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Reinstatement of *Gracilariopsis chorda* (Gracilariaceae, Rhodophyta) Based on Plastid *rbc*L and Mitochondrial *cox*1 Sequences

Myung Sook Kim[1]*, Eun Chan Yang[2], Su Yeon Kim[2], II Kee Hwang[2] and Sung Min Boo[2]

[1]*Research Institute of Basic Sciences, Pusan National University, Busan 609-735*
[2]*Department of Biology, Chungnam National University, Daejeon 305-764*

Two different molecular markers, the plastid *rbc*L and mitochondrial *cox*1 genes, were used to define the taxonomic position of the northwest Pacific Ocean species currently named *Gracilaria chorda*. We analyzed both genes (1,222 bp for *rbc*L and 1,245 bp for *cox*1) from 18 specimens collected in Korea, Japan, and China. Phylogenetic reconstruction revealed that this organism should be classified in the genus *Gracilariopsis*, rather than in the *Gracilaria*. Thus, *Gracilariopsis chorda* (Holmes) Ohmi is the legitimate name for *Gracilaria chorda* Holmes. Within the species, the sequences differed by 8 bp (0.7%) in *rbc*L and 5 bp (0.4%) in *cox*1. Six haplotypes of *cox*1 tended to be geographically organized. *Gp. chorda* is characterized by coarse, elongate terete axes, short filiform branchlets usually at irregular intervals, an abrupt transition in cell size from medulla to cortex, cystocarps without tubular nutritive cells connecting the gonimoblast to the upper pericarp, and relatively large gonimoblast cells of the cystocarp in the specimens collected from Wando in southern Korea.

Key Words: *cox*1, Gracilariales, *Gracilariopsis chorda*, *rbc*L, Rhodophyta, taxonomy

INTRODUCTION

The genus *Gracilariopsis* was differentiated from *Gracilaria* Greville by Dawson (1949) based on the characteristics of the cystocarp, including the sizes of the gonimoblast cells and the nutritive filament cells connecting the pericarp or cystocarp floor. *Gracilariopsis* includes algae having a cystocarp with a dome-shaped gonimoblast composed of small cells and the absence of tubular nutritive cells, whereas *Gracilaria* contains species having large and vacuolated gonimoblast cells and the presence of tubular nutritive cells. Papenfuss (1967), however, compared cystocarp morphology and concluded that the presence or absence of tubular cells cannot be used to discriminate between the two genera. He proposed that the two be merged, as these diagnostic features are not always consistent with those of two type species, *Gp. sjoestedtii* from California and the former *Gracilaria* generitype *G. verrucosa* (Hudson) Papenfuss from England.

*Corresponding author (myungskim@hanmail.net)

Fredericq and Hommersand (1989) resurrected the genus *Gracilariopsis* based on studies of *Gp. lemaneiformis* from California. They emphasized that the gonimoblast cells become linked to gametophytic cells in the floor of the cystocarp by means of secondary pit connections. They also confirmed that tubular nutritive cells are absent in the cystocarp and that the spermatangial parent cells are produced from superficial cortical cells (Fredericq and Hommersand 1990). Recognition of *Gracilariopsis* as a genus distinct from *Gracilaria* received additional strong support from the molecular studies of Bird *et al.* (1992, 1994). In recent years, plastid protein-encoding genes (*rbc*L) have been used to reconstruct the phylogeny of the *Gracilaria*ceae (Gurgel and Hommersand 2004; Gargiulo *et al.* 2006) and to clarify the generic characteristics of the family Gracilariaceae, primarily genera closely related to *Gracilaria* Greville and *Gracilariopsis* Dawson (Gurgel *et al.* 2003).

Gracilaria chorda Holmes was first collected from central Japan and is characterized by a variety of features, including large size, small branches, and very large medullary cells (Holmes 1896). Later, Ohmi (1958) combined this species into *Gracilariopsis chorda* (Holmes)

Table1. List of species, their collection information, and the GenBank accession numbers with haplotypes.

Species	Collection data	Voucher	GenBank accession number and haplotype	
			*rbc*L	*cox*1
Gracilariopsis chorda				
	Sinyangri, Jeju1, Korea (20.Oct.07)	Gr1	EU567330 R3	EU567348 C3
	Sinyangri, Jeju2, Korea (20.Oct.07)	Gr2	EU567331 R3	EU567349 C3
	Sinyangri, Jeju3, Korea (20.Oct.07)	Gr3	EU567332 R5	EU567350 C3
	Sinyangri, Jeju4, Korea (20.Oct.07)	Gr4	EU567333 R3	EU567351 C3
	Sinyangri, Jeju5, Korea (20.Mar.07)	Gr5	EU567334 R3	EU567352 C3
	Sinyangri, Jeju6, Korea (10.Mar.05)	G529	EU567335 R3	-
	Dangmokri, Wando1, Korea (15.Oct.07)	Gr6	EU567336 R2	EU567353 C2
	Dangmokri, Wando2, Korea (15.Oct.07)	Gr7	EU567337 R2	EU567354 C2
	Janghung, Yeosu1, Korea (Nov.07)	Gr8	EU567338 R2	EU567355 C2
	Janghung, Yeosu2, Korea (Nov.07)	Gr9	EU567339 R2	-
	Sageunjin, Kangneung, Korea (20.Jul.06)	Gr10	-	EU567356 C6
	Janghung, Yeosu3, Korea (13.Mar.01)	G217	EU567340 R2	EU567357 C2
	Yeosu Uni., Yeosu4, Korea (06.Dec.02)	G181	EU567341 R2	-
	Hoidong, Jindo, Korea (09.Mar.01)[1]	G039	DQ095785 R2	EU567358 C2
	Sangjokam, Gosung, Korea (02.Mar.03)	G220	EU567342 R2	EU567359 C2
	Daeryeon, China (27.Oct.02)[2]	G210	EF434904 R2	EF434916
	QingdaoBeach, China (20.Dec.06)	Gr11	EU567344 R1	EU567360 C1
	Chiba, Japan (20.Feb.03)	G213	EU567343 R4	EU567361 C4
	Inuwaka, Chiba, Japan (31.Jul.04)	G319	-	EU567362 C2
	Konami, Oki island, Japan (05.May 03)	G279	EU567345 R2	EU567363 C2
	Shimoda, Shizuoka, Japan (18.Feb.03)	G214	EU567346 R4	EU567364 C5
	Shimoda, Shizuoka, Japan (18.Feb.03)	Gr12	EU567347 R4	EU567365 C5
Gracilariopsis lemaneiformis	Yacilla, Paita, Piura, Peru[3]		AY049415	-
Gracilariopsis "lemaneiformis"	Tosa Bay, Shikiku I., Japan[3]		AY049419	-
	Lake Butler, Robe, S.Australia, Australia[3]		AY049422	-
	Swakopsmud, Namibia[3]		AY049410	-
Gracilariopsis heteroclada	Dapdap, Bulusan, Luzon, Philippines[3]		AY049411	-
Gracilariopsis longissima	off Sandfoot Castle, Portland Harbour, Dorset, England[3]		AY049420	-
	Roscoff, France[1]		DQ096786	-
Gracilariopsis tenuifrons	Ilet Caret, Guadeloupe, French West Indies[4]		AY049418	-
Gracilariopsis andersonii	Seal Rock, Lincoln Co., Oregon, USA[3]		AY049414	-
Gracilariopsis costaricensis	South end, Playa Tamarindo, Nicoya Peninsula, Guanacaste, Costa Rica[3]		AY049423	-
Gracilariopsis carolinensis	Kure Beach, Fort Fisher, NC, USA[3]		AY049412	-
Gracilariopsis panamensis	Fort Randolph, Colon City, Panama[3]		AY049405	-
Gracilariopsis changii	Sail Rock, Kenting National Park, Taiwan[5]		DQ119746	-
				-
Outgroup				-
Gracilaria vermiculophylla	Bangpo, Taean, South Korea[2]		DQ095821	-
Gracilaria bursa-pastoris	Italy[6]		AY049373	-

[1]Kim *et al.* 2006, [2]Yang *et al.* 2008, [3]Gurgel *et al.* 2003, [4]Gurgel and Fredericq 2004, [5]Lin *et al.* unpublished, [6]Gargiulo *et al.* 2006.

Ohmi based on the absence of nutritive filaments connecting the gonimoblast and the pericarp. Yamamoto (1978), however, supported Papenfuss' proposal that *Gracilariopsis* should be merged with *Gracilaria* (Papenfuss 1967). Currently, *Gracilariopsis chorda* is treated as a synonym of *Gracilaria chorda* Holmes (Terada and Ohno 2000), although previous studies have supported *Gracilariopsis* as a primary lineage within the *Gracilaria*ceae (Bird *et al.* 1992; Bellorin *et al.* 2002; Gurgel and Fredericq 2004; Iyer *et al.* 2005).

In the present study, we used morphological observations and molecular phylogeny, *rbc*L and *cox*1 genes, to

confirm the taxonomic position of specimens currently named under *Gracilaria chorda*. We also discuss the phylogeny of *Gracilariopsis* based on plastid *rbc*L genes and the possible use of *cox*1 as a DNA barcode in Gracilariacean red algae (Robba *et al.* 2006).

MATERIALS AND METHODS

A total of 22 samples of *Gracilaria chorda* were collected in Korea, China, and Japan (Table 1). Materials for morphological study were collected from Dangmokri, Wando, Korea on October 15, 2007. Vouchers were deposited in the herbarium of the Department of Biology, Chungnam National University, Daejon, Korea (CNUK).

Genomic DNA was extracted from approximately 5 mg of dried thalli ground in liquid nitrogen using the Invisorb® Spin Plant Mini Kit (Invitek, Berlin-Buch, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using primers specific for each gene. The primers used to amplify the plastid *rbc*L region were designed based on previous studies (Freshwater and Rueness 1994; Lin *et al.* 2001; Gavio and Fredericq 2002). The mitochondrial *cox*1 region was amplified using the primers COXI43F and COXI1549R (Geraldino *et al.* 2006). PCR amplification was performed in a total volume of 25 μL containing the same components reported by Yang *et al.* (2008). PCR was carried out with an initial denaturation at 95°C for 4 min, followed by 35 cycles of amplification (denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 1 min), and a final extension at 72°C for 6 min. The PCR products were purified using the High Pure PCR Product Purification Kit (Roche) prior to direct sequencing. Sequencing reactions were performed using BigDye Terminator and 3100 Genetic Analyzers (Applied Biosystems). The (chromatogram) for each specimen was edited using the program Chromas version 1.45. Alignments were performed manually using the Se-Al version 2.0a11 program (Andrew Rambaut).

Bayesian analyses were conducted using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). Metropolis-coupled Markov chain Monte Carlo (MC^3) with the GTR + I + Γ model was used for individual data sets. For each matrix, two million generations of two independent runs were performed with four chains, and trees were sampled every 100 generations. The burn-in period was identified graphically by tracking the likelihoods at each generation to determine whether they had reached a plateau. The 30,002 trees sampled at stationarity were used to infer the Bayesian posterior probability (BPP).

Maximum parsimony (MP) analyses were conducted using PAUP* 4.0b10 software (Swofford 2002). All heuristic searches were performed with 1,000 replicates, employed the random addition of taxa, retained only the best tree, held ten trees at each step, used TBR branch swapping, collapsed zero-length branches, and used MULTREES. Bootstrap support values (MPBS) were calculated using 1,000 replicates with the following options selected: heuristic search, TBR branch swapping, collapse of zero-length branches, and random sequence addition with one replicate.

To assess the level of variation in the *rbc*L and *cox*1 sequences, uncorrected (*p*) pairwise genetic distances were estimated using PAUP*. Inter- and intraspecific *p* distances were calculated and plotted using JMP statistical software (version 4.0.2, SAS Institute Inc.). A statistical parsimony network was drawn for *cox*1 and *rbc*L haplotypes of *Gracilaria chorda* using the program TCS version 1.21 (Clement *et al.* 2000). The nucleotide diversity (Pi) and haplotype diversity (Hd) of each gene were determined using the DnaSP program (Rozas and Rozas 2000).

RESULTS

Morphology of *Gracilariopsis chorda*

Individual organisms cylindrical up to 30 cm tall, reddish brown or reddish purple, consisting of one to few irregularly and sparingly branched indeterminate axes from a discoid holdfast, sometimes with a few shorter proliferous laterals (Fig. 1A); axes broadening to 2 mm diameter and tapering toward the apices, the cortex consisting of globular cells with dense cytoplasm, the medullary cells unpigmented, spherical cells with vacuoles increasing abruptly in size toward the center (Figs 1B-D); cystocarps scattered over the axes and branches, dome-shaped, without a prominent beak (Figs 1E and 1F); pericarp composed of two to three layers of ovoid cells below, followed by six to eight layers of regularly arranged horizontally elongate inner cells that are stretched laterally in mature cystocarps; gonimoblasts pedicellate, attached to the cystocarp floor by palisade-like cells (Figs 1G and 1H), gonimoblast mass generally spherical, composed of sterile pseudo-parenchymatous cells that give rise to straight chains of darkly staining carposporangial initials; gonimoblast cells attached to

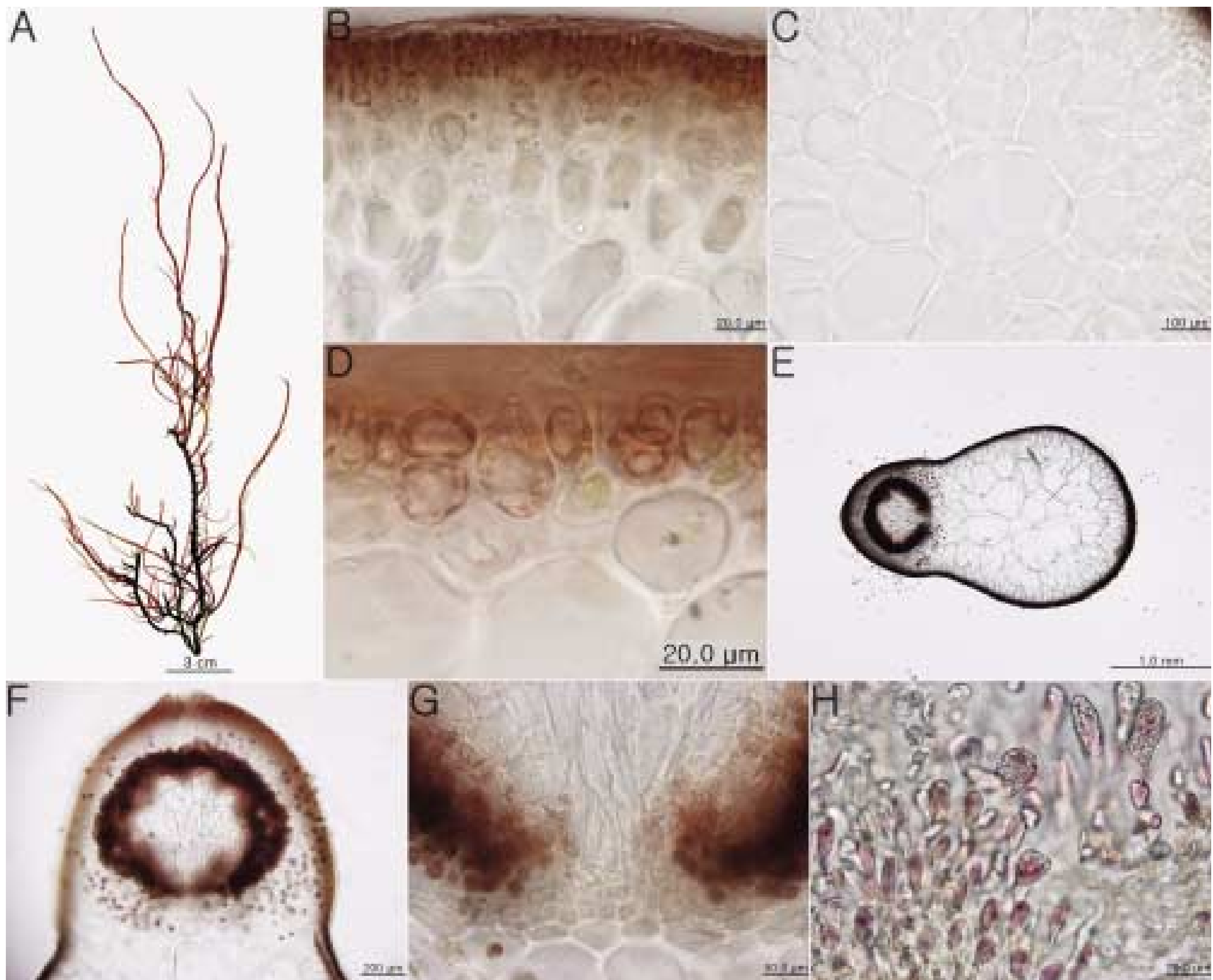

Fig. 1. *Gracilariopsis chorda* (Holmes) Ohmi collected from Dangmokri, Wando, Korea on October 15, 2007. A. Specimen demonstrating numerous short filiform branchlets. B-D. Transverse section of a main axis showing the cortical and basal cells of hairs in the cortex, and the abrupt transition in medullary cell size. E-F. Vertical median section of a mature cystocarp showing relatively large gonimoblast cells. G-H. Enlarged pictures of F showing the floor of the cystocarp with small cells and straight chains of carposporangial initials.

the vegetative floor of the cystocarp by means of ovoid to irregularly shaped conjunctor cells arising individually from basal gonimoblast cells, tubular nutritive cells absent (Figs 1G and 1H).

Characteristics of *rbc*L and *cox*1

MP analysis was used to compare *rbc*L and *cox*1 in the family Gracilariaceae (Table 2). A total of 1,222 base pairs in *rbc*L for 35 taxa from GenBank were aligned, and 18 new sequences were identified (Table 1). 139 sites (11.4%) were variable and 160 sites (13.1%) were parsimoniously informative (Table 2). The MP analysis tree length was 497, and the rescaled consistency index (RC) was 0.528. The sequences differed by up to 93 bp (8.0%) pairwise distance between *Gracilariopsis heteroclada* from the Philippines and *Gp. carolinensis* from the USA, although the average sequence divergence was 4.8%, with a range from 0% [0 bp difference within *Gp. chorda*] to 8.0% (data not shown). Within *Gp. chorda*, the differences were at most 8 bp (0.7%) pairwise divergence. The average sequence divergence was 0.34%, with a range from 0% to 0.65% (Fig. 4).

In *cox*1, a total of 1,245 base pairs were aligned for 41 taxa from GenBank, and 18 new sequences were identified (Table 1). 97 sites were variable (7.8%), and 337 sites (27.1%) were parsimoniously informative (Table 2). The

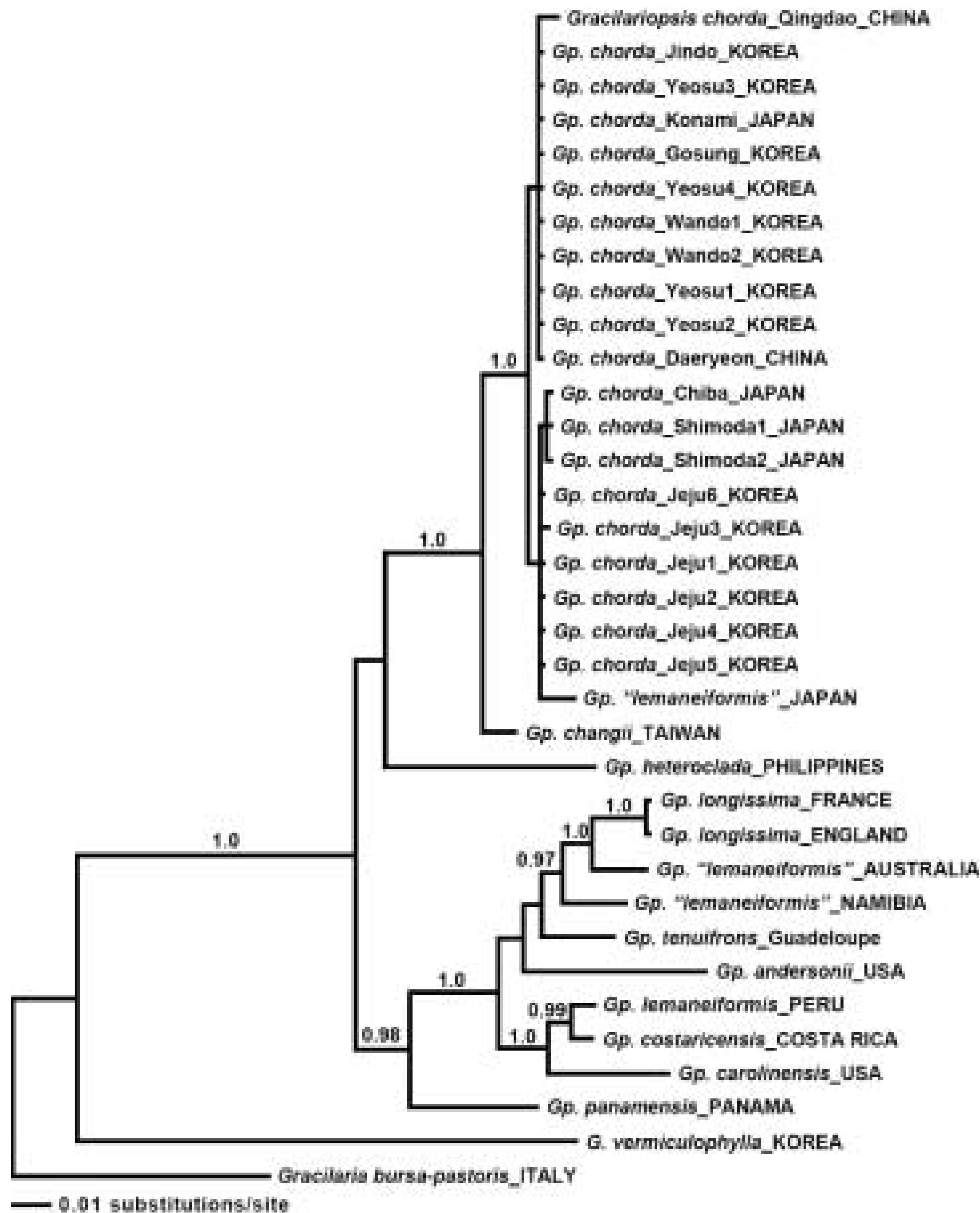

Fig. 2. Bayesian phylogeny estimated using *rbc*L sequences from *Gracilariopsis* analyzed by the GTR + I + Γ evolution model (-lnL = 4381.57). The numbers near each clade refer to Bayesian Posterior Probabilities.

Table 2. Statistics of *rbc*L and *cox*1 MP analyses

	*rbc*L	*cox*1
No. of taxa	35	41
Length (bp)	1,222	1,245
No. of variable sites (%)	139 (11.4)	97 (7.8)
No. of informative sites (%)	160 (13.1)	337 (27.1)
MP tree length	497	1,200
Rescaled consistency index (RC)	0.528	0.474

MP analysis tree length was 1,200, and the RC was 0.474 (tree not shown). The sequences differed by up to 202 bp (16.2%) pairwise distance between *Gp. chorda* from China and *Gracilaria gracilis* from Italy, although the average sequence divergence was 10.2%, with a range from 0% [0 bp difference within *Gp. chorda*] to 16.2% (data not shown). Within *Gp. chorda*, the differences were at most 5 bp (0.4%) pairwise divergence. The average sequence divergence was 0.20% with a range from 0 to 0.4% (Fig. 4).

Haplotype analysis of *Gracilariopsis chorda*

Twenty *Gp. chorda* samples were used for haplotype analysis of *rbc*L (Table 1). The nucleotide and haplotype diversity were 0.00244 and 0.706, respectively (Table 3).

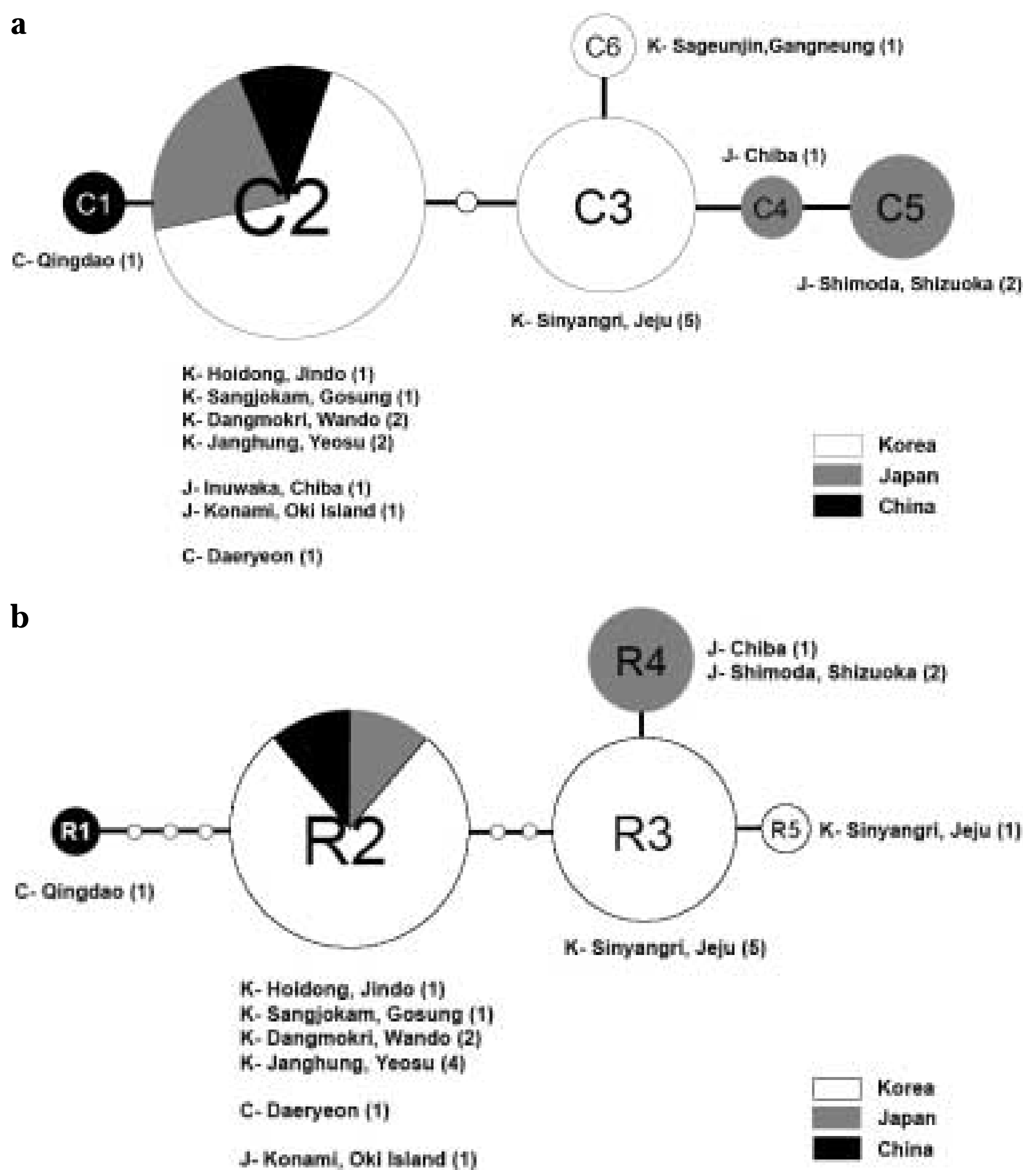

Fig. 3. Statistical parsimony network of mitochondrial *cox*1 haplotypes (a) and plastid *rbc*L haplotypes (b). The small unlabeled circles represent missing haplotypes; each line represents a single mutation. Circle sizes are proportionate to haplotype frequencies.

Table 3. Sequence variability within *Gracilariopsis chorda*

	*rbc*L	*cox*1
No. of taxa	20	19
Length (bp)	1,222	1,245
No. of variable sites (%)	17 (1.39)	5 (0.4)
Nucleotide diversity (Pi)	0.00244	0.00140
No. of haplotypes	5	6
Haplotype diversity (Hd)	0.706	0.725

RbcL analysis resulted in five haplotypes (Fig. 3b; haplotypes R1-R5). Haplotype R2, collected from the southern coast of Korea, China, and Japan, was identified as a basal haplotype and was also the most widespread (10 of 20 individuals). Haplotype R1 was identified from the east coast of China in Qingdao. Haplotypes R3 and R5 were identified from Jeju in Korea, and haplotype R4 from Japan was connected with haplotype R3.

For the *cox*1 analysis, 19 *Gp. chorda* samples were used (Table 1), and six haplotypes (Fig. 3a; haplotypes C1-C6) were identified. The nucleotide and haplotype diversity were 0.00140 and 0.725, respectively (Table 3). Haplotype C2, collected from the southern coast of Korea, was identified as a basal haplotype and was also the most widespread (9 of 19 individuals). Haplotype C1 was identified from the east coast of China in Qingdao. Haplotype C3 was identified from Jeju in Korea, and was connected with haplotype C6 from Gangneung, on the east coast of Korea. Haplotypes C4 and C5 were found in Chiba and

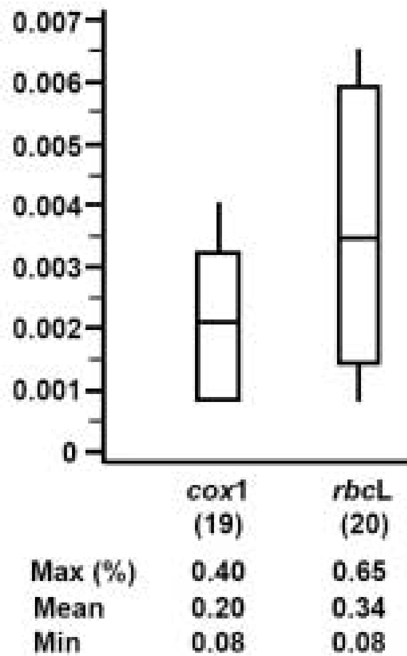

Fig. 4. Pairwise *p* distance distribution of *cox*1 and *rbc*L in *Gracilariopsis chorda*. Lines of box plots indicate mean distance, the surrounding box contains the middle 50% of the data, and whiskers extend to the maximum and minimum values. Numbers in parentheses indicate the number of sequences.

Shimoda, Japan.

Phylogeny of *Gracilariopsis*

The phylogenetic relationships of the *Gracilariopsis* inferred from a Bayesian analysis of 35 *rbc*L sequences including two outgroups is presented in Fig. 4. The *rbc*L tree divides the ingroup taxa into two clades. The first major clade consists of *Gp. chorda* and two Asian *Gracilariopsis*, *Gp. changii* from Taiwan and *Gp. heteroclada* from the Philippines. All *Gp. chorda* specimens formed a monophyletic group supported by a BPP value of 1.0. In the *Gp. chorda* clade, ingroup taxa split into two clades; one clade included specimens from the south coast of Korea (except Jeju), two samples from China, and one from west coast of Japan, and the other clade included samples from Jeju, Korea and the east coast of Japan. *Gp. changii* from Taiwan formed the most closely related sister group with the whole node of *Gp. chorda*. The node was supported at 1.0 by Bayesian posterior probabilities, whereas the node connected with *Gp. heteroclada* from the Philippines was only supported at 0.76 by BPP analysis. The second major clade comprised specimens from countries outside Asia.

The *cox*1 tree of 41 sequences from the Gracilariaceae, including one outgroup, was similar to that from the *rbc*L data set; *Gp. chorda* specimens were monophyletic and supported 100% by the bootstrap for ML (tree not shown).

DISCUSSION

The separate analyses of the *rbc*L and *cox*1 genes produced strongly supported congruent patterns, as demonstrated by the tree inferred from Bayesian analysis of *rbc*L shown in Fig. 2. All of the most recent molecular data have repeatedly confirmed that *Gracilariopsis* as a valid genus (Bellorin *et al.* 2002; Gurgel *et al.* 2003; Gurgel and Fredericq 2004; Iyer *et al.* 2005). The *rbc*L and *cox*1 data indicate that *Gracilaria chorda* represents an evolutionarily distinct species within the genus *Gracilariopsis*, and therefore we propose the following legitimate name in agreement with article 11.3 of the Vienna Code (McNeill *et al.* 2006):

Gracilariopsis chorda (Holmes) Ohmi

Basionym: *Gracilaria chorda* Holmes, in *Jour. Linn. Soc., Bot.*, 31: 253, 1896

Type locality: Enoura, Numazu City, Shizuoka Prefecture, Japan

Type: Saida 6, BM, London (Terada and Ohno 2000)

Although *Gp. chorda* collected from Qingdao, China and *Gp. "lemaneiformis"* from Tosa Bay, Japan (Gurgel *et al.* 2003) are quite variable in their *rbc*L genes (15 bp, 1.2%), all 22 samples of the species from Korea, China, and Japan produced a monophyletic group. RbcL analyses of pairwise base differences among northwest Asian *Gp. chorda* specimens diverge as much as 8 bp (0.7%) and are clearly distinct from *Gp. changii* of Taiwan, from which they differ by a minimum of 18 bp (1.5%). The sister-taxon relationship of *Gp. chorda* is more closely related to *Gp. changii* from Taiwan than to *Gp. heteroclada* from the Philippines, a pairwise base difference of 5.8%.

In a comparison with a previous study, *Gracilaria vermiculophylla* (Yang *et al.* 2008), for example, shows a pairwise base difference divergence of 0.29% in *rbc*L and 0.9% in *cox*1, whereas *Gracilariopsis chorda* diverges 0.3% in *rbc*L and 0.2% in *cox*1. Therefore, *rbc*L is slightly more variable than *cox*1 in the case of *Gp. chorda*. The nucleotide and haplotype diversity are also slightly higher in *rbc*L (Pi = 0.00244 and Hd = 0.706) than in *cox*1 (Pi = 0.00140 and Hd = 0.725). In *G. vermiculophylla*, the parsimony network analysis for *cox*1 reveals a relatively rea-

sonable cryptic diversity of the species, which is not supported by the *rbc*L analysis (Yang *et al.* 2008). In *Gp. chorda*, the parsimony network analysis for *cox*1 found only one additional haplotype (due to the sample from Kangneung, Korea) than for *rbc*L. Most haplotypes show similar patterns based on *rbc*L and *cox*1 analyses and are associated with specific geographical units.

The main finding of this study is that the *rbc*L and *cox*1 sequence data both clearly show the monophyly of *Gracilariopsis chorda* and its isolation from putative relatives of the genus. These results imply that *Gp. chorda* should be recognized as a species of the genus *Gracilariopsis*. *Gp. chorda* is characterized by coarse, elongate terete axes, sparingly to profusely branched usually at irregular intervals, an abrupt transition in cell size from medulla to cortex, cystocarps without tubular nutritive cells connecting the gonimoblast to the upper pericarp or cystocarp floor, and superficial male gametangia (Holmes 1896; Ohmi 1958; Yamamoto 1975 and 1978; Terada and Ohno 2000). Conversely, *Gracilariopsis* species do not always exhibit consistent cystocarp morphology, even though most of the accepted species of *Gracilariopsis* share the same basic vegetative and reproductive morphology (Gurgel *et al.* 2003). Although Papenfuss (1967) mentioned no fundamental difference in gonimoblast cell sizes of cystocarps in *Gp. sjoestedtii* from California compared with those of *G. verrucosa* (Hudson) Papenfuss from England, we observed relatively large gonimoblast cells of cystocarps in the *Gp. chorda* specimen collected from Wando in southern Korea (Fig. 1). The thallus is also highly variable in shape, showing irregular branching and short filiform branchlets similar to specimens from Akkeshi Lagoon (Hokkaido, Japan), which is at the northern limit of the distribution of *Gp. chorda* (Yamamoto 1978). Therefore, we conclude that Gurgel *et al.* (2003) recognized diagnostic characters of *Gracilariopsis*, such as superficial spermatangia, cystocarps lacking tubular cells, and gonimoblasts linked by secondary pit connections to modified gametophytic cells in the floor of the cystocarp, but failed to note the cystocarp gonimoblast cell sizes.

Additional taxon sampling of *Gracilariopsis* from the northwest Pacific Ocean may elucidate the evolutionary relationships of *Gp. chorda* within the genus and identify species diversity among the character-poor species of *Gracilariopsis*.

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