analyzed nonparasitic Gracilariaceae with sper-

TABLE 3. SSU rDNA and ITS sequences of Gracilariaceae included in the analyses.

Entity	Gross morphology/spermatangia type[a]	SSU rDNA GenBank accession no.	ITS GenBank accession no.	Source
G. flabellata V.J.Chapman	Flattened/?[b]	L26207	—	Bird et al. (1992)
G. caudata Araya	Cylindrical/"*verrucosa*"-"*henriquesiana*"	AF168889	AF468908	This work
G. caudata Ceará	Cylindrical/"*verrucosa*"-"*henriquesiana*"	AF468888	ND	This work
G. caudata Coro	Cylindrical/"*verrucosa*"-"*henriquesiana*"	AF472415	AF468909	This work
G. caudata Santa Catarina	Cylindrical/"*verrucosa*"-"*henriquesiana*"	ND	AF468910	This work
G. cervicornis	Compressed/"*textorii*"	AF168897	AF168917	This work
G. chilensis C.J. Bird, McLachlan et E.C. Oliveria	Cylindrical/"*textorii*"	L26217	—	Bird et al. (1992)
G. chilensis	Cylindrical/"*textorii*"	—	AF034265	Goff et al. (1994)
G. cornea Ceará	Cylindrical/"*henriquesiana*"	AF468894	ND	This work
G. cornea Coro	Cylindrical/"*henriquesiana*"	AF168892	ND	This work
G. cornea Santa Lucia	Cylindrical/"*henriquesiana*"	L26212	—	Bird et al. (1992)
G. crassissima	Compressed/"*henriquesiana*"	AF468893	AF468907	This work
G. cuneata	Flattened/"*textorii*"	AF468905	ND	This work
G. curtissiae	Flattened/"*textorii*"	AF468901	ND	This work
G. domingensis Araya	Flattened/"*verrucosa*"-"*henriquesiana*"	AF168903	AF168913	This work
G. domingensis Ceará	Flattened/"*verrucosa*"-"*henriquesiana*"	AF468902	AF472420	This work
G. foliifera var. *angustissima*	Compressed/"*textorii*"	AF468895	AF468912	This work
G. gracilis (Stackhouse) Steentoft, L.Irvine et Farnham	Cylindrical/"*verrucosa*"	L26205, L26210	—	Bird et al.
G. gracilis	Cylindrical/"*verrucosa*"	—	U21312	Goff et al. (1994)
G. lacinulata prox. Bahia	Compressed/"*textorii*"	ND	AF472414	This work
G. lacinulata prox. Cumaná	Compressed/"*textorii*"	AF468896	AF472419	This work
G. mammillaris Coro	Flattened/"*textorii*"	AF468900	AF468916	This work
G. mammillaris São Paulo	Flattened/"*textorii*"	AF168904	AF168914	This work
G. pacifica I.A.Abbott	Cylindrical/"*verrucosa*"	L26206	—	Bird et al. (1992)
G. pacifica	Cylindrical/"*verrucosa*"	—	U21341	Goff et al. (1994)
G. pauciramosa	Flattened/"*henriquesiana*"	AF468887	ND	This work
G. robusta Setchell	Compressed/"*verrucosa*"	—	U21340	Goff et al. (1994)
G. tenuistipitata C.F.Chang et B.M.Xia	Cylindrical/"*textorii*"	—	U21343	Goff et al. (1994)
G. tepocensis prox. Santa Catarina	Compressed/"*textorii*"	AF168891	AF472416	This work
G. tepocensis prox. 2B	Compressed/"*textorii*"	ND	AF472417	This work
G. tepocensis prox. 4B	Compressed/"*textorii*"	ND	AF472418	This work
G. tikvahiae	Cylindrical-compressed/"*textorii*"	M33610	—	Bird et al. (1990b)
G. tikvahiae	Cylindrical-compressed/"*textorii*"	ND	AF168911	This work
Gracilaria sp. Araya	Compressed/"*textorii*"	AF468898	AF468918	This work
Gracilaria sp. Búzios	Flattened/"*textorii*"	AF468899	AF468915	This work
Gracilaria sp. Ceará	Cylindrical/"*henriquesiana*"	AF468890	ND	This work
Gracilaria sp. Elkhorn Slough	Cylindrical/?[b]	—	U21311	Goff et al. (1994)
Gracilaria sp. México	Cylindrical/"*verrucosa*"	AF168886	AF468906	Goff et al. (1994)
Gl. oryzoides Setchell et H.L.Wilson in H.L.Wilson	Parasitic/"*chorda*"	U43557, U43555	U33139	Goff et al. (1996)
Gp. lemaneiformis (Bory) E.Y.Dawson, Acleto et Foldvik	Cylindrical/"*chorda*"	M51986, X51263	—	Bhattacharya et al. (1990)
Gp. lemaneiformis	Cylindrical/"*chorda*"	—	U21243	Goff et al. (1994)
Gp. longissima (S.G.Gmelin) Steentoft, L.Irvine et Farnham	Cylindrical/"*chorda*"	L26208	—	Bird et al. (1992)
Gp. longissima	Cylindrical/"*chorda*"	—	U21339	Goff et al. (1994)
Gp. tenuifrons	Cylindrical/"*chorda*"	AF168884	ND	This work
Gp. tenuifrons	Cylindrical/"*chorda*"	—	U21246	Goff et al. (1994)
Gracilariopsis sp. China	Cylindrical/"*chorda*"	—	U30348	Goff et al. (1994)
Gracilariopsis sp. Ecuador	Cylindrical/"*chorda*"	AF468885	ND	This work
Gracilariopsis sp. North Carolina	Cylindrical/"*chorda*"	L26256	—	Bird et al. (1992)
Gracilariopsis sp. North Carolina	Cylindrical/"*chorda*"	—	U30347	Goff et al. (1994)
Gracilariopsis sp. Perú	Cylindrical/"*chorda*"	—	U21245	Goff et al. (1994)
M. obtusata (Labillardière) J.Agardh	Flattened/?[b]	L26215	—	Bird et al. (1992)

[a]Terminology from Yamamoto (1978, 1984).
[b]Male plants unknown.
[c]Only sequence of ITS1.
ND, not determined.

matangia produced in conceptacles. Thus, the species bearing fused deep conceptacles ("*henriquesiana*" type; Table 3), segregated by some authors in the genus *Hydropuntia*, were not phylogenetically separated from *Gracilaria*, based on the data presented in this work. These three main lineages of Gracilariaceae were always retrieved as a monophyletic group in the Florideophycidae in phylogenetic analyses, including members of Acrochaetiales, Bonnemaisoniales, Halymeniales, Gigartinales, Ceramiales, and Plocamiales (A. Bellorin, data not shown). In MP, ML, and NJ with GTR distances with rate heterogeneity correction, the *Gracilaria*/

TABLE 4. Percentage of sequence divergence in aligned SSU rDNA and ITS among the main taxonomic groups of Gracilariaceae.

	SSU rDNA[a]	ITS matrix 1[b]	ITS matrix 2[c]
Curdiea/*Melanthalia* intergenera	1.47	—	—
Curdiea/*Melanthalia* vs. *Gracilariopsis*/*Gracilariophila*	3.65–6.35	—	—
Curdiea/*Melanthalia* vs. *Gracilaria*	2.53–3.47	—	—
Gracilariopsis vs. *Gracilariophila*	2.94–3.70	—	—
Gracilariopsis vs. *Gracilaria*	2.24–4.65	19.16–26.35	—
Gracilariopsis interspecies	0.47–2.88	—	—
Gracilariopsis intraspecies	0.18	—	—
Gracilariophila vs. *Gracilaria*	4.65–5.12	—	—
Gracilariophila intraspecies	0.76	—	—
Gracilaria interspecies	0.00–1.29	2.09–21.86	2.96–15.48
Gracilaria intraspecies	0.00–0.41	0.30–0.90	0.17–1.22

Positions corresponding to amplification primers and indels were excluded.

[a]1700 positions.

[b]934 positions.

[c]575 positions.

Melanthalia clade was retrieved as the first diverging lineage in the Gracilariaceae (Fig. 1). However, NJ inferences with LogDet/paralinear distances (data not shown) favored the *Gracilariopsis*/*Gracilariophila* clade as the first Gracilariaceae lineage.

In ML and MP the *Gracilariopsis*/*Gracilariophila* clade was separated into four lineages with strong bootstrap support at all nodes (85%–100%): (1) *Gp. lemaneiformis* as the first-diverging lineage, followed by the tricotomy of (2) *Gracilariopsis* sp. from Ecuador, (3) the *Gracilariophila* clade, and (4) the cluster of the Atlantic species of *Gracilariopsis* (*Gp. tenuifrons*, *Gp. longissima*, and *Gracilariopsis* sp. from North Carolina) (Fig. 1A). In the NJ trees, the *Gracilariophila* sequences were related to *Gracilariopsis* sp. Ecuador (bootstrap values 91%–92%), and this clade was retrieved as a sister group of the Atlantic species clade (bootstrap values 100%). Despite these variations, the fact remains that the parasite *Gl. oryzoides* has evolved from a *Gracilariopsis* host.

For the *Gracilaria* species studied, the ML and MP trees retrieved one initial polytomy (Fig. 1B) encompassing most sequences without any clear relationship among them. The following lineages with moderate to strong bootstrap support (70%–100%) were resolved for *Gracilaria* in ML and MP: (1) the clade of *G. caudata*, *G. cornea*, *G. crassissima*, and *Gracilaria* sp. Ceará (named collectively as "Atlantic cylindrical *henriquesiana*" lineage); (2) the clade of *G. gracilis* and *G. pacifica* ("*gracilis*" lineage); and (3) the clade of the two studied populations of *G. domingensis*. The trees produced by NJ were distinctive in some details. First, the sequences of *G. chilensis* and *Gracilaria* sp. México were separated from the rest of *Gracilaria* sequences. When LogDet/paralinear distances were used, the sequences of these species were united into a single clade (data not shown) but without significant bootstrap support (52%). In NJ trees with GTR distances corrected for rate heterogeneity, *G. chilensis* and *Gracilaria* sp. México were not related.

ITS. Nineteen new complete ITS sequences (i.e. ITS1, 5.8S rDNA, and ITS2) and one ITS1 sequence were obtained for Gracilariaceae (Table 3). Size variation among ITS sequences was pronounced. The ITS1 sequences ranged from 105 to 521 bp, the 5.8S rDNA genes from 140 to 163 bp, and the ITS2 from 562 to 778 bp. Sequence variation was also high, and positional homology was inferred with confidence only when moderately conserved motifs, presumably constrained by secondary structure (data not shown), were found. ITS sequence from *Gracilaria* sp. México was almost identical (0.03% divergence) to that from *Gracilaria* sp. Elkhorn Slough studied by Goff et al. (1994) from California. Thus, these two samples are populations of an undescribed species from the northeastern Pacific often misidentified as *G. pacifica*. For *G. tikvahiae*, the ITS sequence we produced was almost identical (0.003% divergence) to that reported for the same population by Goff et al. (1994).

Gracilariopsis-*Gracilaria* divergences on ITS matrix 1 were 19.16% to 26.35% (Table 4). Interspecific divergence in *Gracilaria* ranges from 2.09% to 21.86% and intraspecific divergence from 0.30% to 0.90%. ML, MP, and NJ with JC69 distances gave identical topologies. *Gracilariopsis lemaneiformis* is deeply separated from the *Gracilaria* clade (Fig. 2). Within *Gracilaria*, the following Pacific samples were grouped into a single clade with low bootstrap support (65%–68%): *Gracilaria* sp. México, *Gracilaria* sp. Elkhorn Slough, *G. chilensis*, and *G. tenuistipitata*. All the other *Gracilaria* sequences are from Atlantic material, except for *G. pacifica* and *G. robusta*, and were retrieved in a single clade (bootstrap support 88%–95%). The "Atlantic cylindrical *henriquesiana*" lineage resolved from SSU rDNA sequences, represented by *G. crassissima* and sequences from three populations of *G. caudata* (only *G. caudata* from Península de Araya is shown in Fig. 2), was also supported in ITS sequence comparisons (82%–89% bootstrap values) and was retrieved as the first diverging lineage of the Atlantic taxa. The next diverging clade was the "*gracilis* lineage," encompassing *G. gracilis*, *G. pacifica*, and *G. robusta*, a fleshy species with "*verrucosa*" type spermatangia from the Pacific (bootstrap support 53%–80%). After the separation of these two lineages, one polytomy was formed of the flattened or compressed Atlantic species, with low bootstrap support (54%–57%). In this polytomy, *G. tikvahiae* from Canada was related to the Caribbean entity *G. foliifera* var. *angustissima* with strong bootstrap support (96%–100%), *G. cervicornis* was related to *Gracilaria* sp. Araya with low bootstrap support (67%–69%), and *G. mammillaris* São Paulo was related to *Gracilaria* sp. Búzios, also with low bootstrap support (52%–60%). The sequences of *G. domingensis* and *G. mammillaris* Coro were not specifically related to any other flattened or compressed *Gracilaria*.

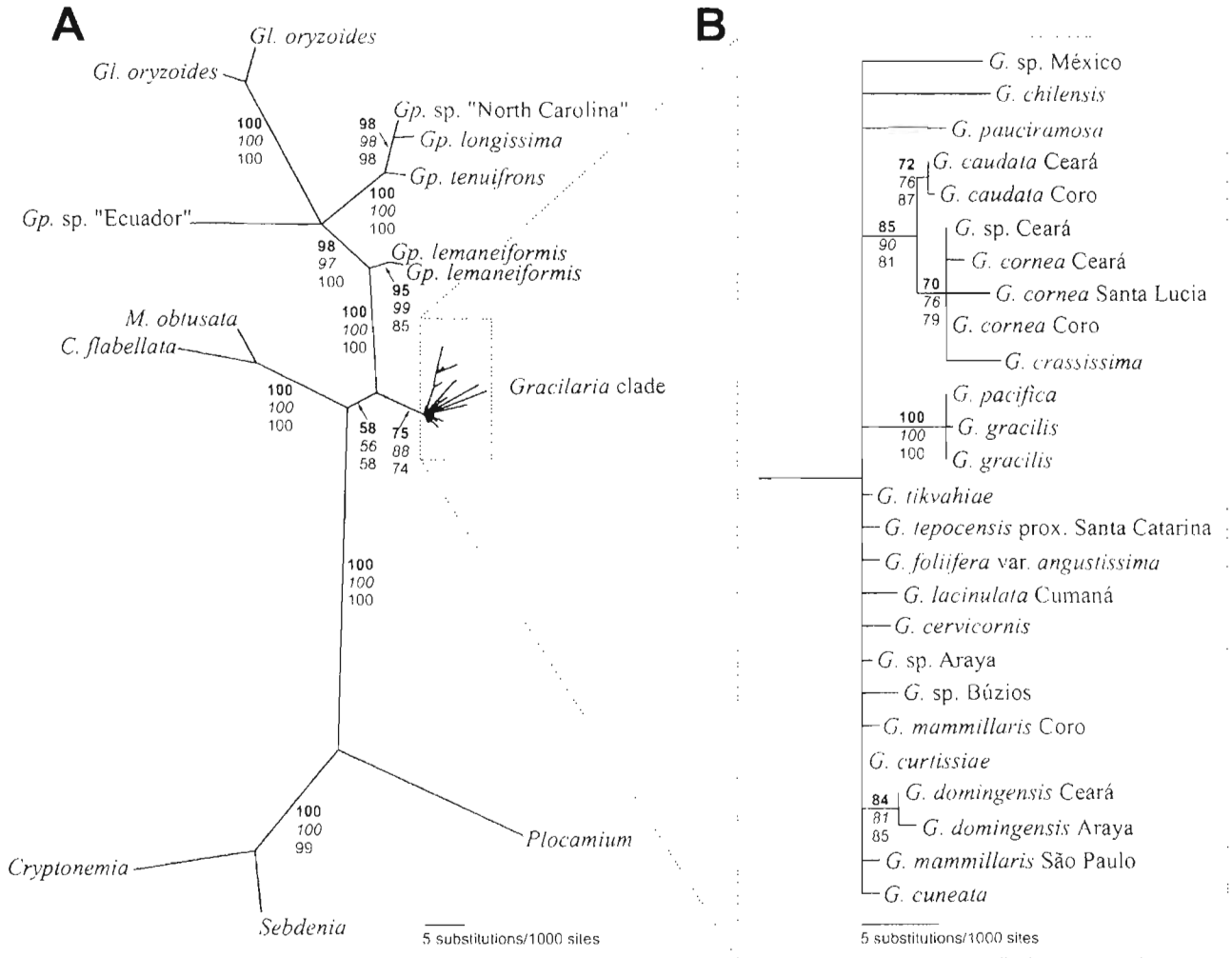

FIG. 1. Bootstrap 50% majority-rule consensus ML tree for SSU rDNA sequences of Gracilariaceae. (A) Gracilariaceae plus outgroup sequences. (B) Detail of *Gracilaria* clade. ML calculations were made under the JC69. Numbers at nodes denote bootstrap values for ML (first row in bold typeface, 200 replicates), MP (second row in italics, 2000 replicates), and NJ (third row in normal typeface, 2000 replicates). Arrows indicate the position of bootstrap values when they do not fit on the branches.

For ITS matrix 2, the interspecific divergences in *Gracilaria* were 2.96% to 15.48% and intraspecific divergences were 0.17% to 1.22% (Table 4). The ML tree had identical topology to that retrieved under MP. NJ trees with K2P with rate-heterogeneity correction, LogDet, and GTR with rate-heterogeneity-correction distances were distinct in some details. All phylogenetic inferences showed that *G. tikvahiae* from Canada, *G. tepocensis* proximate from South America, and *G. lacinulata* proximate and *G. foliifera* var. *angustissima* from the Caribbean were related species (bootstrap support 96%–97%) (Fig. 3), forming the "*tikvahiae*" lineage. All these species are compressed to flattened forms (sometimes cylindrical), with "*textorii*" type spermatangia and very similar morphology. In this lineage, the temperate and subtropical isolates *G. tikvahiae* and *G. tepocensis* proximate were closely related. Relationships among the tropical species, *G. lacinulata* proximate from the Caribbean and northeastern Brazil (as shown by ITS1 sequence of *G. lacinulata* proximate Bahia) and *G. foliifera* var. *angustissima* from the Caribbean were supported by low to moderate bootstrap values (56%–73%). The compressed species *G. cervicornis* and *Gracilaria* sp. Araya were grouped again with moderate to strong support (64%–81%) in all phylogenetic analyses. This clade, named the "*cervicornis*" lineage, was related in ML and MP with low bootstrap support (60%–66%) to *G. domingensis*, a morphologically very plastic species, usually flattened, with "*verrucosa*" or "*henriquesiana*" type spermatangia. However, the sequences of flattened ribbon-like species, *G. mammillaris* São Paulo from Brazil, *G. mammillaris* Coro from Venezuela, and *Gracilaria* sp. Búzios from Brazil, were not related to any group in ML, MP, and NJ. Although ITS1 and ITS2 sequences were compared for a number of species of *Gracilariopsis*, no phylogenetic signal emerged from the multiple alignments attempted.

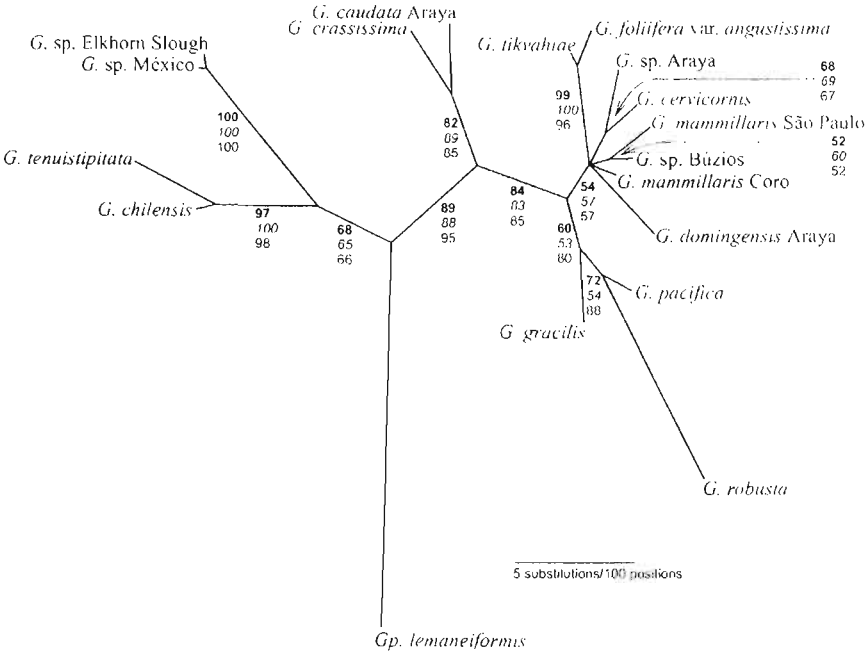

FIG. 2. Bootstrap 50% majority-rule consensus ML tree for ITS matrix 1. ML calculations were made under the JC69. Numbers at nodes denote bootstrap values for ML (first row in bold typeface, 1000 replicates), MP (second row in italics, 2000 replicates), and NJ (third row in normal typeface, 2000 replicates). Arrows indicate the position of bootstrap values when they do not fit on the branches.

We propose a new combination: *Gracilaria pauciramosa* (N. Rodríguez Ríos) Bellorin, M. C. Oliveira et E. C. Oliveira comb. nov.

Basionym: *Polycavernosa pauciramosa* N. Rodríguez Ríos 1989 (*Ernstia* 56:1–7, fig. 1-3).

Homotypic synonym: *Hydropuntia pauciramosa* (N. Rodríguez Ríos) N. Rodríguez Ríos 1991 (*Ernstia* 1:39).

DISCUSSION

Suprageneric and generic lineages of Gracilariaceae. The sequence comparisons of ribosomal genes have shown that Gracilariaceae is a monophyletic clade within the Florideophycidae and has three main lineages, already reported in previous studies (Bird et al. 1992, 1994). In phylogenetic analyses, the additional sequences of *Gracilariopsis* and *Gracilaria* produced in this work always grouped unequivocally with their congeners, preserving these two lineages. However, the sequences of species bearing "*henriquesiana*" type spermatangia (one of the anatomical features used to segregate *Hydropuntia* species by the enthusiasts of this genus) were not always grouped, that is, *G. paucir-*

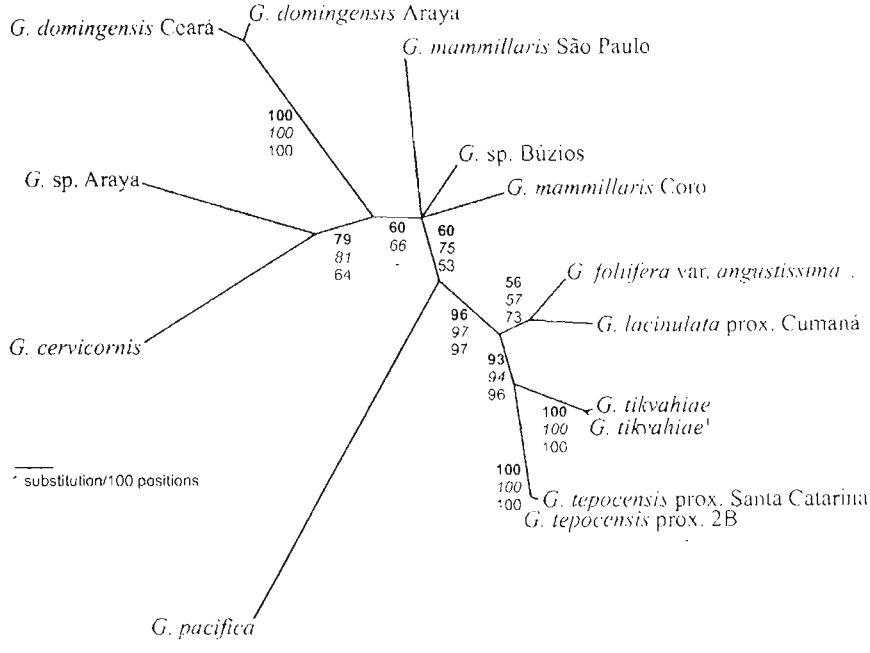

FIG. 3. Bootstrap 50% majority-rule consensus ML tree for ITS matrix 2. ML calculations were made under the JC69. The bootstrapped NJ unrooted tree using K2P distances corrected for rate heterogeneity differed in not having the single node for the *G. domingensis* clade and the *G. cervicornis*-*Gracilaria* sp. Araya clade. Numbers at nodes denote bootstrap values for ML (first row in bold typeface, 1000 replicates), MP (second row in italics, 2000 replicates), and NJ (third row in normal typeface, 2000 replicates). [1]Sequence of ITS from *G. tikvahiae* produced by Goff et al. (1994, not available in GenBank).

amosa and *G. domingensis*, were not related to the rest of the "*henriquesiana*" species studied or to each other. Moreover, all these sequences were clearly included within the *Gracilaria* clade. Another main criterion used to segregate *Hydropuntia* from *Gracilaria*, that is, the production of tubular nutritive cells restricted to the floor of cystocarp (Chang and Xia 1963, Fredericq and Hommersand 1990a), has been repeatedly shown to be equivocal (cf. Bird 1995). Abbott et al. (1991), arguing against *Hydropuntia*, transferred to *Gracilaria* the *Hydropuntia* species known at that time, including the type species, although it is not clear if they studied the type. Thus, the generic status of *Hydropuntia* is doubtful in light of both morphological and molecular data, although sequences of the generitype species, *H. urvillei* Montagne from Torres Strait, Australia, are necessary to propose the formal synonymy between *Gracilaria* and *Hydropuntia* on molecular grounds.

As circumscribed in this work, Gracilariaceae includes four nonparasitic genera, *Curdiea*, *Melanthalia*, *Gracilariopsis*, and *Gracilaria*, although their discrimination is still strongly based on morphology rather than evolutionary information. For example, evolutionary divergence between SSU rDNA sequences of *Curdiea* and *Melanthalia* is on the same order of magnitude as among some species of *Gracilaria* (Table 4). On anatomical grounds, *Curdiea* and *Melanthalia* are closely related (Fredericq and Hommersand 1990a,b), both having sparse secondary pit connections in vegetative tissues, nemathecial production of tetrasporangia, and cystocarps lacking tubular nutritive cells, among other features. These two genera have remained distinct, based mainly on gross morphology. We suspect that if other species from these genera are included in molecular comparisons, the boundaries of *Curdiea* and *Melanthalia* may be obliterated. On the other hand, *Gracilariopsis* is a noteworthy example of incongruity between morphology-based taxonomy and molecular data. *Gracilariopsis* species are morphologically conservative: All have cylindrical thalli and superficial production of spermatangia, and cystocarps lack tubular nutritive cells, among other distinctive features (Fredericq and Hommersand 1989b, Bird 1995). But this homogeneity contrasts with a high degree of evolutionary divergence in SSU rDNA sequences (Table 4). The divergence levels within *Gracilariopsis* may be as large as divergences between *Cryptonemia* and *Sebdenia*, two genera of Halymeniales selected as outgroup taxa (Fig. 1A). Moreover, at least three strongly divergent lineages (*Gp. lemaneiformis*, isolates from the Atlantic, and the unidentified isolate from Ecuador) exist within *Gracilariopsis*. Finally, if we consider the evolutionary position of parasitic *Gl. oryzoides*, *Gracilariopsis* is a paraphyletic assemblage.

One remarkable result of the new set of SSU rDNA sequences compared in this work is the order of divergence within the three main lineages of Gracilariaceae. *Gracilariopsis* was retrieved as the first evolving lineage of Gracilariaceae by Bird et al. (1992, 1994), followed by the sister groups *Curdiea*/*Melanthalia* and *Gracilaria*. Our phylogenetic inferences, based on character-by-character comparisons (MI and MP) and some distance-based analyses, instead resolve the *Curdiea*/*Melanthalia* clade as the first diverging lineage of Gracilariaceae, with *Gracilariopsis* as a sister group of *Gracilaria* (low bootstrap values for this node). Because *Gracilariopsis* species appear to have faster mutation fixation (revealed by the high intraspecific differences: Bird et al. 1994, Goff et al. 1994), the placement of *Gracilariopsis*/*Gracilariophila* clade as the first diverging lineage could be a long-branch attraction effect. From a morphological perspective, the hypothesis that the *Curdiea*/*Melanthalia* lineage divergence first appears likely because the nemathecial disposition of tetrasporangia and the delayed formation of secondary pit connections in vegetative tissues, not shared by other free-living genera of Gracilariaceae, are regarded as primitive features (Fredericq and Hommersand 1990b). Phylogenetic analysis of the *rbc*L gene (M. Hommersand, personal communication) produced trees indicating that the *Curdiea* and *Melanthalia* lineage was the first divergence in the family.

Infrageneric lineages of Gracilariopsis. SSU rDNA sequence comparisons in both *Gracilariopsis* and *Gracilaria* indicate the presence of discrete infrageneric lineages, although there is not enough resolving power in these sequences when closely related *Gracilaria* species are compared. Three lineages of *Gracilariopsis* were deeply separated in phylogenetic inferences. *Gracilariopsis lemaneiformis*, the generitype species from the Pacific, diverged first in all the analyses, followed by the Atlantic species cluster and the material from Ecuador. Besides the entities studied in this work, no other *Gracilariopsis* species are known from the Atlantic. Hereafter, we can consider an Indo-Pacific origin for *Gracilariopsis* as a working hypothesis, with the Atlantic lineage considered as a derived group. However, there are few currently accepted species of *Gracilariopsis* (six in Bird 1995). This relatively low specific diversity, as compared with the sister group *Gracilaria* (nearly 100 recognized species, Oliveira and Plastino 1994), leads us to suspect that this number may be increased if more critical studies are undertaken in the Indo-Pacific. Species recognition within *Gracilariopsis* is difficult because distinctive morphological features are few or hard to recognize (especially anatomic details of the male sorus) and because sexually reproductive specimens are apparently absent in many populations. We believe that the utilization of hybridization and molecular sequencing techniques on widespread samples will reveal cryptic species of *Gracilariopsis*. Evidence supporting this conclusion is the recent recognition of several new taxa: 1) *Gp. longissima*, repeatedly mistaken as *G. gracilis* in Britain; 2) the distinct strains from North Carolina, Peru, and China studied by Bird et al. (1992) and Goff et al. (1994), misnamed as *Gp. lemaneiformis*; and 3) the material from Ecuador studied here. All these entities are possibly undescribed species that need further taxonomic clarification. The

evolutionary relationships among some samples of *Gracilariopsis* and the parasite *G. oryzoides* are also striking. These relationships were studied by Goff et al. (1996), but the taxonomic implications of the paraphyletic nature of *Gracilariopsis* were not discussed. We strongly suggest that sequences from other species of *Gracilariophila* and *Gracilariopsis* should be studied, particularly those species of *Gracilariophila* that parasitize *Gracilaria* and not *Gracilariopsis*. The other parasitic genus, *Congracilaria* H. Yamamoto (1986), found on *Gracilaria*, has typical *Gracilaria* reproductive features (i.e. tubular nutritive cells produced by the gonimoblasts and spermatangia produced in conceptacles), expected for an adelphoparasite. However, these relationships should be addressed using molecular tools.

Infrageneric lineages of Gracilaria. Despite the overall low phylogenetic signal in SSU rDNA sequences in *Gracilaria*, at least two infrageneric lineages were consistently revealed by these sequences, the "Atlantic cylindrical *henriquesiana*" and the "*gracilis*" lineages. ITS sequences were more informative at the species level within *Gracilaria*, confirming the two previously mentioned infrageneric lineages and retrieving the additional "*tikvahiae*" and "*cervicornis*" lineages. The main divergence within *Gracilaria* shown in Figure 2 is between the Pacific (except *G. pacifica* and *G. robusta*) and Atlantic populations. In NJ inferences with LogDet/paralinear distances, the Pacific clade had moderate bootstrap support (84%). These results suggest that the primordial divergences in *Gracilaria* are related more to geographic isolation than to broad morphological differences, such as have been used to delineate infrageneric taxa. The Pacific clade includes *G. chilensis* and *G. tenuistipitata*, which appear to be closely related entities, both slender and terete with "*textorii*" type spermatangial configuration despite their different respective habitats of cold and warm water. They are joined in the Pacific clade by an undescribed species from Baja California and Elkhorn Slough, which also has a stringy thallus, although with "*verrucosa*" type spermatangia. Unfortunately, there are no sequences from flattened Pacific or Indian material to test if these species will group with the cylindrical Pacific ones.

Based on ITS sequences, the first diverging group in the Atlantic clade, including *G. pacifica* and *G. robusta*, is the "Atlantic cylindrical *henriquesiana*" lineage, which encompasses the more "promising commercial agarophytes" from tropical Atlantic: *G. caudata*, *G. cornea*, *G. crassissima*, and an undescribed *Gracilaria* species from northeastern Brazil. All these species are from the tropical Atlantic (except *G. caudata*, which reaches subtropical waters), have cylindrical thalli (except *G. crassissima*), possess deep compound spermatangial conceptacles, have a fusion cell that is highly dissected and ramified, and have pseudo-parenchymatous sporogenous tissue present in cystocarps (cf. Fredericq and Norris 1985, Plastino and Oliveira 1997). Fredericq and Norris (1985), studying *G. cornea* and *G. crassissima* from the Caribbean, used these features to argue for retaining *Hydropuntia* (as *Polycavernosa*). However, their taxonomic conclusions were not followed by many phycologists because they did not study the generitype species and because other authors (Bird and McLachlan 1984) consider that simple versus compound deep male conceptacles may be just a continuous gradation between extremes. For example, species that normally form simple male conceptacles may also form compound ones in some circumstances. We observed this last situation in the extremely morphologically variable *Gracilaria domingensis*. We also found that male plants of some populations of *G. caudata* form consistently "*verrucosa*" type spermatangia and that in other populations the spermatangia are produced in "*henriquesiana*" type conceptacles. The history of the genus *Polycavernosa* has been tortuous, and the last treatment of *Polycavernosa* (as *Hydropuntia*) was the subgeneric rank formally proposed by Tseng and Xia (1999), following a previous pattern established by Yamamoto (1984). However, as we have shown, "*henriquesiana*" type spermatangial conceptacles have appeared independently in several lineages of *Gracilaria* species. Therefore, taxonomic discrimination based uniquely on this feature is not phylogenetically coherent. Another character used to segregate *Polycavernosa/Hydropuntia* species from *Gracilaria* (i.e. the presence of tubular nutritive cells connecting gonimoblasts and pericarp only at the floor of the cystocarps) is also not exclusive to this group (Bird 1995). Although an "Atlantic cylindrical *henriquesiana*" lineage has emerged here despite the inconsistency of the morphological discriminants, it cannot be used to support the subgenus *Polycavernosa/Hydropuntia* as it thus far does not include the type species. These and other *Gracilaria* species will have to be sequenced before a formal subgeneric taxon can be proposed for the "Atlantic cylindrical *henriquesiana*" lineage. In particular, additional sequences from *Polycavernosa* type species and from other Indo-Pacific taxa, as well as *G. damaecornis* J. Agardh from the Caribbean, a cylindrical species with many similarities to *G. cornea*, should be analyzed before any taxonomic conclusions can be reached. Further, relationships with *G. pauciramosa*, a Caribbean flattened species with "*henriquesiana*" type spermatangia (Rodríguez de Ríos 1989, 1991) that was not related to any infrageneric lineage, will require resolution. In distance analyses of SSU rDNA, *G. pauciramosa* diverged before all other *Gracilaria* isolates from the Atlantic.

After the divergence of the "Atlantic cylindrical *henriquesiana*" lineage, there is a separation of the "*gracilis*" lineage and the cluster formed by the flattened and compressed Atlantic species, as revealed in ITS analyses. *Gracilaria gracilis* and *G. pacifica* are very similar morphologically. Both are terete and slender, with deep and simple spermatangial conceptacles and the same general pattern of gonimoblast anatomy (i.e. the typical features of the "*G. verrucosa*" complex). By contrast, *G. robusta* is a fleshy species with compressed lower branches (Abbott and Hollenberg 1976), which does not satisfy the concept of the "*G. verrucosa*" complex, although its male conceptacles are also deep

and simple. The "*gracilis*" lineage is noteworthy because it includes both Atlantic and Pacific species and likely will also include other Pacific and Indian representatives of the "*G. verrucosa*" complex. *Gracilaria gracilis* is apparently a primarily Atlantic entity, with isolated populations in Europe (Atlantic and Mediterranean), Argentina, and Namibia. The sample ascribed to *G. gracilis* from Japan may be another distinct but related species (Rice and Bird 1990, Wattier et al. 1997). Thus, as *G. gracilis* diverged first in this group from a node grouping Atlantic species, the most parsimonious explanation is that *G. pacifica* and *G. robusta* are taxa that evolved from an Atlantic ancestor. The secondary structure model for ITS2 predicted for these three species show that they are closely related.

In the cluster formed by compressed and flattened species from the Atlantic, the "*tikvahiae*" lineage was the most consistently retrieved clade. Initially, we considered that these entities might be populations of a single widespread and very plastic species, with wide tolerance ranges for environmental conditions. However, our molecular comparisons clearly demonstrate that these populations present enough molecular divergence to suggest that they are reproductively isolated and differ at the specific level. Guiry and Freamhainn (1985), through hybridization studies, showed that the North American populations identified as *G. foliifera* or *G. foliifera* var. *angustissima* are conspecific with *G. tikvahiae* from Canada, which led them to infer that the same is valid for the Caribbean populations and by extension for the South American ones as well. Phylogenetic inferences showed that subtropical and temperate representatives (*G. tikvahiae* and *G. tepocensis* proximate) of this lineage are closely related but distinct from tropical ones (*G. lacinulata* and a strain tentatively named as *G. foliifera* var. *angustissima*). Sequences from species morphologically similar to this group, such as *G. multipartita* (Clemente) Harvey from Europe and *G. foliifera* (Forsskål) Børgesen from the Indo-Pacific, should be included to clarify their relationships.

The tropical compressed species *G. cervicornis* and *Gracilaria* sp. Araya are closely related. These species are quite different in gross morphology but similar in reproductive anatomy. Both are widespread in the tropical Atlantic. Although *G. cervicornis* has been well characterized morphologically (Oliveira et al. 1983), we were unable to attribute a correct name to the entity named here as *Gracilaria* sp. Araya. In Venezuela Rodríguez de Ríos (1986) named this species *G. textorii* (Suringar) De Toni. According to this last author, S. Fredericq suggested that this material could be the former *Plocaria flabelliformis* P. Crouan et H. Crouan in Schramm et Mazé (N. Rodríguez, personal communication). Kapraun (1993) adopted the combination *G. flabelliformis* for his collection from Isla de Margarita in Venezuela. The identity of this material will only be clarified with a critical revision of the species proposed by the Crouan brothers, including crossability tests and molecular comparisons on material from the type locality.

The studied populations of *G. domingensis* were not closely related to other flattened species in the alignment. Most of the ribbon-like species, namely *G. curtissiae*, *G. mammillaris* Coro, *G. mammillaris* São Paulo, *G. cuneata*, and an apparently undescribed population from Búzios, Brazil, have similar if not identical SSU rDNA sequences, and ITS sequence comparisons did not resolve their evolutionary relationships. The morphological boundaries among these species are not well established, and we suggest that another region of the genome should be studied, together with critical morphological revisions, to elucidate their systematics and phylogeny.

Species complexes and conclusion. It has long been recognized that distinct gracilarioid species are morphologically so similar that they have been treated as a single taxonomic entity. The "*G. verrucosa*" complex is the best example of this conundrum (Bird and Rice 1990, Bird 1995). Our experience indicates other morphological complexes of gracilarioid algae in the Atlantic, as is the case of the "*tikvahiae*" lineage as defined here, which encompass related but distinct species. Other species complexes include entities that are not necessarily related, for example, the ribbon-like species complex (the two distinct entities named as *G. mammillaris*, *G. cuneata*, *G. pauciramosa*, *Gracilaria* sp. Búzios, among others). Unfortunately, type material of many validly published names that could be applied in these complexes are so fragmentary and usually without spermatangial or cystocarpic specimens that comparison with contemporary collections is very difficult.

In conclusion, although substantial progress has been attained in the last two decades, a reliable recognition of *Gracilaria* species is still an arduous and expensive task. In many situations, critical inspection of morphology will reveal only the "group" of species to which material may be assigned. Therefore, hybridization tests and molecular comparisons are necessary for positive identification. To arrive at a validly published name (if there is one), we need to examine many fragmentary type collections often, and it will be necessary, where possible, to include these type samples in DNA analyses.

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