Gracilaria vermiculophylla: A western Pacific species of Gracilariaceae (Rhodophyta) first recorded from the eastern Pacific

Alexis M. Bellorin,[1]* Mariana C. Oliveira[2] and Eurico C. Oliveira[2]

[1]Departamento de Biología, Escuela de Ciencias, Universidad de Oriente, Cumaná, Venezuela and [2]Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, Caixa Postal 11461, CEP 05422-970, São Paulo, Brazil

SUMMARY

We report *Gracilaria vermiculophylla* (Ohmi) Papenfuss from the Pacific coast of North America based on a morphoanatomical revision and comparison of sequences of the nuclear gene coding for the small subunit of ribosomal RNA and the internal transcribed spacers of populations from Baja California, Mexico and Hiroshima, Japan. It is the first convincing report of this species out of its center of distribution in western north Pacific, where it has been considered as a synonym of former '*G. verrucosa*' records. *G. vermiculophylla* also occurs in central California as indicated by internal transcribed spacer sequences of a previously unknown *Gracilaria* Greville material. In the northeastern Pacific *G. vermiculophylla* is characterized by a robust somewhat vermiform, well-branched cylindrical thallus, with gradual cell size transition from cortex to medulla, deep spermatangial conceptacles, regular chains of carposporangia and carposporangial initials, downwardly oriented tubular nutritive cells, but rarely with upwardly oriented tubular nutritive cells. In some features of its cystocarp anatomy *G. vermiculophylla* is related to *Gracilariopsis* E. Y. Dawson or *Hydropuntia* Montagne and it is a relevant species for discussions about Gracilariaceae genera.

Key words: agarophytes, *Gracilaria*, Gracilariaceae, internal transcribed spacer, phylogeny, small subunit of ribosomal RNA.

INTRODUCTION

The well-known economic importance of gracilarioid algae (*sensu* Rice & Bird 1990) as the main global sources of agar (Oliveira *et al*. 2000) has stimulated a number of authors to search for reliable means to identify species in this group. This interest has also been fueled through decades by the complexity of the taxonomic problems in the gracilarioids, most of which are far from being resolved. Superficially similar species may have totally different agar yields and gel quality and thus there is a need for sound identification at the species level, for utilization and marketing. Since 1980, a vast literature dealing with new species proposals, species merging, nomenclatural changes, reviews of taxonomic criteria, genera characterization and regional floras has been published (reviews in Oliveira & Plastino 1994; Bird 1995). Almost all of the available taxonomic tools, such as anatomy and ontogeny, chemotaxonomy, antigenic studies, chromosome number, crossability, DNA fingerprinting and DNA sequencing, have been applied to species recognition, without much success except for DNA analysis and crossability tests. Recent studies have remarked that there are several species complexes, both in *Gracilaria* and *Gracilariopsis*, in which different species with similar gross morphology could be recognized only with higher-resolution taxonomic techniques (Bird *et al*. 1994; Goff *et al*. 1994; Wattier *et al*. 1997; Bellorin *et al*. 2002; Gurgel *et al*. 2003). Of these species complexes, the best studied has been the *Gracilaria verrucosa* complex (Bird & Rice 1990; Rice & Bird 1990), a group of cylindrical *Gracilaria* species with deep isolated spermatangial conceptacles recorded, erroneously in most cases, for most algal floras in the world.

Since the 1980s we have kept in our laboratory a culture of a species of the *G. verrucosa* complex from Pacific Mexico, whose correct name was suspected to be *G. pacifica* I. A. Abbott (Nunez-Lopez & Valdez 1998; Garza-Sanchez *et al*. 2000). This name has been assumed to be the correct name for the *G. verrucosa* records throughout the northeastern Pacific. Hybridization trials *in vitro* showed that this species (as *G. verrucosa* Baja California) is reproductively isolated from species of the *G. verrucosa* complex from the Caribbean and South America (Plastino & Oliveira 1996). In a previous paper about the phylogeny and systematics of Gracilariaceae based on DNA sequence comparisons (Bellorin *et al*. 2002) we established that this species is distinct from *G. pacifica*. However, no name

*To whom correspondence should be addressed.
Email: almiguel@yahoo.com
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was applied to the material from Mexico until molecular and morphoanatomical data of other species of the *G. verrucosa* complex from other regions was available.

Recently, we have determined the small subunit of ribosomal RNA (SSU rDNA) and internal transcribed spacer (ITS) sequences of *G. vermiculophylla* from Japan, a species of the *G. verrucosa* complex, so far known only from the northwestern Pacific (Tseng & Xia 1999). The sequences we obtained are identical or nearly identical to those from Pacific Mexico. Likewise, no significant differences between these materials were observed in detailed morphoanatomical and reproductive ontogeny studies. Hence, we are extending the original distribution of *G. vermiculophylla* from the western Pacific to the Pacific coast of Mexico and California. We also discuss some reproductive anatomical features used commonly by species and generic discrimination in the Gracilariaceae.

MATERIALS AND METHODS

Anatomical observations were based on herbarium specimens housed in the Phycological Herbarium of the University of São Paulo, Brazil (SPF). The selected materials were soaked in seawater for 24 h and transverse or longitudinal sections were made with a razor blade or a microtome after embedding the material with historesin (Leica Instruments, Heidelberger, Germany). The sections were stained with aqueous 1% aniline blue (AB), aqueous 1.6% toluidine blue O (CI 52040) (TBO) adjusted to pH 1.0 with HCl, or with Wittmann's aceto-iron-hematoxylin-chloral hydrate (WH) (Wittmann 1965). Semipermanent slides of hand-made sections stained with aniline blue or toluidine blue were mounted in aqueous 30% corn syrup (Karo, São Paolo, Brazil). Hand-sections stained with hematoxylin were mounted with 50 : 50 Hoyer's mounting medium (Hommersand & Fredericq 1988). Microtome sections were stored dried.

DNA was extracted from a plant from Miyajima Island, Hiroshima, Japan (collected by E. C. Oliveira on 14 November, 2001; voucher specimen number SPF 56151). DNA extraction and purification, polymerase chain reaction amplification of SSU rDNA and ITS regions, and sequencing were carried out as described in Bellorin *et al*. (2002). The SSU rDNA and ITS sequences were compared with those from *Gracilaria* sp. México (GenBank accession no. AF468886 and AF468906). Phylogenetic inferences under maximum likelihood, maximum parsimony and neighbor-joining methods were maded using PAUP* 4.0 (Swofford 1998), based on datasets of Bellorin *et al*. (2002), updated with new published SSU rDNA and ITS sequences of *Gracilaria*. Robustness of inferred trees was estimated as bootstrap support values (Felsenstein 1985) with 1000–2000 replicates of heuristic searches on the 50% majority-rule consensus trees.

RESULTS

Gracilaria vermiculophylla (Ohmi) Papenfuss, Papenfuss (1966)

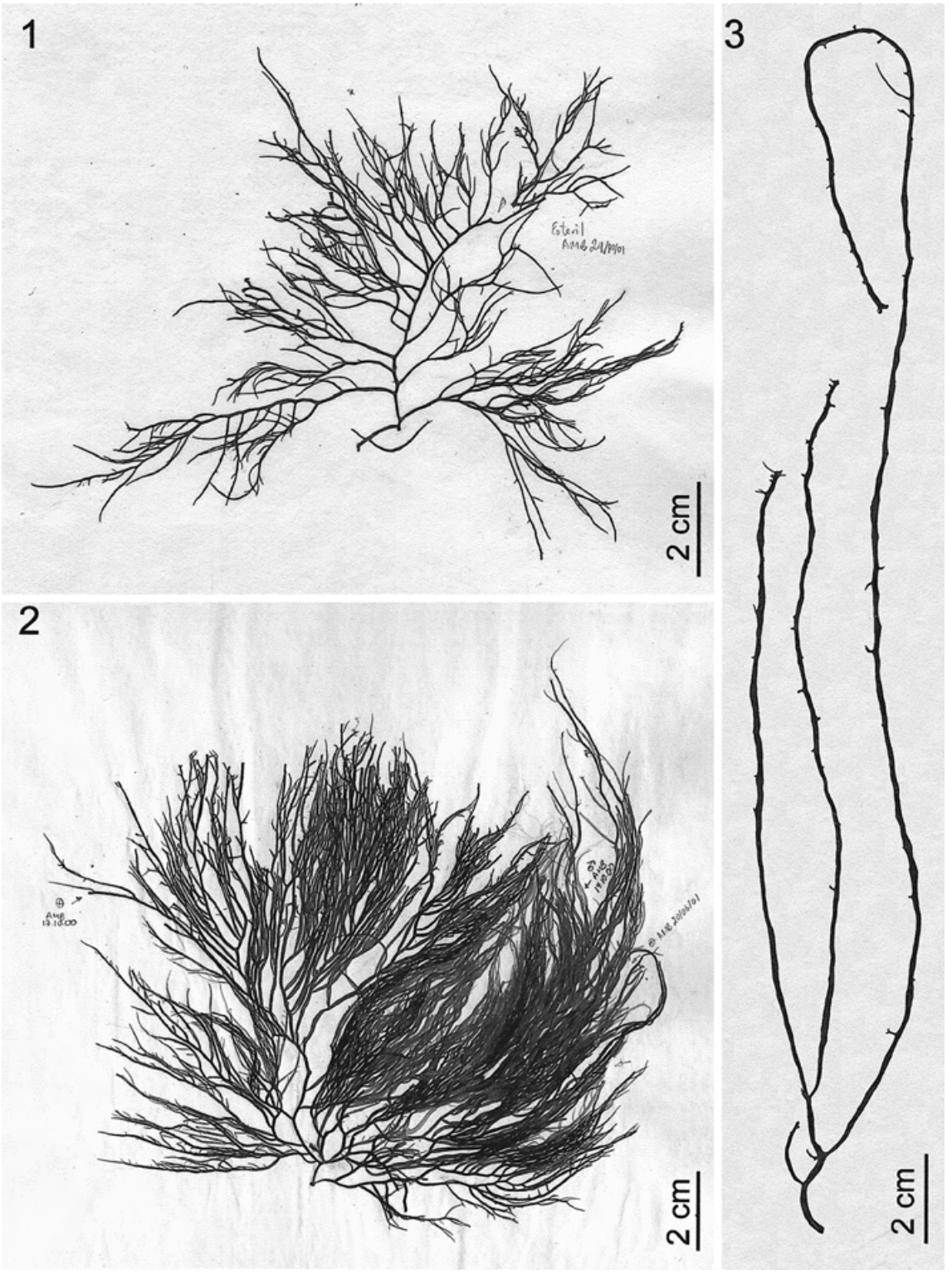
Synomym: *Gracilariopsis vermiculophylla* Ohmi, Ohmi (1956): 271, figs 1–4, pls 1,2.

Geographical distribution: This species was originally described from Akkeshi Lagoon, eastern Hokkaido, Japan (Ohmi 1956), and later for tropical and subtropical regions in the northwestern Pacific (Korea, China, Vietnam; Tseng & Xia 1999). Here we extend the geographic distribution to the northeastern Pacific (Mexico and California), as revealed by the nearly identical ITS sequences among the specimens from Mexico (Bellorin *et al*. 2002; as *Gracilaria* sp. Mexico), California (Goff *et al*. 1994; as *Gracilaria* sp. Elkhorn Slough) and Japan (this work).

Habitat: This species forms extensive beds in the intertidal and upper sublittoral zones, attached to rocks or pebbles, often covered with sand and mud. The collection site in Baja California is an estuary. The records from California are also from an estuarine habitat (Goff *et al*. 1994), and the material from Miyajima Island, Japan, was found in the intertidal, on small pebbles, in the outflow of a small creek.

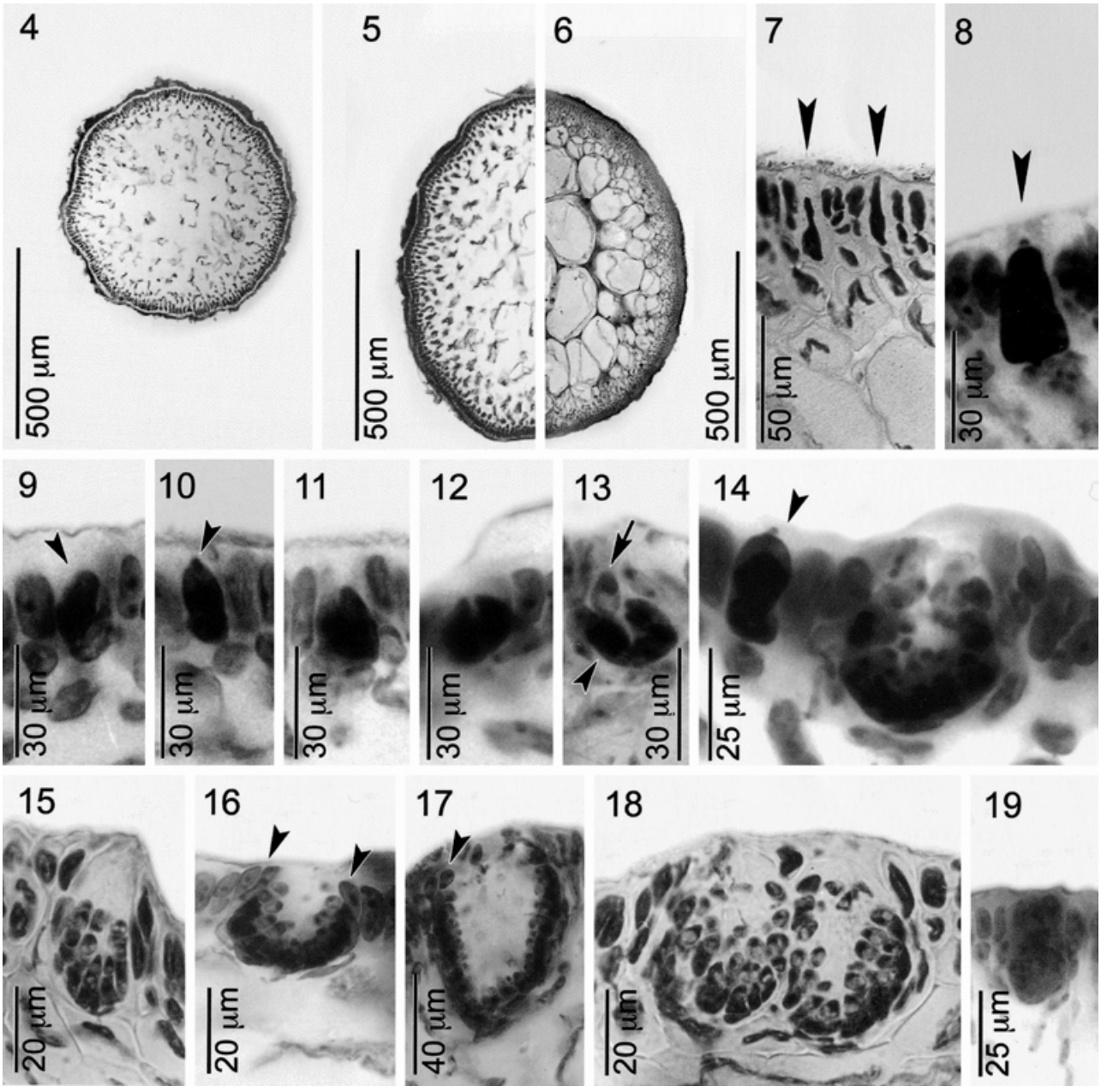
Specimens examined: Estero de Punta Banda (Ensenada, Baja California, Mexico), 9 October 1979, *leg*. M. A. Aguilar Rosas, SPF 55205 (infertile specimens); 28 April 1980, *leg*. R. Aguilar Rosas, SPF 55207 (seven female specimens); 28 May 1986, *leg*. R. Aguilar Rosas, SPF 55206 (two sheets, tetrasporangial, male and infertile specimens); July 1989, *leg*. E. C. Oliveira, SPF 54484 (two sheets, tetrasporangial specimens); July 1989, *leg*. E. C. Oliveira, SPF 54485 (seven sheets, tetrasporangial, female, male and infertile specimens obtained from *in vitro* cultivation); 7 March 2000, *leg*. J. M. Guzmán, SPF 56131, SPF 56139, SPF 56140 (tetrasporangial and female specimens); Miyajima Island (Hiroshima, Japan), 14 November 2001, *leg*. E. C. Oliveira, SPF 56141 (four sheets, female specimens), SPF 56142 (two sheets, male specimens), SPF 56143 (five sheets, tetrasporangial and infertile specimens).

Morphology and vegetative anatomy: This species has a thallus up to 100 cm long and 5 mm in diameter, but usually smaller and cylindrical throughout, with two to five orders of lateral branches of various sizes produced at irregular intervals in an alternate to unilateral pattern. The branches usually lack basal constrictions and are tapered toward the tips (Figs 1–3). Fresh material is fleshy and robust, somewhat vermiform, dark brown, becoming black on drying. The cell size transition from the medulla to the cortex is gradual (Figs 4–6). The cortex has three to four layers of well-pigmented cells (up to eight cell layers in tetrasporangial and spermatangial areas). Outer cortical cells are radially elongated, measuring



Figs 1–3. *Gracilaria vermiculophylla*. 1. Sterile plant obtained *in vitro* from tetrasporangia (SPF 54485). 2. Mixed-phase specimen (tetrasporangia and spermatangia) from Estero de Punta Banda, Mexico (SPF 55206). 3. Large tetrasporangial plant from Estero de Punta Banda, Mexico (SPF 54484).

7–18 × 3–8 μm (Figs 7,8). There is a subcortex of three to five layers of radially elongated cells in cross-sections (Figs 4–7). The medulla consists of large elliptical cells of 34–410 × 12–25 μm in cross-sections, with four to five cell layers (up to 12 cell layers in older axis). Cells are highly vacuolated and have abundant starch grains. Medullary cell walls are 12–20 μm thick (Figs 4–6). Rupture of some medullary cells can be



Figs 4–19. *Gracilaria vermiculophylla*. 4. Cross-section (AB) of female young axis (SPF 55207). 5. Cross-section (AB) of tetrasporangial old axis (SPF 54485). 6. Cross-section (TBO) of female old axis (SPF 56139). 7. Cortex and subcortex (AB) in transverse section showing the basal cells of ephemeral hairs (arrowheads) (SPF 56139). 8. Basal cell of deciduous hair (arrowhead) (WH). Note that undifferentiated terminal cortical cells are multinucleated (SPF 55206). 9. Dividing terminal cortical cell (WH) with a longitudinal oblique concave/convex septum forming a spermatangial initial (arrowhead) (SPF 55206). 10. Differentiated spermatangial initial (arrowhead) (WH), which is a terminal cortical cell (SPF 55206). 11,12. Dividing spermatangial initials by a longitudinal oblique concave/convex septum (WH) (SPF 55206). 13. Formation of spermatangial mother cells (arrowhead) (WH) from a spermatangial initial. Note that one spermatangium (arrow) has been cut off at this stage (SPF 55206). 14. Developing shallow spermatangial conceptacle (WH) with filaments of spermatangial mother cells lining both floor and lateral walls of cavity. Note the enlarged basal cell of a deciduous hair (arrowhead) (SPF 55206). 15. Developing shallow spermatangial conceptacle (AB) with spermatangial mother cells lining only the floor of the cavity. Note that some spermatangial mother cells bear columns of two or more spermatangia (SPF 55206). 16. Developing spermatangial conceptacle (WH). Note the dividing terminal cells of spermatangial/mother cell filaments (arrowhead) (SPF 55206). 17. Deep isolated spermatangial conceptacle (WH), showing a dividing terminal cell of a spermatangial/mother cell filament (arrowhead) (SPF 55206). 18. Coalescing deep male conceptacles resembling 'henriquesiana' type (AB). Some spermatangial mother cells bears two sequentially produced spermatangia (SPF 55206). 19. Cortical decussate/cruciate tetrasporangium (WH) (SPF 54484).

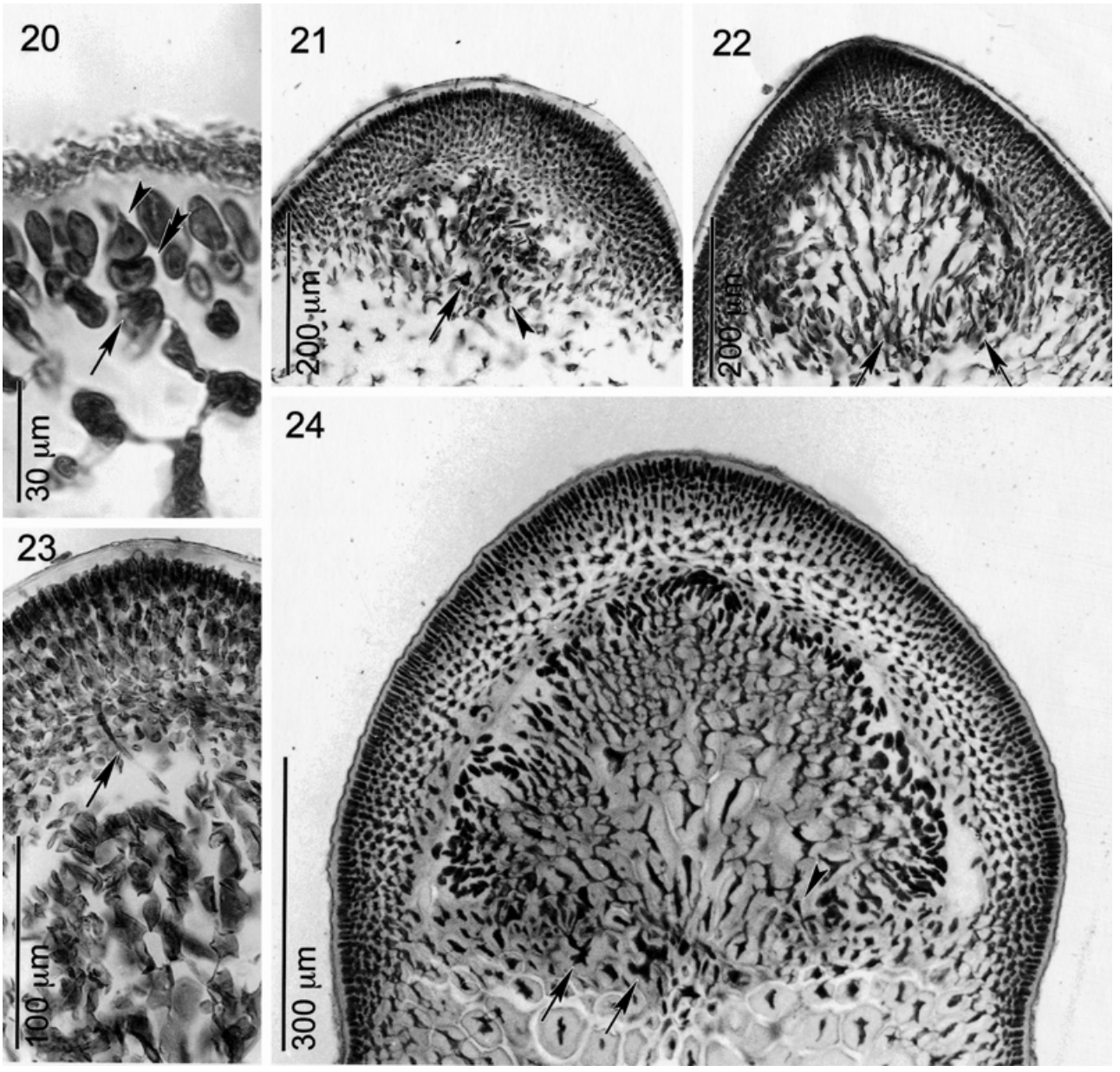
seen on old branches resulting in a partially hollow thallus. Deciduous cortical hairs are produced near the apex, as indicated by the presence of large and multinucleated, darkly stained hair basal cells (Figs 7,8,14).

Spermatangia: Spermatangia are produced in deep conceptacles (up to 90 μm deep, 60 μm wide) that reach the subcortex and open by an external pore ('*verrucosa*' type; Yamamoto, 1984) (Fig. 17). Young conceptacles may appear as shallow depressions with spermatangia limited to the cavity floor (resembling '*textorii*' type) (Fig. 15) or both the cavity floor and lateral walls (Figs 14,16), becoming deeper at maturation. Conceptacles are mostly single, although some may coalesce (Fig. 18). Spermatangial mother cells produced from terminal uninucleate cortical cells ('spermatangial initials', Fredericq & Hommersand 1989a) stain darkly with haematoxylin (Figs 9–10). Differentiated spermatangial initials can be seen near branch apices (approximately 10 mm), linked to subcortical cells. A spermatangial initial first cuts off a spermatangial mother cell by a longitudinal oblique concave/convex division (Figs 11,12). Later on, both the subapical spermatangial initial and the daughter spermatangial mother cell, produce a system of filaments of spermatangial mother cells (Fig. 13). The terminal cells of these fertile filaments are elongated and divide actively, forming new spermatangial mother cells by longitudinally oblique concave/convex divisions (Figs 16–17). In a well-developed conceptacle these dividing cells are positioned at the opening of the conceptacle. Spermatangia are produced distally by an oblique division of the elongated spermatangial mother cells (Fig. 13). Usually only one spermatangium is cut off at a time from each spermatangial mother cell, although two or rarely more (up to four) sequentially produced spermatangia can remain united, resembling chains of spermatangia (Figs 15,16,18).

Female apparatus and cystocarp ontogeny: Typical gracilariacean two-celled carpogonial branches, with short trichogynes, are produced from a terminal cortical cell that acts as the supporting cell (Fig. 20). The supporting cell produces two short filaments of sterile flanking cells. A central, star-like, highly dissected fusion cell is distinguishable only at the initial stages of cystocarp development (Fig. 21). The cortical cells surrounding the fusion cell divide periclinally to form an incipient pericarp prior to gonimoblast production. These incipient pericarp cells are radially elongated, linked by primary pit connections. As the gonimoblast initials are produced from the fusion cell, the cystocarp cavity is produced progressively by a periclinal rupture, which separates the incipient pericarp from the gametophytic cells at the cystocarp floor. Few pericarp cells remain at the cystocarp floor and, therefore, no inner pericarp is formed as has been described for other species (Fredericq & Norris 1985; Fredericq & Hommersand 1989b). The gonimoblast filaments arise first from the fusion cell. Later, fusions between gonimoblastic cells and gametophytic cells at the cystocarp floor give rise to a basal nutritive tissue of secondarily interconnected and highly dissected cells (Fig. 22). At this stage the fusion cell becomes indistinguishable from other cellular fusions. Upwardly oriented tubular nutritive cells (Fredericq & Hommersand 1989a) connecting the gonimoblasts and the pericarp are observed rarely, and only in young cystocarps (Fig. 23). These cells remain linked by a primary pit-connection to sterile gonimoblastic cells close to carposporangial initials. The proximal region of tubular nutritive cells has a triangular outline and the distal region is star-like, because of secondary fusions with several gametophytic cells. Downwardly oriented tubular nutritive cells are also produced (Fig. 24) and the distal region of these cells is difficult to differentiate from other cellular fusions in the cystocarp floor. Mature cystocarps are prominent, up to 1.5 mm high in herbarium specimens, sometimes constricted at the base and rostrate. Inner gonimoblast cells are radially elongated, radiating from the cystocarp floor and connected by abundant secondary pit connections (Fig. 24). The carposporangia mature terminally from radially elongated chains of carposporangial initials (up to five cells) heavily stained with aniline blue. Mature carposporangia measure 15–29 μm. Neither gonimoblast lobes nor upwardly oriented tubular nutritive cells were observed in mature cystocarps. Cellular fusions are still distinguishable at the floor of mature cystocarps (Fig. 24). Three cell types are usually recognized in the pericarp of mature cystocarps: (i) the outer cells (three to four cell layers), which are rounded, except the terminal ones that are radially elongated (from 5–7 to 10–17 μm) and not linked by secondary pit connections; (ii) the intermediate cells (6–10 cell layers), which become star-shaped due to abundant secondary pit connections; and (iii) the innermost cells, (1–4 cells in radial chains), rounded and connected only by primary pit connections (Fig. 24).

Tetrasporangia: Radially elongated tetrasporangia, up to 65 μm long, are produced in the cortex (Fig. 19). Division is decussate cruciate or irregular. The cortical cells surrounding the tetrasporangia divide anticlinally, not forming a traditional nemathecial-type cortex.

Molecular data: The complete unambiguous sequence was obtained for SSU rDNA from *G. vermiculophylla* from Japan (GenBank accession no. AY465828). The ITS sequence was determined with some ambiguous positions (56 from 1640 bp) (GenBank accession no. AY465829). There are no differences between the SSU rDNA sequences of *G. vermiculophylla* from Mexico and Japan in pairwise comparisons. The actual sequence divergence between the ITS sequences in pairwise comparisons, excluding ambiguous positions, was 0.68%. Based on these observations, we assume that



Figs 20–24. *Gracilaria vermiculophylla*. 20. Two-celled carpogonial branch (AB) with a terminal carpogonium (arrowhead) and a hypogynous cell (double arrowhead). One of the sterile flanking filaments produced by the support cell is shown at the left (SPF 54485). 21. Early stage of cystocarp development (AB), showing the fusion cell (arrow) and fusions between some of the inner gonimoblast cells with gametophytic cells at the cystocarp floor (arrowhead). Note that there is no inner pericarp on the cystocarp floor (SPF 56140). 22. Developing cystocarp (AB), with extensive cellular fusions at the cystocarp floor (arrows). Note that the carposporangial initials are differentiated from the distal sterile gonimoblastic cells (SPF 56140). 23. Upwardly oriented tubular nutritive cell (arrow) connecting the gonimoblast with pericarp in a young cystocarp (AB) (SPF 56140). 24. Mature cystocarp (AB) showing the basal cellular fusions (arrows) and a downwardly oriented tubular nutritive cell (arrowhead). Note that the pericarp cells are heavily connected by secondary pit connections, becoming stellate (SPF 56139).

the material studied corresponds to separate populations of a single widespread species. Phylogenetic inferences gave very similar trees to that of Bellorin *et al*. (2002) (results not shown). The sequences of *G. vermiculophylla* from Japan and Mexico were often related to other cylindrical Pacific species, namely *G. chilensis* and *G. tenuistipitata*, but strong bootstrap support was not found for this relationship.

DISCUSSION

Many taxonomic studies on *Gracilaria* have dealt with problems of correct identification of species around the globe that have been called *G. verrucosa*. The history of the binomial *G. verrucosa* (and the former synonym *G. confervoides* (Linnaeus) Greville) is complex and has already been reviewed exhaustively (Bird & Rice

1990; Rice & Bird 1990; Irvine & Steentoft 1995). In brief, the type locality of *G. verrucosa* is the British Isles, although this name was applied somewhat indiscriminately to any stringy and freely ramified cylindrical *Gracilaria* with deep spermatangial conceptacles worldwide (Fredericq & Hommersand 1989a). Nevertheless, a convincing body of evidence from crossability tests, DNA methods and detailed morphology showed that most of these extra-European records are distinct entities from what is considered to be authentic *G. verrucosa* from Britain (McLachlan 1979; Bird *et al*. 1982; Abbott 1985; Zhang & Xia 1985; Plastino & Oliveira 1997). Even in the type locality, the binomial *G. verrucosa* was shown not to be monospecific but to encompass two distinct entities (Bird & Rice 1990; Rice & Bird 1990), one of which belongs to a distinct genus, *Gracilariopsis longissima* (S. G. Gmelin) Steentoft, L. Irvine et Farnham, and the other one to *Gracilaria*, now known as *G. gracilis* (Stackhouse) Steentoft, L. Irvine et Farnham (Steentoft *et al*. 1995). The former name *G. verrucosa* was abandoned because its lectotype belonged to a *Gracilariopsis* species. *G. gracilis* also occurs in Wales, Norway, France, Spain, Argentina and Namibia (Rice & Bird 1990; Bird *et al*. 1994; Steentoft *et al*. 1995).

The most recent reviews of *Gracilaria* in the north Pacific advocate recognition of two species within the *G. verrucosa* complex, both distinct from *G. gracilis*: *G. pacifica* in the North American side, and *G. vermiculophylla* in the western side (Tseng & Xia 1999). The records of *G. verrucosa* along the Pacific coast of North America were first treated as distinct from British Isles material because of sexual incompatibility in hybridization tests by Bird *et al*. (1982), based on a culture isolate from Vancouver Island. Later, Abbott (1985) proposed the binomial *G. pacifica* for the former *G. verrucosa* from California, remarking on the few morphoanatomical differences between the new species from the eastern Pacific and the British entity. Bird *et al*. (1992) ascribed their isolate from Vancouver Island to *G. pacifica* and showed that this species is closely related to *G. gracilis* (as *G. verrucosa*) on the basis of SSU rDNA sequences, which are nearly identical between these two species. The fast-evolving sequences of ITS and Rubisco spacer (Goff *et al*. 1994), as well as microsatellite comparisons (Wattier *et al*. 1997), confirmed later that *G. pacifica* is sufficiently distinct at the species level from *G. gracilis*, although both entities are closely related. Goff *et al*. (1994) also reported *G. pacifica* from Washington State, USA.

In the western side of the north Pacific, on the other hand, the identity of *G. verrucosa* samples remained largely uncertain. Zhang and Xia (1984) compared *G. gracilis* and *G. pacifica* (both as *G. verrucosa*) with the *G. verrucosa* from China and Japan, noting some differences in cystocarp anatomy. The following year these authors (Zhang & Xia 1985) established *G. asiatica* J. F. Zhang et B. M. Xia to accommodate the specimens previously ascribed to *G. verrucosa* in China, also including a collection from Shinori, Hokkaido, Japan. Yamamoto and Sasaki (1988) investigated collections referred to as *G.verrucosa* in Japan, employing crossability tests and found interfertility between two populations identified as *G. verrucosa* from Hokkaido (including the material ascribed to *G. asiatica* from Shinori) and the topotype material of the poorly known *G. vermiculophylla*. They concluded that *G. verrucosa* should be merged with *G. vermiculophylla* rather than be placed under the later name *G. asiatica*, given the nomenclatural priority. The binomial *G. vermiculophylla* was later applied to all former *G. verrucosa* records in the northwestern Pacific, including China, Korea, Vietnam and Japan (Yoshida *et al*. 1995; Yoshida 1998; Tseng & Xia 1999).

The first DNA sequence data of a *G. verrucosa* complex species from Asia was the SSU rDNA sequence of an isolate referred to as *G. verrucosa* from Hokkaido (unspecified locality), Japan (Bird *et al*. 1992). This sequence was nearly identical to others of the *G. gracilis* group (as *G. verrucosa* from Europe and Argentina) and it was suspected that this Japanese isolate belonged to the same species as European and Argentinean specimens (Bird *et al*. 1992). However, a reanalysis of DNA samples in the C. J. Bird collection using four single loci microsatellites and ITS size variation (Wattier *et al*. 1997) showed that the isolate from Japan is distinct from authentic *G. gracilis* from Europe, Argentina and Namibia. At the moment, no name has been applied to this isolate and its taxonomic status remains uncertain.

Recently, one of us (E. C. Oliveira) collected *G. verrucosa* samples from Miyajima Island, Hiroshima, Japan. Our anatomical observation on this species confirmed the presence of deep, separate spermatangial conceptacles typical of the *G. verrucosa* complex, but the cystocarps differed from typical *G. verrucosa* in having few upwardly directed tubular nutritive cells (most were downwardly directed), small protoplasm-rich gonimoblasts cells and rather regular chains of carposporangia (A. Bellorin, unpubl. data, 2002), among other features. We ascribed this material to *G. vermiculophylla* (Ohmi) Papenfuss *sensu* Yamamoto (1978). The SSU rDNA of this material was completely divergent from that of *G. verrucosa* from Japan determined by Bird *et al*. (1992). Instead, the SSU rDNA and ITS sequences of this entity were identical or nearly identical to those of the unknown *Gracilaria* species from California and Mexico previously studied by Goff *et al*. (1994; as *Gracilaria* sp. Elkhorn Slough) and by us (Bellorin *et al*. 2002; as *Gracilaria* sp. Mexico). We conclude that our materials from Mexico and Japan belong to the same species (*G. vermiculophylla*) and that this species is distinct from the *G. verrucosa* from

Japan studied by Bird *et al*. (1992). This finding confirms the previous suspicion that at least two species have been confused under *G. verrucosa* in Japan (Yamamoto & Sasaki 1988). The species studied by Bird *et al*. (1992) appears to be closely related to *G. gracilis* and *G. pacifica*, according to SSU rDNA sequences, while *G. vermiculophylla* has been weakly related to other Pacific species, namely *G. chilensis* and *G. tenuistipitata* (Goff *et al*. 1994; Bellorin *et al*. 2002). This increases considerably the distribution of *G. vermiculophylla* in the Pacific. The question of how many species of the *G. verrucosa* complex occurs in Japan and, by extension, in Asia remains open. Likewise, it should be concluded that the application of binomial *G. vermiculophylla* to all of the *G. verrcuosa* materials in Asia, as proposed by Tseng and Xia (1999), is questionable. The confusion that results from combining superficially similar species into a single taxon was already noticed for *G. blodgettii* in Japan (Terada & Yamamoto 2000), in which *G. shimodensis* R. Terada and H. Yamamoto was confused with the presumably authentic *G. blodgettii* Harvey.

Gracilaria vermiculophylla was first proposed as *Gracilariopsis vermiculophyla* by Ohmi (1956), based on a population from Akkeshi Lagoon, Hokkaido Island, Japan. At that time, the generic concept of *Gracilariopsis* (Dawson 1949) was related to the absence of tubular nutritive cells and other cystocarpic features, without any consideration of spermatangial configuration. Thus, as Ohmi (1956) did not observe tubular nutritive cells in cystocarps of material from Akkeshi Lagoon, he treated his new species as *Gracilariopsis*. When Papenfuss (1966) called for the submergence of *Gracilariopsis* into *Gracilaria*, he transferred *Gracilariopsis vermiculophylla* to *Gracilaria*. Some downwardly directed tubular nutritive cells were later observed in material from Akkeshi Lagoon (Yamamoto 1978) and, as this species presents deep spermatangial conceptacles, a feature associated with the modern *Gracilaria* concept, the placement into *Gracilaria* was not further discussed. The SSU rDNA and ITS sequences of *G. vermiculophylla* from Japan and Mexico determined by us confirm this generic placement. Basic morphological and anatomical features of *G. vermiculophylla* have already been described for Japanese specimens by Ohmi (1956) and Yamamoto (1978). In this work, we provide a description of material from Mexico, describing for the first time the male apparatus and cystocarp formation in the species.

Gracilaria vermiculophylla was considered to be restricted to the type locality in the cold water regions of Japan (Yamamoto 1978, 1984). In fact, its general morphology was described based on specimens from a lagoon in which the plants maintain the vermiform morphology, the scarce to absent tubular nutritive cells in cystocarps and the small-celled gonimoblasts (Yamamoto 1978; Yamamoto & Sasaki 1988). However, when *G. verrucosa* populations from other localities were studied and shown to be conspecific with *G. vermiculophylla* by Yamamoto and Sasaki (1988), it became clear just how morphologically variable this species is. Interestingly, the hybrids between *G. vermiculophyla* from the type locality and the former *G. verrucosa* from Japan showed intermediate numbers of tubular nutritive cells (Yamamoto & Sasaki 1988), which corroborate the marked intraspecific variation of this character (see Bird 1995). The data of Yamamoto (1984) showing that *G. vermiculophylla* is a euryhaline and eurythermal species may explain the wider geographic distribution found in this study.

The weak evolutionary relationships among *G. vermiculophylla*, *G. chilensis* and *G. tenuistipitata* inferred from SSU rDNA and ITS nucleotide sequences (Bellorin *et al*. 2002) were also inferred from Rubisco spacer sequence comparisons (Goff *et al*. 1994, *G. vermiculophylla* as *Gracilaria* sp. Elkhorn Slough). *Gracilaria chilensis* and *G. tenuistipitata* were also considered to be related in comparisons of *rbc*L sequences (Gurgel & Fredericq 2002). These evolutionary relationships are morphologically supported by cystocarp anatomy, although these traits should be considered with caution as they may be quite variable intraspecifically (*G. vermiculophylla* is a good example of this). All of these cylindrical species: (i) rarely produce tubular nutritive cells connecting the gonimoblast to the upper pericarp, although downwardly or laterally oriented tubular nutritive cells are frequent; (ii) have gonimoblast inner cells highly connected by secondary pit connections; (iii) have cellular fusions at the cystocarp floor; and (iv) produce orderly arrays of carposporangial initials and a non-deeply lobed gonimoblast (A. M. Bellorin, unpubl. data, 2002). However, there are some important differences: (i) in *G. chilensis and G. tenuistipitata*, the generative fusion cell can be generally recognized even in mature cystocarps (A. M. Bellorin, unpubl. data, 2002), which is not the case in *G. vermiculophylla*; (ii) in *G. chilensis*, a differentiated inner pericarp (cytologically modified cells derived from cortical gametophytic cells in the cystocarp floor, see Fredericq & Hommersand 1989b) is clearly distinguishable in mature cystocarps, as well as vertically elongated cellular fusions through secondary pit connections between some of the basal gonimoblast cells and inner pericarp cells (Bird *et al*. 1990; Nelson & Ryan 1991; A. M. Bellorin, unpubl. data, 2002). An inner pericarp is not present in *G. vermiculophylla* or *G. tenuistipitata* (A. M. Bellorin, unpubl. data, 2002).

The mature male apparatus of *G. vermiculophylla* is distinct from *G. chilensis* and *G. tenuistipitata* in having deep male conceptacles rather than shallow ones. However, there are no fundamental differences in the ontogeny of male conceptacles between *G. vermiculophylla* and *G. chilensis*. In both species the spermatangial initials are terminal cortical cells that produce a system

of filaments of spermatangial mother cells (Ryan & Nelson 1991). In addition, the young spermatangial conceptacles of *G. vermiculophylla* are shallow and are quite similar to the *textorii* type described in *G. chilensis* (Nelson 1987; Ryan & Nelson 1991). Unfortunately, we do not know the spermatangial ontogeny in *G. tenuistipitata*.

The position of the spermatangial initials was used to define discrete types of male conceptacles (Fredericq & Norris 1985; Fredericq & Hommersand 1989a, 1989b). According to these authors, in the *textorii* type the spermatangial initials are derived from terminal cortical cells, whereas in the *verrucosa* type the spermatangial initials are derived from intercalary cortical cells. *G. vermiculophylla*, however, has *verrucosa*-type spermatangia produced from terminal cortical cells. Moreover, we have observed *verrucosa*-type spermatangia originated from terminal spermatangial initials in other species (A. M. Bellorin, unpubl. data, 2002), thereafter the *verrucosa* and *textorii* types are not necessarily distinct in origin. These results, together with the conflicting observations about the male apparatus ontogeny already reported in the literature (Yamamoto 1973, Fredericq & Norris 1985; Fredericq & Hommersand 1989a; Abbott *et al*. 1991; Ryan & Nelson 1991; Terada & Yamamoto 2000), show that the recognized patterns of spermatangial types are not so clear cut as originally expected, at least among species that have spermatangia within conceptacles. Thus, although usually useful in species recognition, the 'types' of spermatangial conceptacles may not have major evolutionary importance and, in fact, can not be established as synapomorphies (Bird *et al*. 1992; Bellorin *et al*. 2002).

Gracilaria vermiculophylla presents some of the cystocarp features proposed as descriptors for the genus *Gracilariopsis* (Fredericq & Hommersand 1990), as was discussed for *G. chilensis* by Nelson and Ryan (1991). The species also presents some features associated with the generic or subgeneric concept of *Hydropuntia* (Fredericq & Hommersand 1990), a taxon that is currently unsustainable in light of morphological (Bird 1995) and molecular (Bellorin *et al*. 2002) data. Emphasis should be placed on the downwardly oriented tubular nutritive cells that were proposed as being of diagnostic value in the original *Hydropuntia* proposal (Chang & Xia 1963, as *Polycavernosa* C. F. Chang et B. M. Xia). Our studies with *G. vermiculophylla* show that the orientation of these cells may be quite variable among populations. The other *Hydropuntia* basic feature, the compound *henriquesiana*-type spermatangia, is widespread in several unrelated groups of *Gracilaria* species (Bellorin *et al*. 2002), and can not be utilized as a descriptor for any generic or subgeneric taxon.

Gracilaria vermiculophylla and *G. tenuistipitata*, as was previously remarked for *G. chilensis* (Nelson & Ryan 1991; Bird 1995), provide further support for the submergence of *Hydropuntia* into *Gracilaria*. Additionally, as these three species also combine *Gracilariopsis* and *Gracilaria* features, two genera sufficiently distinct according to a strong body of molecular evidence (Bird *et al*. 1992, 1994; Goff *et al*. 1994; Bellorin *et al*. 2002; Gurgel & Fredericq 2002), the morphological boundaries between these genera should be redefined, specially in regard to the inner pericarp differentiation (a proposed feature of *Gracilariopsis* widespread into *Gracilaria*, A. M. Bellorin, unpubl. data, 2002) and the mode of production of carposporangia (as there are several *Gracilaria* species with straight chains of carposporangia, e.g. Terada *et al*. 2000, a proposed feature of *Gracilariopsis*). The next step in the systematics of gracilarioid algae should be the morphological characterization of the infrageneric lineages of *Gracilariopsis* and *Gracilaria* established in molecular comparisons, as well as a revision of the status of many poorly known species.

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