

## MORPHOLOGICAL AND PHYLOGENETIC STUDIES OF *GRACILARIOPSIS CHIANGII*, NEW SPECIES (GRACILARIACEAE, RHODOPHYTA), AN ALGA PRESENTLY KNOWN AS *GRACILARIA CHORDA* IN TAIWAN

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**ABSTRACT.** – “*Gracilaria chorda*” from southern and northern Taiwan were compared with *Gracilaropsis chorda* from Japan, other species of *Gracilaria* and *Gracilaropsis* from the Pacific Ocean. Based on molecular evidence and comparative cystocarp development, it is clear that “*Gracilaria chorda*” from Taiwan does not belong in *Gracilaria* but in *Gracilaropsis*. Accordingly, a new species, *Gracilaropsis chiangii*, new species, is proposed and is characterized by its small size (15–22 cm in height) and only 1-2 (-3) orders of branches from the base to middle part of the thallus. The phylogenetic relationship of *Gracilaropsis chiangii* to the presently described species of *Gracilaropsis* is discussed based on *rbcL* sequence analysis.

**KEY WORDS.** – Systematics; Morphology; *Gracilaria chorda*; Taiwan; Rhodophyta; Gracilariaceae; *Gracilaropsis chiangii*, new species.

### INTRODUCTION

*Gracilaria chorda* was described by Holmes (1895: 253) from material collected at Enoura, Japan. Ohmi (1958) transferred *Gracilaria chorda* to *Gracilaropsis* based on Dawson’s concept (1949) of the genus. Dawson (1949) established his new genus, *Gracilaropsis* mainly upon the development of the carposporophyte, in which the inner gonimoblast cells were densely filled with cytoplasm and nutritive filaments connecting the gonimoblasts to the pericarp were absent. Dawson noted that the spermatangial sori were formed superficially in some, but not in all species of *Gracilaropsis*. Papenfuss (1967) compared the generitype of *Gracilaropsis*, *Gracilaropsis sjoestedtii* Kylin [= *Gracilaropsis andersonii* (Grunow) E.Y. Dawson], with *Gracilaria verrucosa* (Hudson) G. F. Papenfuss [= *Gracilaria gracilis* (Stackhouse) M. Steentoft, L. M. Irvine & W. F. Farnham] from England and concluded that tubular nutritive cells or filaments were not always present in the cystocarps of some species of *Gracilaria*. Accordingly, Papenfuss sank *Gracilaropsis* into *Gracilaria* Greville (1830).

*Gracilaria chorda* was recorded from northeastern and southern (Kenting National Park) Taiwan in the past few decades (Chiang & Wang, 1987; Huang, 1999). However, the author’s recent collections of “*Gracilaria chorda*” from the two localities differed in several respects from *Gracilaria chorda* [currently *Gracilaropsis chorda* (E. M. Holmes) H. Ohmi, see Hau & Lin (2006) and also Kim et al. (2008)]

from Japan based on morphology and molecular analyses. Phylogenetic studies of *Gracilaria* and *Gracilaropsis* have established that species of *Gracilaropsis* form a separate monophyletic clade based on DNA sequence analyses (Bird et al., 1994; Bellorin et al., 2002; Gurgel & Fredericq, 2004; Gurgel et al., 2003a). In this study, the vegetative and reproductive morphology of “*Gracilaria chorda*” from Taiwan were investigated and its taxonomic status assessed based on its morphology and *rbcL* sequence analysis.

### MATERIALS AND METHODS

Collections were made intertidally or by snorkeling at depths of 1–2 m. Algal samples for the morphological study were preserved in 3–5% formalin-seawater or pressed on herbarium sheets, whereas materials used in the molecular studies were desiccated in silica gel or 95% ethanol. Voucher specimens are deposited in the Herbarium of the National Taiwan Ocean University (NTOU). 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). Photomicrographs were taken on an Olympus BX51 microscope with a Q-imaging digital camera (Burnaby, BC, Canada) and habit pictures were captured on an Epson scanner (Tokyo, Japan) and a Nikon Coolpix 995 camera (Tokyo, Japan).

Table 1. List of species used in *rbcL* analysis and accession numbers in GenBank. The number after the accession number is the percentage of the gene sequenced. \*refers to Gurgel et al. (2003a), \*\*refers to Gurgel & Fredericq (2004).

Species	Collection information & GenBank accession number
<i>Curdia coriacea</i> (Hooker et Harvey) J. Agardh	AY049425*, 66.5%
<i>Curdia crassa</i> A. J. K. Millar	AY049427*, 98.1%
<i>Gracilaria bursa-pastoris</i> (Gmelin) P. C. Silva	AY049376**, 91.6%
<i>Gracilaria euchematoides</i> W. H. Harvey	AY049389**, 93.3%
<i>Gracilaria salicornia</i> (C. Agardh) E. Y. Dawson	AY049385**, 98.0%
<i>Gracilaria tenuistipitata</i> C. F. Zhang et B. M. Xia	NC_006137, 100% (Hagopian et al. 2004)
<i>Gracilariopsis andersonii</i> (Grunow) E. Y. Dawson	AY049413*, 94.2%
<i>Gracilariopsis bailinae</i> C. F. Zhang et B. M. Xia	AY049411*, as <i>Gracilariopsis heteroclada</i> , 91.1%
<i>Gracilariopsis carolinensis</i> L. M. Liao et M. H. Hommersand in Gurgel et al.	AY049412*, 96.7%
<i>Gracilariopsis cata-luziana</i> C. F. D. Gurgel, S. Fredericq et J. Norris	AY049406*, 80.2%
<i>Gracilariopsis chiangii</i> , new species, S.-M. Lin	DQ119746, 98.2%, coll. S.-M. Lin, 2.Oct.2002, Sail Rock, Kenting National Park, southern Taiwan.
<i>Gracilariopsis chiangii</i> , new species, S.-M. Lin	FJ235528, 96.7%, coll. S.-M. Lin, 19.May.2007, Wu-Shih-Bi Harbor, Tou-Cheng Township, Yi-Lan County, N.E. Taiwan.
<i>Gracilariopsis chiangii</i> , new species, S.-M. Lin	FJ235529, 96.7%, coll. S.-M. Lin, 18.Mar.2003, Wind Blow Sand, Kenting National Park, southern Taiwan.
<i>Gracilariopsis chorda</i> (Holmes) H. Ohmi	AY049419*, 97.8% (Japan), as <i>Gracilariopsis</i> sp. 3 in Gurgel et al. (2003a)
<i>Gracilariopsis chorda</i> (Holmes) H. Ohmi	AY049421*, 65% (China), as <i>Gracilariopsis</i> sp. 3 in Gurgel et al. (2003a)
<i>Gracilariopsis chorda</i> (Holmes) H. Ohmi	FJ235527, 98.0%, coll. S.-M. Lin, 29.Mar.2007, Awaji Island, Japan.
<i>Gracilariopsis costaricensis</i> E. Y. Dawson	AY049423*, 98.4%
<i>Gracilariopsis funicularis</i> R. Iyer, J. J. Bolton et V. E. Coyne	AY049410*, 98.2% (Namibia), as <i>Gracilariopsis</i> “ <i>lemaniformis</i> ” from Namibia in Gurgel et al. (2003a).
<i>Gracilariopsis hommersandii</i> C. F. D. Gurgel, S. Fredericq et J. Norris	AY049405*, as “ <i>Gp. aff. panamensis</i> ”, 97.1%
<i>Gracilariopsis lemaneiformis</i> (Bory de Saint-Vincent) E.Y. Dawson, O. C. Acleto et N. Foldvik	AY049415*, 97.6%
<i>Gracilariopsis longissima</i> (S.G. Gmelin) M. Steentoft, L. M. Irvine et W. F. Farnham	AF527881*, 97.5%
<i>Gracilariopsis megaspora</i> E. Y. Dawson	AY049422*, 97.8%
<i>Gracilariopsis nhatrangensis</i> L. N. Hau et S.-M. Lin	DQ119744, 78% (Hau et Lin 2006)
<i>Gracilariopsis silvana</i> C. F. D. Gurgel, S. Fredericq et J. Norris	AY049309*, 96.7%
<i>Gracilariopsis tenuifrons</i> (Bird et Oliveira) S. Fredericq et M. H. Hommersand	AY049418*, 97.8%
<i>Melanthalia obtusata</i> (Labillardière) J. Agardh	AY049431*, 99%

DNA samples were prepared using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions. DNA sequencing procedures were as described in Lin et al. (2001). 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 method (MP) available in the computer program PAUP\* v4.0 (Swofford

2003). Support for nodes of the parsimony tree was assessed by calculating 500 bootstrap resamplings of the heuristic searches based on random stepwise additions, MULPARS and TBR. Decay indices analysis (Bremer 1988) followed Lin et al. (2004). Two *rbcL* sequences were generated for the new species, *Gracilariopsis chiangii*, new species, and one for *Gracilariopsis chorda* from Japan and compared with those of the named species of *Gracilariopsis* available in GenBank (see Table 1).

## TAXONOMY

GRACILARIALES S. Fredericq & Hommersand,  
1989

GRACILARIACEAE Nägeli, 1847

GRACILARIOPSIS E. Y. Dawson 1949

*Gracilariopsis chiangii*, new species

Thalli erecti cylindrici, purpurei vel atrorubentes, ex axibus primariis 1–8 ex haptero discoideo exorientia constantes, axibus primariis 15–22 cm longis, 0.8–1.5 mm in diametro, aetate dilatantibus ad 3–6 mm in diametro et cavescens, quoque axe ex ordinibus 1–2(–3) ramorum leviter curvatorum constante, ramis ferentibus aut paucos multosve (20–60) ramulos laterales 0.5–6.0 mm longos aut ramulos 5–7 in sua regione media fasciculatos; plantae masculae non visae; cystocarpia ad basin 1.1–1.3 mm in diametro, 0.87–1.10 mm alta, tholiformia, ad basin non constricta; cellulae nutritiae tubulares nullae; carposporangia 30–40  $\mu$ m longa, 22–28  $\mu$ m lata, in catenis ramosis portata; tetrasporangia 45–50  $\mu$ m longa, 25–30  $\mu$ m in diametro, superficialia, cruciatim divisa.

**Diagnosis.** – Thalli erect, terete, purple to dark red, consisting of 1–8 main axes from a discoid holdfast, 15–22 cm long and 0.8–1.5 mm in diameter; axes increasing to 3–6 mm in diameter and becoming hollow with age, each axis composed of 1–2 (–3) orders of slightly curved branches which bears few to many (20–60) lateral branchlets 0.5–6.0 cm long, or 5 to 7 branchlets clustered in the mid-region of a branch; male plants not seen; cystocarps 1.1–1.3 mm in diameter at base by 0.87–1.10 mm high, dome-shaped, not constricted at the base; tubular nutritive gonimoblast cells absent; carposporangia, 30–40  $\mu$ m long by 22–28  $\mu$ m wide, borne in branched chains; tetrasporangia, 45–50  $\mu$ m long  $\times$  25–30  $\mu$ m in diameter, superficial, cruciately divided.

**Etymology.** – Named in honor of Professor Y.-M. Chiang for his significant contribution to the taxonomy of the Gracilariaceae and marine floristic surveys of Taiwan.

**Holotype.** – In Institute of Marine Biology, National Taiwan Ocean University (NTOU), Taiwan, Republic of China. 19 May 2007, no. GP-5-19-2007-WSB-1 (Figure 1A); isotypes, GP-5-19-2007-WSB-2~4.

**Type locality.** – Wu-Shih-Bi Harbor, Tou-Cheng Township, Yi-Lan County, northeastern Taiwan, Republic of China. (27°51'N 121°51'E).

**Distribution, Habitat and seasonality.** – Sparsely distributed along the southern and northeastern coastlines of Taiwan, growing on coral reef or rocks at 1–2 m deep. The collections were made seasonally from Jul.2002 through May 2007.

**Specimens examined.** – 1) Wu-Shih-Bi Republic of China Harbor, Tou-Cheng Township, Yi-Lan County, N.E. Taiwan, Republic of China: coll. S.-M. Lin, 19 May 2007 (female and sterile); 2) Kenting National Park, S. Taiwan, Republic of China: Sail Rock, coll. S.-M. Lin, 22 Jul.2002 (tetrasporic), 2 Oct.2002 (tetrasporic), 27 Mar.2004 (tetrasporic); Nan-Wan, coll. S.-L. Liu, 13 Jan.2006 (female and tetrasporic); Wind Blow Sand, coll. S.-M. Lin, 18 Mar.2003 (silica gel material only).

**Habit and vegetative structure.** – Thalli (Figs. 1A, B; 2D) are 15–22 cm in height, erect, terete, cartilaginous, arising from a small, discoid holdfast (0.5–1.2 mm in diameter), consisting of 1–8 main axes, 0.8–1.5 mm in diameter but increasing to 3–6 mm in diameter when medulla becomes hollow with age (Fig. 2C), light to purplish-red or light to dark brown in color. Thalli adhere imperfectly to herbarium paper when dried. Main axes consist of 1–2 (–3) orders of branches from the base that bear few or 20–60 lateral branchlets (0.5–6.0 cm in length) (Fig. 1B), or form 5–7 densely clustered branches in the mid-region of an axis (Fig. 2D). Branches are composed of 1–2 (–3) layers of pigmented, ovoid cortical cells, 8–10 by 10–15  $\mu$ m in dimensions, 1–2 layers of subcortical cells, 15–20  $\mu$ m in diameter, and a medulla, 0.6–1.5 mm in diameter, composed of large, thin-walled cells (Figs. 2A, B). Tips of branches are sometimes broken, bearing 2–5 short regenerating branches, 3–5 mm in length (Fig. 1B, arrows).

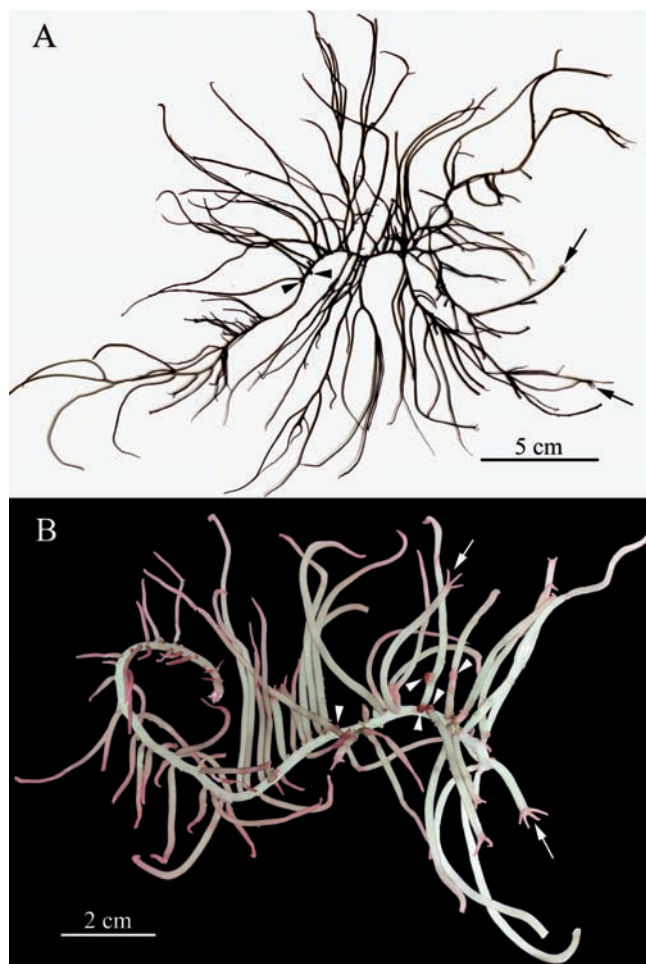


Fig. 1. *Gracilariopsis chiangii* Lin, new species. Habit: A, Holotype: female plant (GP-5-19-2007-WSB-1); B, Liquid preserved, female plant.

**Reproductive structures.** – The tetrasporophytes and gametophytes are isomorphic; however, male plants were not seen. Tetrasporangia are superficial, initiated from outer cortical cells (Fig. 2E) through oblique, longitudinal cell divisions of terminal cells. They later expand and divide transversely (Fig. 2F) and then undergo longitudinal division to produce four cruciately arranged tetraspores at maturity, 45–50  $\mu\text{m}$  long by 25–30  $\mu\text{m}$  in diameter (Fig. 2G). Cystocarps, 1.1–1.3 mm in diameter at base by 0.87–1.10 mm in height, are scattered over the fertile branches, usually at bases of lateral branches (Fig. 3A). Mature cystocarps are dome-shaped, broad-based and are not constricted at their bases. Carpogonial branches are borne on an intercalary supporting cell and consist of a carpogonium and hypogynous cell flanked by a pair of sterile filaments (Fig. 3B). Early post-fertilization stages were not found. The formation of the pericarp and fusion cell is more or less complete prior to gonimoblast initiation. Gonimoblast lobes are initiated before a cavity forms between the pericarp and fusion cell

(Fig. 3C). As the development of gonimoblast lobes continue, the pit-connections between the pericarp cells at the level of the fusion cell break down to initiate a schizogenous cavity (Fig. 3D), which enlarges as the pericarp becomes thicker and the gonimoblasts grow (Figs. 3E, 4A). During early stages of gonimoblast development, the lowermost cells link to the darkly staining cells in the floor of the cystocarp by means of secondary pit connections (Figs. 3E, 4A). Later, the innermost gonimoblast cells are united by numerous secondary pit connections, whereas the lower gonimoblast cells continue pit-connecting to the floor cells (Fig. 4B). Tubular nutritive cells are completely absent. Mature pericarps are 10–14 cell layers thick (Fig. 4C). Carposporangia are formed in branched chains and are 22–28  $\mu\text{m}$  wide by 30–40  $\mu\text{m}$  long (Figure 4D).

### MOLECULAR ANALYSIS

Fifteen *rbcL* sequences of species of *Gracilariopsis*

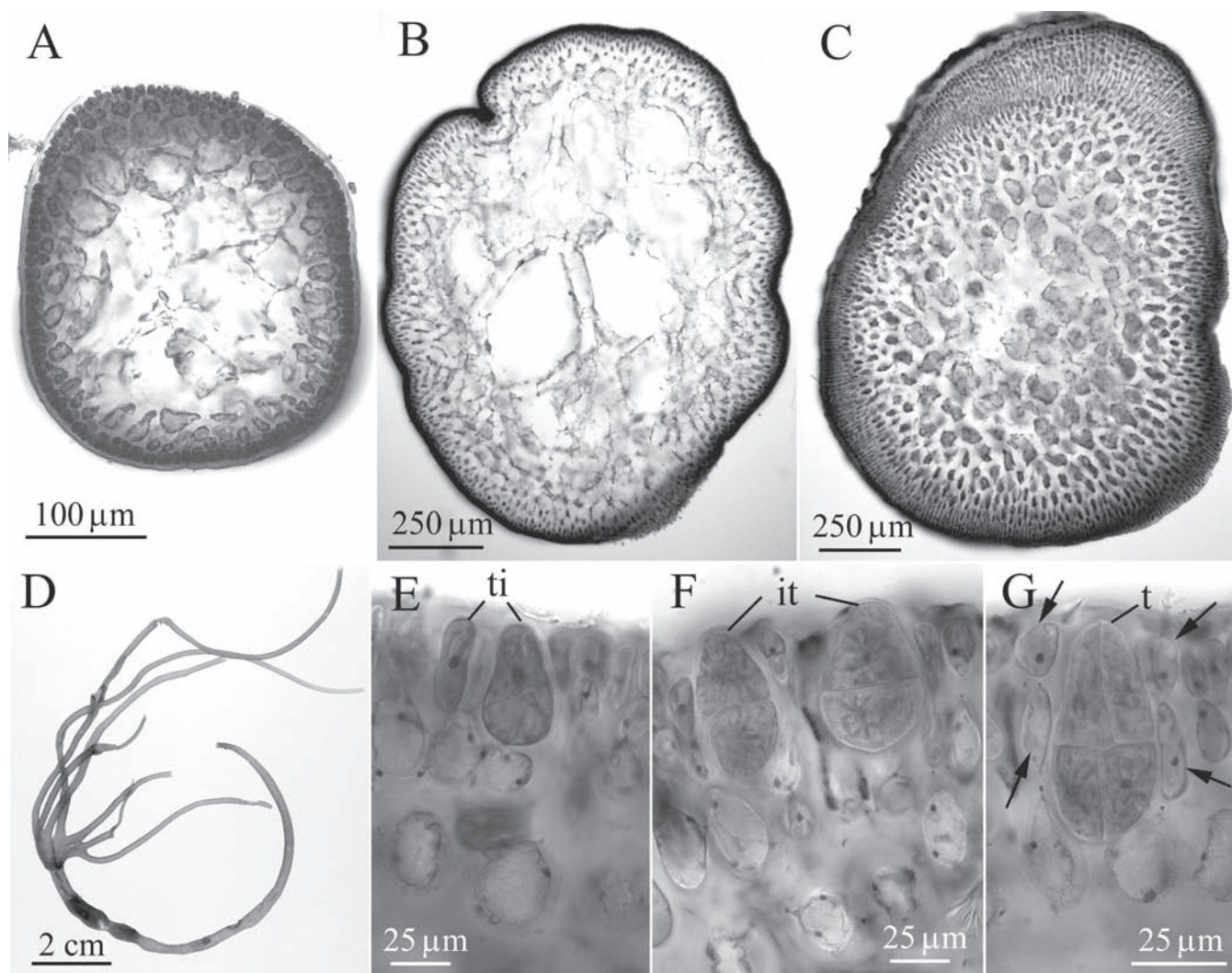


Fig. 2. *Gracilariopsis chiangii*, new species. Vegetative and tetrasporangial structures: A, Transverse section through a branchlet showing small-celled cortex and vacuolated cells of the medulla in the center; B, Transverse section through middle part of a main branch showing small-celled cortex and large vacuolated cells of the medulla; C, Transverse section through base of a main branch showing thickened cortex and compact medulla; D, Habit of a sterile plant showing intestine-like main axe bearing many, terminal branchlets; E–F, Tetrasporangial development: E, tetrasporangial initial (ti); F, immature tetrasporangia (it); G, a fully developed tetrasporangium (t) flanked by slightly modified cortical cells (arrows).

worldwide that possess the *chorda*-type spermatangial conceptacle and a set of four additional representatives belonging to the genera *Gracilaria* and *Hydropuntia* were selected for analysis, together with two species of *Curdia* and one species of *Melanthalia*, which served as the outgroup (see Table 1, Fig. 5). No insertion or deletion mutations were found in the *rbcL* sequences used in this study, allowing for unambiguous alignment of all sequences. The final *rbcL* data matrix was restricted to 1407 sites as the first 60 bases of the sequenced gene at the 5' end were not complete. Parsimony analysis revealed two most parsimonious trees with tree length 1116 steps, CI = 0.5708 and RI = 0.6002; there were 346 informative characters out of 1407 included sites (24.59%).

Bootstrap proportion values (500 replicates, above) and decay indices (below) derived from maximum parsimony analysis are shown on the nodes. The proposed new species, *Gracilariopsis chiangii*, new species, from Taiwan and three collections of *Gracilariopsis chorda* from China and Japan were closely associated and clustered in a single clade with moderate bootstrap support (80%), and have 22 base pairs of the *rbcL* sequence pairwise difference (ca. 1.54%). The remaining species of *Gracilariopsis* included in the dataset were distantly related to species from the western Pacific Oceans. Interspecific *rbcL* sequence divergences (pairwise distance) among the analyzed species within the *chorda*-type clade varied from 0.65 to 7.50%.

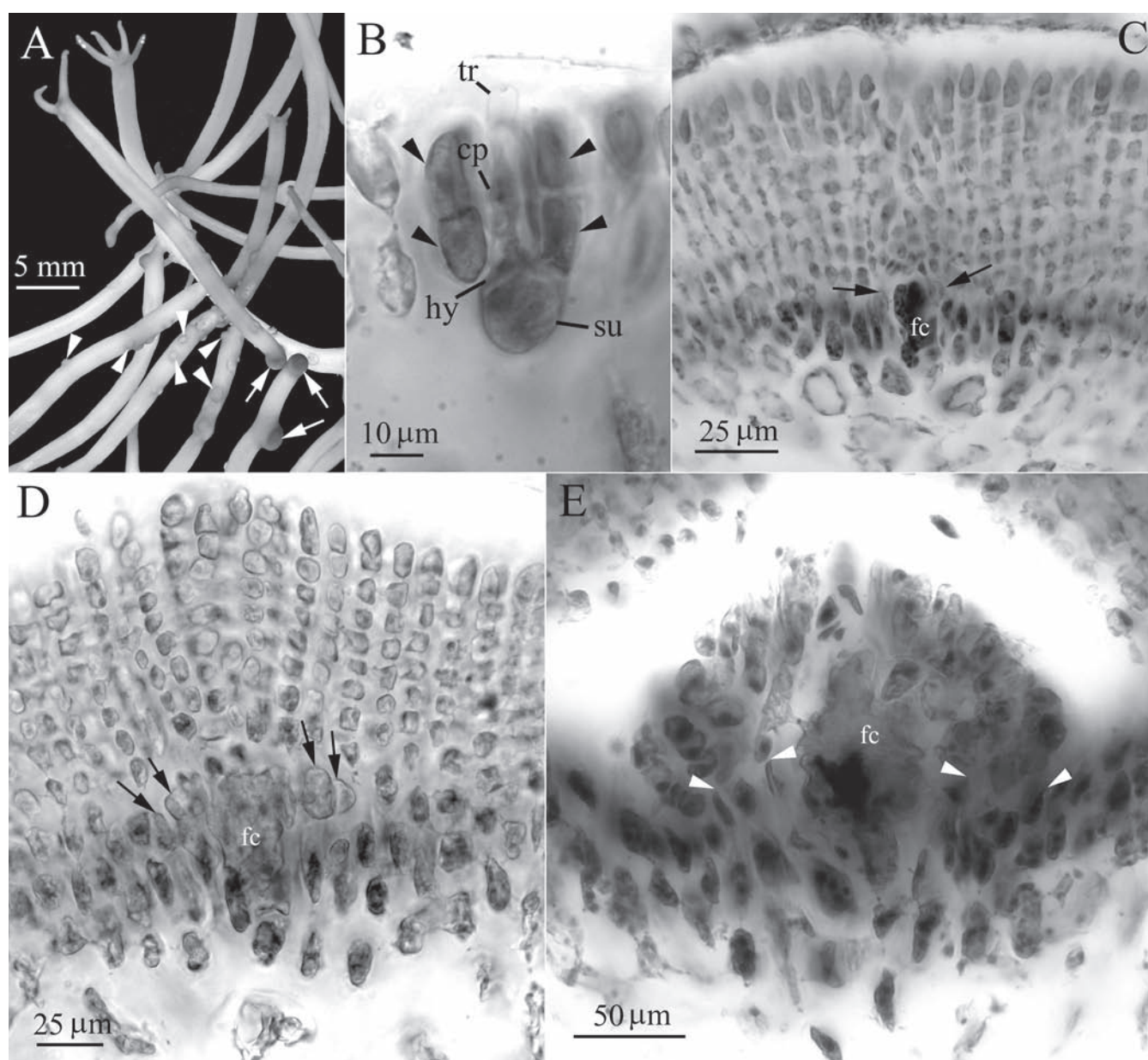


Fig. 3. *Gracilariopsis chiangii*, new species. Cystocarp development: A, Close up of a branch bearing young (arrowheads) and mature (arrows) cystocarps; B, Carpogonial branch apparatus showing sterile cells (arrowheads), supporting cell (su), hypogynous cell (hy) and carpogonium (cp) with trichogyne (tr); C, Early post-fertilization stage showing pericarp and gonimoblast initials cut off from a fusion cell (fc). Note that the cavity of cystocarp has not yet formed; D, A later stage showing a fusion cell cutting off more gonimoblast initials (arrows) bilaterally; E, Young carposporophyte showing a fusion cell (fc) bearing densely compacted gonimoblast cells, some pit-connections (arrowheads) to inner pericarp cells.

## DISCUSSION

The *rbcL*-based phylogenetic analysis of the 15 worldwide species of *Gracilariopsis* resulted in a single clade using the genera *Curdia* and *Melanthalia* as the outgroup (see Fig. 5). This result is in agreement with the tree topology shown in Hau & Lin (2006, Fig. 1) based on the same molecular marker, *rbcL* gene, and *rbcL* topology shown in this study, and is also congruent with the tree in Iyer et al. (2005). *Gracilariopsis chiangii*, new species, from Taiwan is closely related to *Gracilariopsis chorda* from Japan and China as well as the *rbcL* pairwise base difference, but is

widely separated from all other species reported for the genus based on plastid *rbcL* DNA sequence analysis, in which their *rbcL* pairwise divergence between *Gracilariopsis chiangii*, new species, and *Gracilariopsis chorda* from Japan is about 1.54%.

I examined the specimens recorded under “*Gracilaria chorda*” that are deposited in the Herbarium of National Museum of Natural Science by Professor Y.-M. Chiang, his associates and the herbarium curators, and determined that all the specimens of “*Gracilaria chorda*” from Taiwan fit within the description of *Gracilariopsis chiangii*, new species, as proposed in this study. This Herbarium survey confirmed that *Gracilariopsis chorda* is not present in Taiwan.

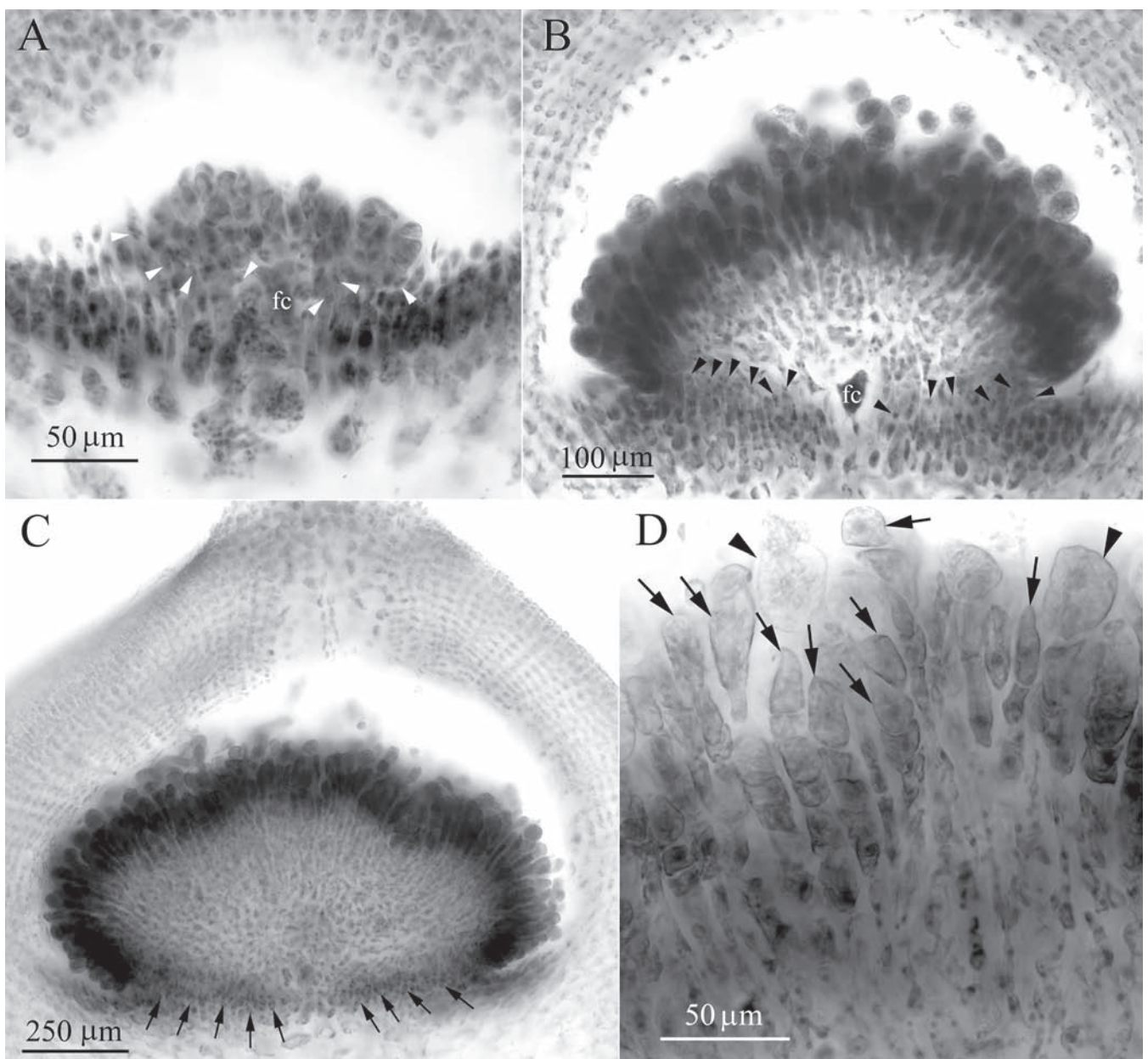


Fig. 4. *Gracilariopsis chiangii*, new species. Cystocarp development: A, Cross-section of immature cystocarp showing fusion cell (fc) and non-vacuolated inner gonimoblast cells pit-connecting (arrowheads) to inner pericarp cells; B, Transverse section of a nearly mature cystocarpic showing dense, small-sized gonimoblast filaments and pit-connections (arrowheads) between lower gonimoblast cells and inner pericarp cells; C, Cross-section of a fully mature cystocarp showing dense, small-sized gonimoblast filaments and pit-connections (arrows) between lower gonimoblast cells and inner pericarp cells; E, Close up of developing (arrows) and mature (arrowheads) carposporangia.

*rbcL*

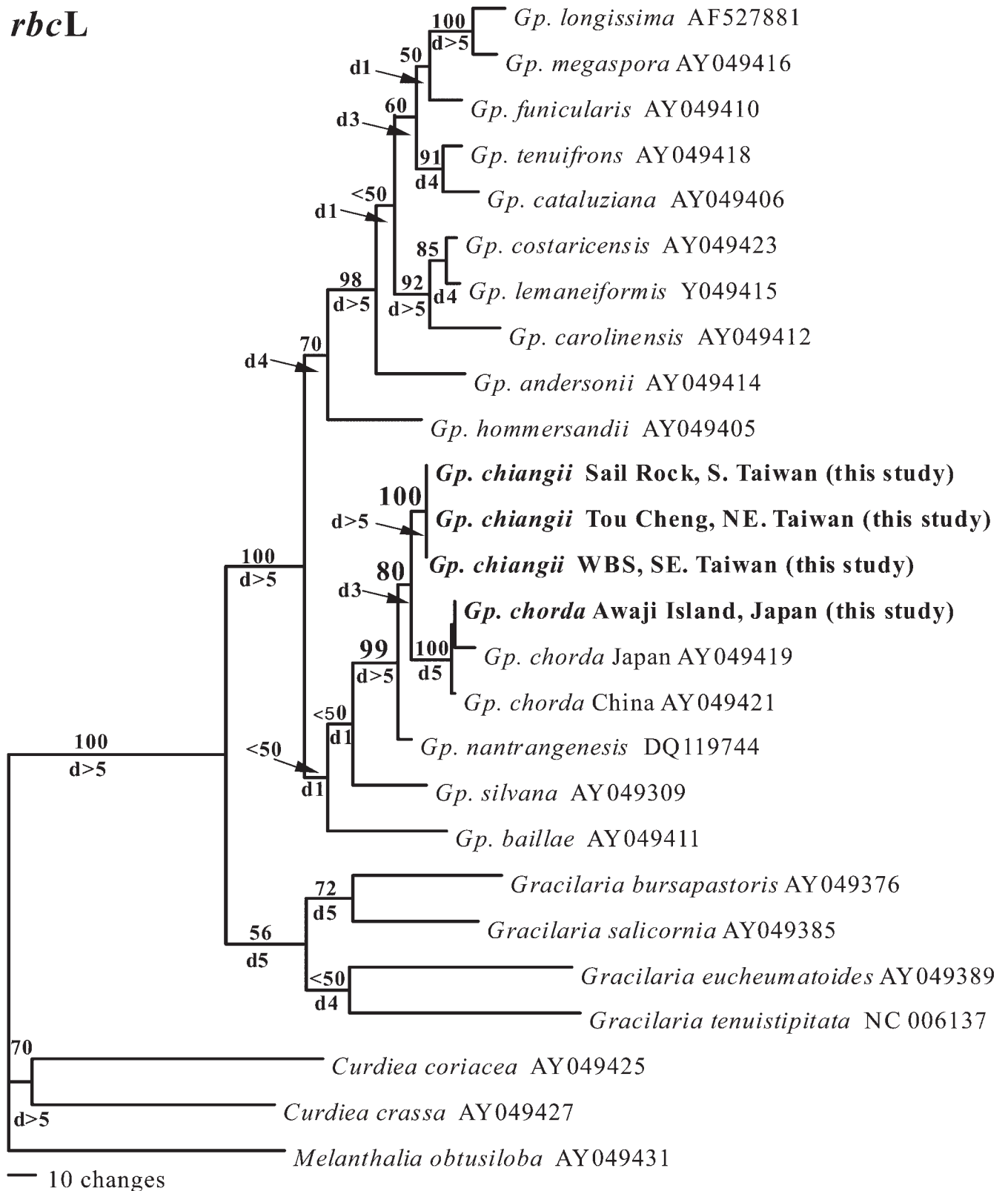


Fig. 5. One of two most parsimonious trees from analysis of the *rbcL* sequence data. Bootstrap proportion values are shown above nodes and decay indices are shown below nodes. Branch lengths are proportional to the amount of sequence change.

Fourteen species of *Gracilariopsis* have been reported from the Pacific Ocean (Gurgel et al., 2003b; Hau & Lin 2006). Hau & Lin (2006) made some detailed morphological comparisons among all described species and agreed to Ohmi's (1958) taxonomic treatment of *Gracilariopsis chorda*, which possesses a continuous superficial layer of spermatangia, the so-called chorda-type spermatangia conceptacles proposed by Yamamoto (1978). When Holmes (1895) described his new species, *Gracilariopsis chorda*, it was based on a partial specimen, 2–3 feet (ca. 60–90 cm) in length, from Enoura, Japan. According to Yamamoto (1978), the thallus size of *Gracilariopsis chorda* can grow up to 200 cm in height! *Gracilariopsis chiangii*, new species, can be distinguished from *Gracilariopsis chorda*, and some other large species [i.e. *Gracilariopsis lemaneiformis* (Bory de Saint-Vincent) E. Y. Dawson, *Gracilariopsis bailinae* (C. F. Zhang ex B. M. Xia) C. F. Zhang et B. M. Xia] by its much smaller size and sparse or luxuriant short lateral branches as seen in Figs. 1A–B, 2D.

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