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Applying DNA barcoding to Korean Gracilariaceae (Rhodophyta)

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Abstract – The red algal family Gracilariaceae is commercially valuable due to its use in biotechnology and microbiology research as a phycocolloid agar. *Gracilaria* species are difficult to identify by morphological features alone, and the taxonomy of the gracilarioids occurring in Korea is uncertain. We tested the effectiveness of DNA barcoding in the identification and discovery of Gracilariaceae species in Korea. Our analysis of cytochrome *c* oxidase 1 (COI) sequences from 75 specimens yielded a total of five species: the four *Gracilaria* and one *Gracilariopsis* species were *Gracilaria incurvata*, *G. parvispora*, *G. textorii*, *G. vermiculophylla*, and *Gracilariopsis chorda*. Intraspecific uncorrected divergences ranged between 0% and 0.9%, and interspecific divergences were 9.2-16.1%. These findings prompted further taxonomic studies on the Gracilariaceae to better understand species diversity, with more extensive specimen sampling from the known distributional areas in the Asian-Pacific region. The results indicated the validity of mtDNA COI sequence data in identifying species via marine biomonitoring of family members and also in understanding the species boundaries of this group. Our study demonstrates that DNA barcoding can provide an efficient method for species-level identifications and contribute powerfully to taxonomic and biodiversity research.

COI / DNA barcoding / *Gracilaria* / *Gracilariopsis* / Rhodophyta

Résumé – Les Gracilariacées (algues rouges) sont exploitées commercialement pour en extraire de l'agar qui sert en recherche biotechnologique et microbiologique. Les espèces de *Gracilaria* sont difficiles à identifier sur la base de critères morphologiques et la taxonomie des gracilarioides présents en Corée est incertaine, Nous avons testé l'efficacité du code-barres ADN pour l'identification et la découverte d'espèces de Gracilariacées en Corée. Notre analyse des séquences codant la cytochrome oxydase 1 (COI) provenant de 75 spécimens ont permis de délimiter quatre espèces de *Gracilaria* et une espèce de *Gracilariopsis Gracilaria incurvata*, *G. parvispora*, *G. textorii*, *G. vermiculophylla*, and *Gracilariopsis chorda*. Les distances génétiques (non corrigées) intraspécifiques s'échelonnent entre 0 et 0,9 % et les interspécifiques varient de 9,2 à 16,1 %. Ces résultats ont suscité des études taxinomiques plus poussées pour mieux comprendre la diversité des espèces, avec un échantillonnage plus large incluant des spécimens de toute l'aire de répartition de ces espèces dans le Pacifique asiatique. Les résultats indiquent que le marqueur mitochondrial COI est valide pour identifier les espèces lors de suivit de la diversité marine et aussi pour comprendre les limites spécifiques au sein de cette famille. Notre étude a démontré que le code-barres ADN constitue une méthode efficace pour identifier les Gracilariacées et contribue efficacement à la taxonomie et aux recherches sur la biodiversité.

COI / code-barres ADN / *Gracilaria* / *Gracilariopsis* / Rhodophyta

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INTRODUCTION

The monophyly of the red algal family Gracilariaceae has been confirmed by 18S rDNA (Bellorin *et al.*, 2002) and *rbc*L (Gurgel & Fredericq, 2004) sequence analyses: the three main lineages are *Curdiea/Melanthalia*, *Gracilaria/Gracilariopsis*, and *Gracilaria sensu lato*. In particular, the genus *Gracilaria* has a worldwide distribution, with more than 150 valid species (Guiry & Guiry, 2010). *Gracilaria* species and related genera are commercially important, as they are agarophytes, which are used as human and shellfish food, as well as in biotechnology and microbiology research as a raw material from which phycocolloid agar is extracted (Byrne *et al.*, 2002; Cohen *et al.*, 2004). In the northwest Pacific Ocean, seven gracilarioid species (3 flattened and 4 cylindrical) have been reported, predominantly on the Korean coast: *Gracilaria cuneifolia* (Okamura) Lee *et* Kurogi, *G. gigas* Harvey, *G. incurvata* Okamura, *G. parvispora* Abbott, *G. textorii* (Suringar) Hariot, *G. vermiculophylla* (Ohmi) Papenfuss, and *Gracilariopsis chorda* (Holmes) Ohmi. However, in Korea, taxonomic studies of this group are insufficient, and species diversity is not fully clarified.

Gracilaria species are often difficult to identify from morphological features alone (Bellorin *et al.*, 2008). Within the Gracilariaceae, some species share a common but variable morphology and consequently are often misidentified. Thus, DNA sequence analysis is the most widely used molecular technique to infer phylogenetic relationships at the species level within the Gracilariaceae (Iyer *et al.*, 2005; Gargiulo *et al.*, 2006; Bellorin *et al.*, 2008). It has provided a useful taxonomic tool to distinguish organisms that are difficult to identify by more traditional means, as well as resolved evolutionary relationships among taxa. Given the morphological difficulties and new possibilities of accurate identification using molecular biology techniques, the taxonomic status of gracilarioids is in flux (Bellorin *et al.*, 2002). Using *rbc*L sequence data, Kim *et al.* (2008a, b) determined that a gracilarioid alga from Korea and Japan was *G. parvispora* and found that a *Gracilaria chorda* from Korea was actually *Gracilariopsis chorda*.

DNA barcoding is a diagnostic species identification technique that uses a short, standardized DNA region for which genetic variation between species typically exceeds that within species, to provide a rapid and efficient method of species-level identification in taxonomic and biodiversity research (Hajibabaei *et al.*, 2007). DNA barcoding using the mitochondrial cytochrome *c* oxidase I (COI) gene, a 660-base fragment at the 5' end of COI (*cox*1), is now well established for red algae (Saunders, 2005, 2009).

We tested the effectiveness of DNA barcoding in identifying and discovering gracilarioid species in Korea. We investigated whether COI barcodes provide sufficient resolution to identify specimens of morphologically identifiable species in the family Gracilariaceae. Because this family has been well studied taxonomically for at least two decades, it provides a template against which to test the accuracy of DNA barcoding.

MATERIALS AND METHODS

Table 1 lists the collection data for the samples included in this study. Total DNA was extracted from dried thalli ground in liquid nitrogen using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufac-

Table 1. Collection and taxonomic details

Taxon	*Collection information*	*Voucher*	*GenGank*
Gracilaria incurvata Okamura	Hagwi: Jeju: Korea (14. Apr. 2010)	HG100404-5	HQ322018
	Hansuri: Jeju: Korea (05. May. 2004)	CN-G353	HQ322020
	Hansuri: Jeju: Korea (05. May. 2004)	CN-G352	HQ322021
	Hansuri: Jeju: Korea (25. May. 2009)	G062	HQ322022
	Hansuri: Jeju: Korea (26. Jun. 2010)	G134	HQ322019
	Jongdal: Jeju: Korea (04. Apr. 2010)	JD100404-14	HQ322060
	Sukdawon: Hadori: Jeju: Korea (11. Aug. 2010)	G157	HQ322016
	Misaki: Japan (29. Apr. 2010)	MI03	HQ322017
	Misaki: Japan (29. Apr. 2010)	MI02	HQ322023
	Misaki: Japan (30. Apr. 2010)	MI58	HQ322024
	Misaki: Japan (30. Apr. 2010)	MI57	HQ322025
	Misaki: Japan (30. Apr. 2010)	MI56	HQ322026
Gracilaria parvispora Abbott	Daejung: Jeju: Korea (06. Jun. 2005)	GB0601	HQ322047
	Dukchonri: Geomundo: Korea (12. Jun. 2010)	GM51	HQ322030
	Hado: Jeju: Korea (08. Aug. 2010)	G153	HQ322033
	Hamduck: Jeju: Korea (21. Jul. 2009)	G074	HQ322034
	Jongdal: Jeju: Korea (04. Apr. 2010)	JD100404-20	HQ322038
	Jongdal: Jeju: Korea (29. May. 2010)	G143	HQ322029
	Sinyang: Jeju: Korea (27. May. 2010)	G154	HQ322032
	Sinyang: Jeju: Korea (27. May. 2010)	G150	HQ322028
	Sinyang: Jeju: Korea (27. May. 2010)	G152	HQ322027
	Misaki: Japan (30. Apr. 2010)	MI59	HQ322037
	Misaki: Japan (30. Apr. 2010)	MI60	HQ322036
	Misaki: Japan (30. Apr. 2010)	MI61	HQ322035
Gracilaria textorii (Suringar) Hariot	Chagwido: Jeju: Korea (14. Jun. 2009)	GT0616	HQ322064
	Hagwi: Jeju: Korea (14. Apr. 2010)	HG100414-4	HQ322063
	Hansuri: Jeju: Korea (23. May. 2010)	G064	HQ322070
	Hyodon: Jeju: Korea (17. Jan. 2010)	G095	HQ322069
	Jongdal: Jeju: Korea (04. Apr. 2010)	JD100404-9	HQ322062
	Pyosun: Jeju: Korea (17. Jan. 2010)	PS100117-10	HQ322061
	Pyosun: Jeju: Korea (17. Mar. 2010)	PS100317-2	HQ322051
	Udo: Jeju: Korea (17. Jun. 2009)	GT0615	HQ322065
	Udo: Jeju: Korea (28. Feb. 2010)	CJ100228-51	HQ322071
Gracilaria vermiculophylla (Ohmi) Papenfuss	Bangpo: Taean: Korea (16. Jul. 2003)	CN-G223	HQ322057
	Dukchonri: Geomundo: Korea (12. Jun. 2010)	GM74	HQ322054
	Godo: Geomundo: Korea (12. Jun. 2010)	GM01	HQ322053
	Haengwon: Jeju: Korea (15. Apr. 2010)	HW100415-46	HQ322043
	Hagwi: Jeju: Korea (14. Apr. 2010)	HG100414-1	HQ322044
	Iho: Jeju: Korea (02. Feb. 2006)	GV0202	HQ322040
	Jongdal: Jeju: Korea (04. Apr. 2010)	JD100404-8	HQ322042
	Keumneung: Jeju: Korea (15. Jan. 2010)	G099	HQ322048
	Kimnyeong: Jeju: Korea (11. Jun. 2005)	GC0601	HQ322077
	Molunde: Busan: Korea (15. Nov. 2009)	G068	HQ322059
	Pyosun: Jeju: Korea (17. Mar. 2010)	PS100317-1	HQ322052
	Sangjokam: Gosung: Korea (02. Mar. 2002)	CN-G221	HQ322058
	Sinyang: Jeju: Korea (18. Jan. 2010)	G100	HQ322039
	Sungsan: Jeju: Korea (04. Apr. 2010)	SS100404-8	HQ322049

Table 1. Collection and taxonomic details *(cont'd)*

Taxon	*Collection information*	*Voucher*	*GenGank*
	Woljung: Jeju: Korea (15. Oct. 2005)	GV1001	HQ322045
	Asamushi: Japan (26. Apr. 2005)	GV0405	HQ322046
	Inuwaka: Chiba: Japan (31. Jul. 2004)	CN-G317	HQ322055
	Konami: Oki Island: Japan (05. May. 2003)	CN-G281	HQ322056
	Nokonosima: Fukuoca: Japan (04. Mar. 2010)	YG012	HQ322041
	Shimoda: Shizuoka: Japan (18. Mar. 2003)	CN-G212	HQ322086
	China (28. Jan. 2000)	G3002	HQ322068
Gracilariopsis chorda (Holmes) Ohmi	Biyangdo: Jeju: Korea (20. May. 2010)	BY19	HQ322089
	Biyangdo: Jeju: Korea (20. May. 2010)	BY18	HQ322090
	Dangmokri: Wando: Korea (15. Oct. 2007)	CN-Gr6	HQ322081
	Hoidong: Jindo: Korea (09. Mar. 2001)	CN-G039	HQ322088
	Janghung: Korea (Nov. 2007)	CN-Gr8	HQ322080
	Jongdal: Jeju: Korea (04. Apr. 2010)	JD100404-21	HQ322076
	Jongdal: Jeju: Korea (06. Aug. 2010)	G161	HQ322031
	Jongdal: Jeju: Korea (24. Jan. 2008)	G3030	HQ322066
	Jongdal: Jeju: Korea (29. May. 2010)	G121	HQ322079
	Ojori: Jeju: Korea (23. Jan. 2008)	G3028	HQ322067
	Ojori: Jeju: Korea (23. Jan. 2008)	G3029	HQ322050
	Sangjokam: Gosung: Korea (02. Mar. 2003)	CN-G220	HQ322083
	Chiba: Japan (20. Feb. 2003)	CN-G213	HQ322085
	Japan (29. Mar. 2007)	GC0304	HQ322078
	Konami: Oki Island: Japan (05. May. 2003)	CN-G279	HQ322082
	Misaki: Japan (30. Apr. 2010)	MI41	HQ322075
	Misaki: Japan (30. Apr. 2010)	MI42	HQ322074
	Misaki: Japan (30. Apr. 2010)	MI62	HQ322073
	Misaki: Japan (30. Apr. 2010)	MI63	HQ322072
	Shimoda: Shizuoka: Japan (18. Feb. 2003)	CN-G214	HQ322084
	Daeryeon: China (27. Oct. 2007)	CN-G210	HQ322087

turer's instructions. The COI-5' region was amplified via polymerase chain reaction (PCR) as outlined in Saunders (2009) using the forward primers GazF1 (Saunders, 2005), GHalF (Saunders, 2008), and GWSFn (personal communication with Dr. Saunders) variously combined with the reverse primers GazRI (Saunders, 2005), GWSRx (pers. com. with Dr. Saunders), and COX1R1 (Saunders, 2008). Amplified products were cleaned before sequencing using an ABI PRISM BigDye Terminator Cycle Sequencing Kit, following the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Forward and reverse sequence reads from the respective PCR primers (Saunders, 2009) were edited using Chromas ver 1.45, and a multiple sequence alignment was constructed in BioEdit. The alignment included 81 taxa (75 from this study and six from GenBank: *Gracilaria tikvahiae* McLachlan (FJ499546), *G. salicornia* (C. Agardh) E.Y. Dawson (FJ499537), *G. pacifica* I.A. Abbott (FJ499511), *G. vermiculophylla* (Ohmi) Papenfuss (FJ499590), *Gracilariopsis longissima* (S.G. Gmelin) M. Steentoft, L.M. Irvine *et* W.F. Farnham (FJ499660), and *Gp. andersonii* (Grunow) E.Y. Dawson

(FJ499637)) with 661 nucleotide positions. Analyses were conducted using PAUP* 4.0b10 (Swofford, 2003) and MEGA 4.02 (Tamura *et al.*, 2007), with distances corrected via a general time-reversible model. Neighbor-joining (NJ) by MEGA 4.02 was used to provide a visual display of COI-5' variation within and between species.

For morphological observations, all collections photographed using a Stylus 1030 SW digital camera (Olympus, Tokyo, Japan). Digital images were edited and assembled on plates using Photoshop 5.5 (Adobe, San Jose, CA, USA). Vouchers are housed at the herbarium of Jeju National University, Jeju-do, Korea (JNUB).

RESULTS

Molecular results

We obtained the COI sequences of 75 gracilariacean specimens, in addition to the six sequences from GenBank (Table 1). The amplified COI region ranged from 670 to 685 bp, depending on the primer combination used, of which we analyzed 661 bp corresponding to the sequences from GenBank. An unweighted pair group method with arithmetic mean (UPGMA) phenogram (Fig. 1) based on these sequences illustrates the levels of divergence within and between morphologically identified species. The 75 individuals resolved into five expected clusters that were assignable to *Gracilaria incurvata* (n=12; 7 isolates from Korea and 5 from Japan), *G. parvisphora* (n=12; 9 from Korea and 3 from Japan), *G. textorii* (n=9; all from Korea), *G. vermiculophylla* (n=21; 15 from Korea, 5 from Japan, 1 from China), and *Gracilariopsis chorda* (n=21; 12 from Korea, 8 from Japan, 1 from China). The within-species variation of *Gracilaria* was generally between 0% and 0.9% divergence, and 0.3% within *Gracilariopsis*. Between species variation within *Gracilaria* ranged from 9.2% (between *G. parvispora* and *G. textorii*) to 13.9% (between *G. vermiculophylla* and *G. textorii*) divergence. Intergenus variation between *Gracilaria* and *Gracilariopsis* ranged from 14.1% (between *G. vermiculophylla* and *Gp. chorda*) to 16.1% (between *G. incurvata* and *Gp. chorda*).

Distribution, ecology, and morphology

Gracilaria incurvata occurs along the southern and eastern coasts of Korea, including Jeju Island. *Gracilaria incurvata* grows in the intertidal areas of Jeju Island, attached to rocks or stones in tide pools, which are often covered with sandy mud in protected, calm waters. The alga has a flattened form, membranous texture, and purplish-red color. A blade arises from a small discoid holdfast with a short subcylindrical stipe and is 4-5 times dichotomously branched, with incurved apices and twisted branches. Branch margins are entire or sometimes proliferous. *G. incurvata* is distinguished by having narrow blades (0.5-1 cm), 7-12 cm height, and 3-6 cell layers comprising the 350-450-μm medulla. The thallus is thick, with a fleshy texture (Figs 2-7).

Gracilaria parvispora is commonly collected along the coast of Jeju Island, Korea. It attaches to rocks and stones in sand in the sublittoral zone of

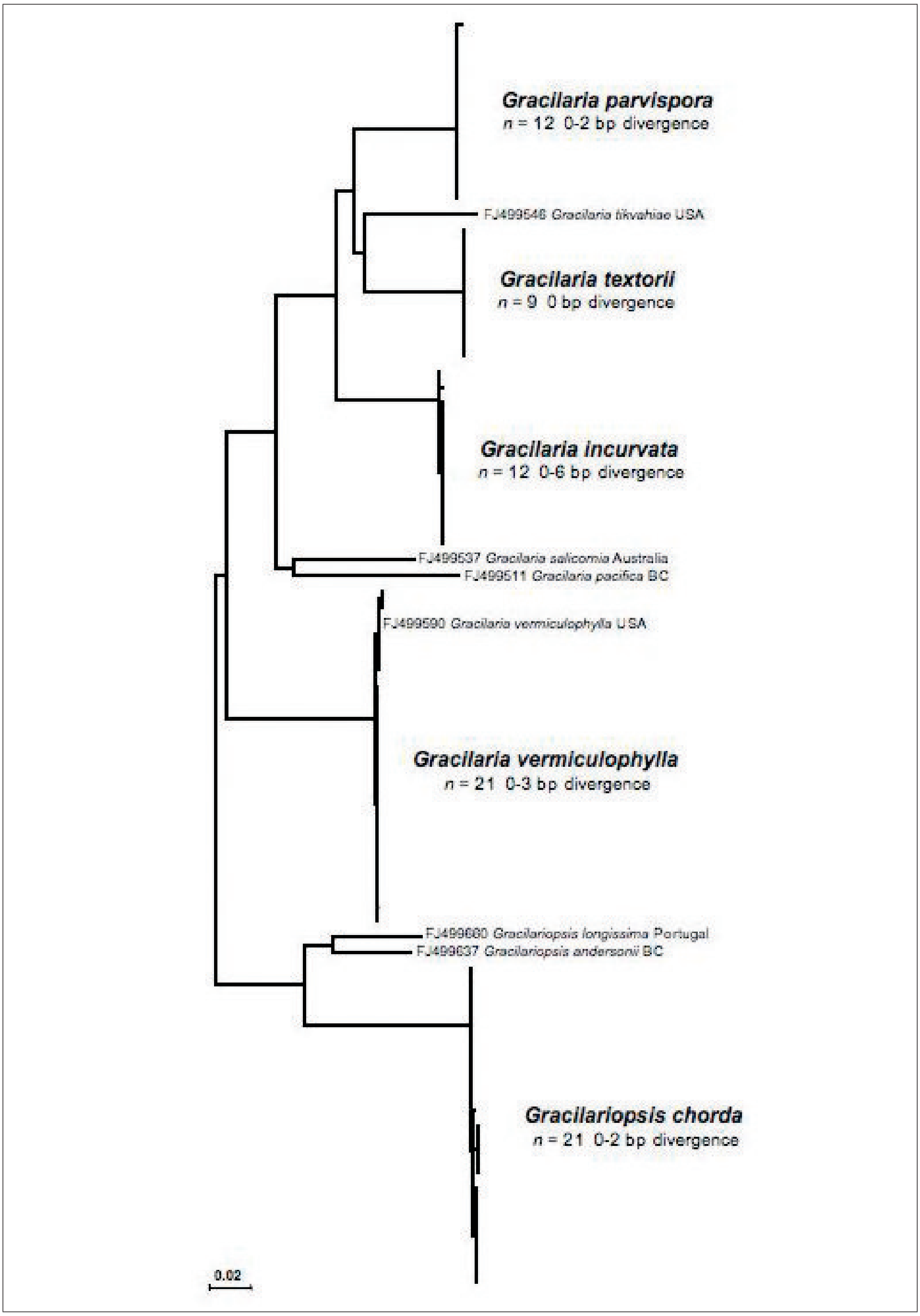
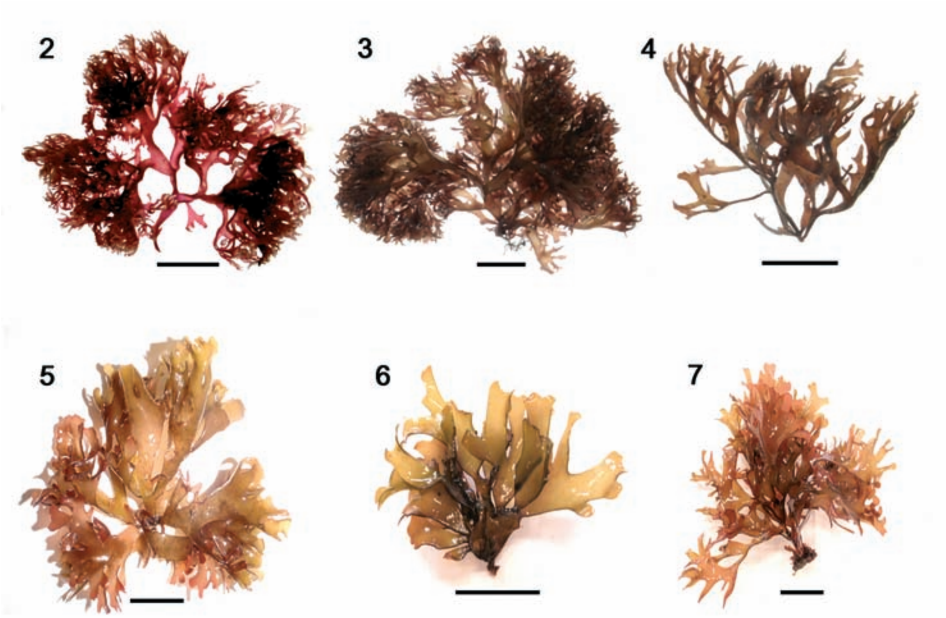


Fig. 1. Unrooted phylogram generated using neighbor-joining analyses, from COI sequences of samples collected in this study (taxa in bold) and acquired from GenBank. Scale bar: substitutions/site.

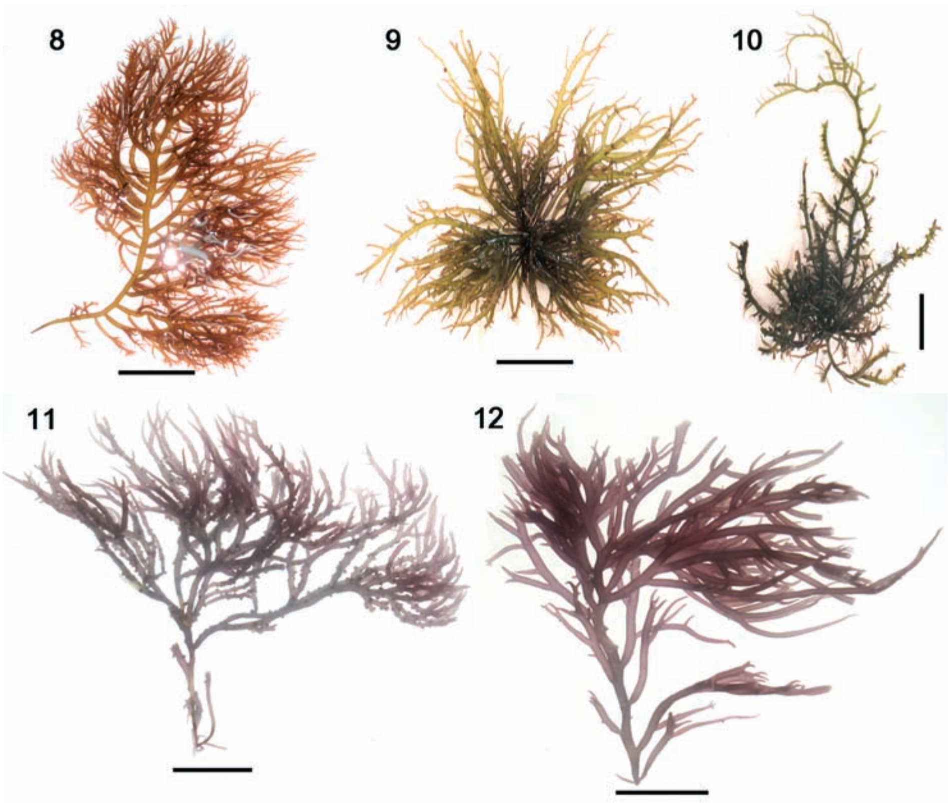


Figs 2-7. *Gracilaria incurvata* Okamura. **2-4.** Tetrasporophyte (Hagwi, 30 vii 2009). **5-7.** Tetrasporophyte (Misaki, Japan, 30 iv 2010). Scale bars: 3 cm.

calm areas near the open sea. The species grows underwater at low tide and in places with flowing water. Thalli are erect, coarse, compressed, and solitary. They arise from a small disk, usually 15-23 cm long and 2–3.5 mm in diameter, with 2–4 orders of branches, the last order being short, slender, and spinelike. The alga is succulent, percurrent, easily broken, and brittle when dry. Thalli are purplish-red or sometimes greenish-red when fresh. Branches are irregularly alternate, occasionally furcated, and strongly mixed with unilateral or secund branching in whole or in part (Figs 8-12).

Gracilaria textorii is widely distributed on the Korean coast and is one of the most common red algal species in Korea. *Gracilaria textorii* grows in the upper sublittoral zone and below low water, attached to rocks or stones. The species appears to be common in exposed sites, but it is also abundant in tide pools during low tide. The alga has a flattened, erect form, coriaceous texture, and is brownish-red to yellowish-red or greenish-red in color. The plant grows up to 10-20 cm high and is 2-5 cm wide and 750-950 µm thick. The blade, arising from a small discoid holdfast with a short subcylindrical stipe, expands into irregularly subdichotomous or flabellate-cuneate branches. Branch apices are rounded or attenuated and bifurcate. Branch margins are smooth and entire, normally nonproliferous but occasionally composed of numerous branchlets. This flattened species is highly variable in morphology (Figs 13-20).

Gracilaria vermiculophylla is common along the entire Korean coastline of the northwest Pacific Ocean region. The alga grows on small pebbles in upper intertidal zones with freshwater influx and muddy estuarine environments. Thalli are erect, solitary, or caespitose and cylindrical throughout, usually 7-30 cm long and 1-2 mm in diameter, with two or four orders of branches. Branches are



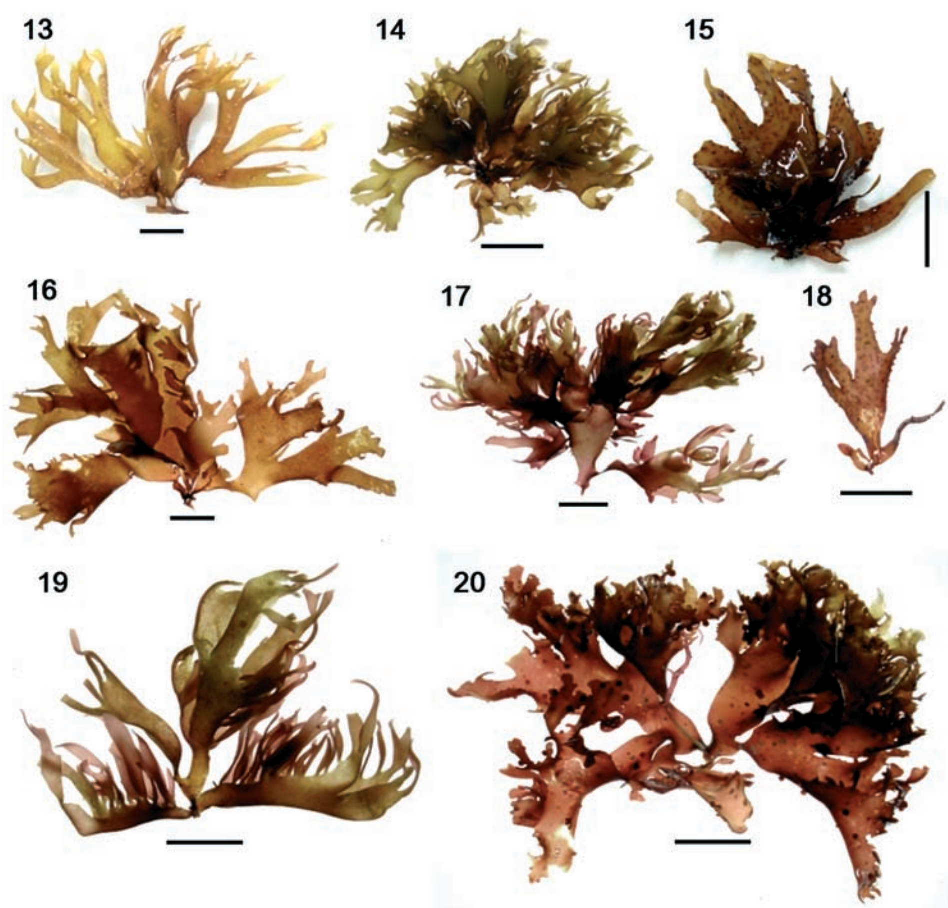
Figs 8-12. *Gracilaria parvispora* Abbott. **8.** Vegetative thallus (Sinyang, 27 v 2010). **9-10.** Tetrasporophyte (Misaki, Japan. 30 iv 2010). **11.** Carposporophyte (Heongwon, 22 viii 2009). **12.** Tetrasporophyte (Heongwon, 22 viii 2009). Scale bars: 3 cm for Figs 8 and 11, 5 cm for Figs 9 and 10, 2 cm for Fig. 12.

irregularly alternate or secund, taper gradually toward the apices, and are slightly or abruptly constricted at the base (Figs 21-26).

Gracilariopsis chorda occurs along the southern part of Korea, including Jeju Island and grows on pebbles, shells, rocks, and ropes of cultivation rafts, from the low tide mark down to the upper sublittoral zones, in comparatively calm waters. *Gracilariopsis chorda* is characterized by its large size, small branches, and very large medullary cells. Thalli are coarse, elongate terete axes up to 30 cm tall, 2 mm in diameter, reddish-brown or reddish-purple in color, and consist of one to a few irregularly and sparingly branched indeterminate axes from a discoid holdfast, sometimes with a few shorter proliferous laterals. The main axis is more or less traceable, or distinctive, not abundantly branched, generally slightly or markedly constricted at the base, and sometimes compressed as it broadens (Figs 27-31).

DISCUSSION

This study represents the first DNA barcoding contribution to the taxonomy of gracilarioid algae from Korea. The DNA barcoding data obtained permitted us to verify four *Gracilaria* and one *Gracilariopsis* species from Korea.



Figs 13-20. *Gracilaria textorii* (Suringar) Hariot. **13.** Tetrasprororphyte (Udo, 22 vi 2009). **14-16.** Tetrasporophyte (Udo, 22 vi 2009). **15.** Carposporophyte (Hansu-ri, 25 v 2009). **17-19.** Tetrasporophyte (Sagye, 24 vi 2009). **18.** Carposporophyte (Udo, 23 vi 2009). **20.** Carposporophyte (Udo, 23 vii 2009). Scale bars: 3 cm for Figs 13-19, 2 cm for Fig. 20.

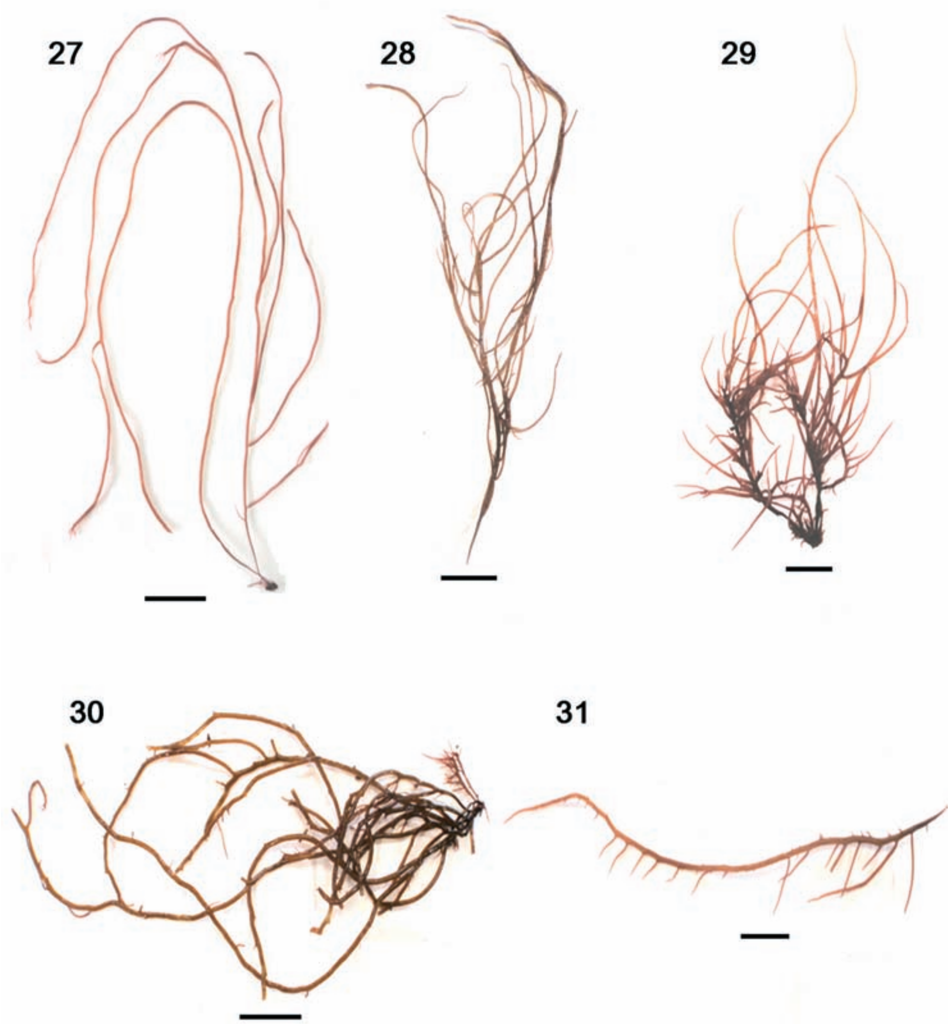
We analyzed the COI sequences of 75 specimens of the Gracilariaceae and found that 5 species were unambiguously distinguishable, as their barcode sequences formed distinct, nonoverlapping clusters in a NJ analysis.

About 40 flattened or compressed species of *Gracilaria* can be found throughout the world (Hoyle, 1994; Millar, 1997; Gurgel *et al.*, 2004), of which about 10 (5 in China, 6 in Japan) are reported from the Northwest Pacific region (Skelton *et al.*, 2004). In Korea, two flattened *Gracilaria* species were confirmed in this study: *G. textorii* and *G. incurvata* showed average inter-species divergences of 9.9%~10.3%, whereas the respective average within-species divergences were 0% and 0.9%, respectively. *Gracilaria textorii* is one of the most common red algal species in Korea (Lee & Kang, 2001) and is distributed along the Pacific coast of Asia and North America, extending into the Indian Ocean (Guiry & Guiry, 2010). All of our *G. textorii* collections from Korea had identical DNA barcodes, indicating the presence of a single species, characterized by a flattened

Figs 21-26. *Gracilaria vermiculophylla* (Ohmi) Papenfuss. **21.** Carposporophyte (Donggwi, 16 ix 2009). **22.** Tetrasporophyte (Donggwi, 16 ix 2009). **23-24.** Tetrasporophyte (Gimnyeong, 31 vii 2009). **25.** Tetrasporophyte (Geomundo, 12 vi 2010). **26.** Tetrasporophyte (Heongwon, 15 iv 2010). Scale bar: 2 cm for Figs 21-24, 5 cm for Figs 25 and 26.

frond with irregular dichotomous branching, large medullary cells with starch grains, and hemispherical cystocarps scattered on both sides of the blade (Yamamoto, 1978; Tseng & Xia, 1999).

Zhang and Xia (1994) reported a rare species, *G. cuneifolia* collected as a drift sample from Hainan Province, China, with a thin, membranous, narrow frond and shallow, saucer-shaped spermatangial configurations. Kim *et al.* (2006) provided molecular evidence of *G. textorii* and *G. cuneifolia* from Korea and Japan using plastid-coding *rbc*L and *psb*A genes. Their molecular phylogenetic analyses revealed the uniqueness of *G. cuneifolia*, which has a considerable amount of gene sequence divergence from its putative relatives. In addition, both the *rbc*L and *psb*A sequences clearly separated four samples of *G. incurvata* in Japan from *G. textorii*. However, we recently collected *G. incurvata* from the type locality in Misaki, Kanagagwa Prefecture, Japan, and analyzed this species using COI DNA barcoding. We found that five specimens of *G. incurvata* from Misaki and two of *G. cuneifolia* from Korea were identical. Three of the flattened species from Korea are difficult to identify because of variations in frond morphology and inconsistent branching patterns. *G. incurvata* is very similar to *G. textorii* and



Figs 27-31. *Gracilariopsis chorda* (Holmes) Ohmi. **27.** Vegetative plant (Biyangdo, 20 v 2010). **28.** Vegetative plant (Geomundo, 12 vi 2010). **29-31.** Vegetative plant (Misaki, Japan, 30 iv 2010). Scale bars: 5 cm for Figs 27 and 29-31, 3 cm for Fig. 28.

G. cuneifolia in having flattened thalli and shallow spermatangial conceptacles (Yamamoto, 1978); however, it is distinguished by narrower blades, smaller medulla cells relative to *G. textorii*, incurved apices, and twisted branches. *G. cuneifolia* is decidedly membranous in texture, with 2-3 medulla cell layers, whereas the thallus of *G. incurvata* has a fleshy texture and 3-6 medulla cell layers (Lee & Kurogi, 1977). *Gracilaria incurvata* occurs in calm, protected waters, whereas *G. textorii* usually occurs in exposed areas, and *G. cuneifolia* in deep water, where it is usually collected as a drift specimen. Hoyle (1994) divided *Gracilaria* species into three categories of flattened thalli for comparative purposes, based on the morphology of the margins: proliferous, nonproliferous, and species that are usually nonproliferous but sometimes have proliferations. Inasmuch as *G. incurvata* is usually found entire, but sometimes with proliferations, it falls within the third category.

Gracilaria parvispora is reported from Hawaii to East Asia, growing on rocks and stones in sand in the sublittoral zone of calm areas near the open sea (Abbott, 1985). The plastid *rbc*L gene sequence analysis of Kim *et al.* (2008a) of Korean, Japanese, and Hawaiian *G. parvispora* samples showed that the taxon from all three countries was identical. In this COI DNA barcoding study, we examined three *G. parvispora* samples from Misaki, Japan, and nine from Jeju, Korea. The DNA barcoding data of all of these samples were identical, except those from Hamduck and Hado, Jeju, Korea. Two samples differed in two base pairs from the other 10, which had identical sequences. Three from the same shore region of Misaki, Japan, were identical to the seven others from different sites of Korea. *Gracilaria parvispora* has several distinguishing characteristics, including compressed thalli with prominent unilateral branching, conspicuous absorbing filaments in the cystocarps, small, star-shaped cells in the pericarp, and shallow spermatangia conceptacle. *Gracilaria parvispora* from Korea was the first recorded occurrence outside Hawaii since the species was recognized as a new species (Abbott, 1985; Kim *et al.*, 2008a).

Gracilaria vermiculophylla is distributed from the northwest and southeast Pacific Ocean to the northeastern Atlantic Ocean (Bellorin *et al.*, 2004; Rueness, 2005; Yang *et al.*, 2008). Recently, this species was recorded in Peter the Great Bay, Russia (Skriptsova & Choi, 2009) and British Columbia (BC), Canada (Saunders, 2009). *G. vermiculophylla* grows well in the low to upper intertidal zones and is commonly associated with freshwater influx and confined to muddy estuarine habitats. Saunders (2009) discussed the ecological impacts of invasive species and *G. vermiculophylla* has proved to be a problem in Bamfield, BC, algal communities. Bellorin *et al.* (2004) found that identifying gracilarioids on the basis of terete form is difficult, owing to morphological plasticity and convergence, although *G. vermiculophylla* is characterized by traversing filaments in cystocarps, large gonimoblasts having small, densely protoplasmic cells, and vermiform upper branches (Terada & Yamamoto, 2002). We analyzed 21 *G. vermiculophylla* samples via COI DNA barcoding: 15 from Korea, five from Japan, and 1 from China. Of these, 15, including one from Japan and the Chinese specimen, had identical COI sequences. Seven others from Korea (2), Japan (4), and the United States (1) had intraspecific variations of between 0 and 3 bp.

The most recent molecular data have repeatedly confirmed *Gracilariopsis* as a valid genus (Gurgel *et al.*, 2003; Bellorin *et al.*, 2008). Most of the accepted species of *Gracilariopsis* share the same basic vegetative and reproductive morphology, and almost all have the following main features: slender, elongate terete axes, abrupt transition in cell size from medulla to cortex, cystocarps without tubular nutritive cells, and superficial male gametangia (Gurgel *et al.*, 2003; Kim *et al.*, 2008b). Bellorin *et al.* (2008) noted that the importance of informative DNA sequence comparisons to solve species boundaries is paramount, due to the difficulty in recognizing species within *Gracilariopsis* based on morphology. We included 21 *Gp. chorda* samples (12 from Korea, 8 from Japan, and 1 from China) in this DNA barcoding study and obtained a 0.3% average within-species divergence. Two base pairs differed among four samples that came from the same site in Misaki, Japan, and eight samples from Korea and Japan.

Molecular data have largely supported morphological concepts, but morphology alone cannot reveal the true diversity hidden within some species groups (Byrne *et al.*, 2002; Bellorin *et al.*, 2008). In a test of the ability of COI to identify accurately specimens of the Gracilariaceae in BC, Saunders (2009) reported successful identifications to the species level for representatives of this morphologically confused taxon and uncovered *G. vermiculophylla*, which was not known previously from those waters. The 75 specimens examined in our study were unambiguously identified establishing that DNA barcoding is an effective tool for gracilarioid species recognition in the Korean flora as well.

The economic ramifications of accurate gracilarioid identifications are important, since the Gracilariaceae provide the main source of agar (Oliveira *et al.*, 2000). Morphologically convergent species may have different agar yields and other qualities necessitating accurate identification for the best use and marketing of the product (Bellorin *et al.*, 2004). However, taxonomic expertise in the Gracilariaceae is limited, and morphological identification is often difficult. Thus, a DNA-based identification system using COI profiles could provide an important tool for species identification in gracilarioid marine biomonitoring. Geographically widely sampled specimens are vital to assess the possible extensive COI diversity, as observed by Saunders (2009). Our results highlight the utility of mtDNA COI sequence characters to identify species, and thus potentially also to understand the species boundaries of this group of the Gracilariaceae. In a forthcoming study, we will sample specimens over a broad geographic range of the Asia-Pacific area for DNA barcoding studies, as the coupling of detailed morphological and ecological investigations with barcode results is critical for a valid documentation of species richness.

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