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GRACILARIOPSIS MCLACHLANII SP. NOV. AND *GRACILARIOPSIS PERSICA* SP. NOV. OF THE GRACILARIACEAE (GRACILARIALES, RHODOPHYCEAE) FROM THE INDIAN OCEAN[1]

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Two new species of *Gracilariopsis* from the Indian Ocean are proposed—*Gracilariopsis (Gp.) mclachlanii* Buriyo, Bellorin et M. C. Oliveira sp. nov. from Tanzania and *Gracilariopsis persica* Bellorin, Sohrabipour et E. C. Oliveira sp. nov. from Iran—based on morphology and DNA sequence data (*rbc*L gene and SSU rDNA). Both species fit the typical features of *Gracilariopsis*: axes cylindrical throughout, freely and loosely ramified up to four orders, with an abrupt transition in cell size from medulla to cortex, cystocarps lacking tubular nutritive cells and superficial spermatangia. Nucleotide sequence comparisons of *rbc*L and SSU rDNA placed both species into the *Gracilariopsis* clade as distinct species from all the accepted species for this genus, forming a deeply divergent lineage together with some species from the Pacific. The new species are very difficult to distinguish on morphological grounds from other species of *Gracilariopsis*, stressing the importance of homologous molecular marker comparisons for the species recognition in this character-poor genus.

***Key index words: Gracilaria*; Gracilariaceae; *Gracilariopsis*; phylogeny; *rbc*L; Rhodophyceae; SSU rDNA**

***Abbreviations:* BPI, Bayesian phylogenetic inferences; *G.*, *Gracilaria*; *Gp.*, *Gracilariopsis*; ML, maximum likelihood; MP, maximum parsimony; *rbc*L, LSU of the RUBISCO gene; TrN + I + G, Tamura and Nei model with proportion of invariable sites and shape parameter of the gamma distribution**

The taxonomy of Gracilariaceae, a confused and difficult group over decades, has been clarified with the advent of DNA sequencing. Several homologous markers have been compared among samples of Gracilariaceae covering a wide range of phylogenetic resolution. For generic and species relationships, the most utilized are the nuclear-encoded SSU rRNA gene (Bird et al. 1992, 1994, Bellorin et al. 2002, Iyer et al. 2005b) and the plastid-encoded LSU of RUBISCO gene (*rbc*L; Gurgel et al. 2003b, Gurgel and Fredericq 2004, Rueness 2005, Gargiulo et al. 2006, Kim et al. 2006). For closely related species and population relationships, the fast-evolving internal transcribed spacers (ITSs) of rRNA (Goff et al. 1994, Bellorin et al. 2002, Byrne et al. 2002), the spacer between *rbc*L and the SSU RUBISCO genes (*rbc*L-*rbc*S spacer; Goff et al. 1994, Byrne et al. 2002, Iyer et al. 2005b, Rueness 2005), and the mitochondrial *cox* 1 gene (Yang et al. 2008) and intergenic *cox* 2–3 spacer (Byrne et al. 2002, Rueness 2005) are preferred. Recently, Kim et al. (2006) used the plastid-encoded *psb*A to compare flattened species from the western Pacific.

As expected, as more molecular data are available, together with critical examination of reproductive morphology and life cycle studies (Liao and Hommersand 2003, Bellorin et al. 2004, Rueness 2005), many problematic species boundaries and distributional ranges have been gradually clarified, and often new species proposed (Gurgel et al. 2003a,b, 2004, Byrne et al. 2002, Iyer et al. 2005a). The descriptions of new taxa are likely to increase as these methods are extended to areas not yet studied in detail, particularly in Asia and Africa.

Molecular evidence has also been used to clarify the generic concepts within Gracilariaceae, mainly the nonparasitic genera closely related to *Gracilaria*, namely, *Gracilariopsis* and *Hydropuntia* (*Polycavernosa*). The available molecular data have fully supported *Gracilariopsis* as a valid genus (Bird et al. 1992,

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Bellorin et al. 2002, Gurgel and Fredericq 2004, Iyer et al. 2005b), while *Hydropuntia* has received considerably less support (Gurgel and Fredericq 2004). In our opinion, the splitting of *Hydropuntia* from *Gracilaria* was based on disputable morphological criteria (Chang and Xia 1963, Fredericq and Hommersand 1990), as have been previously discussed (Abbott et al. 1991, Bird 1995, Bellorin et al. 2004). In fact, conflicting results about the usefulness of splitting this genus, even on *rbc*L sequence grounds, are still being published (Gargiulo et al. 2006).

The genus *Gracilariopsis* is noteworthy because almost all of its species have a highly conserved gross and reproductive morphology, very similar to that of *Gp. andersonii* described by Fredericq and Hommersand (1989b; as *Gp. lemaneiformis*). However, *Gracilariopsis* species are strongly divergent on molecular grounds, even when highly conserved sequences, such as SSU rDNA, are compared (Bird et al. 1992, Bellorin et al. 2002). This cryptic diversity was overlooked in most traditional studies, and historically, binomials such as *Gp. lemaneiformis/Gp. sjoestedtii* considered as cosmopolitan species have been misapplied for virtually every *Gracilariopsis* collection all over the world (Gurgel et al. 2003b). In many of these instances, molecular evidence and, to a lesser degree, detailed morphological observations have led to the erection of distinct species with narrower distributions to replace the old names with broader distributions (Steentoft et al. 1995, Gurgel et al. 2003a,b, Iyer et al. 2005a).

In this study, we propose two new species of *Gracilariopsis* for previously undetermined samples from the Indian Ocean, based on *rbc*L and SSU rDNA nucleotide sequence data. A detailed description of the vegetative and reproductive morphology is also provided. Additionally, *rbc*L and SSU rDNA sequences from some other species from the Pacific and Atlantic oceans are presented.

MATERIALS AND METHODS

Specific information about the collections examined is given in the Results. Fresh, dried, or formalin-fixed materials were hand-sectioned, stained with 1% aniline blue solution, and mounted in semipermanent glass slides with Karo syrup. Some preparations were stained with Wittmann's aceto-iron-hematoxylin-chloral hydrate (Wittmann 1965) and mounted with 50:50 Hoyer's medium in distilled water according to the procedure of Hommersand and Fredericq (1988). Herbaria specimens as well as microscope slides studied are housed in the Phycological Herbarium of São Paulo University (SPF).

Total DNA was extracted from field-collected specimens dried in silica gel (Table S1 in the supplementary material). The DNA extraction, PCR amplification for SSU rDNA, and sequencing protocols were identical to those described in Bellorin et al. (2002). Partial sequences of *rbc*L (1,401 positions; 95.5% of the total sequence) were amplified using the primers F*rbc*L start and R1381 (Freshwater and Rueness 1994) under the following PCR parameters: 96°C for 4 min, 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 h 30 min, and 72°C for 10 min. Samples for PCR reactions (50 μL each) were prepared using 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer, 1.25 U of *Taq* DNA polymerase (Promega, Madison, WI, USA). For *rbc*L sequencing, the amplification primers plus the internal primers F492, F753, F993, R492, R753, and R1150, as listed in Freshwater and Rueness (1994), were utilized. Purified PCR products using MicroSpin HR columns (Amersham, Pharmacia Biotech, Piscataway, NJ, USA) were sequenced on ABI PRISM™ 310 Genetic Analyzer or 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Multiple alignments for *rbc*L and SSU rDNA sequences were manually constructed in BioEdit 7.0.4.1 (Hall 1999), including the sequences produced in this work and most of the available *rbc*L and SSU rDNA sequences of *Gracilariopsis* species in GenBank (Table S1). In the case of published *rbc*L sequences, wherever possible, sequences with a larger percentage of determined positions were chosen. Alignment of SSU rDNA sequences was made using the secondary structure-based data set of Bellorin et al. (2002) as a model. For phylogenetic inferences, the positions corresponding to amplification primers and gaps were removed. The outgroups used were *Gracilaria (G.) chilensis*, *G. tenuistipitata,* and *G. vermiculophylla*, which are the species of *Gracilaria* more closely related to *Gracilariopsis* (Bellorin et al. 2004, Gurgel and Fredericq 2004).

Phylogenetic inferences for *rbc*L and SSU rDNA alignments using maximum likelihood (ML) were constructed in PAUP* v.4.0.10 (Swofford 2002), under the optimality settings for likelihood analyses estimated with Modeltest 3.7 (Posada and Crandall 1998) by Akaike information criterion. Fifty percent majority-rule consensus trees were derived from ML bootstrap analyses, involving 1,000 bootstrap replicates of heuristic searches, with random sequence addition, stepwise addition, tree-bisection-reconnection swapping algorithm, COLLAPSE and MULTREES options. Additionally, maximum-parsimony (MP) bootstrap analyses were made on PAUP* using 2,000 heuristic searches with random sequence addition, stepwise addition, tree-bisection-reconnection, MaxTree setting automatically increased by 100, COLLAPSE and MULTREES. Bayesian phylogenetic inferences (BPI) were conducted on MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003), using four simultaneous Markov chains, and the parameters of the best-fit sequence evolution model determined with Modeltest. We performed 1,000,000 generations starting with a random tree, sampling one tree every 10 generations. The ''burn-in'' point was the generation 22,000. A 50% majority-rule consensus tree was assembled in PAUP* with the trees generated after the burn-in. The frequency of each clade among all saved trees was considered as the credibility value for each clade.

RESULTS

Gracilariopsis mclachlanii Buriyo, Bellorin et M. C. Oliveira sp. nov. (Figs. 1–3).

Thallus maturus ad 15–60 cm (−150 cm) alti, frondes erecti teretes gracilisque, ad 1–3 mm latus, ramificatio irregularis at 4 ordines, compositus ex strato externo 1–2 cellularum corticalium ad 5–12 μm diametro, et 1–3 stratis cellularum subcorticalium, et medulla cellularum globosarum 100–400 μm diametro. Spermatangia superficiaria, in strato 9–16 μm profundo. Cystocarpia usque ad 1 mm diametro et usque ad 1 mm alta, sine filamenta travertentia (trabeculis) inter gonimoblastus et pericarpium et fundum cystocarpia. Gonimoblastus latus, leviter constricto ad bases, carposporangia

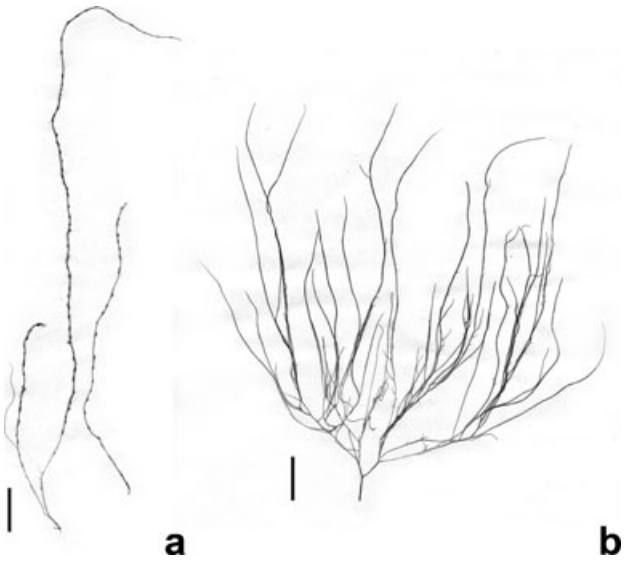


FIG. 1. *Gracilariopsis mclachlanii* sp. nov. (a) Holotype of *Gp. mclachlanii* from Sazadongo Nungwi Mnaraní, Zanzibar, Tanzania (SPF 56287). Scale bar, 2 cm. (b) Habit of a sterile plant from Sazadongo Nungwi Mnaraní, Zanzibar, Tanzania, 18.iv.2003, leg. A. Buriyo (SPF 56291). Scale bar, 2 cm.

terminalia 15–25 μm diametro. Pericarpium ex 8–13 stratis cellularis, usque 130 μm crassum. Tetrasporangia intra corticem locata, decusate cruciate divisa, 20–60 × 15–32 μm. Characteres ceteri more generis.

Holotype: A cystocarpic plant from Tanzania, Zanzibar, Unguja Island, Nungwi Maraní (A. Buriyo, 27.vii.2002, SPF 56287; 5°43′ S, 39°17′ E).

Additional specimens examined: Tanzania, Zanzibar, Unguja Island, Nungwi Maraní (A. Buriyo, 18.iv.2003, male, SPF 56288; tetrasporangial, SPF 56284; female, SPF 56290; sterile, SPF 56291); Tanzania, Zanzibar, Pemba Island, Njini Kinyu, Pangamkungu (A. Buriyo, 18.iii.2003, tetrasporangial, SPF 56292 and SPF 56293).

Etymology: The species is named in honor of Prof. Jack McLachlan (National Research Council, Canada) for his outstanding research on Gracilariaceae.

Distribution and ecology: The species grows on intertidal and subtidal rocky, sandy, or sandy-muddy substrates partially covered by sand, as well as on beds of dead *Halimeda* segments and shells on areas with good water circulation. Due to its long and slender nature, fronds lay prostrate over the substrate and sometimes partially embedded in sandy substrate. The basal part is always buried in the sand.

Habit and vegetative structure: Plants are 15–60 cm (–150 cm) tall at maturity, solitary or caespitose; axes and branches cylindrical throughout, 1–3 mm in diam., arising from small disk-like holdfast. Thalli usually have a distinctive central axis often unbranched at the base; branches scattered, alternate to irregularly disposed up to four orders, shallowly constricted at bases and tapering gradually toward apices (Fig. 1). Color varies from yellowish-green to pale red and dark red probably depending on the degree of exposure to sunlight. The cortex is composed of two layers of small isodiametric or anticlinally elongate cells, measuring 5–12 μm diam., with dense content, heavily pigmented, connected only with their parental cells by primary pit connections.

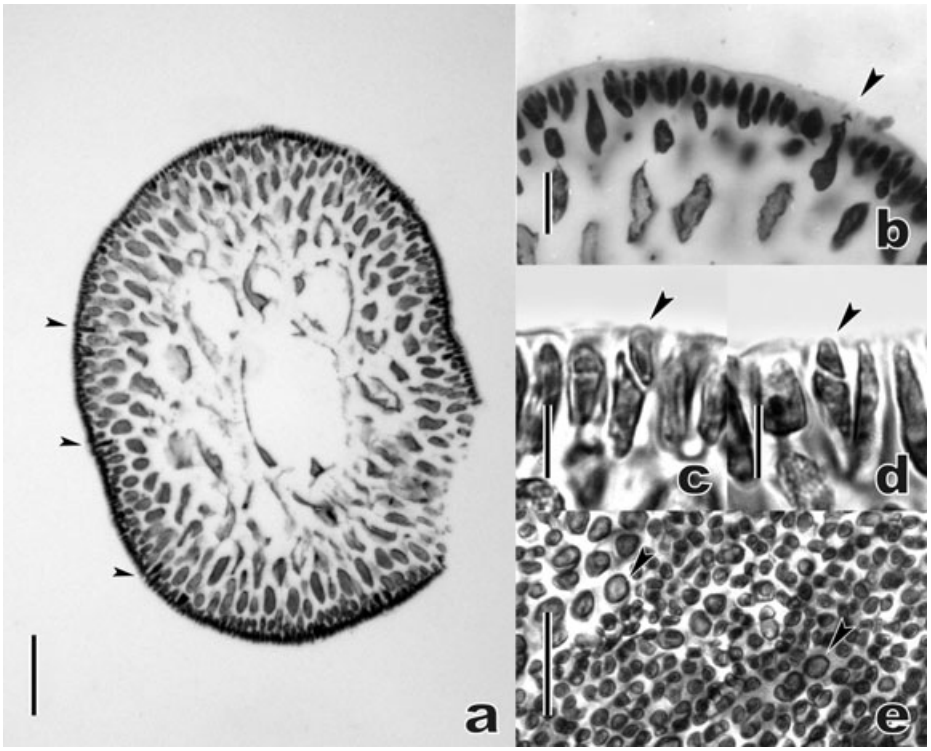


FIG. 2. *Gracilariopsis mclachlanii* sp. nov. (a) Transverse section of a main axis showing the abrupt transition in cell size from medulla to cortex and basal cells of hairs (arrowheads) in the cortex (SPF 56288). Scale bar, 100 μm. (b) Male cortex as seen in transverse section showing a basal cell of hair (arrowhead; SPF 56288). Scale bar, 20 μm. (c–d) Detail of superficial spermatangia produced by oblique concavo-convex septa (arrows) from parent spermatangial mother cells (SPF 56288). Scale bar, 10 μm. (e) Superficial view of male cortex showing superficial spermatangia interspersed with larger sterile cortical cells (arrowheads; SPF 56288). Scale bar, 25 μm.

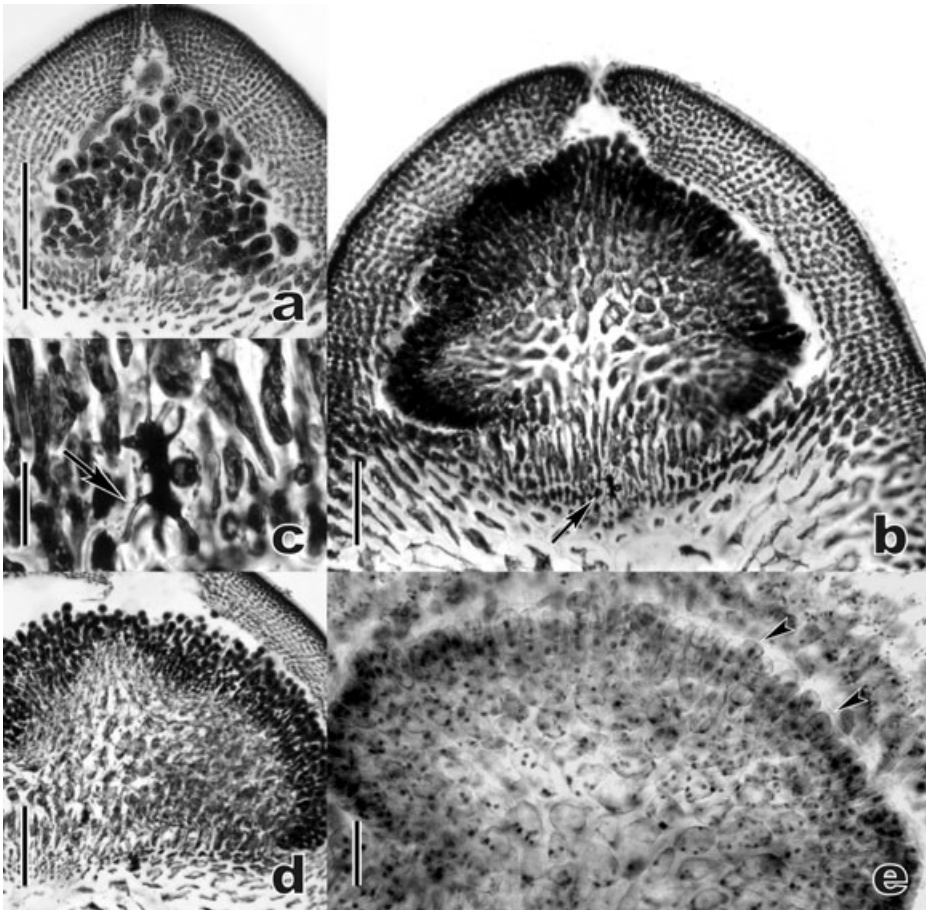


FIG. 3. *Gracilariopsis mclachlanii* sp. nov. (a) Vertical median section of a young cystocarp (SPF 56290). Scale bar, 200 μm. (b) Vertical median section of a mature cystocarp showing the generative fusion cell at the cystocarp floor (arrow; SPF 56287). Scale bar, 100 μm. (c) Detail of generative fusion cell showing an enlarged secondary pit-connection with a gametophytic cell (arrow; SPF 56287). Scale bar, 50 μm. (d) Vertical median section of a fully developed cystocarp showing the shallowly lobate carposporophyte and the carposporangia produced in straight chains (SPF 56290). Scale, 200 μm. (e) Vertical section of a mature cystocarp stained with Wittmann's aceto–iron–hematoxylin–chloral hydrate showing the uninucleate carposporangia initials and carposporangia (arrowheads) and multinucleate inner sterile goninoblasts cells (SPF 56287). Scale bar, 100 μm.

The central medulla is composed of large globose cells, 100–400 μm in diam. in transverse sections, thick walled and highly vacuolated, lacking pigments (Fig. 2a). A subcortex composed of 1–3 layers of anticlinally elongated cells in cross-sections is located between the cortex and the medulla in fully developed thalli. The transition of cell size from medulla to cortex is abrupt. Large basal cells of deciduous hairs are frequent near the surface.

Reproductive structures: Spermatangia are formed in superficial sori on the cortex, from anticlinally elongated terminal spermatangial mother cells (i.e., ''*chorda*'' type, Yamamoto 1975; Fig. 2, b–d). The spermatangia are small (3–5 μm in diam.) and colorless, often appearing triangular in transverse sections, cut off by an oblique concavo-convex or transverse septum from the parent cell (Fig. 2, c–d). In superficial views, fully developed spermatangial sori appear as irregular pale patches covering nearly the whole surface of thallus, interrupted by small groups of sterile terminal cortical cells as well as larger basal cells of hairs (similar to those described in *Gp. tenuifrons* by Bird and Oliveira 1986, as *G. tenuifrons*; Fig. 2e). The fertile cortex (cuticle, spermatangia, and spermatangial mother cells together) is 9–16 μm thick.

The cystocarps are scattered on thallus surface, except on basal and apical regions; they are prominent at maturity and not constricted at the base, measuring 0.7–1.0 mm in diam., and up to 1 mm high in herbarium specimens. The mature carposporophytes are slightly constricted at the bases and shallowly lobed (Fig. 3, a–b). The inner sterile gomimoblast cells are small, 25–110 μm in diam., multinucleate, and connected by abundant secondary pit connections (Fig. 3, b, d–e). Remnants of the generative fusion cell, highly dissected and cytoplasm rich, were often observed at the center of the cystocarp floor in median vertical sections (Fig. 3, b–c). The carposporangia are formed terminally from radially elongated long chains of carposporangial initials (up to 6 cells); the carposporangial initials are uninucleate and stain densely with aniline blue. The mature carposporangia are uninucleate and 15–25 μm in diam. (Fig. 3e). Tubular nutritive cells connecting the gonimoblasts to outer pericarp or to cystocarp floor were not observed. The outer pericarp is a 90–130 μm thick, formed by 8–13 cell layers; ostioles are seldom prominent.

Tetrasporangia occur singly, embedded in the cortex over the entire frond, except at basal and apical regions, the sporangia are decussate cruciate and ovoid, with considerable variation in size (20–60 × 15–32 μm).

Gracilariopsis persica Bellorin, Sohrabipour et E. C. Oliveira sp. nov. (Figs. 4–6).

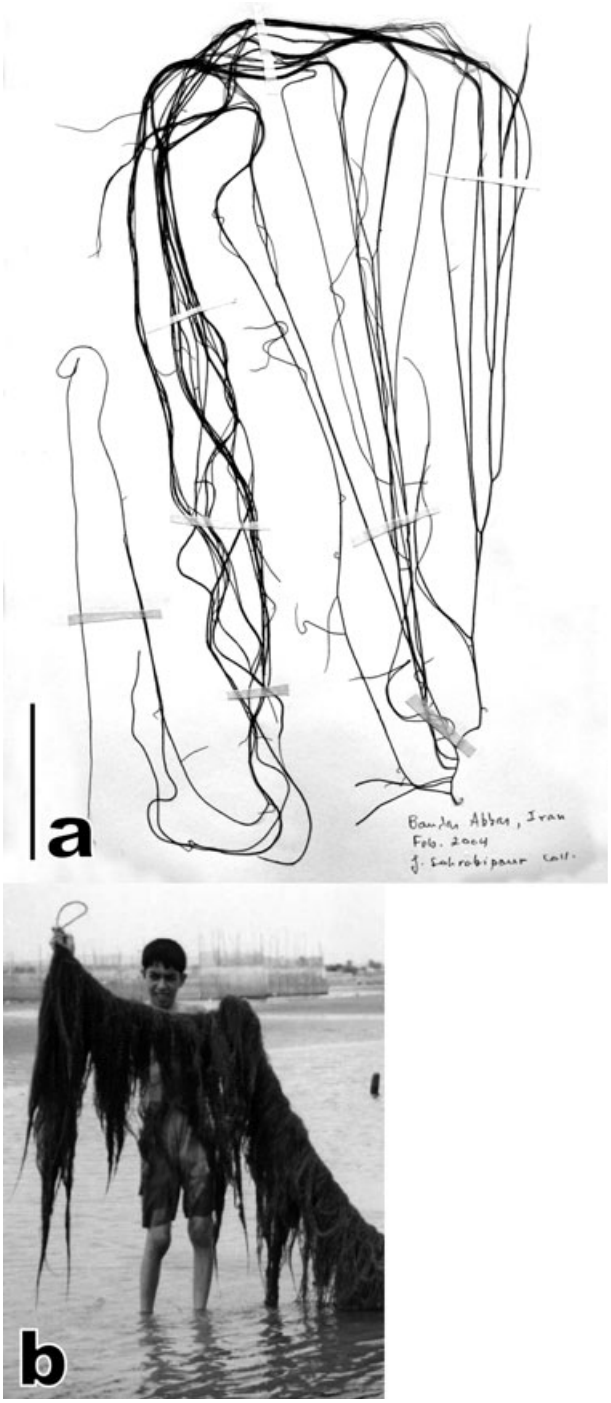


FIG. 4. *Gracilariopsis persica* sp. nov. (a) Holotype of *Gp. persica* from Bandarabbas, Iran (SPF 56294). Scale bar, 5 cm. (b) Specimens attached to a culture rope in Bandarabbas, Iran.

Thallus maturus ad 30–60 cm (−250 cm) alti, teretes gracilisque et 1–2 mm diametro, ad 1–4 ordines ramosus, libere ramificatio, compositus ex strato externo 1–2 cellularum corticalium 5–8 μm lato et 10–16 μm alti, et 1–4 stratis cellularum subcorticalium, et medulla cellularum globosarum magnarum (100–400 μm diamtero). Spermatangia superficiaria, in strato 8–14 μm profundo. Cystocarpia 0.7–1.1 mm diametro et usque 1.2 mm alta, pericarpium 90–120 μm crassum, ex 8–12 stratis cellularum constitutum, gonimoblastus latus, sine filamenta travertentia (trabeculis) inter gonimoblastus et pericarpium et fundum cystocarpia. Carposporangia terminalia 15–30 μm diametro. Tetraspoprangia per corticem dispersa, decusate cruciate divisa, 23–65 × 12–45 μm. Characteres ceteri more generis.

Holotype: A tetrasporangial plant from Iran, Persian Gulf, Bandarabbas (J. Sohrabipour, ii.2004, SPF 56294; 27°10′ N, 58°16′ E).

Additional specimens examined: Iran, Persian Gulf: Tang (J. Sohrabipour and R. Rabei, ii.2003, cystocarpic, SPF 56295), Bandarabbas (J. Sohrabipour and R. Rabei, 27.xii.2003, cystocarpic, SPF 56296; tetrasporangial, SPF 56297; 13.i.2004, male, SPF 56298. J. Sohrabipour, 6.vi.2004, cystocarpic, SPF 56299; male, SPF 56300; tetrasporangial, SPF 56301. J. Sohrabipour and R. Rabei, ii.2004, cystocarpic, SPF 56302).

Distribution and ecology: Iran—Bandarabbas, Qeshm Island, Hormuz Island, Bandar-e-lengel, and Khark Island. Plants grow attached to pebbles or artificial substrates on sandy-loam or sandy bottoms, at the intertidal or sublittoral regions of coasts not exposed to strong wave actions or currents. The fronds are present from mid-October to May.

Habit and vegetative structure: The plants are solitary or caespitose, up to 60 cm (–250 cm) long, cartilaginous, leathery or stringy elastic in texture, purple, red-greenish pale to yellowish; erect axes are terete throughout and 1–2 mm in diam., arising from small disk-like holdfast. The thalli are freely and loosely branched to 1–4 orders; branches and branchlets not abruptly constricted at bases, tapering gradually toward apices (Fig. 4). Mature thalli with an abrupt transition from medulla to cortex in cell size in transverse sections (Fig. 5, a–b); cortex consisting of two layers of subquadrate to anticlinally elongate cells, measuring 5–8 × 10–16 μm in cross-section; a subcortex distinct from medulla underlines the cortex, composed of 1–4 layers of larger, isodiametric to anticlinally elongate cells in transverse sections (Fig. 5c). Medulla consisting of large and highly vacuolated cells, 100–400 μm in diam. in transverse sections. Basal cells of deciduous hairs were occasionally observed in the cortex.

Reproductive structures: Spermatangia are superficial, formed on the whole surface except apical and basal regions of branches; first as irregular patches in superficial views interspersed with sterile cortical cells (Fig. 5f), later the spermatangial sori extend over the whole surface of branches as irregular patches interrupted by sterile cortical cells and by basal cell of hairs (similar to *Gp. tenuifrons*, Bird and Oliveira 1986). Each spermatangium is a small (3–4 μm in diam.) and colorless cell, cut off from anticlinally elongate spermatangial mother cell by a transverse or oblique septum (Fig. 5, d–e). The

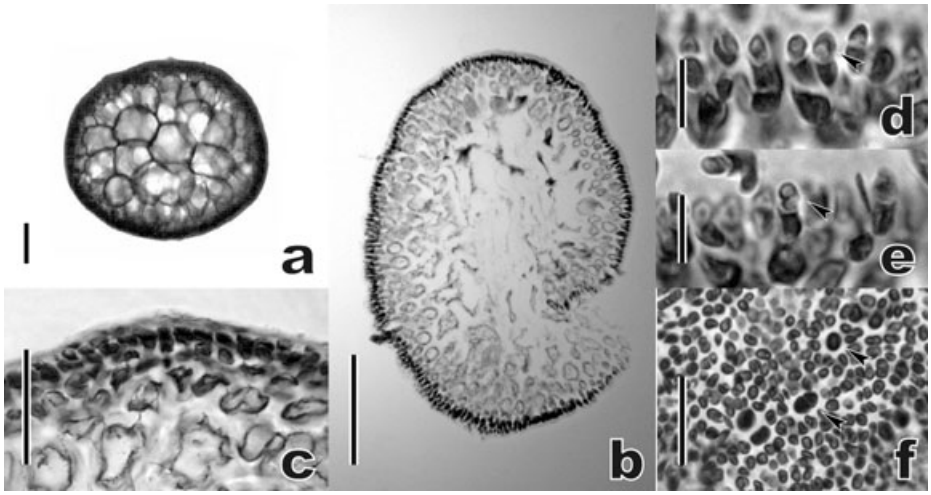


FIG. 5. *Gracilariopsis persica* sp. nov. (a) Cross-section of main axis (fresh material) showing the abrupt transition in cell size from medulla to cortex. Scale bar, 200 μm. (b) Transverse section of a main axis (SPF 56296). Scale bar, 200 μm. (c) Cortex and subcortex as seen in transverse section (SPF 56297). Scale bar, 50 μm. (d–e) Male cortex in transverse section showing the spermatangia (arrowheads) cut off from spermatangial mother cells by transverse septa (SPF 56298). Scale bar, 10 μm. (f) Superficial view of male cortex showing the spermatangia interspersed with sterile cortical cells (arrowheads) (SPF 56300). Scale bar, 25 μm.

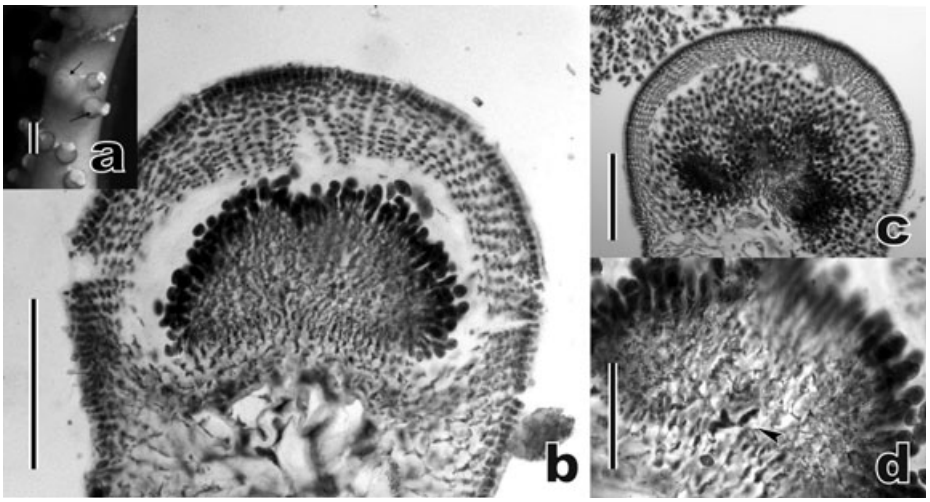


FIG. 6. *Gracilariopsis persica* sp. nov. (a) Mammillate cystocarp with eroded ostioles (arrows). Scale bar, 0.5 mm. (b) Vertical median section of young cystocarp stained with Wittmann's aceto-iron-hematoxylin-chloral hydrate (SPF 56302). Scale bar, 200 μm. (c) Fully developed cystocarp with carposporangia filling the cystocarp cavity (SPF 56299). Scale bar, 200 μm. (d) Carposporophyte stained with Wittmann's aceto-iron-hematoxylin-chloral hydrate showing the remnant of a generative fusion cell at the basal portion (arrowhead) (SPF 56302). Scale, 200 μm.

cuticle, spermatangia, and spermatangial mother cells altogether form a fertile layer 8–14 μm thick.

Cystocarps are produced on the entire surface of thallus, except basal and apical regions; they are prominent, slightly to markedly constricted at the bases, at maturity measuring 0.7–1.1 mm in diam., and up to 1.2 mm high in herbarium specimens (Fig. 6a). Fully developed carposporophytes are not constricted at the bases. The sterile inner gomimoblast cells are small (30–105 μm in diam.) and connected by abundant secondary pit connections (Fig. 6b). The carposporangial initials form short chains of up to three cells, which stain heavily with aniline blue. Mature carposporangia measure 15–30 μm in diam. The carposporophyte appears lightly lobed at the surface, and the released carpospores often completely fill the cystocarp cavity (Fig. 6c). Tubular nutritive cells are absent. The pericarp is 90–120 μm thick and is composed of 8–12 cell layers, with an often mammillate ostiole. The remnant of the generative fusion cell is often discernible in median sections of the cystocarp (Fig. 6d).

Tetrasporangia are produced in the cortex over the entire frond, except basal and apical regions; they are radially elongate, measuring 23–65 × 12–45 μm; division is decussate cruciate.

DNA sequences. The *rbc*L gene and SSU rDNA were sequenced for *Gp. mclachlanii* from two localities in Tanzania, and for *Gp. persica* from Bandarabbas, Iran (Table S1). Additionally, the SSU rDNA and *rbc*L sequences of three other species of *Gracilariopsis* were determined in this work—namely, *Gp. lemaneiformis* from Ancón, Peru; *Gp. chorda* from Japan; and *Gp. funicularis* from a previously undetermined

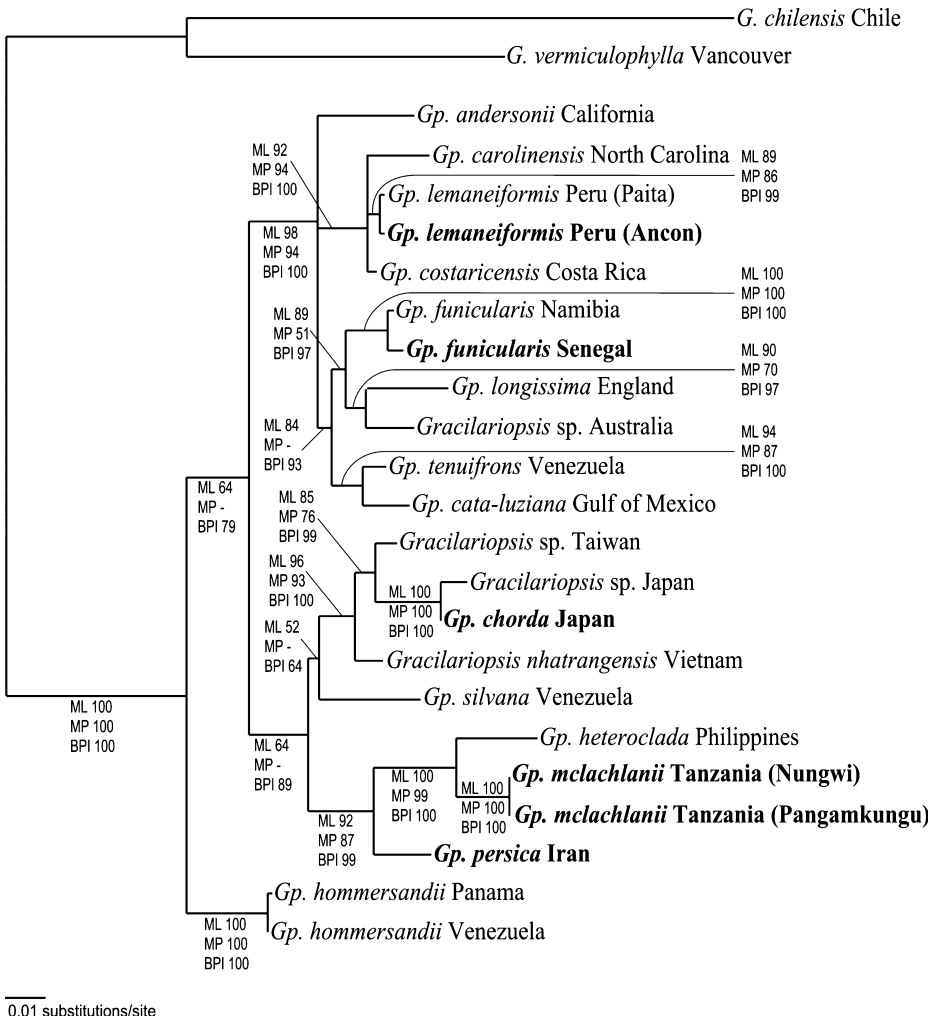


FIG. 7. Fifty percent majority-rule consensus tree derived from maximum-likelihood (ML) analyses (1,000 bootstrap replicates) of *rbc*L sequences under the TrN + G model (K = 6; base frequencies: A = 0.3038, C = 0.1522, G = 0.2151, T = 0.3290; rate matrix: A − C = 1, A − G = 4.1474, A − T = 1, C − G = 1, C − T = 9.0675, G − T = 1; gamma distribution shape parameter = 0.1699). Bootstrap supports under ML and maximum parsimony (MP), as well as Bayesian credibility clade value, are shown. Species names in bold correspond to new sequences determined in this work. BPI, Bayesian phylogenetic inferences; *G.*, *Gracilaria*; *Gp.*, *Gracilariopsis*.

sample from Senegal. The SSU rDNA of *G. tenuistipitata* from China was also sequenced for the first time. The multiple alignment for the *rbc*L gene contained 22 sequences of *Gracilariopsis* (including at least one sequence for each of the currently accepted species) plus two outgroup sequences (840 positions long). For SSU rDNA, the multiple alignment contained 14 sequences of *Gracilariopsis* and three outgroup sequences (1,709 positions long). Phylogenetic inferences using ML clearly showed the distinctness of *Gp. mclachlanii* and *Gp. persica* (Figs. 7–8). MP and BPI retrieved basically the same topologies both for *rbc*L and SSU rDNA (Figs. 7–8). In all analyses, the new species form a well-supported clade together with *Gp. heteroclada*.

DISCUSSION

Gracilariopsis was segregated from *Gracilaria* by Dawson (1949) based on features of the cystocarps, mainly the absence (*Gracilariopsis*) versus the presence (in *Gracilaria*) of tubular nutritive cells connecting the carposporophyte with the pericarp or cystocarp floor, and the nature of the sterile gonimoblasts cells: small and cytoplasm rich in *Gracilariopsis* versus large and vacuolated in *Gracilaria*. Later, Papenfuss (1967) stressed that these diagnostic features are inconsistent in comparisons between type species of both genera, *Gp. sjoestedtii* from California and the former *Gracilaria* generitype, *G. verrucosa*, from England, and proposed to consider *Gracilariopsis* as a synonym of *Gracilaria*. Yamamoto (1975, 1978) noted that *Gracilariopsis*-like species lacking tubular nutritive cells in cystocarps from Japan also have superficial spermatangia, a feature observed in very few species of gracilarioids (Dawson 1949), but he did not recognize *Gracilariopsis* as being distinct from *Gracilaria*.

Further compelling morphological evidence for considering *Gracilariopsis* as a distinct genus was presented by Fredericq and Hommersand (1989a,b), based on detailed morphological and developmental studies of the generitype species of both genera. These authors added new distinctive features to *Gracilariopsis*, mainly the mode of production of spermatangia (superficial in *Gracilariopsis* vs. in shallow

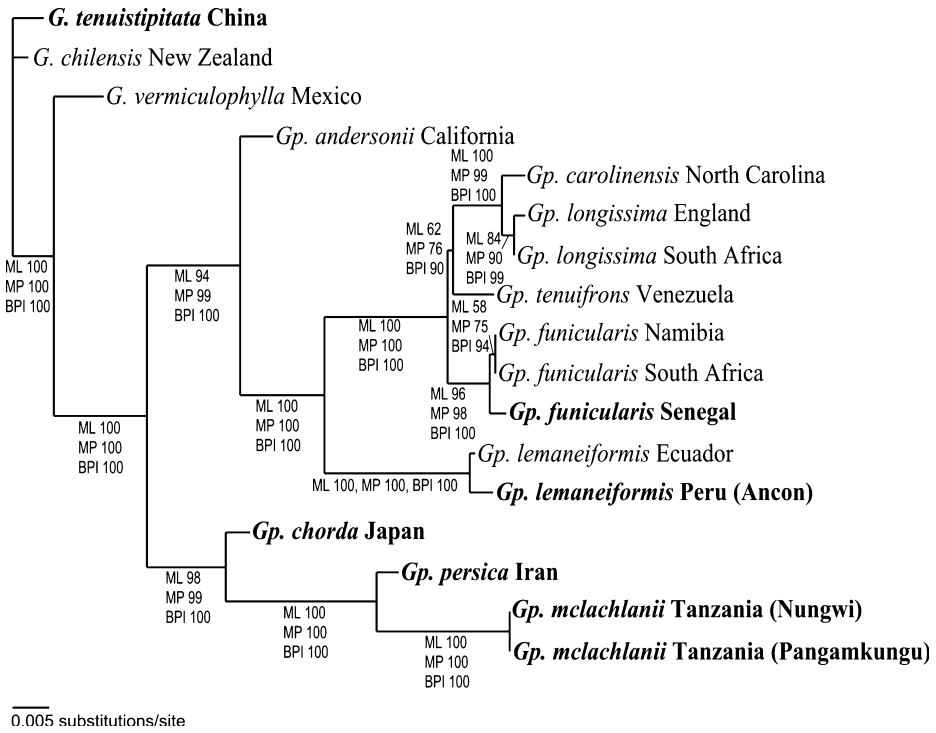


FIG. 8. Fifty percent majority-rule consensus tree derived from maximum-likelihood (ML) analyses (1,000 bootstrap replicates) of SSU rDNA sequences under the TrN + I + G model (K = 7; base frequencies: A = 0.2541, C = 0.2052, G = 0.2868, T = 0.2538; rate matrix: A − C = 1, A − G = 2.2231, A − T = 1, C − G = 1, C − T = 3.6051, G − T = 1; proportion of invariable sites = 0.7554; gamma distribution shape parameter = 0.8168). Bootstrap supports under ML and maximum parsimony (MP), as well as Bayesian credibility clade value, are shown. Species names in bold correspond to new sequences determined in this work. BPI, Bayesian phylogenetic inferences; *G.*, *Gracilaria*; *Gp.*, *Gracilariopsis*.

or deep cavities in *Gracilaria*) and some differences observed in the early postfertilization development and cystocarp anatomy. Nucleotide SSU rDNA sequences, which were available in the early 1990s, supported the distinctness of *Gracilariopsis* (Bird et al. 1990, 1992). It was also shown that two distinct species were largely confused under *G. verrucosa* collections from England, and that one of them, in fact, belonged to *Gracilariopsis* (Bird and Rice 1990, Bird et al. 1992, Steentoft et al. 1995). More recent molecular data have repeatedly confirmed *Gracilariopsis* as being a valid genus (Bellorin et al. 2002, Gurgel et al. 2003a,b, Gurgel and Fredericq 2004, Iyer et al. 2005a,b), and its acceptance has become almost universal (although some authors still do not recognize it today). However, some of the distinctive features of *Gracilariopsis* proposed by Dawson (1949) and Fredericq and Hommersand (1989b, 1990) were shown to be inconsistent, mainly those referring to cystocarp anatomy (summarized by Bird 1995). Admittedly, since Yamamoto's (1978) observations, it was clear that the most reliable morphological character for *Gracilariopsis* is the superficial production of spermatangia. In fact, when Fredericq and Hommersand (1989b) reinstated *Gracilariopsis*, only four species were unequivocally ascribed to it on the basis of documented superficial spermatangia—namely, *Gp. lemaneiformis*, a species first described from Peru but later recorded indiscriminately all over the world and by that time considered a taxonomic synonym of the generitype *Gp. sjoestedtii* (Abbott 1983); *Gp. costaricensis*, a species only known from the Pacific coast of Costa Rica (Dawson 1949); *Gp. chorda* from Japan (Yamamoto 1978); and *Gp. tenuifrons* from the western Atlantic (Bird and Oliveira 1986). Later, a species with superficial spermatangia from southern China, *Gp. heteroclada* (valid name for *Gp. bailiniae*, Zhang and Xia 1991) was added. On the other hand, the species from England and European coasts with superficial spermatangia were described under *Gracilariopsis* as *Gp. longissima*, after careful field and morphological studies (Steentoft et al. 1995).

Recently, it was shown in a global review of the genus based on *rbc*L sequence comparisons (Gurgel et al. 2003a,b) that the many records of *Gp. lemaneiformis*/*Gp. sjoestedtii* over the world are incorrect and that *Gp. lemaneiformis* occurs only in Peruvian coasts. Materials from the North American Pacific Ocean presumed to be *Gp. lemaneiformis* belong to *Gp. andersonii* (the valid name instead of *Gp. sjoestedtii*; Gurgel et al. 2003b), and this species was proposed as the generitype. Gurgel et al. (2003a,b) also described another four new species: *Gp. carolinensis* from North Carolina, a strain largely known (Dawson 1953, Bird and Oliveira 1986, Bird et al. 1992) but up to that time not formally described; *Gp. cata-luziana* from the Gulf of Mexico; and *Gp. hommersandii* and *Gp. silvana* from southern Caribbean. Other strains previously misidentified as *Gp. lemaneiformis* from China, Australia, and Mexico were not determined and probably correspond to species yet not described. The monographic work of Gurgel et al. (2003a,b) is very relevant because the *rbc*L genes of all the accepted species of *Gracilariopsis* were sequenced, including some other undetermined strains. Iyer et al. (2005a,b) showed that *Gp. longissima* is also present in South Africa and

described another new species from Namibia and South Africa, *Gp. funicularis*. Recently, a species from Vietnam was described, *Gp. nhatrangensis* Hau and Lin (2006) and an undescribed strain was reported from Taiwan (S. M. Lin, unpublished data, smlin@nttu.edu.tw).

To summarize, there are, to date, 12 accepted species of *Gracilariopsis*. There are some additional species validly described by Dawson (1949) from the eastern Pacific and a species from Vietnam (Pham-Hoàng 1969), which were listed by Gurgel et al. (2003a). However, the status of such species, only recorded in the original descriptions, needs to be reviewed in the light of modern concepts and molecular techniques.

Most of the accepted species of *Gracilariopsis* share the same basic vegetative and reproductive morphology, and few discrete differences could be proposed as being clear-cut among them. Almost all these species have the following main features: slender, elongate terete axes, sparingly to profusely branched usually in a indefinite fashion, abrupt transition in cell size from medulla to cortex, cystocarps without tubular nutritive cells; wherever the male gametangia have been described, they are superficial (Dawson 1949, Yamamoto 1975, Bird and Oliveira 1986, Zhang and Xia 1988, Fredericq and Hommersand 1989b, Steentoft et al. 1995, Gurgel et al. 2003a,b, Iyer et al. 2005a). The only species that severely departs from this pattern is *Gp. silvana*, a flattened species recently described from Venezuela on the basis of *rbc*L sequences and on morphological examinations of female and vegetative plants (Gurgel et al. 2003a). *Gp. chorda*, a species often characterized by coarse axes (up to 5 mm thick; Yamamoto 1978, Terada and Ohno 2000) is also readily distinguishable. Considering the difficulty of recognizing species within *Gracilariopsis* based on morphology, due to the absence of morphological discontinuities, the importance of informative DNA sequence comparisons to solve species boundaries and relationships in this group of algae becomes paramount.

The two species described here agreed very well with the morphological (both vegetative and reproductive) concept of *Gracilariopsis* and also belong to this genus on the basis of *rbc*L and SSU rDNA sequences. For the reasons discussed above, it is difficult to propose discrete or quantitative distinguishing features for each. Perhaps, the superficial arrangement of spermatangia in the thallus surface could be useful to distinguish some species, as was argued for *Gp. costaricensis* (Dawson 1949), *Gp. tenuifrons* (Bird and Oliveira 1986), and *Gp. longissima* (Steentoft et al. 1995). In this regard, *Gp. mclachlanii* and *Gp. persica* are somewhat similar to *Gp. tenuifrons* in having a fertile male cortex interspersed with small groups or single sterile cortical cells and basal cells of hairs. However, we are of the opinion that a fully developed male cortex in superficial view is similar in most *Gracilariopsis* species, so the discriminating value of this character may be very limited. A feature not documented so far in *Gracilariopsis* is the mode of cleavage of spermatangia from spermatangial mother cells through concavo-convex oblique septa observed in *Gp. mclachlanii*, which could be distinctive of this species. Nevertheless, in the absence of adequate description of the male cortex in most species and even the complete lack of such data in some others recently described (e.g., *Gp. cata-luziana*, *Gp. Hommersandii*, and *Gp. silvana*), no secure conclusions can be drawn. In Tanzania, *Gp. mclachlanii* can be confused in gross vegetative morphology with the cylindrical *Gracilaria* species, *G. edulis*. Other cylindrical species in Tanzania (*G. arcuata* and *G. salicornia*) are coarser and very different from *Gp. mclachlanii* in gross morphology. In Iran, *Gp. persica* seems to be the only cylindrical species with a slender and freely ramified thallus, which makes its identification simple.

Gp. mclachlanii and *Gp. persica* are the first species of *Gracilariopsis* critically described from the Indian Ocean. Previous records of *Gracilariopsis* in the Indian Ocean, namely, ''*Gp. lemaneiformis*'' citations, are probably incorrect (Silva et al. 1996) but could be conspecific with the species described here. There are still many Indian and Pacific coasts where the ''gracilarioids flora'' has not been studied in detail, and therefore one cannot say that the proposed species are endemic or have a broader geographic distribution.

It is worth mentioning that the SSU rDNA sequence of a previously undetermined *Gracilariopsis* strain from Ecuador studied by Bellorin et al. (2002) is nearly identical to that of the authentic *Gp. lemaneiformis* from Peru studied in this work (0.2% dissimilarity), so these materials are probably conspecific, and, accordingly, the distributional range of *Gp. lemaneiformis* should be extended to Ecuador. A similar case was found regarding a strain of *Gracilariopsis* from Senegal studied in this work and *Gp. funicularis*, a species recently described by Iyer et al. (2005a) from Namibia and South Africa. The *rbc*L and SSU rDNA sequences of the strain from Senegal are nearly identical to those of *Gp. funicularis* (0.65% dissimilarity for *rbc*L, and 0%–0.2% dissimilarity for SSU rDNA). Accordingly, we are assuming that these materials are conspecific and extend the distributional range of this species.

Most of the species of *Gracilariopsis* are suitable for commercial agar production (Oliveira et al. 2000), and, in fact, *Gp. mclachlanii* and *Gp. persica* are being considered locally as a source of agar. So, it is very important to extend the sampling for critical morphological and molecular studies for those algae to the areas that have not been studied so far, to assess correct and stable names for such economically valuable species.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. Sequences of DNA of *Gracilaria* (*G.*) and *Gracilariopsis* (*Gp.*) representatives included in the phylogenetic analyses. Sequences from this study in bold.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1529-8817.2008.00552.x.

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