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A new flattened species of *Gracilaria* (Gracilariales, Rhodophyta) from Taiwan

Showe-Mei LIN [a*] *and Olivier DE CLERCK* [b]

[a] *Department of Natural Science Education, National Taitung University, Taitung 950, Taiwan*

[b] *Research Group Phycology, Ghent University, Krijgslaan 281 / S8, 9000 Gent, Belgium*

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Abstract – A new, flattened species of *Gracilaria*, *G. huangii*, is described from southern Taiwan in the warm water region of the west Pacific Ocean. *Gracilaria huangii* is mainly characterized by numerous lobes or bladelets along the margins and on the surface of the blades, and a lack or near absence of tubular nutritive cells. The molecular analysis based on the plastid encoded *rbc*L gene of the flattened species with spiny margins and the *textorii* – type spermatangial conceptacles supports the proposal of this new species.

***Gracilaria huangii* / Gracilariaceae / *rbc*L / Rhodophyta / Taiwan / Taxonomy**

Résumé – Une nouvelle espèce aplatie de *Gracilaria* (Gracilariales, Rhodophyta) récoltée à Taiwan. Une espèce nouvelle de *Gracilaria, G. huangii* est décrite du sud de l'île de Taïwan. *Gracilaria huangii* est principalement caractérisée par de nombreux lobes ou feuillets le long des marges et sur la surface des lames, et une absence ou presque-absence des cellules nutritives tubulaires. L'analyse moléculaire basée sur le *rbc*L des espèces aplaties avec les marges épineuses et les conceptacles spermatangiaux de type *textorii* appuie la proposition de cette nouvelle espèce.

***Gracilaria huangii* / Gracilariaceae / *rbc*L / Rhodophyta / Taiwan / Taxonomie**

INTRODUCTION

The economical importance of *Gracilaria* Greville (1830) for the agar and colloid industry has been a major driving force for understanding the taxonomy and systematics of this red algal genus. *Gracilaria* has been intensively studied in the past decades (Dawson, 1949; Ohmi, 1958; Chang & Xia, 1976; Yamamoto, 1978; Fredericq & Hommersand, 1989; Bird *et al.*, 1994; Bellorin *et al.*, 2002; Liao & Hommersand, 2003; Gurgel & Fredericq, 2004; Iyer *et al.,* 2004). The

* Correspondence and reprints: smlin@nttu.edu.tw
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phylogenetic relationships among *ca* 150 species within the genus *Gracilaria* have been largely inferred using DNA sequencing of the ribosomal DNA cistron as well as the plastid encoded large subunit of the RUBICO gene (Battacharya *et al.*, 1990; Bird *et al.*, 1994; Bellorin *et al.*, 2002; Gurgel & Fredericq, 2004; Gurgel *et al.*, 2004; Iyer *et al.*, 2005). Liao & Hommersand (2003) examined the types of genera presently placed in synonymy with *Gracilaria* and identified 10 species groups, based mainly on the formation of spermatangial conceptacles and cystocarp development. The importance of the reproductive features has been supported by *rbc*L sequence analysis (Gurgel & Fredericq, 2004).

Eighteen species of *Gracilaria* have been recorded from Taiwan, including four flattened species, *Gracilaria textorii* (Suringar) De Toni, *G. punctata* (Okamura) Yamada, *G. spinulosa* (Okamura) Chang *et* Xia and *G. vieillardii* (Chiang, 1985; Lewis & Norris, 1987; Huang, 1999). The record of *Gracilaria textorii* (Suringar) De Toni was shown to represent a misidentification and the existence of *G. punctata* is in a doubt (Lin, 2006). *Gracilaria vieillardii* was proposed by Silva (in Silva *et al.*, 1987) as a substitute name for *G. denticulata* (Kützing) Weber-van Bosse, originally described from New Caledonia but a later homonym of *G. denticulata* Schmitz ex Mazza, which is restricted to southeastern Africa. Because of the absence of morphological details for material from its type locality, the records of *G. vieillardii* throughout the Western Pacific Ocean remain provisional (Yamamoto, 1978; Chiang, 1985; Withell *et al.*, 1994). After comparing the *rbc*L sequences of all the flattened species of *Gracilaria* recorded from Taiwan, Lin (2006) identified an unnamed, flattened *Gracilaria* species that superficially resembles *G. denticulata* from South Africa, calling for a detailed study. The vegetative and reproductive morphology of this new, flattened Gracilaria from Taiwan are documented in detail in this study and its taxonomic status is discussed based on its morphology and rbcL sequence analysis.

MATERIAL AND METHODS

Collections were made by SCUBA or snorkeling. Algal samples for the morphological study were preserved in 3-5% of formalin-seawater or pressed on herbarium sheets, whereas materials used in the molecular study were desiccated in silica gel. Voucher specimens are deposited in the Herbaria of the National Taitung University, Taiwan (NTTU), and the Herbarium of Ghent University, Belgium (GENT). Hand sections were stained with 1% aniline blue acidified with 1% HCl and mounted in 25-30% Karo® syrup (Englewood Cliffs, USA), or treated with aceto-iron-hematoxylin-chloral hydrate and mounted in 50% Hoyer's mounting medium as described in Lin *et al.* (2004).

DNA samples were prepared using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the instructions of the manufacturer. DNA sequencing procedures were as described in Lin *et al.* (2001, 2004). New sequence data and those available from GenBank were compiled and aligned with Sequencher (Gene Codes Corp., Ann Arbor, MI, USA) and exported for phylogenetic analysis. Phylogenetic analyses were performed using the maximum parsimony (MP) available in the computer programs PAUP* v4.b10 (Swofford 2003). An *rbc*L sequence for the flattened species of *Gracilaria denticulata* Schmitz ex Mazza from South Africa was newly generated in this study and compared with that of the proposed new species. The sequences available from GenBank and Lin (2006) are listed in Table 1.

Table 1. List of species used in *rbc*L analysis and accession numbers in GenBank. The number after the accession number is the percentage of the gene sequenced. *refers to Gurgel & Fredericq (2004).

Species	*Collection information/GenBank accession number*
Gracilaria arcuata	AY049383*, 96.3%
Gracilaria beckeri	AY049377*, 96.3%
Gracilaria bursa-pastoris	AY049376*, 91.6%
Gracilaria canaliculata	AY049377*, 87.9%
Gracilaria capensis	AY049378*, 96.5%
Gracilaria cervicornis	AY049365*, 95.6%
Gracilaria denticulata	Coll. O. De Clerck, 15/06/2003, The Bluff, Durban, KwaZulu-Natal, South Africa, DQ296121, 98.2%
Gracilaria flabelliformis	AY049343*, 98.8%
Gracilaria gracilis	AY0494000*, 98.0%
Gracilaria hayi	AY049319*, 95.6%
Gracilaria huangii sp. nov.	Lin 2006, AY737438, 98.1%, as *Gracilaria* sp.
Gracilaria huangii sp. nov.	Lin 2006, AY737439, 96.8%, as *Gracilaria* sp.
Gracilaria huangii sp. nov.	Lin 2006, AY737440, 98.1%, as *Gracilaria* sp.
Gracilaria intermedia	AY049336*, 97.6%
Gracilaria lacinulata	AY049344*, 97.1%
Gracilaria mammillaris	AY049323*, 97.1%
Gracilaria multipartita	AY049322*, 98.6%
Gracilaria occidentalis	AY049322*, 98.6%
Gracilaria pacifica	AY049397*, 97.7%
Gracilaria salicornia	AY049385*, 98.0%
Gracilaria smithsoniensis	AY049321*, 97.3%
Gracilaria spinulosa	Lin 2006, AY737442, 98.3%
Gracilaria spinulosa	Lin 2006, AY737444, 98.3%
Gracilaria textorii	AY049325*, 97.5%
Gracilaria vieillardii	Lin 2006, AY737436, 99.5%
Gracilaria vieillardii	Lin 2006, AY737437, 98.3%
Gracilariopsis bailiniae	AY049411*, 91.1%, as *Gracilariopsis heteroclada*
Gracilariopsis lemaneiformis	AY049415*, 97.6%
Hydropuntia caudata	AY049358*, 76.4%
Hydropuntia cornea	AY049338*, 98.8%
Hydropuntia crassissima	AY049351*, 98%
Hydropuntia usneoides	AY049346*, 98%

Eleven *rbc*L sequences of flattened *Gracilaria* species, possessing the *textorii*-type of spermatangial conceptacles, from South Africa, Taiwan and Japan, and a set of 22 additional representative taxa belonging to the genera *Gracilaria* and *Hydropuntia* were selected for analysis**,** together with two species of *Gracilariopsis* that served as the outgroup (see Table 1, Fig. 21). The final *rbc*L data matrix was restricted to 1407 sites due to incompleteness of the first 60 bases of the sequenced gene. Parsimony heuristic searches and calculation of bootstrap proportion values (BP) were made as described in Lin *et al.* (2001). Decay indices (Bremer, 1988) representing the number of steps less parsimonious than minimal at which branches were no longer resolved were determined based on strict consensus analysis of cladograms found by relaxing parsimony sequencing one step at a time, up to five steps. Bootstrap proportion values (1000 replicates) and decay indices derived from maximum parsimony analysis are shown on the nodes (Fig. 21).

RESULTS

Gracilaria huangii Lin *et* De Clerck, sp. nov.

Thallus erectus, usque ad 4-8 cm altum, roseus ad artoruber; axibus dichotomis irregulariter, complanatis, 5-13 mm latis, exorientes stipes, 5-18 mm altus; haptero parvo circularis; laminae spinis vel lobis marginalibus, paginis lobis minutis vel laminis minimis; tetrasporangia superficialia, divisa cruciata; spermatia dispersa super laminam, valdosi textorii-typi; cystocarpia hemisphaerica, aliquantum constricta basim, cellulae tubulosae nutriciae raro, carposporangia uninucleata, portata in series cateniformis ramosa.

Thalli erect 4-8 cm long, rose to dark red, consisting of irregularly dichotomously branched, flattened blades, 5-13 mm in width, arising from a stipe, 5-18 mm in length, with a discoid holdfast; blades associated with marginal spines or lobes, numerous, tiny lobes or bladelets arising from surfaces of blades; tetrasporangia superficial, cruciately divided; spermatangia scattered over surfaces of male gametophytes in shallow, *textorii*-type conceptacles; cystocarps hemispherical, slightly constricted at the base, tubular nutritive cells rare, carposporangia uninucleate borne in branched chains.

Etymology: "*huangii*" in honor of Dr. Feng-Kuo Huang, a mathematician, collecting partner and strong supporter of Showe-Mei Lin.

Holotype: In Department of Natural Science Education, National Taitung University (NTTU), 16 January 2004, no. SML ss-1-26-04-1-1 (Fig. 1).

Type locality: Sail Rock, Kenting National Park, southern Taiwan (21°90'N, 120°47'E).

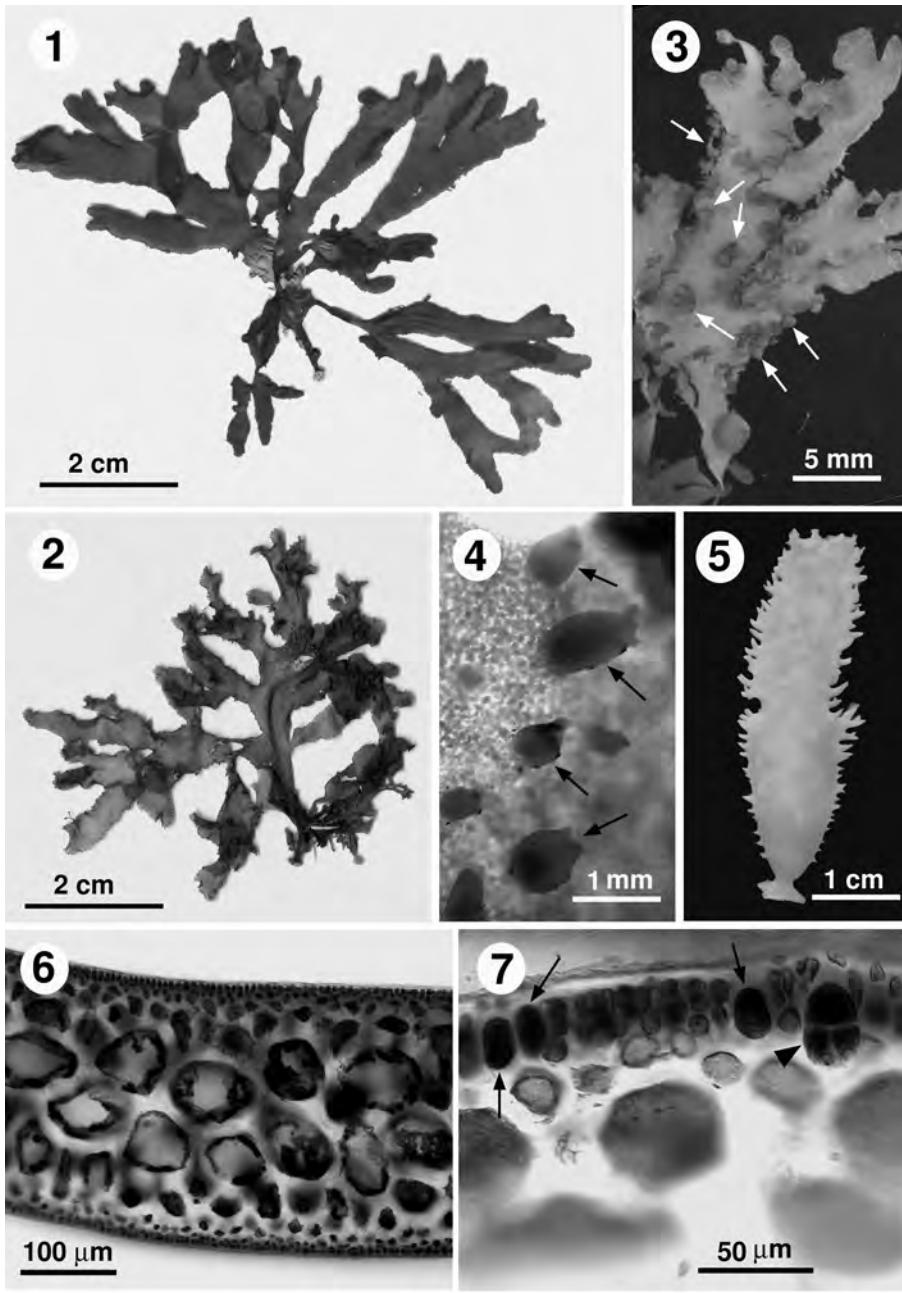
Distribution: Sparsely distributed along the northeastern and southeastern coastlines of Taiwan, and the Kenting National Park.

Habitat and seasonality: The collections were made seasonally from early winter to late summer, November-August. Plants were found in tide pools or grew subtidally, 1-2 m deep, and were attached on reef rocks in association with *Gracilaria spinulosa*, *G.* "*punctata*" and *G. vieillardii*. The seawater temperature in the waters of Taiwan ranges from 18° to 25 °C.

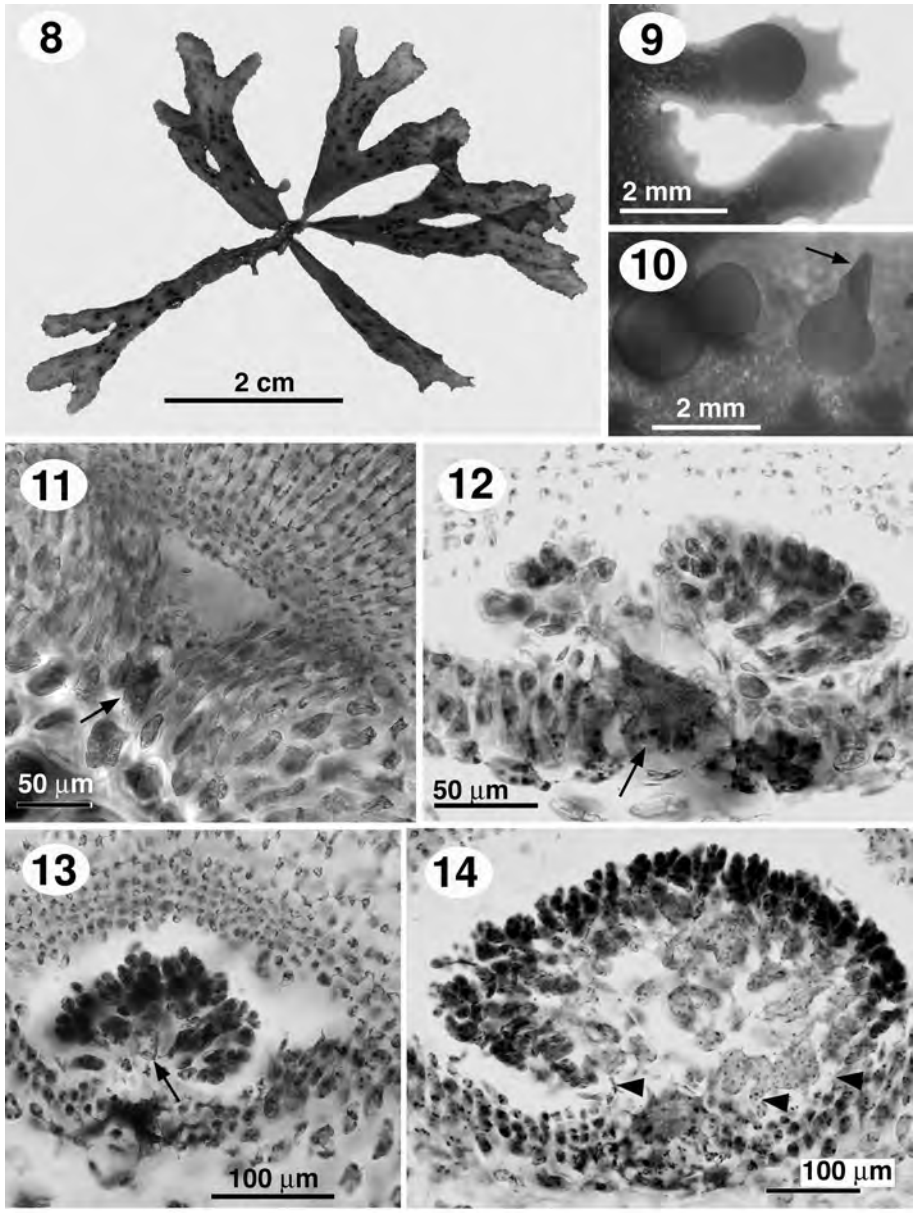
Specimens examined: Kenting National Park, southern Taiwan: 1) Sail Rock, coll. Allen Liu, 26.i.2004 (tetrasporic, female and male, holotype and isotypes); coll. S.-M. Lin, 29.xi.2001 (sterile), 14.iii.2002 (female), 29.iii.2002 (sterile), 27.iii.2004 (female and tetrasporic), 17.iv.2005 (female and tetrasporic); 2) Houwan, coll. S.-M. Lin, 13.xii.2001 (sterile); 3) Banana Bay, S.-M. Lin, 29.iii.2002 (tetrasporic). Northeastern Taiwan: 1) Yeliu, coll. Allen Liu, 27.vii.2002 (female); 2) Keelung, coll. S.-M. Lin, 1.v.2002 (sterile).

Habit and vegetative structure: Thalli (Figs 1, 2, 8) are erect, 4-8 cm in length, consisting of 3-5 irregularly dichotomously branched, flattened blades, 4-15 mm wide, arising from a short stipe, 3-18 mm long, with a discoid holdfast, 2-4 mm in diameter. The color of blades is usually rose to dark red, occasionally, turning green when exposed to sunlight. The margins of the blades are associated with spines or lobes (Fig. 3) and numerous, tiny lobes or bladelets, 1-3 mm long by 1-4 mm wide are also found on the surfaces of blades (Fig. 4). The flattened blades are 250-500 μm thick (Fig. 6), composed of 1-2 layers of pigmented cortical cells, 5-6 μm in diameter, 1-2 layers of subcortical cells, 12-20 μm in diameter, and 1-3 layers of medullary cells, 75-160 μm by 150-170 μm in diameter (Fig. 6). In general, tetrasporophytes (Fig. 1) are slightly larger than the male and female gametophytes (Figs 2, 8).

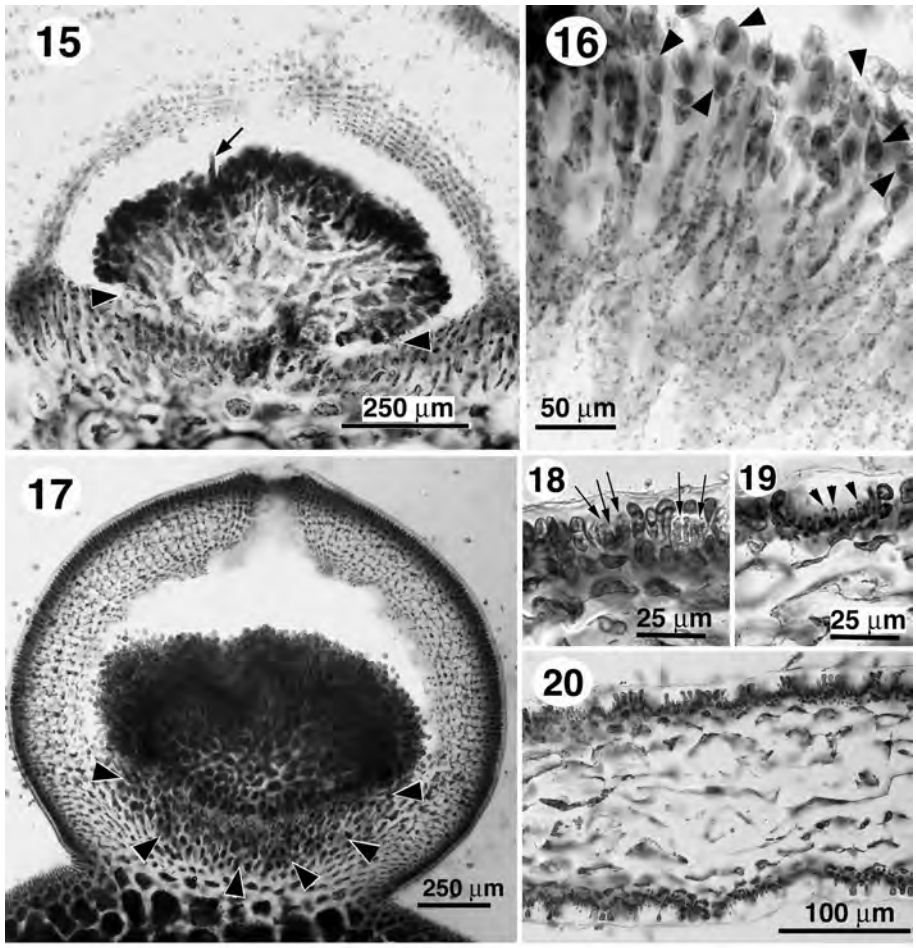
Reproductive structures: The tetrasporophytes and gametophytes are isomorphic and the gametophytes are dioecious. Reproductive structures are scattered over both surfaces of the blades. Tetrasporangia are initiated superficially from



Figs 1-4, 6-7: *Gracilaria hunagii* sp. nov. (Sail Rock, southern Taiwan); Fig. 5: *Gracilaria denticulata* (South Africa). **1.** Holotype: tetrasporic plant. **2.** Isotype: male plant. **3.** Close up of a fresh, tetrasporic plant showing numerous bladelets (arrows). **4.** Close up of tiny bladelets on the surface of a blade (arrows). **5.** Habit of a young thallus with denticulate margins. **6.** Cross-section of vegetative thallus. **7.** Cross-section through a tetrasporic plant showing a mature tetrasporangium (arrowhead) and some immature ones (arrows).



Figs 8-14: *Gracilaria huangii* sp. nov. Female reproductive structures (Sail Rock, southern Taiwan). **8**. Habit of a female plant. **9.** Close up of a cystocarp on a marginal bladelet. **10.** Close up of cystocarps on surface of blade, one bearing a tiny bladelet (arrow) on the top. **11.** An early post fertilization stage showing a fusion cell (arrow) and a newly formed cavity. **12.** A multinucleate fusion cell (arrow) bearing gonimoblast clusters. **13.** Cross-section of a young cystocarp showing a vacuolated inner gonimoblast cell (arrow). **14.** A further stage of carposporophyte showing multinucleate, vacuolated inner gonimoblast cells, some pit-connecting (arrowheads) to vegetative cells on the floor.



Figs 15-20: *Gracilaria huangii* sp. nov. Female reproductive structures and development of spermatangia (Sail Rock, southern Taiwan). **15.** Transverse section of an immature cystocarp showing a nutritive tube filament (arrow) and pit-connections (arrowheads) between lower gonimoblast cells and vegetative cells in the floor. **16.** Close of carposporangia (arrowheads). **17.** Transverse section of a mature cystocarpic showing darkly staining, vegetative cells (arrowheads) in the floor. **18.** Transverse section of young spermatangial conceptacles showing short filaments of spermatangial mother cells (arrows). **19.** Transverse section of an immature spermatangial conceptacle showing newly formed spermatangia (arrowheads). **20.** Transverse section of a male plant showing mature spermatangial conceptacles.

terminal cells (Fig. 7, arrows) cut off through an oblique, longitudinal cell division of outer cortical cells, and then expand and divide twice to produce four cruciately arranged tetraspores at maturity (Fig. 7, arrowhead). The cystocarps are borne on both surfaces of the fertile blades (Fig. 8) and on the marginal bladelets (Fig. 9). On one occasion, the top of a cystocarp was found to bear a tiny bladelet (Fig. 10, arrow). Carpogonial branches were not found in the examined materials. Presumably, soon after fertilization, the sterile branches flanking the carpogonial branch fuse directly onto the fertilized carpogonium. Fusion-cell formation progressively

involves the sterile branches and neighboring vegetative cells (Fig. 11, arrow) and a cavity is formed distally to the fusion cell through break-down of the primary pit-connections between inner cortical cells (Fig. 11). At an early stage of gonimoblast development, the incorporated, multinucleate fusion cell (Fig. 12, arrow) cuts off uninucleate gonimoblast cells in clusters. The innermost cells of the gonimoblasts soon become multinucleate and vacuolate (Fig. 13, arrow). Later, the vacuolated cells between different gonimoblast clusters are confluent by numerous secondary pit-connections (Fig. 14). As the gonimoblast development proceeds, the vegetative cells in the floor of the cystocarp become darkly stained and slightly elongated and are secondarily pit-connected with the inner cells of gonimoblasts near the floor (Figs 14, 15, arrowheads). Tubular nutritive cells are nearly absent in the cavity of cystocarp, although one such cell was found among the 50 cystocarps sectioned during this study (Fig. 15, arrow). As the cystocarp matures, the darkly staining cells in the floor of the cystocarp divide several times increasing the thickness of the floor at the base of the carposporophyte (Fig. 17). These cells probably play an important role during the formation of carposporangia. Carposporangia are uninucleate, 18-25 μm wide by 20-30 μm long and are borne in branched chains (Fig. 16). Mature cystocarps are hemispherical and slightly constricted at the base, 1.2-2.2 mm in diameter with the inner gonimoblast cells 75-115 μm long by 40-60 μm wide (Fig. 17). Spermatangial parent filaments are initiated from outer cortical cells (Fig. 18), then produce and release spermatangia (Fig. 19, arrowheads) successively to form a shallow depression. Mature spermatangia are scattered over the surface of male gametophytes in shallow, *textorii*-type conceptacles (Fig. 20).

Molecular analysis: Parsimony analysis revealed two most parsimonious trees (MPT) with tree length of 1072 steps, CI = 0.5019 and RI = 0.6194; there were 302 informative characters out of 1407 included sites (22%). The only difference between the two tree is that *G. huangii* is either clustered with *G. veillardii* from Taiwan or a sister clade to *G. spinulosa*. The flattened species of *Gracilaria* having *textorii*-type spermatangial sori from South Africa and Taiwan were closely associated and clustered in a single clade with *G. textorii* from Japan as the sister taxon (Fig. 21). The rest of the taxa included in the dataset were distantly related to the flattened species from Indo-Pacific Oceans. Interspecific *rbc*L sequence divergences among the species within the *textorii*-type clade varied from 1.20 to 4.96%. The plants with marginal teeth clustered in four different groups within the *G. textorii* assemblage: a *G. denticulata* clade, a *G. vieillardii* clade, a *G. spinulosa* clade, and a *G. huangii* clade sister to *G. vieillardii*. The position of the proposed new species, *Gracilaria huangii*, was between *G. vieillardii* with 4.9% sequence divergence and the *G. spinulosa* clade with 3.8% sequence divergence, but without a strong bootstrap support.

DISCUSSION

The *rbc*L-based phylogenetic analysis of flattened species of *Gracilaria* with spiny margins in the Indo-Pacific region resulted in four distinct clades, representing *G. denticulata*, *G. spinulosa*, *G. vieillardii* and *G. huangii* (Fig. 21). *Gracilaria huangii* from Taiwan is closely related to *G. spinulosa* and *G. vieillardii* from the same region, whereas *G. denticulata* from South Africa is closely associated with *G. beckeri* and *G. capensis* from South Africa. All of these species consistently formed a single clade sister to *G. textorii* from Japan in the analyses, although the bootstrap support was rather low (62%). Furthermore, the phylogenetic relationships inferred from *rbc*L sequence analysis showed the flattened *Gracilaria* species from South Africa, Taiwan and Japan to form an

assemblage distinct from the flattened species from the Atlantic Ocean (Fig. 21). The general topology of the tree is congruent with the results of Gurgel *et al.* (2004, Fig. 1) based on the same molecular marker, *rbc*L gene.

Gracilaria huangii is characterized by three to five flattened blades from a single stalk, blades consisting of 1-2 layers of pigmented cortical cells, 1-2 layers

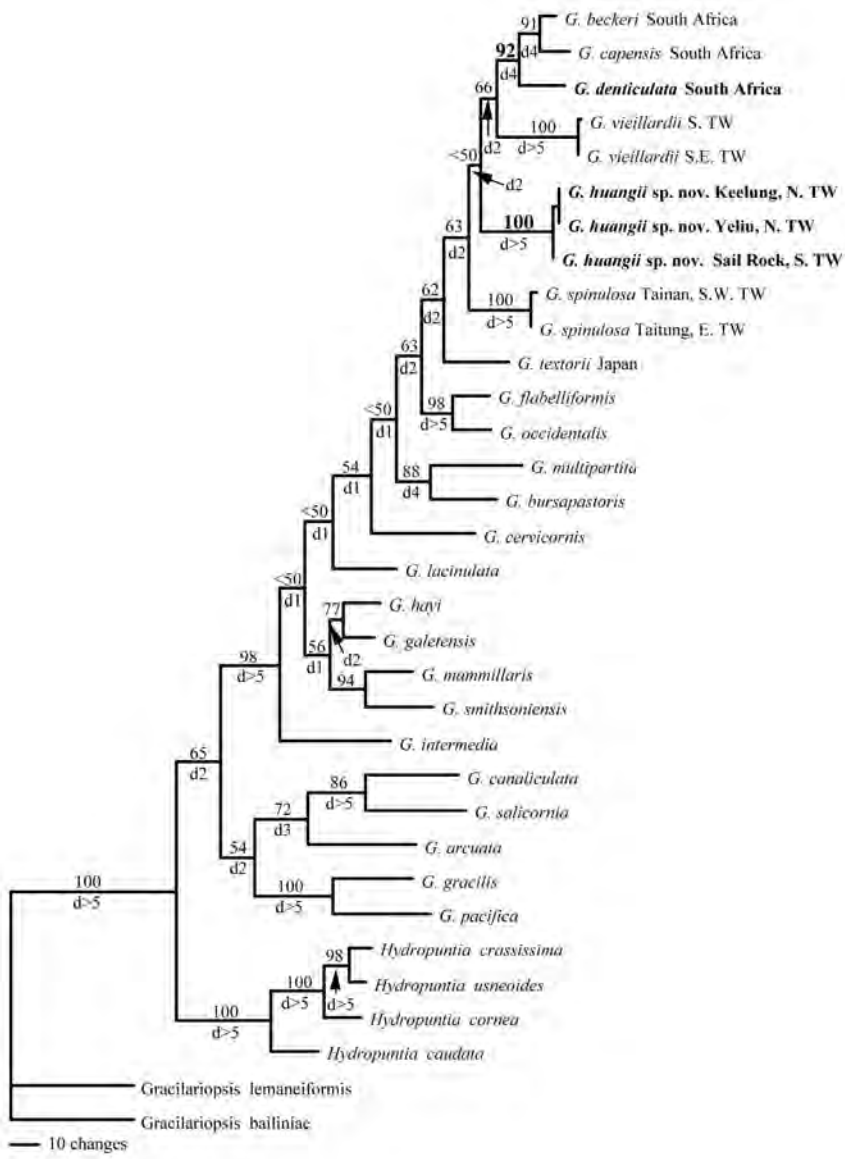


Fig. 21. One of two most parsimonious trees from analysis of the *rbc*L sequence data. Bootstrap proportion values are shown above nodes and thick bold branches correspond to 100% support; decay indices are shown below nodes. Branch lengths are proportional to the amount of sequence change.

of subcortical cells and 1-3 layers of medullary cells, numerous lobes or bladelets along the margins and on surfaces of the blades, a darkly staining and thickened floor in the cavity of the cystocarp and a virtual lack of tubular nutritive cells. Although *Gracilaria huangii* is morphologically similar to *G. vieillardii* and *G. spinulosa* from Taiwan, *G. denticulata* from South Africa and *G. srilankia* Withell, Millar *et* Kraft 1994 from Ceylon, the four species can be separated based on vegetative and reproductive features (see Table 2). *Gracilaria vieillardii* from Taiwan is characterized by its prostrate habit and relatively smaller thallus (4-5 cm) (Lin, 2006), whereas *G. denticulata* from southeastern Africa is a larger plant (up to 12 cm in length, see Fig. 5 in this study) bearing thicker blades consisting of up to seven layers of medullary cells (Iyer *et al.*, 2004). *Gracilaria spinulosa* was originally described from southwestern Taiwan (the type locality: Tainan) and can be distinguished by its bushy habit and densely branched thallus (Lin, 2006). Although *G. vieillardii* has been reported from South Africa (Iyer *et al.*, 2004; De Clerck *et al.*, 2005) and Asian Pacific areas (Chiang, 1985; Chang & Xia, 1976; Yamamoto, 1978), one should be aware that the type locality of *G. vieillardii* is New Caledonia, and, as yet, its morphology has not been carefully re-examined. Descriptions of *Gracilaria vieillardii* based on specimens from anywhere other than the type locality may not agree with the type of *G. vieillardii* (Withell *et al.*, 1994; Lin, 2006). Therefore, some records of *G. vieillardii* from the Indo-Pacific region remain provisional. Although *G. srilankia* is morphologically similar to *G. huangii* and *G. spinulosa*, the former can be distinguished by possessing the *verrucosa*-type spermatangial conceptacles and by a lack of marginal spines (Withell *et al.*, 1994).

Table 2. Morphological and reproductive structure comparison of *Gracilaria huangii* to the other flattened *Gracilaria* species with marginal spines and related species from Indo-Pacific regions.

Species	*Thallus habit*	*Spermatangial conceptacles*	*Tubular nutritive cells in the cystocarp*	*References*
G.. huangii	Blades erect, 4-8 cm long by 5-13 mm wide, 3-5 irregularly dichotomously branched blades arising from a short stipe	*Textorii*-type	Extremely rare	This study
G. denticulata	Blades erect, up to 12 cm long, mostly singly arising from a short stipe	*Textorii*-type	Commonly observed at upper and lower portions	Iyer *et al.*, 2004; this study
G.. spinulosa	Blades bushy, erect, 3-9 cm long by 2-7 mm wide, arising from a discoid holdfast	*Textorii*-type	Commonly observed at upper and lower portions	Lin, 2006
G.. vieillardii	Blades prostrate or arising laterally and radially near the apex of stipe	*Textorii*-type	Mostly observed at lower portions	Withell *et al.*, 1994; Lin, 2006
G.. srilankia	Blades without any marginal spines, 3-4 cm long by 3-5 mm wide	*Verrucosa*-type	Lack of information	Withell *et al.*, 1994

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