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A Morphological and Molecular Study on the Gracilariaceae (Gracilariales, Rhodophyta) around the Hakata Bay, Northern Kyushu, Japan

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Detailed morphological study on the plants of the Gracilariaceae around the Hakata Bay, northern Kyushu, Japan revealed that five species of the family were recognizable in this area. According to the previous description, they were assignable to *Gracilaria chorda, G. cuneifolia, G. parvispora, G. textorii* and *G. vermiculophylla*. Of these, only *G. chorda* was separated from the other four species by its lack of spermatangial cavities and nutrient tubular cells in cystocarps. As *rbcL* gene sequence analyses and the resultant phylogenetic trees (maximum parsimony, maximum likelihood, Bayesian inference) support this distinction, the four species were retained in the genus *Gracilaria* while *G. chorda* was considered to belong to *Gracilariopsis*, a genus that consists of species with superficial spermatangia and without nutrient tubular cells. In our phylogenetic trees, *G. vermiculophylla* was recovered as a basal species to all other *Gracilaria* species, which were positioned in the same evolutionary lineage. Interestingly, our specimens of *G. vermiculophylla* showed a mixture of spermatangial conceptacles, monocavitied (the *verrucosa* type) and multicavitied (the *polycarvernosa* type) on the same blade. However, the multicavitied conceptacle is a major character of other genus *Hydropuntia* within the Gracilariaceae. Further studies are thus necessary to clarify the relationship among these genera and to establish a better classification system of the family.

Keywords: Gracilariaceae, *Gracilaria*, *Gracilariopsis*, Hakata Bay, phylogenetic analyses

INTRODUCTION

The Gracilariaceae is a red algal family found worldwide in the intertidal to subtidal zone. Some members are remarkable for their economic importance as agarophytes and some are used as human food or to feed aquatic animals (Bird, 1995). Eight genera are now recognized as distinct in the Gracilariaceae, with more than one hundred species in the genus *Gracilaria* (Algaebase, 2010). Taxonomic concepts of this group are principally based on the internal features of mature cystocarps and the spermatangial arrangements (Fredericq and Hommersand, 1990). Because of the great morphological plasticity, the taxonomy of this family is notoriously difficult. To overcome this difficulty, molecular analyses have been used by several authors (e.g. Bellorin *et al*., 2002; Gurgel and Fredericq, 2004).

So far in Japan, twenty–three species of the Gracilariaceae have been reported (Yoshida *et al*., 2005): twenty–two species of *Gracilaria* and one species of *Congracilaria*. However, these species have been distinguished solely on the morphological characteristics (Yamamoto, 1978; Terada and Yamamoto, 2002). The aims of the present study are to clarify the specific distribution and diversification of the Gracilariaceae found around the Hakata Bay, northern Kyushu, Japan not only on morphological basis but on *rbc*L gene sequence analysis, and to contribute to the establishment of a better classification system of the Gracilariaceae in Japan.

MATERIALS AND METHODS

Morphological observation

Specimens were collected from tide pools or reef flats around the Hakata bay (Fig. 1, Table 1). The specimens for morphological investigation were fixed and

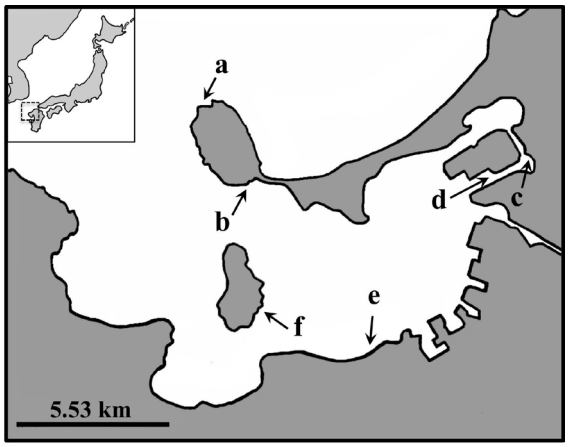

Fig. 1. Map depicting the collection sites in the Hakata Bay. a. Katsuma. b. Shikanoshima. c. Mishima. d. Island City. e. Komogawa canal. f. Nokonoshima.

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Table 1. List of taxa used in this study and their GenBank accession numbers

Taxa	Collection data including reference	Voucher	GenBank No.
Gracilaria chorda Holmes	Japan, Fukuoka, Island City, 11.v.2009	NM114	HQ880629
	Japan, Fukuoka, Katsuma, 11.v.2009	NM130	HQ880630
	Japan, Fukuoka, Katsuma, 24.v.2009	NM182	HQ880631
	Japan, Fukuoka, Nokonoshima, 9.v.2009	NM160	HQ880632
	Japan, Fukuoka, Nokonoshima, 22.vi.2009	NM231	HQ880633
	Japan, Fukuoka, Nokonoshima, 22.vi.2009	NM232	HQ880634
	Japan, Chiba, Katsuura, 5.viii.2009	NM290	HQ880635
Gracilaria cuneifolia (Okamura) Lee et Kurogi	Korea, Jeju (Kim *et al*. 2006)		DQ095788
	Japan, Fukuoka, Katsuma, 24.v.2009	NM109	HQ880636
Gracilaria parvispora I.A. Abbott	Korea, Jeju (Kim *et al*. 2008b)		EF434924
	Japan, Oki Island, Kamo Bay (Kim *et al*. 2008b)		EF434944
	Japan, Fukuoka, Island City, 28.iv.2009	NM104	HQ880637
	Japan, Fukuoka, Nokonoshima, 9.v.2009	NM119	HQ880638
	Japan, Fukuoka, Katsuma, 24.v.2009	NM194	HQ880639
Gracilaria salicornia (C.Agardh) Dawson	Thailand, Phuket, Pa Klok, 19.x.2009	NM301	HQ880640
Gracilaria tenuistipitata Chang et Xia	Vietnam (Lin, S.M. unpublished)		DQ119743
Gracilaria textorii (Suringar) De Toni	Japan, Shizuoka, Shimoda (Kim *et al*. 2006)		DQ095814
	Japan, Fukuoka, Nokonoshima, 9.iv.2009	NM070	HQ880641
	Japan, Fukuoka, Katsuma, 24.v.2009	NM184	HQ880642
	Japan, Fukuoka, Shikanoshima, 25.ii.2009	NM037	HQ880643
Gracilaria vermiculophylla (Ohmi) Papenfuss	Russia, lagoons of Khasan district (Skriptsova and Choi 2009)		GQ292862
	USA, NC, Carteret County (Hommersand and Freshwater 2009)		AY049427
	USA, VA, Hog Island Bay (Gurgel e*et al*. 2004)		AY049312
	Korea, Jeju (Yang *et al*. 2008)		DQ095816
	Japan, Chiba (Yang *et al*. 2008)		EF434912
	Japan, Hokkaido (Hommersand and Freshwater 2009)		EU600293
	Japan, Hiroshima, Fukuyama, Tojiri, 17.vi.2008	GVF1	HQ880644
Gracilaria vermiculophylla (Ohmi) Papenfuss	Japan, Fukuoka, Mishima, 13.x.2008	NM007	HQ880645
	Japan, Fukuoka, Nokonoshima, 11.xii.2008	NM017	HQ880646
	Japan, Fukuoka, Nokonoshima, 10.iii.2009	NM043	HQ880647
Gracilariopsis chorda (Holmes) Ohmi	Korea, Jeju (Kim *et al*. 2008a)		EU567330
	Japan, Chiba (Kim *et al*. 2008a)		EU567343
Gracilariopsis lemaneiformis (Bory) Dawson, Acleto et Foldvilk	Peru, Piura, Paita, Yacilla (Gurgel *et al*. 2003)		AY049415
Outgroups			
Curdiea crassa Millar	Australia, North of Sydney, Bongin Bingin Bay (Gurgle *et al*. 2003)		AY049427
Melanthalia obtusata (Labillardière) J. Agardh	Australia, Victoria, Warrambool (Gurgle *et al*. 2003)		AY049431

stored in 5% formalin/seawater or immediately pressed onto herbarium sheets. Voucher specimens were deposited in the Marine Algal Herbarium of the Faculty of Agriculture, Kyushu University, Fukuoka, Japan. For microscopic observation, cross–sections were made by hand using a stainless steel razor blade and stained with 1% cotton blue in 50% glycerol/seawater.

DNA isolation, PCR amplification, and sequencing

Genomic DNA extraction from the dried herbarium sheets was performed with a DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) following the manufacturer's protocol. The *rbc*L region on the chloroplast genome was selected for PCR amplification. PCR primers were designed based on available *rbc*L sequences from Genbank: *Gracilaria bursa–pastoris* (Gmelin) Silva AY049373, *G. incurvata* Okamura DQ095790, *G. textorii* (Suringar) De Toni AY049325, *G. vermiculophylla* (Ohmi) Papenfuss EF434912 and *G. chorda* [as *Gracilariopsis chorda* (Holmes) Ohmi] EU158085 by manual method using GENETYX–Mac v.11 program (Software Development Co., Tokyo, Japan). Also some PCR primers were modified from those published by Freshwater and Rueness (1994). The primer sequences are presented in Table 2. The PCR reaction mixture contained 1 μL of total DNA template, 14.38 μL of Milli–Q water, 2.5 μL of 10x Ex *Taq* Buffer, 2 μL of 2.5 mM dNTP mixture, 2.5 μL of 5 μM of each of the primer pair and 0.12 μL of Takara Ex *Taq* (Takara Bio Inc., Shiga, Japan) in a total volume of 25 μL. PCR amplifications were carried out with 30 cycles of denaturation at 94ºC for 1 min, primer annealing at 54ºC for 1 min, extension at 72ºC for

Table 2. Primer sequence for template PCR amplification and sequencing of the *rbc*L in this study

Primer name	Sequence 5'–3' (F: forward; R: reverse)
F–rbcL start†	TGTGTTGTCGACATGTCTAACTCTGTAGAAG
F–rbcL startB‡	TCGACATGTCTAACTCTGTAGAAG
FGr–1‡	TCTGGTGTAATTCCATAYGCWAAAAT
FGr–2‡	GTAGAACGTGAGCGTATGGA
FGr–3‡	TGTAAATGGATGCGTATGGC
R–rbcS start†	TGTGTTGCGGCCGCCCTTGTGTTAGTCTCAC
RbcS1‡	AAAAGYYCCTTGTGTTARTCTCAC
RGr–1‡	GCTGTTGGAGTTTCWACAAAATCA
RGr–2‡	TCCAWACAACAGTCCAAGTA

‡ Present study; † Frestwater and Rueness (1994). The letter of W in primer sequence represents a combination of A and T nucleotides.

2 min and termination by 10 min at 72ºC. All PCR products were electrophoresed in 1% agarose for the product yield and size. Some low yield PCR products were reamplified applying 2 μL of the first PCR product as template with the same amplification condition.

The PCR products for sequencing were purified by polyethylene glycol (PEG) precipitation to remove the non–incorporated primers and nucleotides. Sequences of purified PCR products were determined using ABI prism 3100 automatic sequencer (Applied Biosystems, CA, USA) and DNA Sequencing Kit (BigDye terminator v1.1 cycle sequencing kit, ABI) with the PCR primers and other internal primers for sequencing. Primers used during sequencing are listed in Table 2.

Phylogenetic analysis

Due to incomplete data for the seventy–seven bp initial sequences of the 5' region, we used 1390 bp for phylogenetic analyses. Multiple alignments of *rbc*L sequences were prepared in GENETYX–Mac v.11 program and further modified manually. Sixteen additional sequences of the Gracilariaceae species were downloaded from GenBank and included in these alignments (Table 1). *Curdiea crassa* Millar and *Melanthalia obtusata* (Labillardiére) J. Agardh were selected as outgroup taxa for our analyses on the basis of their close phylogenetic relationship within the Gracilariaceae (Gurgel *et al*., 2003). In order to assess phylogenetic signal in the sequence, the g_1 skewness statistic was calculated from 10,000 random tree length distribution in PAUP* v.4.0 b10 (Swofford, 2002) and compared with critical value in Hillis and Huelsenbeck (1992). Also, the *rbc*L data were tested for substitutional saturation by plotting the uncorrected p–distances against the maximum likelihood–corrected distances. Saturation plots were done separately for *rbc*L datasets with and without outgroup taxa. Maximum parsimony (MP) and maximum likelihood (ML) were generated by PAUP* and Bayesian inference (BI) was performed with the program MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003). MP trees were reconstructed under the heuristic search option, 2000 random sequence additions, MULTREES, and tree bisection–reconnection (TBR) branch swapping. To evaluate relative robustness of the clade found in the most parsimonious trees, bootstrap values (Felsenstein, 1985) were calculated 1000 replicates, with 10 random sequence additions. Prior to ML and BI analysis, the best–fit model of nucleotide substitution was selected by likelihood scores computation under the Akaike information criterion (AIC) framework in jModelTest v.0.1.1 (Posado, 2008). After assuming the best evolutionary model, the most likely ML trees were conducted using the estimates model parameters with the following options: starting tree obtained by stepwise addition, and TBR branch swapping. Robustness of the tree topology was evaluated by 1000 bootstrap replications with full heuristic search option. For BI analysis, two runs of 2,000,000 generations were performed, storing 1 tree every 10 visited trees. The burn–in period, which differed insignificantly in the two runs, was determined using Tracer v1.5 (Rambaut and Drummond, 2003). Bayesian posterior probability (BPP) was calculated to support the reliability of each internal branch for BI tree.

RESULTS

Morphological observation

By diligent collections around the Hakata Bay, we have recognized five taxa of the Gracilariaceae. Following the descriptions of Japanese species of the family by Yamamoto and Yoshida (1998), these taxa were identifiable to *Gracilaria chorda, G. cuneifolia, G. parvispora, G. textorii* and *G. vermiculophylla*. The morphological features of these taxa are as follows:

Gracilaria chorda Holmes 1895, p. 253

Specimens examined: (1) Shikanoshima, 9.iii.2009, vegetative NM 046; (2) Katsuma, 11.v.2009, cystocarpic NM 128, 130, 24.v.2009, cystocarpic NM 182, tetrasporangial NM 178; (3) Island City, 29.iv.2009, spermatangial NM 090, 092, 11.v.2009, cystocarpic NM 109, tetrasporangial NM 114; (4) Nokonoshima, 9.v.2009, spermatangial NM 160, 7.vi.2009, spermatangial NM 227, 22.vi.2009, cystocarpic NM 232, tetrasporangial NM 231, 6.ix.2009, cystocarpic NM 238.

Plants attach to rock in intertidal or subintertidal zone, 1–2 m deep. Plants are found from spring to early summer (April to July), and mature plants appear from May to June. Plants are cylindrical, growing in tufts or solitary, up to 1 m tall, frequently with percurrent axes, 1.5–3 mm in diam. Blades grow from a small discoid holdfast, 3–8 mm in diam. (Fig. 2a and b). Blades are succulent and pale brown or reddish brown in color. Branches are usually alternate or sometimes unilateral in upper part. Branches are moderately constricted at the base. Short–spinous proliferations (up to 1 cm long) form all sides of axes and branches (Fig. 2c).

In section, blades consist of 1 layer of pigmented cortical cells, 7–13 μm high, 4–9 μm broad, 2–3 layers of subcortical cells, 10–38 μm high, 5–11 μm broad (Fig. 2d) and 6–8 layers of colorless medullary cells, 75–300 μm in diam. The transition from medulla to cortex is abrupt. Spermatangia are produced from cortical cells in contin-

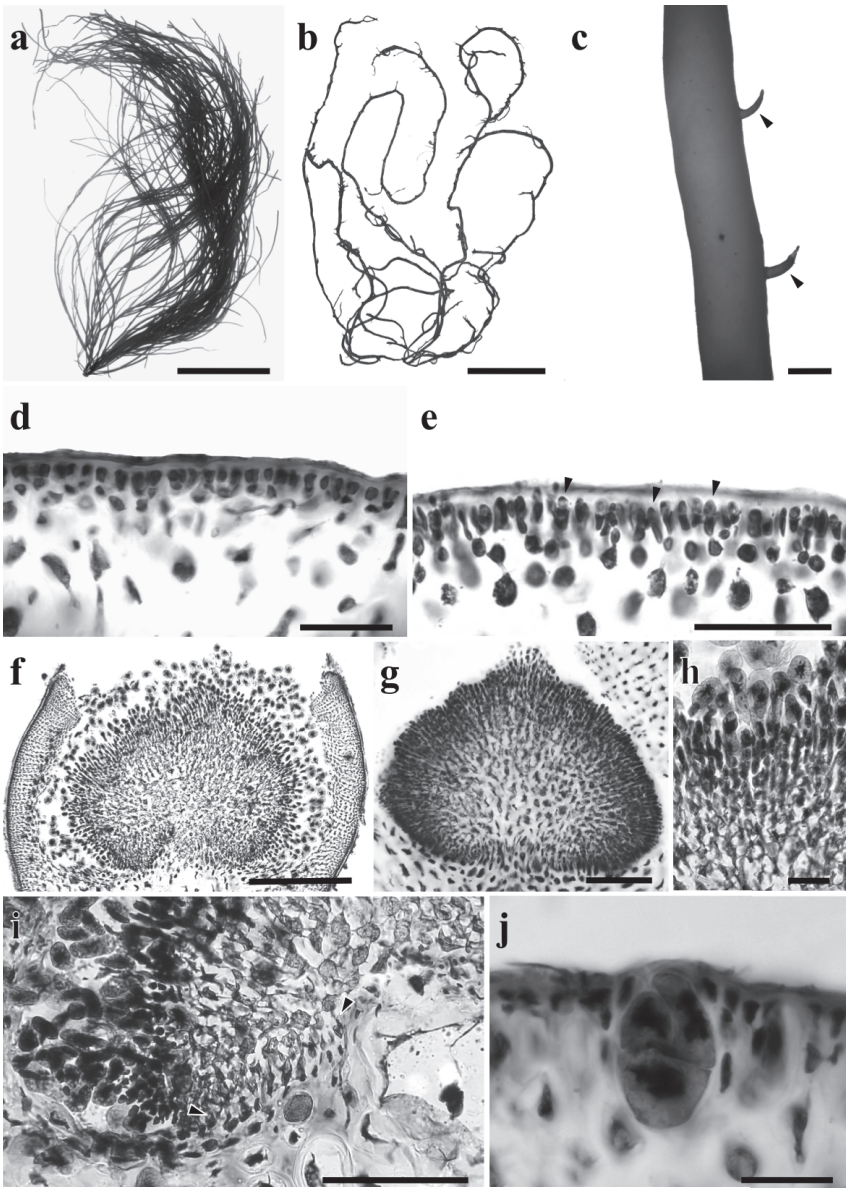

Fig. 2. *Gracilaria chorda*. (a) Attached and (b) unattached form of vegetative plant. Scales, 15 and 20 cm respectively. (c) Short–spinous proliferations (arrowhead). Scale, 1 cm. (d) Cross–section of vegetative plant showing cortical and subcortical layers. Scale, 50 μm. (e) Cross–section of male gametophyte showing superficial arrangement of spermatangia (arrowhead). Scale, 100 μm. (f) Median section of mature cystocarp. Scale, 400 μm. (g) Gonimoblasts consisting of small packed cell without nutrient tubular cells. Scale, 300 μm. (h) Close–up of carposporangia containing with stellate–shaped pigments. Scale, 50 μm. (i) Gonimoblasts attaching with cells lining on cystocarp floor by secondary pit connections (arrowhead). Scale, 120 μm. (j) Transverse section showing tetrasporangium. Scale, 30 μm.

uous or discontinuous cluster over the blades (Fig. 2e). Cystocarps are globose, up to 1.5 mm in diam., sometimes rostrate and basally constricted in mature stage, scattered over the blades except for basal and apical portions (Fig. 2f). Gonimoblasts consist of tightly packed small cells, without any nutrient tubular cells connected to pericarp (Fig. 2g). Carposporangia are borne in unbranched chains, 12–30 μm high by 10–25 μm broad, (Fig. 2h). The basal cells of the gonimoblasts link to the cells lining on the floor by secondary pit connections (Fig. 2i). Tetrasporangia are produced among cortical and subcortical cells, scattered over the blades, 40–55 μm high by 20–35 μm broad, cruciately divided (Fig. 2j).

Gracilaria cuneifolia (Okamura) I.K. Lee and Kurogi 1977, p. 177, figs 1–3

Basionym: Rhodymenia cuneifolia Okamura 1934, p. 16, pl. VII.

Specimens examined: Katsuma, 24.v.2009, vegetative NM 190, 7.vi.2009, vegetative NM 228.

Plants attach to pebbles or rocky coral reefs on the sandy bottom in the lower intertidal zone, 1–2 m deep, sometimes in association with *Gracilaria textorii* (Suringar) De Toni and *Grateloupia imbricata* Homles. Plants thrive on spring season (March to May). Plants are flattened, growing in clump, slightly prostrate or erect, 4–6 cm tall and 1–15 mm wide, arising from discoid holdfast with a short stipe 3–6 mm tall (Fig. 3a). Plants are

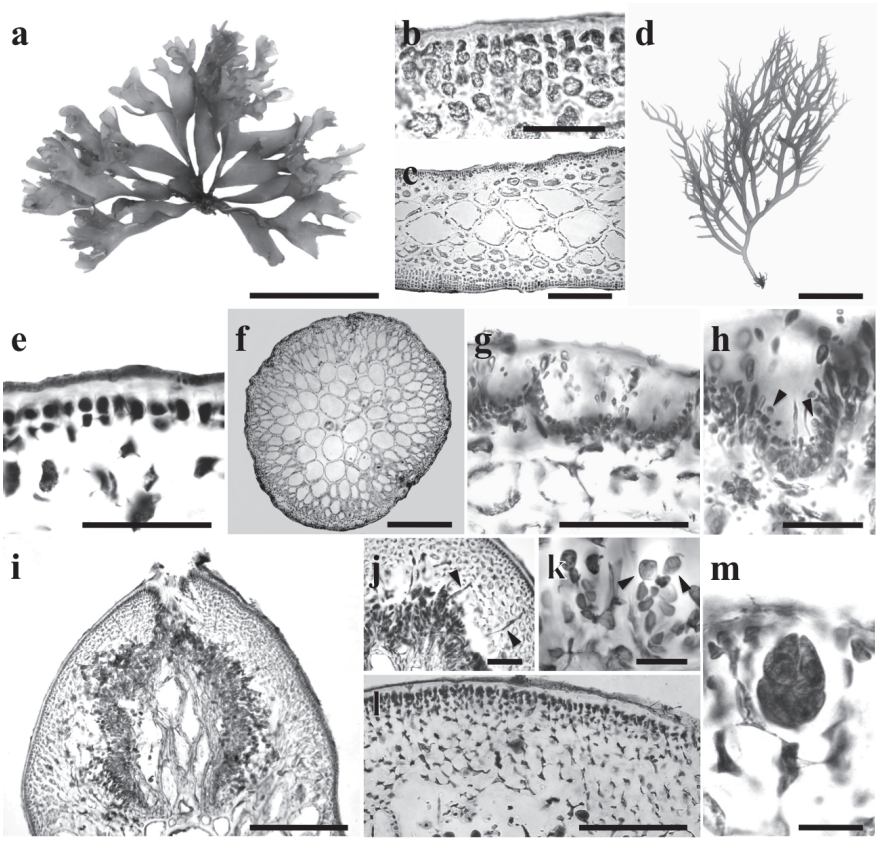

Fig. 3. (a–c) *Gracilaria cuneifolia*. (a) Vegetative plant. Scale, 5 cm. (b) Cross–section of vegetative plant showing cortical and subcortical layers and (c) transition of cells. Scales, 50 and 300 μm respectively. (d–m) *Gracilaria parvispora*. (d) Vegetative plant. Scale, 5 cm. (e) Cross–section of vegetative plant showing cortical and subcortical layer and (f) transition of cells. Scales, 80 and 100 μm respectively. (g) Cross–section of male gametophyte showing shallow pits, *textorii–type* spermatangial conceptacle. Scale, 50 μm. (h) Close–up of spermatangial conceptacles showing spermatangia (arrowhead) produced from conceptacle floor. Scale, 30 μm. (i) Cross–section of mature cystocarp showing large cell gonimoblasts. Scale, 400 μm. (j) Gonimoblasts with nutrient tubular cells connecting to pericarp. Scale 100 μm. (k) Carpospores (arrowhead) arranged in short branched chain. Scale, 50 μm. (l) Star–shaped pericarp cells joining with pit connection. Scale, 200 μm. (m) Cruciately divided tetrasporangium surrounded by elongated cell. Scale, 30 μm.

membranous in texture and reddish pink or greenish yellow in color. Blades are dichotomously or irregularly dichotomously branched. Upper parts are densely branched 4–5 times, with branches slightly incurved or twisted. The apices of blades are bifurcated, with blunt or acute tips. Blades margins are entire and smooth.

In section, blades are 550–750 μm thick in the middle part and are composed of 1–2 layers of pigmented cortical cells, 7–11 μm high, 3–6 μm broad, 1–2 layers of subcortical cells, 10–18 μm high, 8–14 μm broad and 2–4 layers of colorless medullary cells, 100–240 μm high, 90–300 μm broad (Fig. 3b). The transition from the medulla to cortex is abrupt (Fig. 3c). Gametophytes and tetrasporangial plants were not observed.

Gracilaria parvispora I.A. Abbott 1985, p. 119, fig. 1
Specimens examined: (1) Katsuma, 24.v.2009, cystocarpic NM 162, 194, 197, tetrasporangial NM 196, vegetative NM 179; (2) Island City, 28.iv.2009, tetrasporangial NM 104; (3) Nokonoshima, 9.v.2009, vegetative NM 118, 119, 7.vi.2009, cystocapic NM 213, 214, 22.vi.2009, spermatangial NM 241, 242, tetrasporangial NM 247, 6. vii.2009, cystocarpic NM 250, 253.

Plants are found in tide pools, or growing on pebbles or rocks in intertidal or subtidal zone, to 1–2 m deep. Plants thrive from early spring to early summer (March to August), and mature plants appear in May to July. Plants are compressed in axes and somewhat cylindrical in branches, 1–2.5 mm in diam., usually 12–30 cm tall, arising from small discoid holdfast (Fig. 3d). Plants are succulent with coarse texture and brownish yellow or pale brown in color, occasionally dark green. Branches are unilaterally protruded form axes, 3–6 times branching, sometimes mixed with irregularly alternate branches and are tapering to acute apices.

In section, blades are composed of 1–2 layers of pigmented cortical cells, which are oval or elongated, 7–22 μm high, 5–10 μm broad, 1–2 layers of subcortical cells, 18–25 μm high, 8–15 μm broad (Fig. 3e) and 4–8 layers of thick–walled medullary cells, 140–550 μm in diam. The transition from medulla to cortex is gradual (Fig. 3f). Spermatangial conceptacles are scattered over the blade of male gametophytes, forming shallow pits, 25– 55 μm deep, 20–50 μm broad (Fig. 3g), with spermatangia produced in the lower part of the conceptacle (Fig. 3h). Cystocarps are spherical and slightly rostrate, 1–2 mm in diam. (Fig. 3i). Nutrient tubular cells, which connect gonimoblasts to pericarp cells, are conspicuous

(Fig. 3j). Gonimoblasts consist of large cells, producing short–chained carposporangia, 10–20 μm long by 7–15 μm broad (Fig. 3k). Star–shaped pericarp cells are linked by pit connections (Fig. 3l). Tetrasporagia are scattered over the blade, ovoid or oblong in shape, 25–55 μm high by 20–45 μm broad, cruciately divided (Fig. 3m).
Remark: This alga has been known as *G. bursa–pastoris* in Japan (Yamamoto and Yoshida, 1998; Yoshida *et al*., 2005). However, recently Kim *et al*. (2008b) have shown that the taxon was not identical to *G. bursa–pastoris* but to *G. parvispora*, and proposed to use this name for the alga in Japan and Korea. We followed this proposal.

Gracilaria textorii (Suringar) De Toni 1895, p. 27
Basionym: Sphaerococcus textorii Suringar 1868, p. 259.
Specimens examined: (1) Shikanoshima, 25.ii.2009, vegetative NM 037, 9.iii.2010, spermatangial NM 271, 272, vegetative NM 273, 10.iv.2009, tetrasporangial NM 077; (2) Katsuma, 24.v.2009, cystocarpic NM 172, 176, tetrasporangial NM 184; (3) Island City, 11.v.2009, vegetative NM 105,107; (4) Komogawa canal, 7.v.2009, cystocarpic NM 223, 225; (5) Nokonoshima, 9.iv.2009, spermatangial NM 070, 6.vi.2009 cystocapic NM 252.

Plants attach to rocks in intertidal to subtidal zone, to 1–2 m deep, associated with other algae such as *Grateloupia imbricata, Laurencia okamurae* Yamada, *Ulva pertusa* Kjellman, etc. Plants are found from late winter to late spring (February to July). Mature plants appear in April and May. Plants are flattened, growing in tufts, up to 20 cm tall, usually 6–18 cm tall and 4–50 mm wide. Blades grow from a small discoid holdfast with a short subcylindrical stipe, 1–4 mm tall, brownish–red or yellow in color. Blades are fan shaped or palmate in middle part, becoming dichotomously or irregularly dichoto-

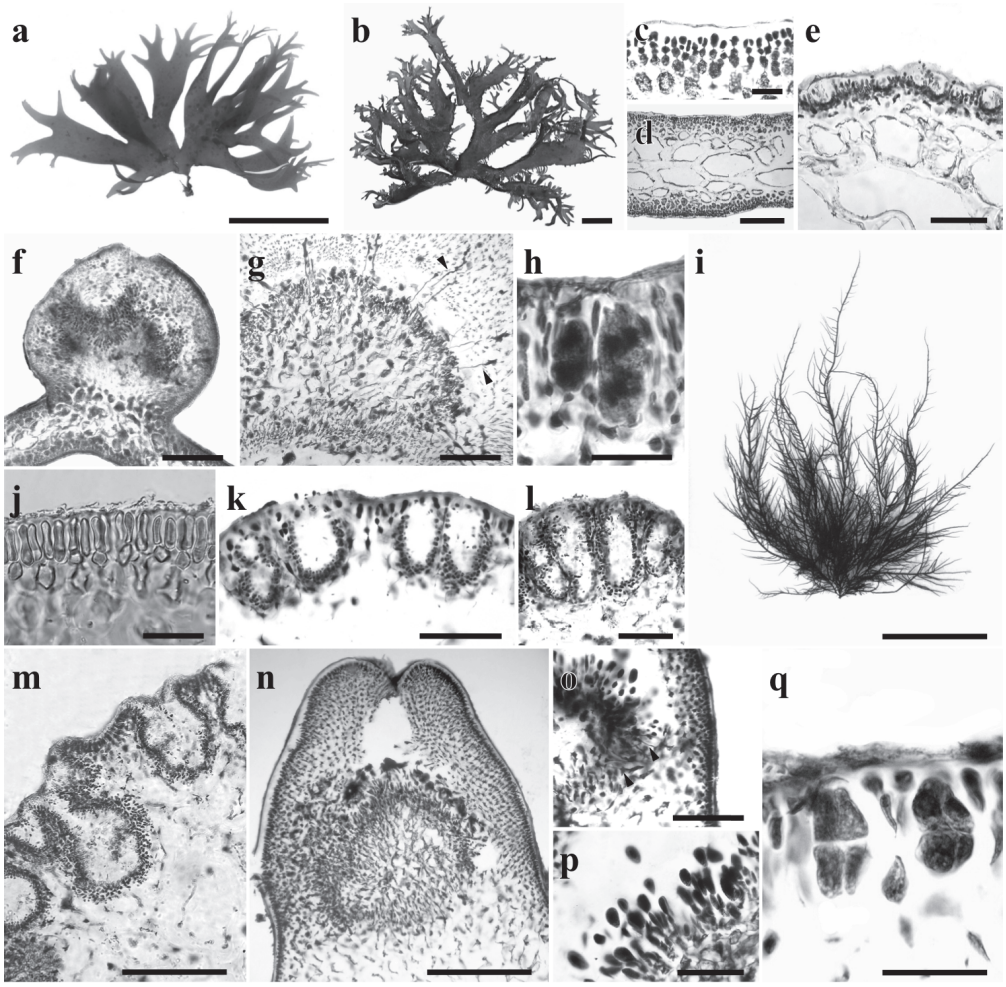

Fig. 4. (a–h) *Gracilaria textorii*. (a) Vegetative plant with entire and smooth margins. Scale, 10 cm. (b) Vegetative plant with proliferations. Scale, 5 cm. (c) Cross–section of vegetative plant showing cortical and subcortical layer and (d) transition of cells. Scales, 40 and 300 μm respectively. (e) Spermatangial conceptacle in shallow pit. Scale, 80 μm. (f) Median section of mature cystocarp. Scale, 500 μm. (g) Nutrient tubular cells (arrowhead) connecting to pericarp. Scale, 200 μm. (h) Cross–section showing cruciated tetrasporangia. Scale, 40 μm. (i–q) *Gracilaria vermiculophylla*. (i) Tetrasporangial plant. Scale, 15 cm. (j) Transverse section of vegetative plant showing cortical and subcortical layer. Scale, 40μm. (k) Transverse section of spermatangial conceptacles in deep–pot shaped, *verrucosa* type, (l) Confluent spermatangial conceptacles, (m) The *polycavernosa–type* spermatangial conceptacles. Scales, 80, 100 and 150 μm respectively. (n) Cross–section of mature cystocarp. Scale, 400 μm. (o) Nutrient Tubular cells (arrowhead) at basal portion of cystocarp. Scale, 300 μm. (p) Carposporangial chain with terminal carpospore. Scale, 100 μm. (q) Tetrasporangia in modified cortex. Scale, 40 μm.

mously divided with expanded tops, sometimes twisted. Fresh plants are fragile but become coriaceous while drying. Blade tips are bifurcated, with blunt or somewhat acuminate apices. The margins are mostly entire (Fig. 4a) or proliferous (Fig. 4b).

Blades in section are 350–750 μm thick in the middle part, consisting of 1–2 layers of pigmented cortical cells, 7–20 μm high, 4–10 μm broad, 2–3 layers of subcorital cells, which are somewhat larger cell, 18–30 μm long, 20–30 μm wide (Fig. 4c) and 3–4 layers of colorless medullary cells, 40–150 μm high, 50–380 μm broad. The transition from medulla to cortex is abrupt (Fig. 4d). Spermatangial conceptacles are scattered throughout the blade, forming shallow pits, 20–40 μm deep, 25–40 μm broad (Fig. 4e). Cystocarps are spherical, slightly constricted at the base, 1.5–2 mm in diameter (Fig. 4f), borne on the middle and upper parts of plants. Nutrient tubular cells are moderately occurred in the cystocarp cavity (Fig. 4g). Carposporangia are arranged in branched chains, 10–25 μm long by 5–20 μm broad. Tetrasporangia are cruciately divided, 35–60 μm high by 20–32 μm broad (Fig. 4h), scattered in both sides of blade cortexes.

Gracilaria vermiculophylla (Ohmi) Papenfuss 1967, p. 101

Basionym: Gracilariopsis vermiculophylla Ohmi 1956, p. 271, figs 1–4; pls 1,2.

Specimens examined: (1) Shikanoshima, 25.ii.2009, spermatangial NM 035; (2) Mishima, 13.x.2008, cystocarpic NM 007, 011, spermatangial NM 008, tretasporangial NM 010; (3) Nokonoshima, 12.xi.2008, vegetative NM 001, 11.xii.2008, spermatangial NM 017, tetrasporangial NM 014, 015, 10.iii.2009, spermatangial NM 043, 045, 061, 9.v.2009, cystocarpic NM 138, 140, tetrasporangial NM 100, 101.

Plants attach to rocks or pebbles on the sandy bottom in the intertidal to subtidal zone, 1– 2 m deep, sometimes in association with *Ahnfeltiopsis flabelliformis* (Harvey) Masuda, *Chondracanthus tenellus* (Harvey) Hommersand, *Ulva pertusa*, etc. Plants thrive in spring, and mature plants appear from February to July. Plants are cylindrical, forming in clump, up to 80 cm tall, usually 15–30 cm tall and 0.8–2.2 mm in diam. (Fig. 4i). Blades grow from a small discoid holdfast, 3–5 mm in diam., dark red, reddish brown and pale brown in color. Blades are usually 3–4 times alternately, or sometimes irregularly or unilaterally branched. Short branchlets, alternately or irregularly branched, prominently protrude all side of axes. Apices are acute and spine–like.

In section, blades consist of 1–2 layers of cortical cells, 10–25 μm high, 5–10 μm broad, 1–2 layers of subcortical cells, 20–35 μm high, 25–30 μm broad (Fig. 4j) and 6–8 layers of medullary cells, somewhat rounded, 400–550 μm in diam. The transition from medulla to cortex is abrupt. Spermatangial conceptacles are scattered over the blades, forming solitarily in deep pot, 70–200 μm deep, 50–100 μm broad (Fig. 4k), or at times gathering with adjacent conceptacles becoming confluent ones (Figs. 4l and m); Cystocarps are globose, 550–1200 μm in diam., slightly rostrate and somewhat constricted at base (Fig. 4n), forming on entire blades except basal or apical parts. Nutrient tubular cells are present, but not many (Fig. 4o). Carposporangia are arranged in unbranched chains, 25–35 μm long by 20–28 μm broad (Fig. 4p). Tetrasporagia are cruciately divided, 30–50 μm high by 25–45 μm broad, embedded in the cortex, scattered over the blade (Fig. 4q).

Molecular phylogenetic analyses

Thirty–five *rbc*L sequences, nineteen of which were newly generated in the present study, were employed to reconstruct the phylogenetic trees under the best–fit model of nucleotide substitution of GTR+G with 6 substitution types (AC=1.0728, AG=6.7783, AT=2.7965, CG=1.3598, CT=16.1133 and GT=1.0000), assumed nucleotide frequencies of A=0.3065, C=0.1428, G=0.2188 and T=0.3319, rate at variable sites following the Gamma distribution with shape parameter=0.2260. Our *rbc*L sequences included 1390 bp in data set, with 941 bp (67.7%) being constant, 113 bp (8.1%) being variable and 336 bp (24.2%) being phylogenetically informative. There were no insertions or deletions in this database marker. The g_1 value (g_1=−0. 5602) for *rbc*L dataset was lower than the predicted critical value at both 95% and 99% confidence level in Hillis and Huelsenbeck (1992), implying that significant phylogenetic signal exists for the data. In saturation analysis, two saturation plots for datasets with and without outgroup taxa displayed a near–linear correlation between ML–corrected and uncorrected p–distances, suggesting little saturation (Figs. 5a and b).

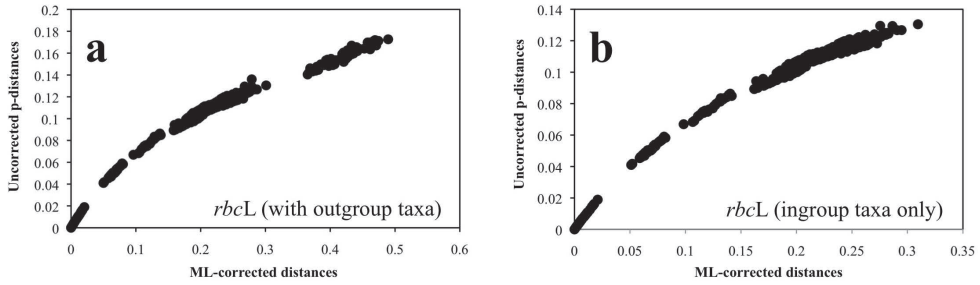

Fig. 5. Saturation plots of the uncorrected p–distances and ML corrected distances for *rbc*L datasets with and without outgroup taxa. ML corrected distances were calculated in PAUP* as determined a best–fitting evolution model suggested by AIC in jModelTest.

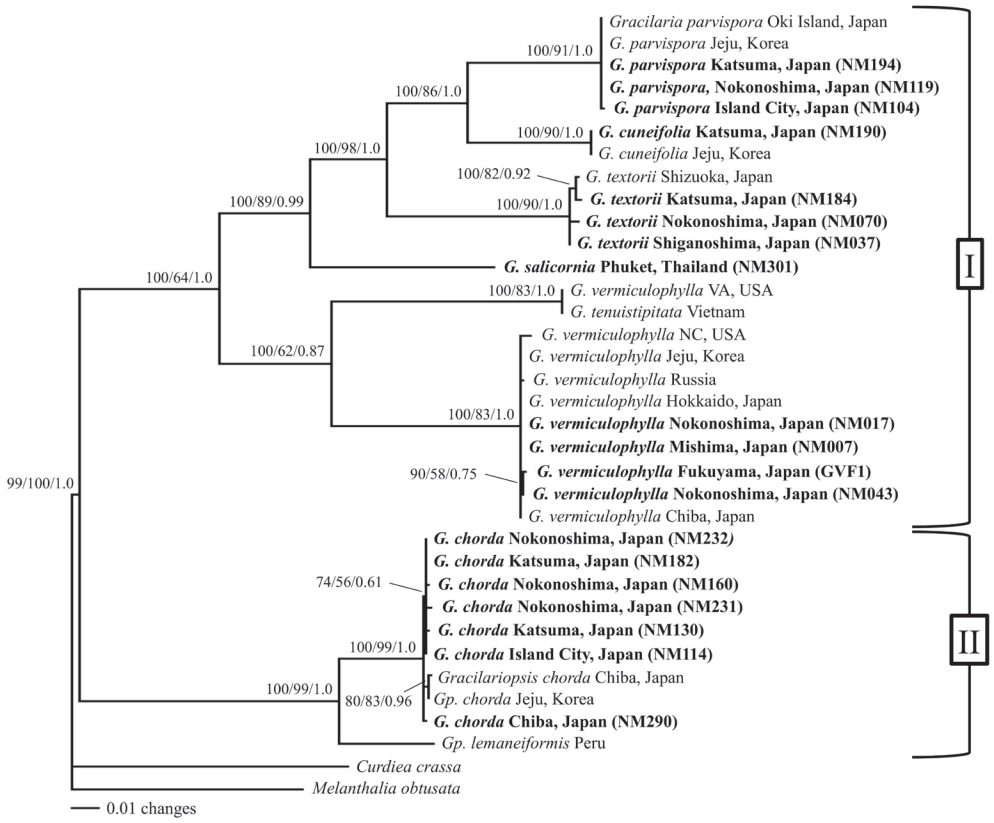

Fig. 6. Phylogram inferred from ML analysis of the *rbc*L gene in *Gracilaria, Gracilariopsis* and related taxon. Bootstrap proportions from maximum parsimony, maximum likelihood and Bayesian posterior probability support are noted above each branch respectively. Species name in thick bold correspond to new sequences in this study.

Furthermore, neither of saturation plots seems likely to have reached a plateau, indicating that *rbc*L sequences for this present study still retain enough phylogenetic signals.

The topologies obtained from the three methods for phylogenetic inference were largely congruent with only minor differences in the placement of taxa. All the phylogenetic trees resulted in two distinct major clades (Fig. 6, I and Ⅱ). The genetic divergence among the species in the major clade I ranged from 5.68% to 11.58%, meanwhile it differed from 4.61% to 5.11% in the major clade Ⅱ. Within the major clade I, six distinct clades were recognized: *G. vermiculophylla* clade, *G. textorii* clade, *G. parvispora* clade, *G. cuneifolia* clade, *G. salicornia* clade and *G. tenuistipitata* clade. The first divergent was made by *G. vermiculophylla* clade. The genetic distance among the plants of *G. vermiculophylla* from Japan, Korea, Russia and NC, USA was up to 0.43% except for the plant from VA, USA, and these formed a monophyletic group. Among *G. textorii* plants all from Japan, the difference was up to 0.50%, and they formed a well–supported clade. The variation among the plants of *G. parvispora* from Japan and Korea was minimal (0.07%). *G. cuneifolia* from Hakata Bay and Korea were identical in the *rbc*L sequences. On the other hand, our specimens of *G. chorda* were positioned in the major clade Ⅱ with *Gracilariopsis chorda* from Chiba, Japan and Jeju, Korea and *Gp. lemaneifomis* from Peru. In the major clade Ⅱ, two clades were disticnct: *G. chorda* clade and *G. lemaneiformis* clade. The variation of *rbc*L sequences among the plants of *G. chorda* from Hakata Bay and Chiba, Japan and those (as *Gp. chorda*) from Chiba, Japan and Korea was up to 0.43%, and they formed a monophyletic group with high bootstrap support.

DISCUSSION

Of the five species recognized around the Hakata Bay, the four species are separable into two groups from the reproductive features. One group consists of the species having nutrient tubular cells in mature cystocarps and spermatangial cavities. In this group are included *Gracilaria parvispora, G. textorii* and *G. vermiculophylla* (Figs. 3 and 4). The other group consists of only *G. chorda*, which has neither nutrient tubular cells nor spermatangial cavities (Fig. 2). Although reproductive plants of *G. cuneifolia* were not found in present study, the alga was considered to belong to the former group from the previous description (Yamamoto and Yoshida, 1998). According to the studies by Fredericq and Hommersand (1989) and Gurgel *et al*. (2003), the species of the Gracilariaceae with superficial spermatangia and without nutrient tubular cells in cystocarps are included in the genus *Gracilariopsis*. As long as this generic distinction is applied to our species, *G. chorda* will be included in the latter genus. Our *rbc*L gene

sequence analyses and the resultant phylogenetic trees strongly support this consideration (Fig. 6), as the results displayed a clear monophyly of *G. chorda* and *Gp. lemaneiformis* from Peru with high bootstrap value (MP=99%, ML=100% and BI=1.0%). The genetic distance between *G. chorda* and other species of *Grcilaria* was more than 116 bp (8.34%). Therefore, we come to the same conclusion of Kim *et al*. (2008a) that *G. chorda* should be moved to the genus *Gracilariopsis*.

Our phylogenetic analyses also verified a monophyletic group composed of *Gracilaria cuneifolia, G. parvispora, G. textorii* and *G. vermiculophylla*. As shown in Fig. 6, *G. cuneifolia* was clustered in the same evolutionary lineage with *G. parvispora* and *G. texorii* and had a closer relationship to *G. parvispora* than to *G. texorii*. Irrespective of a similarity in spermatangial arrangement (the *textorii* type), the flattened species, *G. cuneifolia* and *G. textorii*, were quite different from *G. parvispora* in gross morphological features (habit form, medullary cell arrangement and gonimoblast cell size). These facts suggest that the *textorii–type* spermatangial conceptacles may be synapomorphy in the evolutionary history of *G. cuneifolia, G. parvispora* and *G. textorii*.

Gracilaria vermiculophylla from Japan, Korea, Russia and USA formed a well–supported monophyletic clade (Fig. 6) except for the plant from VA, USA, which formed a clade with *G. tenuistipitata* from Vietnam. The VA plant of *G. vermiculophylla* might belong to *G. tenuistipitata* or other taxon. Ohmi (1956) originally designated *G. vermiculophylla* as a member of *Gracilariopsis* based on the specimens from Hokkaido, Japan, and this alga was subsequently moved to *Gracilaria* by Papenfuss (1967). This alga is characterized by the presence of small number of nutrient tubular cells, the spermatangia in deep cavities and the small cells with dense protoplast in gonimoblast tissues (Terada and Yamamoto, 2002; Rueness, 2005). Interestingly, the plants of *G. vermiculophylla* from the Hakata Bay demonstrated striking morphological variations in the configurations of spermatangial conceptacles. They had two types of spermatangial conceptacles, monocavitied (the *verrucosa* type) and multicavitied (the *polycarvernosa* type), on the same blades. The former type was only found in younger plants, while both types were occasionally found on the same section of older plants. This is the first finding of such a mixture in the Japanese plants of *G. vermiculophylla*. Abbott *et al*. (1991) have already found a mixture of the two types of spermatangia on the same blade in *G. mixta* Abbott, Zhang and Xia from south China. Withell *et al*. (1994) also described the occurrence of the same phenomena on older plants of *G. harveyana* J. Agardh from Australia. Considering these facts, the *polycarvernosa* type of spermatangial conceptacle might be developed from the *verrucosa* type. However, the *polycarvernosatype* spermatangial conceptacle is an important criterion of the genus *Hydropuntia*. Further investigations for some additional species (e.g. *G. mixta*, *G. harveyana* and some *Hydropuntia* species) are apparently required to shed light on the specific circumstances of *Gracilaria* possessing a mixture of spermatangial conceptacles and the relationship among the members of Gracilariaceae distributed in Pacific Ocean regions.

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