

Molecular Phylogeny of the Genus *Chondracanthus* (Rhodophyta), Focusing on the Resurrection of *C. okamurae* and the Description of *C. cincinnus* sp. nov.

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Abstract – Determining the taxonomic status of the red algal genus *Chondracanthus* based on morphological characters is challenging due to the similarity and high degree of plasticity of the thallus. Since the taxonomic history of several *Chondracanthus* species remains unclear, we analyzed the plastid *rbcL* and mitochondrial COI genes of the specimens from Korea and Japan, in combination with morphological observations, to examine their phylogenetic relationships. Our results confirmed the distinction of *C. okamurae*, which is separated from *C. intermedius*, and identified a novel species, *C. cincinnus* sp. nov. Three species (*C. okamurae*, *C. intermedius* and *C. cincinnus*) formed a monophyletic clade with *C. tenellus*. *C. okamurae* is distinguished by linear, narrow, cylindrical to compressed, slightly recurved axes, and a high-intertidal to subtidal distribution. It was collected from Korea and Japan, while *C. intermedius* was identified from Japan only. A new species, *Chondracanthus cincinnus* sp. nov., is characterized by linear, compressed, strongly recurved axes, and a low-intertidal to subtidal distribution. Based on the molecular phylogeny using *rbcL* and COI data, we herein resurrect *C. okamurae* as a distinct species and identify *C. cincinnus* as a new species.

Key words – *Chondracanthus*, *C. cincinnus* sp. nov., *C. okamurae*, molecular phylogeny, Rhodophyta

1. Introduction

Recently conducted taxonomic studies and molecular analyses have assigned a more precise taxonomic status to specimens identified by morphological characters alone (Hind et al. 2014; Kang et al. 2015; Yang et al. 2015). Using molecular analyses, species diversity may be clearly resolved into regional groups. For example, five species of *Gelidium* J.V. Lamouroux were added to the flora of marine algae in Korea (Kim et al. 2011,

2012), while nine species of *Martensia* Hering were decreased to two entities in this region (Kang et al. 2015; Lin et al. 2013). Molecular analyses have also been used to uncover cryptic species having simple morphology and convergent characters (Saunders 2008; Yang and Kim 2015).

Chondracanthus Kützting is one of nine genera in the red algal family Gigartinaceae, which was resurrected by Hommersand et al. (1993). The generitype is *Chondracanthus chauvinii* (Bory) Kützting from the western Pacific region of South America. *Chondracanthus* is currently characterized by its erect thalli, which are pinnately branched or foliaceous; a proliferation bearing numerous vegetative or reproductive branchlets; procarps initiated on ordinary branchlets; and tetrasporangial sori located in the inner cortex (Hommersand et al. 1993). However, the similarity and high degree of plasticity of the thallus shape have led to a longstanding controversy over the taxonomic status of *Chondracanthus* species (Hommersand et al. 1993; Hughey and Hommersand 2008). For example, *C. johnstonii* (E.Y. Dawson) Guiry and *C. macdougalii* (E.Y. Dawson) Guiry was revealed to be the same species as *C. squarulosus* (Setchell et Gardner) J.R. Hughey, P.C. Silva et M.H. Hommersand, which were manifested as different forms induced by different environmental conditions (Hughey and Hommersand 2008).

Chondracanthus currently includes 21 valid species, distributed in tropical to warm temperate water (Guiry and Guiry 2015). The present center of distribution is presumed to lie in the eastern North Pacific Ocean (Hommersand et al. 1993), where two new species were recently reported using molecular evidence, namely *C. bajaclifornicus* and *C. kjeldsenii* (Hughey and Hommersand 2008). The majority of species in *Chondracanthus* have been transferred from *Gigartina*

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(Hommersand et al. 1993), including *C. intermedius* (Suringar) Hommersand and *C. tenellus* (Harvey) Hommersand. These two species are both distributed in the western Pacific Ocean, and *C. intermedius* occurs in the eastern Pacific and Australia region as well.

Hommersand et al. (1994, 1999) initiated a molecular systematic study on the genus *Chondracanthus* and related taxa by analyzing plastid *rbcL* sequences. Afterwards, Schneider and Lane (2005) reported a new species from Bermuda using *rbcL* sequences. This species had been assigned in the past to *C. acicularis* (Roth) Fredericq and *C. teedei* (Mertens et Roth) J.V. Lamouroux because of morphological similarity and overlapping distributions. Combining intensive sampling and re-examination of herbarium specimens with *rbcL* and ITS sequences, Hughey and Hommersand (2008) identified two new species and corrected many misidentified species in previous studies conducted in Pacific North America. Norris (2014), using morphological observations, described a new species from the Gulf of California that had been referred to as *C. intermedius* in this region.

In Korea and Japan, three species of *Chondracanthus* occur in intertidal to subtidal habitats (Kang 1968; Lee 2008; Lee and Kang 2001; Mikami 1965; Yoshida 1998). Abbott (1998) reported a new species, *Chondracanthus okamurae*, based on the Okamura's specimens from Japan, but it was later merged into *C. intermedius* as a synonym by Masuda et al. (1999). Recently, the name of *C. chamissoi* (*C. Agardh*) Kützing, originally endemic to South America, was assigned to the specimens of *C. teedei* from Korea and Japan using molecular evidence (Yang et al. 2015). *C. intermedius* and *C. tenellus* were originally described in Japan and are frequently collected along the coastline in Korea and Japan. These two species have similar morphological features, but the more erect and dense fronds of *C. tenellus* make that species distinguishable from *C. intermedius* (Mikami 1965).

In this study, we used the plastid-encoded large subunit of RuBisCO (*rbcL*) and mitochondrial cytochrome *c* oxidase subunit I (COI) sequences to assess the species diversity of *Chondracanthus* in Korea and Japan.

2. Materials and Methods

Taxon sampling and morphology

Representative *Chondracanthus okamurae* specimens were collected in high-intertidal to subtidal zones of Korea and Japan (Table 1). Field-collected samples were put in an icebox

with seawater, and transported to the laboratory. Small parts of the thallus were desiccated in silica gel for molecular study, while remaining parts were mounted on herbarium sheets. The herbarium specimens were deposited at the Herbarium of Jeju National University Biology (JNUB), Jeju, and the National Institute of Biological Resources (NIBR), Incheon, Korea. Materials for morphological observations were fixed in 5% formalin/seawater. Tissues were sectioned using a freezing microtome (NK-101-II; Nippon Optical Works Co., Ltd., Tokyo, Japan), and sectioned preparations were stained with 1% aqueous aniline blue, acidified with a drop of 1% HCl. The stained sections were mounted in 40% Karo corn syrup. Photomicrographs were taken using a QImaging 1394 camera (QImaging, Surrey, BC, Canada) attached to a BX50 microscope (Olympus, Tokyo, Japan). All images were imported into the Adobe PhotoShop 5.5 software (Adobe Systems Inc., San Jose, CA, USA) for plate assembly.

DNA extraction and sequencing

41 specimens were used for DNA extraction (Table 1). All DNA was extracted from approximately 5 mg dried thallus ground in liquid nitrogen using a DNeasy Plant Mini kit (QIAGEN, Germany), following the manufacturer's instructions. The extracted DNA was stored at -20°C and used as a template to amplify the *rbcL* and COI genes.

Specific primer pairs for the amplification and sequencing of each gene were as follows: for *rbcL*, F145, R898, F762, and R1442 (Kim et al. 2010); for COI, GazF2 (Lane et al. 2007) and GazR1 (Saunders 2005). All PCR amplifications were performed with Swift MaxPro thermal cyclers (ESCO, Singapore) using the AccuPower PCR Premix (Bioneer, Daejeon, Korea). PCR reactions were carried out with an initial denaturation at 96°C for 4 min, followed by 35 cycles of amplification (denaturation at 94°C for 1 min, annealing at 50°C for 1 min and an extension at 72°C for 2 min) with a final extension at 72°C for 7 min for the *rbcL* gene; and with an initial denaturation at 96°C for 4 min, followed by 40 cycles of amplification (denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 1 min) with a final extension at 72°C for 7 min for the COI gene. PCR products were purified using the Accuprep PCR Purification Kit (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. Sequencing of the forward and reverse strands of purified PCR products were performed by Macrogen (Seoul, Korea). Both electropherogram outputs from each sample were edited with Chromas version 1.45 (Queensland, Australia).

Table 1. Samples of *Chondracanthus* spp. used in the present study

Species	Voucher	Collection site and date	GenBank accession no.		
			<i>rbcL</i>	COI	
<i>C. acicularis</i>	Gigar404	Sebastian Inlet: Florida: USA (3 August 2013)	KR909558	KR909519	
	Gigar405	Sebastian Inlet: Florida: USA (3 August 2013)	KR909559	KR909520	
	Gigar406	Sebastian Inlet: Florida: USA (3 August 2013)	KR909560	KR909521	
<i>C. cincinnus</i>	Gigar031	Jakdo, Yeosu, Korea (26 July 2012)	KR909561		
	Gigar032	Daesambudo, Yeosu, Korea (25 July 2012)	KR909562	KR909522	
	Gigar213	Chujado, Jeju, Korea (1 October 2013)	KR909563	KR909523	
	Gigar318	Otaru, Hokkaido, Japan (23 February 2014)	KR909564		
	Gigar319	Otaru, Hokkaido, Japan (23 February 2014)	KR909565		
	Gigar320	Otaru, Hokkaido, Japan (23 February 2014)		KR909524	
	Gigar412	Gapado, Jeju, Korea (20 March 2015)	KR909566		
	Gigar414	Gapado, Jeju, Korea (20 March 2015)	KR909567		
	<i>C. okamurae</i>	Gigar166	Ieodo, Jeju, Korea (18 June 2013)	KR909568	KR909525
Gigar170		Wando, Korea (15 January 2013)	KR909569	KR909526	
Gigar215		Hamduck, Jeju, Korea (9 January 2013)	KR909570	KR909527	
Gigar216		Ieodo, Jeju, Korea (18 June 2013)	KR909571	KR909528	
Gigar247		Katsuura, Chiba, Japan (23 March 2014)	KR909572	KR909529	
Gigar323		Katsuura, Chiba, Japan (23 March 2014)	KR909573	KR909530	
Gigar409		Sinyang, Jeju, Korea (23 December 2014)	KR909574	KR909531	
Gigar413		Gapado, Jeju, Korea (20 March 2015)	KR909575	KR909532	
Gigar415		Gapado, Jeju, Korea (20 March 2015)	KR909576	KR909533	
<i>C. intermedius</i>		Gigar125	Misaki, Kanagawa, Japan (10 April 2013)	KR909577	KR909534
		Gigar128	Shimoda, Shizuoka, Japan (12 April 2013)	KR909578	KR909535
		Gigar246	Katsuura, Chiba, Japan (23 March 2014)	KR909579	KR909536
		Gigar324	Shimoda, Shizuoka, Japan (27 March 2014)		KR909537
		Gigar325	Shimoda, Shizuoka, Japan (28 March 2014)	KR909580	KR909538
		Gigar326	Shimoda, Shizuoka, Japan (28 March 2014)	KR909581	KR909539
<i>C. tenellus</i>	Gigar033	Oedo, Namhaedo, Korea (19 May 2012)	KR909582	KR909540	
	Gigar124	Shimoda, Shizuoka, Japan (12 April 2013)	KR909583	KR909541	
	Gigar200	Seorim, Jeju, Korea (3 July 2012)		KR909542	
	Gigar201	Seorim, Jeju, Korea (3 July 2012)		KR909543	
	Gigar202	Chujado, Jeju, Korea (17 August 2012)	KR909584	KR909544	
	Gigar204	Sungsan, Jeju, Korea (8 May 2012)		KR909545	
	Gigar205	Chiba, Japan (9 April 2013)		KR909546	
	Gigar207	Songjeong, Busan, Korea (20 December 2012)		KR909547	
	Gigar208	Wando, Korea (15 January 2013)		KR909548	
	Gigar209	Wando, Korea (15 January 2013)		KR909549	
	Gigar210	Wando, Korea (16 January 2013)		KR909550	
	Gigar211	Jookbyeon, Uljin, Korea (28 April 2012)		KR909551	
	Gigar212	Jookbyeon, Uljin, Korea (28 April 2012)	KR909585	KR909552	
	Gigar399	Chiba, Japan (9 April 2013)	KR909586	KR909553	
	Gigar401	Gijang, Busan, Korea (20 September 2013)	KR909587	KR909554	
	Gigar402	Guryoungpo, Pohang, Korea (29 September 2012)	KR909588	KR909555	
Gigar403	Haeundae, Busan, Korea (3 November 2012)	KR909589	KR909556		
Gigar416	Gapado, Jeju, Korea (20 March 2015)	KR909590	KR909557		

59 *rbcL* sequences (including 25 accessions from GenBank) and 48 COI sequences (including 20 accessions from GenBank)

of *Chondracanthus* with outgroups were collated using the multiple-sequences editing program BioEdit (Hall 1999) and

aligned visually. Outgroup taxa for *rbcL* and COI analyses included available representatives of the Gigartineae genus, *Gigartina* Stackhouse and *Rhodoglossum* J. Agardh.

Phylogenetic analyses

Maximum likelihood (ML) phylogenetic analyses were performed with RAxML software (Stamatakis 2006) using the GTR + Γ + I model. To identify the best tree, we constructed 200 independent tree inferences using the $\#$ option with default $-I$ (automatically optimized Subtree Pruning-Regrafting rearrangement) and $-c$ (25 distinct rate categories) software options. Statistical support for each branch was obtained from 1000 bootstrap replications using the same substitution model and RAxML program setting.

Maximum parsimony (MP) trees were constructed with PAUP* 4.0b.10 software (Swofford 2002) using a heuristic search algorithm with the following settings: 1000 random sequence additions, TBR branch swapping, MulTrees and unweighted characters and branches with a maximum length of zero collapsed to yield polytomies. Bootstrap values for the resulting nodes were assessed using 1000 bootstrap replicates with 10 random sequences additions, TBR and MulTrees.

3. Results

Molecular analyses of *rbcL* and COI

A total of 59 sequences consisting of 18 *Chondracanthus* taxa and 2 outgroups were aligned using a 1294-nucleotide (nt) region of the *rbcL* gene. Variable sites occurred at 299 positions (23%), and 185 positions (14%) were parsimoniously informative. The ML and MP trees of *rbcL* sequences were identical except for the bootstrap values (Fig. 1).

Nine *Chondracanthus okamurae* sequences from Korea and Japan were identical, which formed a monophyletic clade with the sequence of *C. tenellus* from Taiwan (AF146196, 89% bootstrap probability (BP) for ML and 92% BP for MP) with 0.3% sequence divergences. The sister group was formed by other individuals of *C. tenellus* (73% BP for ML) with 0.8–1.9% sequence divergence. Nine newly generated sequences of *C. tenellus* from Korea and Japan were identical except for two nucleotide differences. These sequences formed a monophyletic clade with the sequence of *C. tenellus* from Japan (AF146197, 95% for ML and 84% for MP), but they differed with 1.1–1.2% sequence divergence. A new species, *Chondracanthus cincinnus*, was collected from Korea and Japan. Seven *C. cincinnus* sequences were identical with

strong support in all analyses (98% for ML and 90% for MP) and differed with *C. intermedius* from Japan with 0.4–0.5% sequence divergence. Seven sequences of *C. intermedius* including those from NCBI (U02942) formed a clade (63% for ML and 73% for MP) with 0.4% sequence divergence.

The COI gene (669 nt) was aligned from 11 *Chondracanthus* taxa. Among 130 variable sites, 128 positions (19%) were parsimoniously informative. Six sequences of *C. intermedius* had 0–0.6% intraspecific divergence, which was closely related to *C. cincinnus* with 1.1% sequence divergence (Fig. 2). Three *C. cincinnus* samples from Korea and Japan were identical. The COI tree showed that all *C. okamurae* from Korea and Japan were grouped together with an intraspecific divergence of 0.6%. Eighteen *C. tenellus* specimens formed a node with 0.2% intraspecific divergence. *Chondracanthus tenellus* differed from *C. okamurae*, *C. cincinnus*, and *C. intermedius* clade with 2.1–2.9% interspecific divergence.

Morphology

Chondracanthus okamurae Abbott (Fig. 3)

Basionym: *Gigartina intermedia sensu* Okamura (1909, pp. 172–173, pl. 35, Figs. 1–5).

Holotype: The specimens illustrated in Fig. 3 of Okamura (1909, pl. 35) as *Gigartina intermedia* (SAP).

Type locality: Japan, central Honshu (Abbott 1998: 104).

Specimens examined: Hamduck, Jeju, Korea, 09 January 2013, NIBRRD0000000002; Wando, Korea, 15 January 2013, JN-Gigar170; Jeodo, Jeju, Korea, 18 June 2013, JN-Gigar 216; Sinyang, Jeju, Korea, 23 December 2014, JN-Gigar 409; Gapado, Jeju, Korea, 20 March 2015, JN-Gigar 413; Katsuura, Chiba Pref., Japan, 23 March 2014, JN-Gigar 323.

The thallus is cartilaginous and flexible in texture, purplish red to black with bluish iridescence in color, 1–3 cm high, and attached firmly to the substratum by small discoid holdfasts forming a low-growing tuft (Figs. 3a–b). Axes are cylindrical at the base (Fig. 3d), linear, slender and compressed (Figs. 3d–e), 0.3–1.3 mm wide and 480–620 μ m thick, irregularly branched, slightly recurved, adhering to each other by a secondary attachment (Fig. 3c), and attenuated upwardly with acuminate apices. Axes and branches consist of three layers in cross-section: cortical, subcortical, and medulla layer. The five- to six layer cortex consists of small, pigmented, and globose cells 4–8 μ m \times 7–9 μ m in size that bears ultimate narrowly elongate cells (Fig. 3g). The subcortical layer is anastomosing with elongate stellate cells (Fig. 3g). The medulla is composed

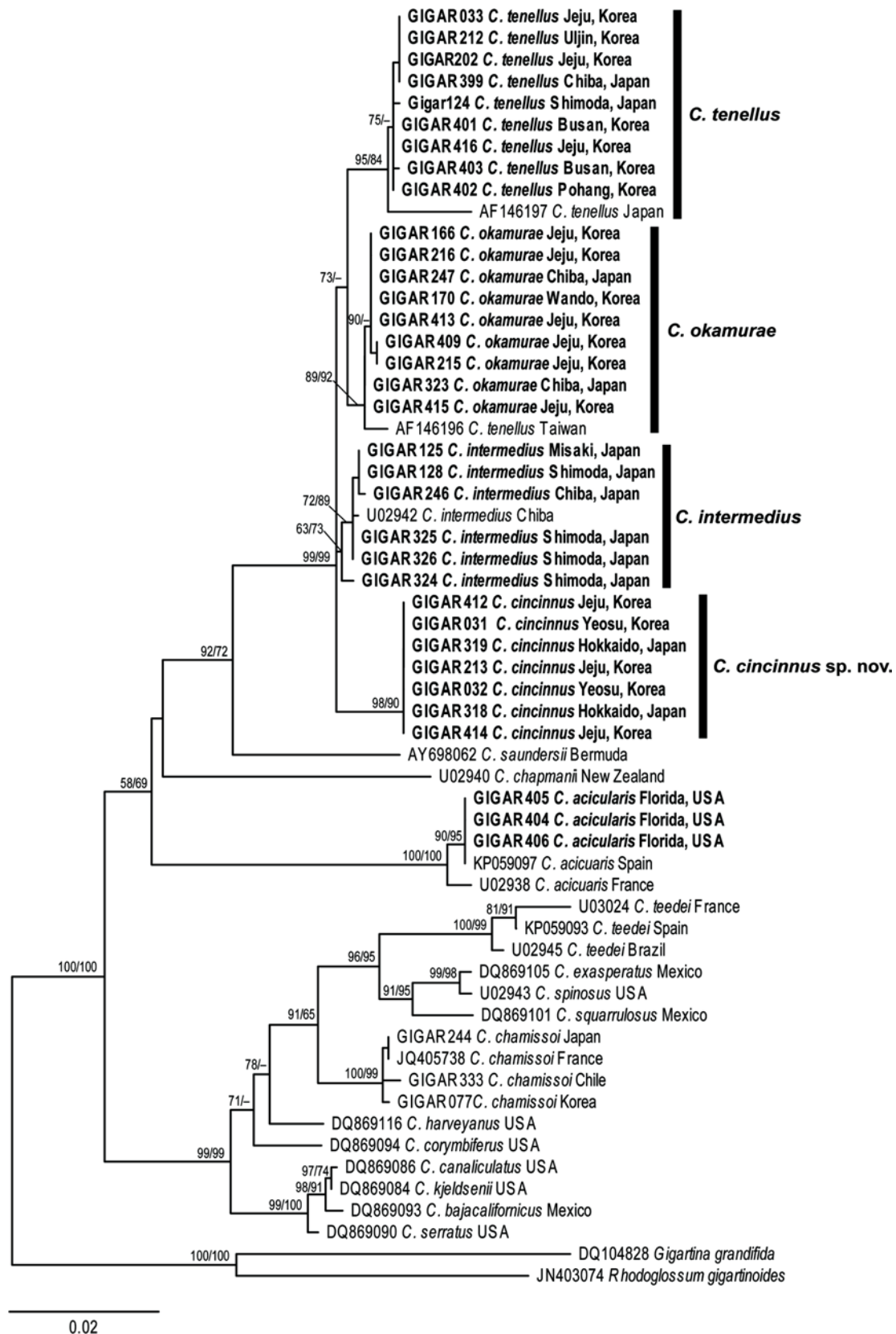


Fig. 1. Maximum likelihood phylogenetic tree of *Chondracanthus* inferred from *rbcL* sequences. Bootstrap values for ML and MP are shown for each node. Bold type indicates sequences generated in the present study. Scale indicates substitutions per site

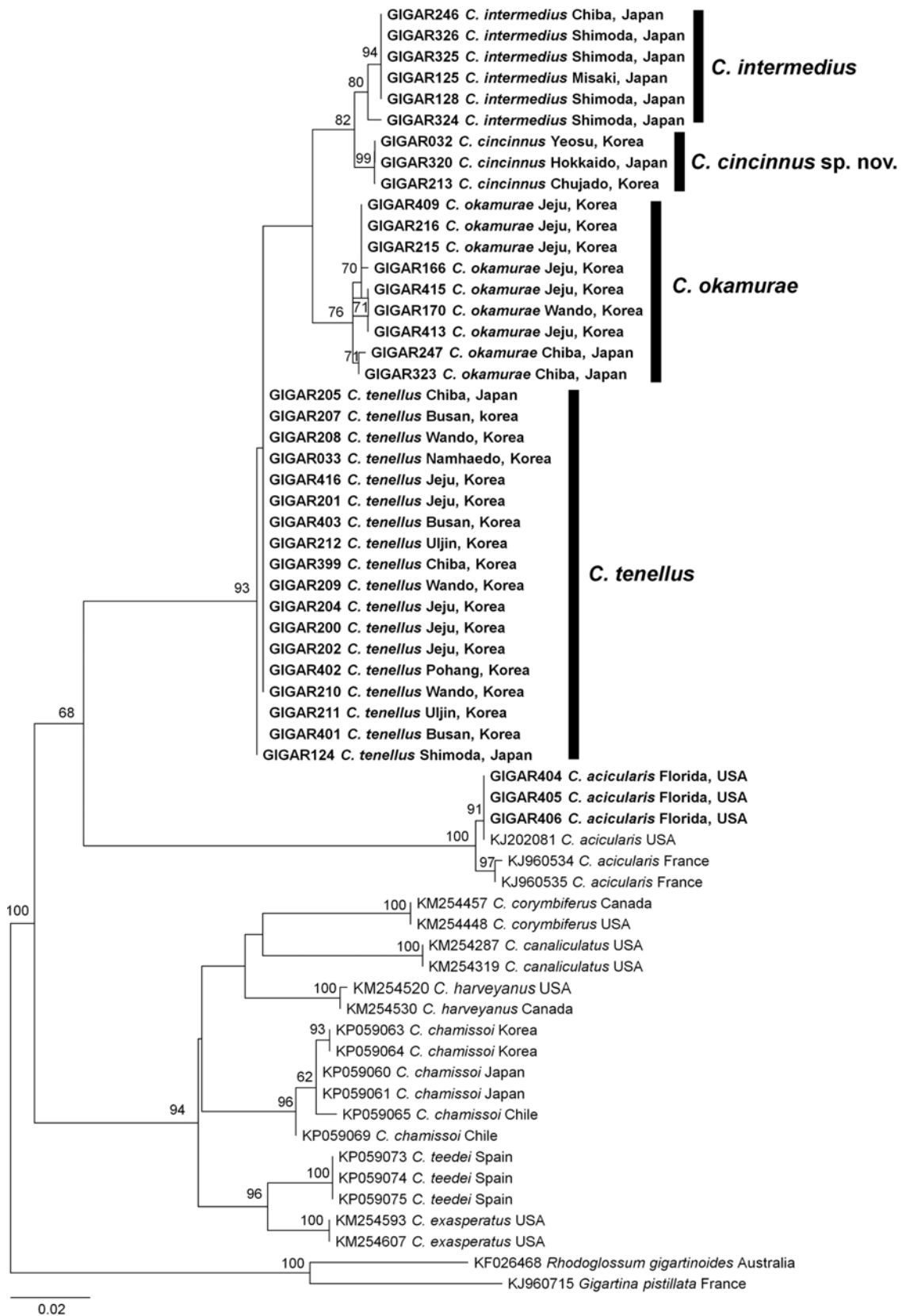


Fig. 2. Maximum likelihood phylogenetic tree of *Chondracanthus* inferred from COI sequences. Bootstrap value are shown for each clade. Bold type indicates sequences generated in the present study. Scale indicates substitutions per site

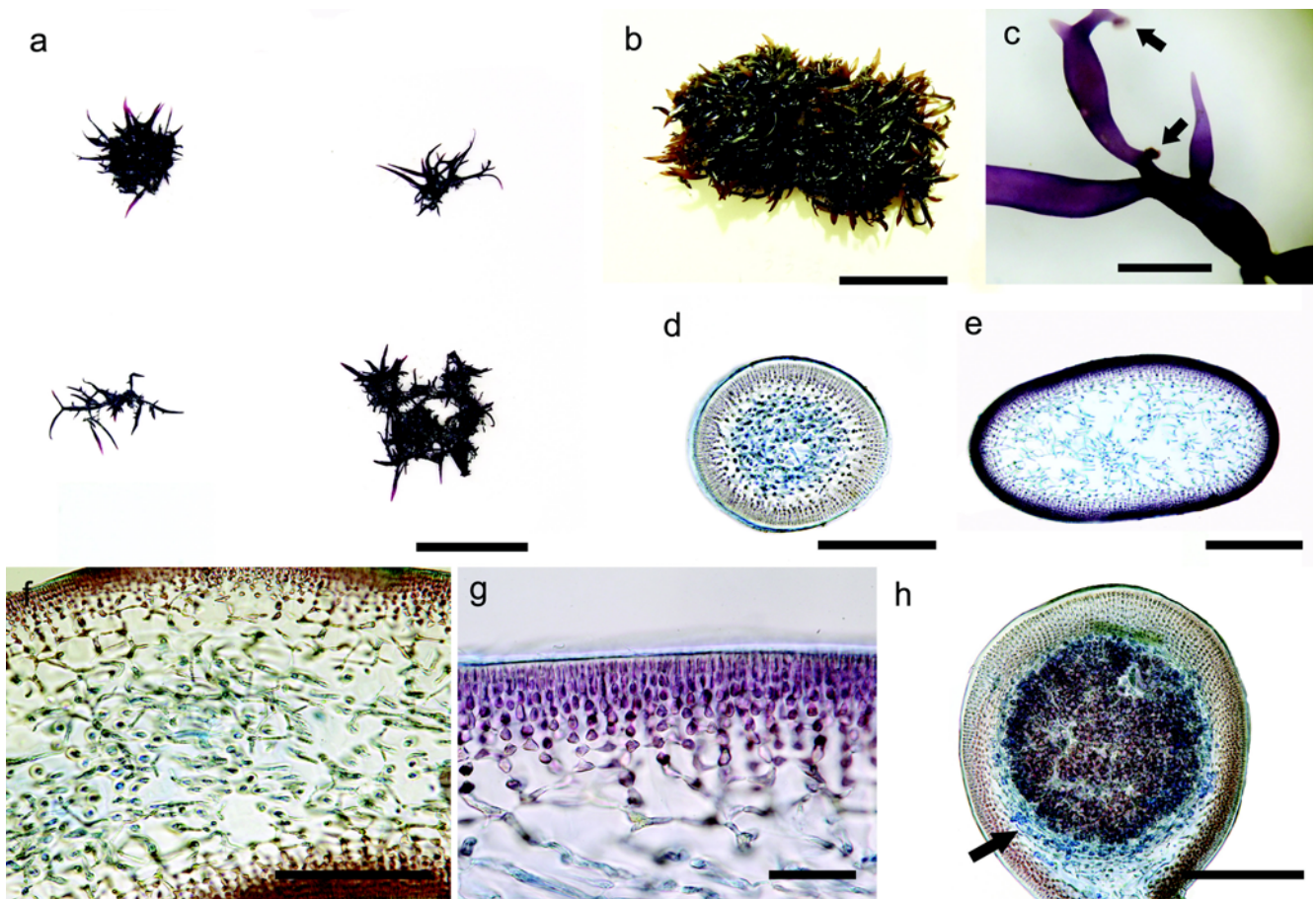


Fig. 3. *Chondracanthus okamurae* Abbott. (a) The specimen collected from Hamduck, Jeju, Korea (9 January 2013). (b) The thallus forming low growing tuft. (c) The thallus with a secondary attachment. (d) Transverse section of low part of thallus. (e) Transverse section of middle part of thallus. (f) Transverse section of thallus showing filamentous medullary cells. (g) Transverse section of thallus showing cortical and subcortical layers. (h). Transverse section of cystocarp showing central carposporangial mass surrounded by medullary filaments. Scale bars: (a–b) 3 cm; (c) 2 mm; (d, f, h) 200 μ m; (e) 300 μ m; (g) 50 μ m

of loosely entangled elongate filaments that are colorless and 40–70 μ m in diameter (Fig. 3f).

The cystocarps are globose, sessile, 350–490 μ m in diameter, and protruding externally on margins of axes and branches (Fig. 3h). The mature cystocarps are composed of large central carposporangial masses, 260–330 μ m in diameter, and surrounded by medullary filaments (Fig. 3h). The carpospores are subglobose and 10–15 μ m in diameter. The thallus of *Chondracanthus okamurae* grows on rocks in the high-intertidal to subtidal zone. Some thalli have cystocarps in August. Tetrasporangia and spermatangia were not observed.

***Chondracanthus cincinnus* M.Y. Yang & M.S. Kim, sp. nov. (Fig. 4)**

Holotype: JN-Gigar412 collected on 20 March 2015, deposited at JNUB (Herbarium of the Department of Biology,

Jeju National University, Korea).

Type locality: Gapado, Jeju, Korea (33°10′02.87″N, 126°16′30.12″E)

Isotype: JN-Gigar414 (NIBRRD0000000001).

Etymology: *cincinnus* = curl; thallus are strongly curled back.

Specimens examined: Jakdo, Yeosu, Korea, 26 July 2012, JN-Gigar031; Daesambudo, Yeosu, Korea, 25 July, 2012, JN-Gigar032; Chujado, Jeju, Korea, 1 October 2013, JN-Gigar213; Otaru, Hokkaido, Japan, 23 February 2014, JN-Gigar318–320.

The thallus is cartilaginous in texture, purplish red to black with bluish iridescence in color, 2–4 cm high, creeping, and attached firmly to the substratum by small discoid holdfasts (Figs. 4a–b). Axes are linear, compressed throughout, 0.6–2.0 mm wide and 350–800 μ m thick, irregularly branched, strongly recurved, adhering to each other by a secondary

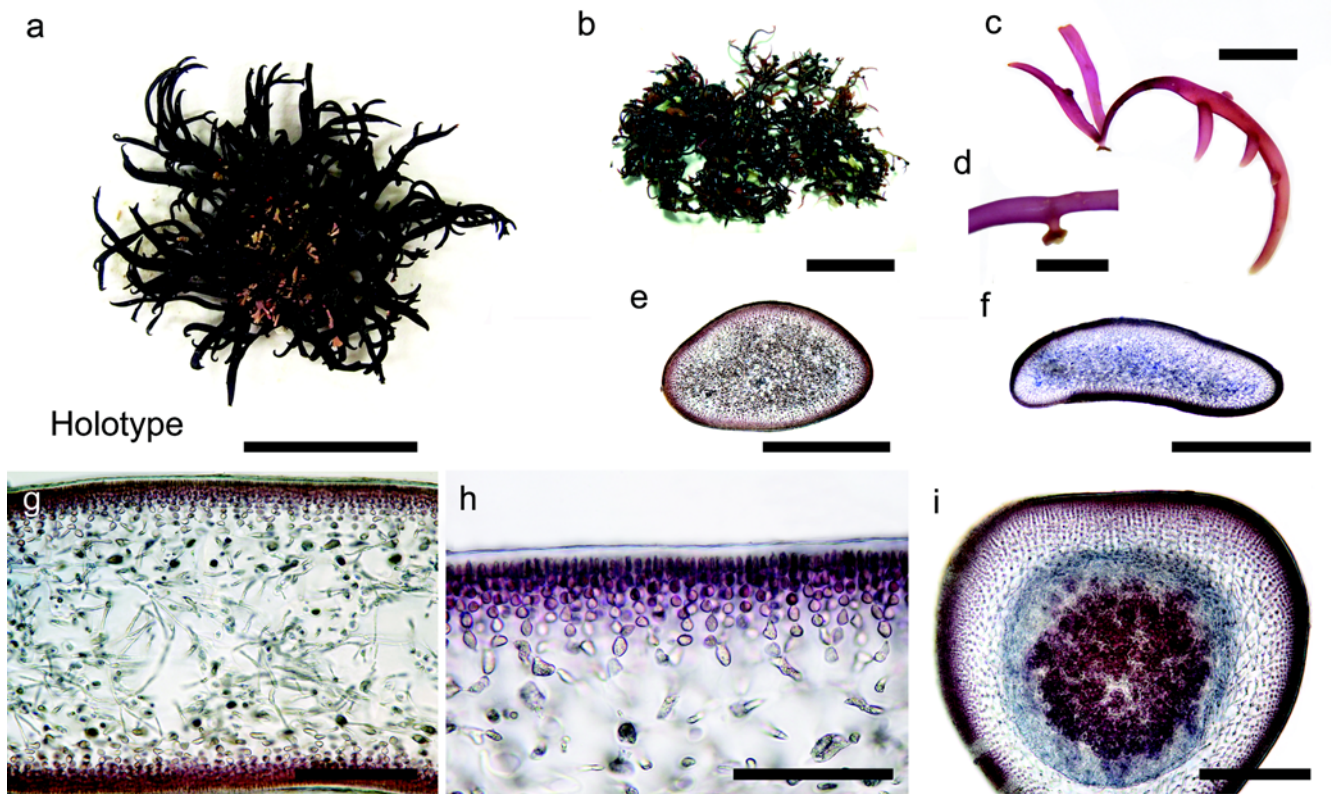


Fig. 4. *Chondracanthus cincinnus* sp. nov. (a) An image of the holotype specimen JN-Gigar412 deposited at the herbarium of Jeju National University (JNUB) in Jeju, Korea. (b) The creeping thallus with the cystocarps. (c) The axes strongly recurving. (d) The thallus with a secondary attachment. (e) Transverse section of low part of thallus. (f) Transverse section of middle part of thallus. (g) Transverse section of thallus showing filamentous medullary cells. (h) Transverse section of thallus showing cortical and subcortical layers. (i) Transverse section of cystocarp showing central carposporangial mass surrounded by dense medullary filaments. Scale bars: (a) 2 cm; (b) 3 cm; (c) 1 cm; (d) 50 mm; (e) 300 μ m; (f) 1000 μ m; (g) 200 μ m; (h) 50 μ m; (i) 100 μ m

attachment, and attenuated upwardly with acuminate apices (Figs. 4c–f). Axes and branches consist of three layers in cross-section: cortical, subcortical, and medulla layer. The five- to six layer cortex consists of small, pigmented, and globose cells 4–8 μ m \times 7–9 μ m in size that bears ultimate narrowly elongate cells (Fig. 4h). The subcortical layer is anastomosing with elongate stellate cells (Fig. 4h). The medulla is composed of loosely entangled elongate filaments that are colorless and 40–80 μ m in diameter (Fig. 4g).

The cystocarps are sessile, subglobose with a flat surface on the top, 1000–1100 μ m in diameter, and protruding externally on the margins of axes and branches (Fig. 4i). The mature cystocarps are composed of large central carposporangial masses, 500–650 μ m in diameter, and surrounded by dense anastomosing medullary filaments (Fig. 4i). The carpospores are subglobose and 14–20 μ m in diameter. Thallus of *Chondracanthus cincinnus* grows on rocks in low-intertidal to subtidal zones. Some thalli have cystocarps in July to

February. Tetrasporangia and spermatangia were not found.

Chondracanthus intermedius (Suringar) Hommersand (Fig. 5)

Basionym: *Gigartina intermedia* Suringar.

Type locality: Japan (Suringar 1867).

Specimens examined: Misaki, Japan, 19 April 2013, JN-Gigar125; Shimoda, Shizuoka, Japan, 12 April 2013, JN-Gigar128; Shimoda, Shizuoka, Japan, 27 March 2014, JN-Gigar324; Shimoda, Shizuoka, Japan, 28 March 2014, JN-Gigar325–326; Katsuura, Chiba Pref., Japan, 23 March 2014, JN-Gigar246.

The thallus is cartilaginous in texture, purplish of greenish red to black with bluish iridescence in color, 2–4 cm high, attached firmly to the substratum by discoid holdfasts forming a low-growing tuft (Fig. 5a). Axes are lanceolate, compressed throughout, 1.9–5.0 mm wide and 730–1100 μ m thick, subpinnately or bifurcate branched, recurved, adhering to

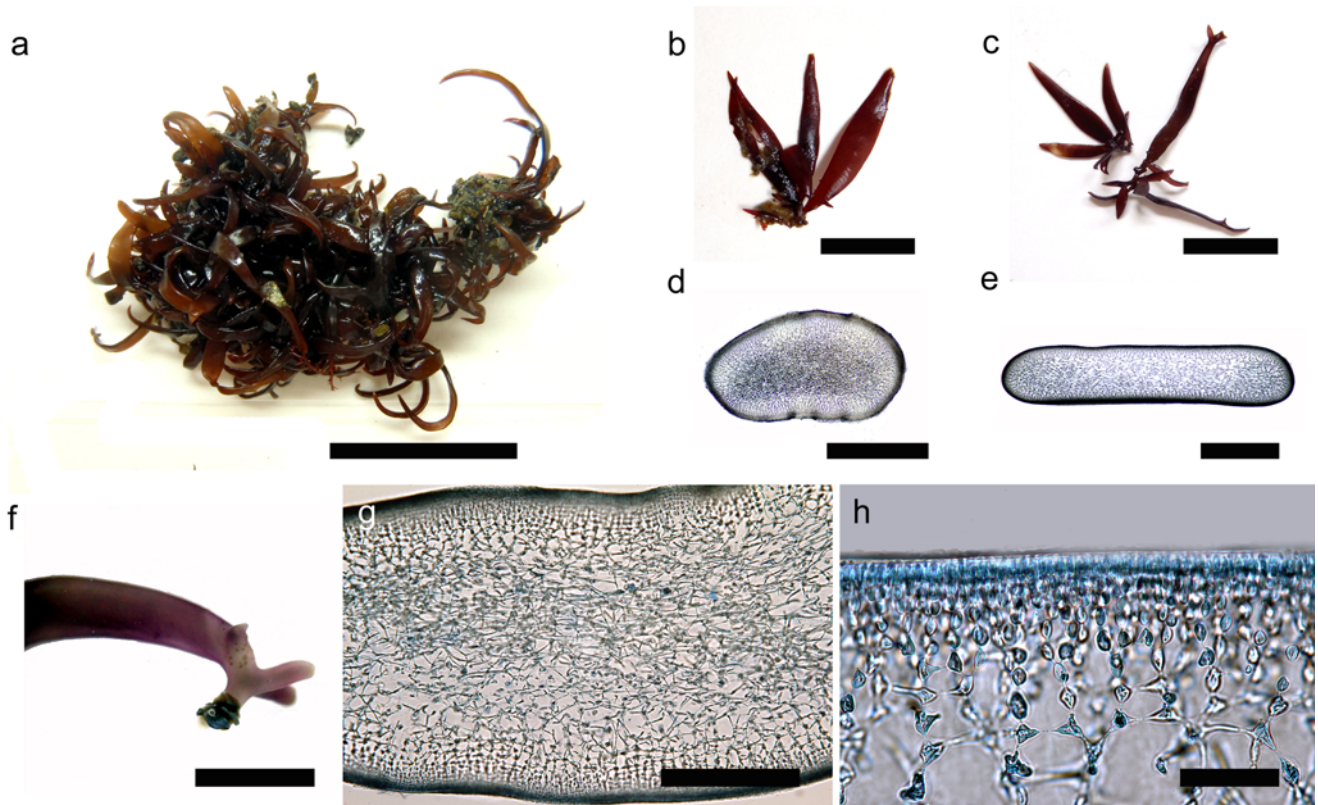


Fig. 5. *Chondracanthus intermedius* (Suringar) Hommersand. (a) The specimen collected from Misaki, Kanagawa, Japan (10 April 2013). (b–c) Specimens collected from Shimoda, Shizuoka, Japan (27, 28 March 2014). (d) Transverse section of low part of thallus. (e) Transverse section of middle part of thallus. (f) The thallus with a secondary attachment. (g) Transverse section of thallus showing filamentous medullary cells. (h) Transverse section of thallus showing cortical and subcortical layers. Scale bars: (a) 2 cm; (b–c) 1 cm; (d–e) 1 mm; (f) 50 mm; (g) 200 μ m; (h) 50 μ m

each other by a secondary attachment, and attenuated upwardly with acuminate apices (Figs 5b–f). The axes and branches consist of three layers in cross-section: cortical, subcortical, and medulla layer. The seven- to nine cortex layers consists of small, pigmented, and globose cells $4\text{--}8\ \mu\text{m} \times 7\text{--}9\ \mu\text{m}$ in size that bears ultimate narrowly elongate cells (Fig. 5h). The subcortical layer is anastomosing with elongate stellate cells (Fig. 5h). The medulla is composed of entangled elongate filaments that are colorless and $25\text{--}50\ \mu\text{m}$ in diameter (Fig. 5g). Reproductive plants were not found.

4. Discussion

Based on the molecular analyses coupled with our comprehensive morphological observations, we resurrected *C. okamurae* and identified a novel species, *C. cincinnus*, from Korea and Japan. These two species form a distinct clade, closely related to *C. intermedius* and *C. tenellus* in *rbcL* phylogeny (Fig. 1). The clade is consistent with the COI tree despite the fact that

this tree does not include many species (Fig. 2). The close relationships among *C. tenellus* and *C. intermedius* are corroborated by previous studies (Hughes and Hommersand 2008). *C. okamurae* and *C. cincinnus* are clearly divided from *C. tenellus* and *C. intermedius*, which show interspecific divergences from 0.4% to 2.0% in the *rbcL* data. Although the interspecific divergence between *C. cincinnus* and *C. intermedius* is rather low (0.4%), several lower divergences are also observed between *C. kjeldsenii* and *C. canaliculatus* (0.1%), *C. exasperatus* and *C. spinosus* (0.3%), and *C. canaliculatus* and *C. bajacalifornicus* (0.4%). The COI data show relatively high interspecific divergences among four species with 7.3–7.8% divergence with *C. chamissoi*, compared to the *rbcL* data (1.1–2.9%) (Yang et al. 2015).

Our specimens from Korea and Japan correspond to the morphology of *Chondracanthus okamurae*, characterized by linear, narrow, compressed axes, forming low masses, being cylindrical at the base, attenuated upwardly, and slightly recurved irregular branches (Fig. 3). Our specimens grow on rocks from

high-intertidal to subtidal levels. *C. okamurae* was identified as a distinct species by Abbott (1998) based on the Okamura (1909)'s specimens, which were originally identified as *C. intermedius*. However, Masuda et al. (1999), based on morphological variation, concluded that *C. okamurae* is a synonym of *C. intermedius*. Hughey and Hommersand (2008) confirmed that *C. intermedius* from Japan is different from *C. tenellus* using morphology and molecular analyses. Our study separates *C. okamurae* from *C. intermedius* and *C. tenellus* based on the molecular evidence. Therefore, we herein resurrect *C. okamurae* as an independent entity in the genus *Chondracanthus*. *C. okamurae* is distinguished by its small thallus with a cylindrical base and narrow axes, which produced a small and globose cystocarps (Table 2).

The taxonomic status of *Chondracanthus intermedius* needs to be confirmed by comparison between *C. intermedius* and *C. okamurae* worldwide. Norris (2014) recognized more than one species in collections of *C. intermedius* from the Gulf of California, and consequently reported *C. zertucheii* J.N. Norris et Fredericq as a new species, which differed from *C. intermedius* and *C. okamurae* by having wider, semi-erect branches, and a compressed lowermost thallus (Norris 2014). Malaysian *C. intermedius* is similar to *C. okamurae* because of a high-intertidal distribution although it is considered to be an extreme representative from the morphological spectrum of *C. intermedius* (Masuda et al. 1999). Our specimens identified as *C. okamurae* are monophyletic with *C. tenellus* from Taiwan (AF146196) in the *rbcL* tree (Fig. 1). It is presumed that Taiwanese *Chondracanthus* may be misidentified, and *C. okamurae* occurs in Taiwan as well. Our specimens from Korea are identified as *C. okamurae* based on the molecular and morphological data, and we report *C. okamurae* in the Korean algal flora. On the other hand, we found *C. intermedius* in Japan only, which is characterized by having wider axes at the broadest portion up to 5 mm, which is consistent with the description by Mikami (1965).

Chondracanthus cincinnus grows in low-intertidal to subtidal zones, while *C. okamurae* is distributed in high-intertidal to subtidal zones. Molecular phylogenetic analyses of *rbcL* and COI sequences consistently demonstrated the distinctness of *C. cincinnus* from congeners. *C. cincinnus* is characterized by linear, compressed throughout, strongly recurved axes that produce irregular branches, and the cystocarps are surrounded by dense medullary filaments. *C. cincinnus* differs from *C. okamurae* in that the latter species has a cylindrical base, small cystocarps, and small a carposporangial mass (Table 2).

C. cincinnus is closely related genetically to *C. intermedius* (Figs. 1–2), but *C. intermedius* is characterized by lanceolate, wide, and thick axes (Table 2). The plant size and irregular branching pattern of *C. cincinnus* are similar to *C. zertucheii*, which is established based on the collections of *C. intermedius* from the Gulf of California (Norris 2014). However, *C. cincinnus* is distinguished from *C. zertucheii*, which have semi-erect thalli bearing short lateral branches and no secondary attachment, whereas *C. cincinnus* is prostrate with strongly recurved axes bearing secondary attachment discs (Fig. 2). Phylogenetic relationships between *C. cincinnus* and *C. zertucheii* need to be studied.

Chondracanthus tenellus is distributed in the coast of Korea and Japan together with *C. okamurae*, *C. intermedius* and *C. cincinnus*. The clade of *C. tenellus* is separated from those of three other species in this region, and this clade is supported by strong bootstrap values (Fig. 1). Compared to three creeping species (*C. okamurae*, *C. intermedius*, and *C. cincinnus*), *C. tenellus* is larger, having erect fronds which are alternately or oppositely branched, and lacks a secondary attachment (Table 2). *Chondracanthus chamissoi* has been recently assigned to the specimens previously called *C. teedei* in this region (Yang et al. 2015). *C. chamissoi* is easily distinguished by its broadly flattened axes covering short spin-like ramuli and large cystocarps (Table 2).

Recent studies of algal taxonomy require the assistance of molecular analyses (Hind et al. 2014; Saunders and McDonald 2010; Schneider and Lane 2005; Yang et al. 2015). Molecular analyses provide valid evidences allowing for the exploration of cryptic diversity (Saunders and McDonald 2010), correct identifications (Schneider and Lane 2005), and investigation of the distributional range (Yang et al. 2015). According to Schneider and Lane (2005), molecular data about *C. acicularis* is needed from Florida and North Carolina because specimen from Bermuda (AY698062) was corrected as a new species, *C. saundersii*. In this study, we confirm that the specimen of *C. acicularis* collected from Florida is monophyletic with European *C. acicularis* (Fig. 1). However, its distribution has not yet been confirmed by molecular analyses because it has been reported worldwide.

In conclusion, we confirm the resurrection of *C. okamurae*, and describe *C. cincinnus* as a new species from Korea and Japan with molecular evidences. The specimens identified as *C. intermedius* from Korea are assigned as being *C. okamurae*, about which there had been confusion for a long time. Our results reveal that *C. intermedius* occurs in Japan only. These findings demonstrate the benefits of an integrated

Table 2. Morphological comparison of *Chondracanthus cincinnus*, *C. okamurae* and related species

	<i>C. cincinnus</i>	<i>C. okamurae</i>	<i>C. intermedius</i>	<i>C. tenellus</i>	<i>C. chamissoi</i>	<i>C. zertucheii</i>
Height	2–4 cm	1–3 cm	2–4 cm	5–8 (–12) cm	9–25 cm	up to 4 cm
Color	purple-red with bluish iridescence	purple-red with bluish iridescence	dark red-purple	livid purple with bluish iridescence	reddish purple with sometimes yellowish	dark greenish red to purplish red
Axes	linear, compressed, strongly recurved, creep	linear, cylindrical to compressed, slightly recurved, creep	lanecolate or linear, compressed, recurved, creep	linear, cylindrical to compressed, slightly recurved, erect or creep	slightly or broadly flattened, covered in short spine-like ramuli	few sublanceolate, flattened, recurved, semierect or creep
Branching	irregularly	irregularly	subpinately or bifurcate	irregularly pinnate with alternate and opposite intermixed	alternately or oppositely	irregularly, short lateral branchlets
Secondary attachment	present	present	present	absent	absent	absent
Width of axes	0.6–2 mm	0.3–1.3 mm	1.9–5.0 mm	1–2(–3) mm	2–6 mm	(1)2–3(–4) mm
Thick of axes	350–800 μ m	480–620 μ m	730–1100 μ m	–	–	–
Cortical	5–6 layers	5–6 layers	7–9 layers	5–7 layers	5–6 layers	–
Cystocarp	subglobose, 1000–1100 μ m in diameter	globose, 350–490 μ m in diameter	–	globose or subspherical	hemispherical, 950–1700 μ m in diameter	globose, up to 1000 μ m in diameter
Carposporangial mass	500–650 μ m in diameter, thickly surrounded by medullary filaments	260–330 μ m in diameter, surrounded by medullary filaments	–	surrounded by medullary filaments	600–1000 μ m in diameter	–
Carpospore	subglobose, 14–20 μ m in diameter	subglobose, 10–15 μ m in diameter	–	subglobose, 12.5–20 μ m in diameter	subglobose, 32–45 μ m in diameter	–
Tetrasporangia	–	–	–	–	crucially divided	–
Habitat	low intertidal to subtidal	high intertidal to subtidal	–	tide pool	Intertidal to subtidal	mid to low intertidal
Type locality	Jeju, Korea	Japan	Japan	Japan	Chile	Baja California, Mexico
References	this study	Okamura 1909; Abbott 1998; this study	this study	Harvey 1860; Mikami 1965	Yang et al. 2015a	Norris 2014

molecular and morphological data to document marine algal diversity. A more careful observation of collections is needed for the exact identification of *Chondracanthus*, and this will probably extend knowledge of their geographical distribution as well.

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