



Cryptic species diversity of the red algal genus *Callophyllis* (Kallymeniaceae, Gigartinales) from Korea

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Abstract

The genus *Callophyllis* is recorded as six separate species with imprecise species delimitation in Korea. To elucidate the species boundaries of Korean *Callophyllis*, we performed morphological observations and molecular analyses, and included three Japanese *Callophyllis* species from the type locality. From the results of molecular analyses using plastid *rbcl* and mitochondrial COI-5P genes, we confirmed ten *Callophyllis* species, including five cryptic ones: *C. adhaerens*, *C. adnata*, *C. crispata*, and *C. japonica* from Korea and Japan; *C. hayamensis* as an unrecorded species from Korea; *C. cartilaginea*, *C. mollitia*, *C. repens*, *C. serratifolia*, and *C. undulata* as new species from Korea. There were no Korean specimens that matched *C. adnata* or *C. crispata* from Japan, except Korean *C. japonica*, which formed a genetic group with the Japanese species. We obtained the interspecific divergences among the five cryptic species as 0.6–4.5% in *rbcl* and 2.8–8.4% in COI-5P. We recognized that the species diversity of *Callophyllis* has been underestimated from the north-western Pacific region. The species boundary of *Callophyllis* from Korea and Japan will be a cornerstone to revealing the phylogenetic affinity of the genus distributed in both hemispheres of the western Pacific.

Key words: *Callophyllis*, COI-5P, cryptic species, *rbcl*, taxonomy

INTRODUCTION

The red algal genus *Callophyllis* was established by Kützing (1843) for *Callophyllis variegata* (Bory de Saint-Vincent) Kützing from Chile and *C. laciniata* (Hudson) Kützing from Europe (Norris 1957), and it is distributed in the cold and temperate regions of both hemispheres (Arakaki et al. 2011). The genus *Callophyllis* is distinguished from other kallymeniacean genera by its medullary structure that is composed of large and conspicuous isodiametric cells, and the female reproductive structure that has no separate auxiliary cell systems (Norris 1957, Womersley and Norris 1971). At first, species delimitation was carried out by observing the simple vegetative features of *Callophyllis* species distributed in the west coast of North America (Setchell 1923), but the identification of species

was conducted using the reproductive structures such as the position and size of cystocarps, number and shape of ostioles, and presence or absence of tetrasporangia sori (Dawson 1954, Norris 1957, Abbott and Norris 1966). Abbott and Norris (1966) indicated that identification based on only vegetative features has led to errors when determining species diversity, because considerable variation occurs in some *Callophyllis* species from the Pacific coast of North America.

The advancement of the molecular method for the taxonomic study of red algae has allowed us to overcome the limit of morphological observations, to recognize species diversity with the discovery of new species, and to infer the phylogenetic relationships among taxa

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(Saunders 2008, Koh et al. 2013, Schneider et al. 2014). In the genus *Callophyllis*, Harper and Saunders (2002) carried out molecular analysis using the nuclear LSU rDNA gene marker. Consequently, they confirmed the monophyletic clade of the genus *Callophyllis* in the family Kallymeniaceae and transferred three *Callophyllis* species to *Euthora* and *Pugetia*: *C. cristata* to *Euthora*, *C. firma*, and *C. chilensis* to *Pugetia* (Harper and Saunders 2002). Arakaki et al. (2011) studied the species delimitation of *Callophyllis* from the central-southern Chile using the plastid *rbcL* gene and newly reported *C. conceptionensis* and *C. macrostiolata*. Clarkston and Saunders (2012) established the new genus, *Salishia*, by examining the mitochondrial COI-5P gene and other DNA markers. Furthermore, Clarkston and Saunders (2013) resolved the species diversity of *Callophyllis* that is distributed in the northeastern Pacific coastline, including a new species, *C. schneideri*, and Klochkova et al. (2013) described *Ionia cornu-cervi* as a member of *Callophyllis* using *rbcL* analysis combined with morphological evidence. Although the species delimitation of the genus *Callophyllis* from the eastern Pacific of both hemispheres was well-established, based on the morphology and molecular evidence, it remains an unresolved taxonomic group in the western Pacific region.

In the western Pacific region of the northern hemisphere, there are currently eight recorded *Callophyllis* species (Yoshida 1998), including six species from Korea (Lee and Kang 2001) and three in China (Xia 2004): *Callophyllis adhaerens* Yamada, *C. adnata* Okamura, *C. crispata* Okamura, *C. hayamensis* Yamada, *C. japonica* Okamura, *C. mageshimensis* Tanaka, *C. palmata* Yamada, and *C. rhynchocarpa* Ruprecht. *C. adhaerens* has branches that adhere to each other or other things, which was recognized as a unique characteristics (Yamada 1932). *C. adnata* is characterized by its softly membranous and horizontally attached by radical discs (Okamura 1932). *C. crispata* has an erect, palmate or flabellate thallus, 10–20 cm high, and 20–30 mm wide, with a crenulated margin (Okamura 1896). *C. crispata* and *C. palmata* have a similar external habit, including the coriaceous substance, but they are distinguished by the position of the cystocarps (Okamura 1916, 1932, Yamada 1930). *C. japonica* is distinguished by its pinnately subdichotomous thallus in irregular, growing spinescent protuberances at the margin, and the location of cystocarps (Okamura 1936). The above recorded species, except *C. rhynchocarpa*, were considered endemic in the northwestern Pacific (Guiry and Guiry 2014).

The species of *Callophyllis* mostly grow in the subtidal zone, but specimens have hardly been collected from this

area, because of the difficulty of approaching deep water. Therefore, we have continuously surveyed subtidal habitats around Jeju Island, Korea, to discover cryptic species of the red algae. The aims of this study are to clarify the species delimitations of *Callophyllis*, based on its morphology and molecular analyses of plastid *rbcL* and mitochondrial COI-5P, to discover cryptic species diversity in the subtidal zone of the Korean coast by applying molecular tools, and to understand the phylogenetic relationships of *Callophyllis* species that occur in the northwestern Pacific.

MATERIALS AND METHODS

The *Callophyllis* specimens examined in the present study are listed in the Table 1. The collections of samples were conducted in the intertidal and subtidal zones by SCUBA diving in Korea and Japan, including the type locality, Enoshima. Samples were transported to the laboratory, and photographed by a D-80 camera (Nikon, Tokyo, Japan). The samples for DNA extraction were detached from the thallus to make silica-gel-dried tissue samples. The voucher specimens were made into a pressed one on the herbarium paper and deposited in the herbarium of the Jeju National University (JNUB), Jeju, and in the National Institute of Biological Resources (NIBR), Incheon, Korea. Samples for anatomical study were preserved in 4–5% formalin in seawater. After describing the external morphology by using a dissecting microscope SZ61 (Olympus, Tokyo, Japan), sections were done by hand using a razor or a freezing microtome (NK-101-II; Nippon Optical Works Co. Ltd., Tokyo, Japan). In addition, specimens for squash preparations were soaked for 1–2 h in a 5% solution of 5 N KOH, then rinsed in distilled water for 1 h (Schneider et al. 2014). Sections and squash preparations were stained with 1% acidified aniline blue and mounted in 30% corn syrup. The Wittmann's aceto-iron-hematoxylin chloral hydrate staining method (Wittmann 1965) was used for precise observations of the carpogonial branches with 50% Hoyer's mounting medium (Lin et al. 2012). Photomicrographs were taken using a QImaging 1394 camera (QImaging, Surrey, BC, Canada) attached to a BX50 microscope (Olympus, Tokyo, Japan).

DNA was extracted using DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Extracted DNA was processed to the polymerase chain reaction (PCR) to amplify *rbcL* and COI-5P genes. The primer combinations used to amplify each gene region were *rbcL*_F145-*rbcL*_R898 and *rbcL*_F762-

Table 1. Collection information and GenBank accession number of *Callophyllis* specimens analyzed in this study

Taxa	Collection information	Habitat (depth / substratum)	Voucher	GenBank accession No.	
				<i>rbcL</i>	COI-5P
<i>Callophyllis adhaerens</i> Yamada	Munseom, Jeju, Korea; 17 Jun. 2009		JN-CA09sp002	KM675242	KM675335
	Jungmun, Jeju, Korea; 16 Mar. 2010		JN-CA10ad002	KM675240	KM675340
	Mara Is., Jeju, Korea; 17 Jul. 2010		JN-CA10ad004	KM675241	KM675336
	Udo, Jeju, Korea; 12 Jun. 2011		JN-CA11ad003	KM675239	KM675337
	Biyangdo, Jeju, Korea; 30 Jul. 2011		JN-CA11ja001		KM675339
	Udo, Jeju, Korea; 11 Jun. 2011		JN-CA11sp012		KM675338
	Biyangdo, Jeju, Korea; 7 Apr. 2012	12-15 m depth / rock	JN-CA12sp006		KM675341
	Jongdal, Jeju, Korea; 6 Jun. 2012	10-12 m depth / rock	JN120606-05		KM675342
	Jongdal, Jeju, Korea; 21 Jun. 2012	10-12 m depth / rock	JN120621-01		KM675343
	Gochome, Shimoda, Japan; 26 Mar. 2014	8-10 m depth / rock	JN140326-013		KM675344
Gochome, Shimoda, Japan; 26 Mar. 2014	8-10 m depth / rock	JN140326-014		KM675345	
Gochome, Shimoda, Japan; 26 Mar. 2014	8-10 m depth / rock	JN140326-015		KM675346	
Enoshima, Fujisawa, Japan; 28 Mar. 2014	Intertidal drift	JN140328-069		KM675347	
Enoshima, Fujisawa, Japan; 28 Mar. 2014	Intertidal drift	JN140328-070		KM675348	
Sangmo, Jeju, Korea; 5 Apr. 2011		JN-CA11sp006		KM675295	
Jongdal, Jeju, Korea; 6 Jun. 2012	5-10 m deep / rock	JN120606-01		KM675296	
Dueokdo, Wando, Korea; 27 Jul. 2012	10 m deep / rock	JN120727-45		KM675329	
Dueokdo, Wando, Korea; 27 Jul. 2012	10 m deep / rock	JN120727-60		KM675328	
Onjuku, Isumi, Japan; 24 Mar. 2014	Intertidal drift	JN140324-017		KM675349	
Ohara, Isumi, Japan; 24 Mar. 2014	Intertidal drift	JN140324-100		KM675350	
Ohara, Isumi, Japan; 24 Mar. 2014	Intertidal drift	JN140324-101		KM675351	
Ohara, Isumi, Japan; 24 Mar. 2014	Intertidal drift	JN140324-102		KM675352	
Enoshima, Fujisawa, Japan; 28 Mar. 2014	Intertidal drift	JN140328-071		KM675353	
Enoshima, Fujisawa, Japan; 28 Mar. 2014	Intertidal drift	JN140328-072		KM675354	
Jochoon, Jeju, Korea; 20 May 2011	25-30 m depth / rock	JN-CA11an002		KM675355	
Jochoon, Jeju, Korea; 20 May 2011	25-30 m depth / rock	JN-CA11an003		KM675356	
Udo, Jeju, Korea; 23 Jul. 2009		JN-CA09ja003		KM675361	
Udo, Jeju, Korea; 30 Jan. 2010		JN-CA10ja001		KM675366	
Shinyang, Jeju, Korea; 14 Jun. 2010		JN-CA10ja003		KM675368	
Udo, Jeju, Korea; 21 Feb. 2010		JN-CA10ja007		KM675370	
Jongdal, Jeju, Korea; 6 Jan. 2012	5-8 m deep / rock	JN-CA12ja005		KM675265	
Jongdal, Jeju, Korea; 6 Jan. 2012	5-8 m deep / rock	JN-CA12ja006		KM675264	
Jongdal, Jeju, Korea; 6 Jan. 2012	5-8 m deep / rock	JN-CA12ja007		KM675263	
Goseong, Jeju, Korea; 3 Mar. 2012	Intertidal / rock	JN-CA12ja009		KM675261	
Goseong, Jeju, Korea; 3 Mar. 2012	Intertidal / rock	JN-CA12ja010		KM675254	
Gimnyeong, Jeju, Korea; 10 Mar. 2012	Intertidal / rock	JN-CA12ja011		KM675253	
Hanlim, Jeju, Korea; 6 Apr. 2012	Intertidal / rock	JN-CA12ja012		KM675252	
Ganjeolgot, Ulsan, Korea; 19 Aug. 2011	Intertidal / rock	JN-GJG-20		KM675280	
Hanlim, Jeju, Korea; 22 April 2012	Intertidal / rock	JN120422-02		KM675260	
Hado, Jeju, Korea; 15 May 2012	Intertidal / rock	JN120515-19		KM675259	
Jongdal, Jeju, Korea; 6 Jun. 2012	5-8 m deep / rock	JN120606-03		KM675258	
Jongdal, Jeju, Korea; 6 Jun. 2012	5-8 m deep / rock	JN120606-04		KM675257	
Jongdal, Jeju, Korea; 6 Jun. 2012	5-8 m deep / rock	JN120606-19		KM675251	
Enoshima, Fujisawa, Japan; 28 Mar. 2014	Intertidal drift	JN140328-075		KM675255	
Enoshima, Fujisawa, Japan; 28 Mar. 2014	Intertidal drift	JN140328-076		KM675256	

Table 1. Continued

Taxa	Collection information	Habitat (depth / substratum)	Voucher	GenBank accession No.	
				<i>rbcL</i>	COI-5P
<i>Callophyllis mollitia</i> sp. nov.	Pyeoseon, Jeju, Korea; 4 Feb. 2010		JN-CA10an001	KM675301	KM675330
	Udo, Jeju, Korea; 30 Jan. 2010		JN-CA10sp001	KM675304	KM675331
	Haengwon, Jeju, Korea; 15 Apr. 2010		JN-CA10sp006	KM675303	
	Chagwido, Jeju, Korea; 6 Jun. 2011		JN-CA10sp007	KM675302	
	Jongdal, Jeju, Korea; 6 Jan. 2012	10 m deep / invertebrates	JN-CA12sp001	KM675300	
	Jongdal, Jeju, Korea; 6 Jan. 2012	10 m deep / invertebrates	JN-CA12sp002	KM675299	
	Jongdal, Jeju, Korea; 6 Jan. 2012	10 m deep / invertebrates	JN-CA12sp003	KM675298	
	Jongdal, Jeju, Korea; 6 Jan. 2012	10 m deep / invertebrates	JN-CA12sp004	KM675297	KM675332
	Daesambudo, Yeosu, Korea; 25 Jul. 2012	10-15 m deep / corallines	JN120725-06		KM675333
	Daesambudo, Yeosu, Korea; 25 Jul. 2012	10-15 m deep / corallines	JN120725-07		KM675334
<i>Callophyllis repens</i> sp. nov.	Shinchang, Jeju, Korea; 17 Jul. 2012	25-30 m deep / rock, coralline	JN120717-20	KM675294	
	Shinchang, Jeju, Korea; 17 Jul. 2012	25-30 m deep / rock, coralline	JN120717-21	KM675293	
<i>Callophyllis serratifolia</i> sp. nov.	Munseom, Jeju, Korea; 28 Mar. 2010		JN-CA10cr001	KM675290	KM675305
	Gwideok, Jeju, Korea; 31 May 2011		JN-CA11sp002		KM675306
	Gwideok, Jeju, Korea; 31 May 2011		JN-CA11sp003		KM675307
	Munseom, Jeju, Korea; 13 May 2012		JN120513-22	KM675287	KM675308
	Munseom, Jeju, Korea; 13 May 2012	8-12 m deep / rock	JN120513-24	KM675286	KM675309
	Munseom, Jeju, Korea; 2 Jun. 2012	8-15 m deep / rock	JN120602-04	KM675289	
	Munseom, Jeju, Korea; 2 Jun. 2012	8-15 m deep / rock	JN120602-05	KM675288	KM675310
	Munseom, Jeju, Korea; 2 Jun. 2012	8-15 m deep / rock	JN120602-06	KM675283	KM675311
	Geomundo, Yeosu, Korea; 25 Jul. 2012	10-15 m deep / rock	JN120725-49		KM675312
	Geomundo, Yeosu, Korea; 25 Jul. 2012	10-15 m deep / rock	JN120725-51		KM675313
<i>Callophyllis undulata</i> sp. nov.	Geomundo, Yeosu, Korea; 25 Jul. 2012	10-15 m deep / rock	JN120725-52		KM675314
	Jakdo, Yeosu, Korea; 26 Jul. 2012	10-15 m deep / rock	JN120726-08		KM675285
	Jakdo, Yeosu, Korea; 26 Jul. 2012	10-15 m deep / rock	JN120726-09	KM675284	
	Jakdo, Yeosu, Korea; 26 Jul. 2012	10-15 m deep / rock	JN120726-10	KM675282	
	Baekdo, Yeosu, Korea; 26 Jul. 2012	10-15 m deep / rock	JN120726-45	KM675281	
	Cheongsando, Wando, Korea; 27 Jul. 2012	10-15 m deep / rock	JN120727-42		KM675316
	Cheongsando, Wando, Korea; 27 Jul. 2012	10-15 m deep / rock	JN120727-43		KM675317
	Gochoe, Shimoda, Japan; 27 Mar. 2014	10-15 m deep / rock	JN140327-043		KM675318
	Gochoe, Shimoda, Japan; 27 Mar. 2014	8-10 m deep / rock	JN130427-044		KM675319
	Udo, Jeju, Korea; 11 Jun. 2011	25-30 m deep / rock	JN-CA11sp008		KM675320
<i>Callophyllis undulata</i> sp. nov.	Udo, Jeju, Korea; 11 Jun. 2011	25-30 m deep / rock	JN-CA11sp009		KM675324
	Udo, Jeju, Korea; 11 Jun. 2011	25-30 m deep / rock	JN-CA11sp011		KM675326
	Udo, Jeju, Korea; 12 Jun. 2011	25-30 m deep / rock	JN-HU110612-01	KM675291	KM675321
	Udo, Jeju, Korea; 12 Jun. 2011	25-30 m deep / rock	JN-HU110612-02	KM675292	KM675322
	Udo, Jeju, Korea; 12 Jun. 2011	25-30 m deep / rock	JN-HU110612-03		KM675323
	Udo, Jeju, Korea; 12 Jun. 2011	25-30 m deep / rock			

rbcl_R1442 (Kim et al. 2012), and *GazF1-GazR4* in *COI-5P* (Saunders 2008). PCR amplification was conducted in a total volume of 20 μ L with AccuPower PCR PreMix (Bioneer, Daejeon, Korea) using Peltier Thermal Cycler, PTC-100 (BIO-RAD, CA, USA). Each PCR profile of *rbcl* and *COI-5P* was followed by Saunders and Moore (2013). PCR products were purified using an AccuPrep Purification Kit (Bioneer), and then all sequencing was outsourced to Macrogen (Seoul, Korea). Both electropherogram outputs from each sample were edited using Chromas ver. 1.45 (Technelysium Pty Ltd., Helensvale, QLD, Australia). The total *rbcl* and *COI-5P* sequences were organized using the multiple-sequence editing program BioEdit (Hall 1999) and aligned visually.

A neighbor-joining analysis of *COI-5P* alignment with distance corrected under a Kimura 2-parameter model was completed using MEGA ver. 5.10 (Tamura et al. 2011). For *rbcl* phylogeny of the genus *Callophyllis*, our specimens were analyzed with sixteen *Callophyllis* species from GenBank including the generitype *Callophyllis variegata* (HQ910494), and three outgroups: *Pugetia fragilissima* (JX969789), *Salishia frima* (HQ910506), and *Kallymenia reniformis* (KF280990). Maximum likelihood (ML) analyses were performed using RAxML software (Stamatakis 2006). We used the “-f a” option for rapid bootstrap analysis, and best likelihood tree searching using “-# 1000” and 200 independent tree inferences with the “number of run” option, with default-I and an automatically optimized subtree pruning and regrafting (SPR) rearrangement to identify the best tree. To generate bootstrap values for the phylogeny, the statistical support for each branch was obtained from 1,000 replications, using the same substitution model and RAxML program settings. In addition, we performed Bayesian phylogenetic inferences (BI) using MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck 2003) with the Markov Chain Monte Carlo (MCMC) to get the Bayesian posterior probabilities.

RESULTS

Molecular analyses

In total, we analyzed 50 *rbcl* and 59 *COI-5P* sequences of *Callophyllis* specimens from Korea and Japan (Table 1) and confirmed ten species. We aligned 1,290 nucleotide base pairs of *rbcl* gene. Of all sites, 450 (34.9%) were variable and 356 (27.6%) were phylogenetically informative. In the ML phylogenetic tree of *rbcl* sequences (Fig. 1), nine species were confirmed: *C. adhaerens* from Korea,

C. japonica from both countries, *C. adnata* from Japan, *C. hayamensis* from Korea as a new record, and five cryptic new species—*C. cartilaginea*, *C. mollitia*, *C. repens*, *C. serratifolia*, and *C. undulata*. However, we failed PCR amplification of *rbcl* for *C. crispata* from Japan. The interspecific divergence of *rbcl* gene ranged from 0.6 % to 9.9 % in the genus *Callophyllis*. *C. hayamensis* showed 0.8–0.9% interspecific divergence with *C. serratifolia* sp. nov., and 1.1–1.2% with *C. undulata* sp. nov. There was 0.6–0.8% interspecific divergence between *C. serratifolia* sp. nov. and *C. undulata* sp. nov. In *rbcl* analysis, *C. repens* sp. nov. formed a basal node of the clade consisting of *C. hayamensis*, *C. serratifolia* sp. nov., and *C. undulata* sp. nov., but we failed to confirm in *COI-5P* analysis. *C. japonica* was sister to *C. cartilaginea* sp. nov. with 1.4–1.5% interspecific divergence. The intraspecific divergence of *C. japonica* was 0.3–1.0% with Korean specimens, Japanese specimens from the type locality, and other Japanese specimen (HQ910507) in NCBI. *C. mollitia* sp. nov. formed a clade with *C. adnata* from the type locality in Japan, showing 3.1–3.2% interspecific divergence. *C. adhaerens* did not show phylogenetic affinity with any other species from GenBank. The maximum likelihood (ML) and Bayesian inference (BI) of *rbcl* analyses resulted in a phylogenetic tree in which most *Callophyllis* species from the northwestern Pacific were strongly supported, more than 95% in bootstrap (BS) and 1.00 in posterior probabilities (PP), and their topology was congruent at an interspecific level (Fig. 1).

The neighbor-joining tree of *COI-5P* (701 bp) is shown in Fig. 2. We resolved nine *Callophyllis* species delimitation, except for the sequences of *C. repens* sp. nov. in *COI-5P* analysis. The genetic variations within species ranged 0–1.0% in *C. japonica*, including one specimen from Japan, 0–1.6% in the clade of *C. serratifolia* sp. nov. included seven specimens from both countries, and 0–0.2% in *C. undulata* sp. nov. The interspecific variation ranged 2.8–9.5% in the genus *Callophyllis*. *C. serratifolia* sp. nov. and *C. undulata* sp. nov. formed a sister group like *rbcl* analysis, showing 2.8–3.3% interspecific divergence. *C. serratifolia* sp. nov. exhibited 1.2–1.6% intraspecific divergence between Korean and Japanese specimens. *C. hayamensis* was sister to *C. cartilaginea* sp. nov., with 4.5% interspecific divergence. *C. japonica* showed more interspecific divergence for *COI-5P* with *C. cartilaginea* sp. nov., 5.7–5.9%, than that through *rbcl* analysis, 1.4–1.5%. The intraspecific divergence of *C. japonica* from Korea and Japan was 1.0%. The intraspecific divergence of *C. mollitia* sp. nov. and *C. adhaerens* was identical, but *C. adnata* from the type locality in Japan is 0.3%. *C. crispata* from Japan

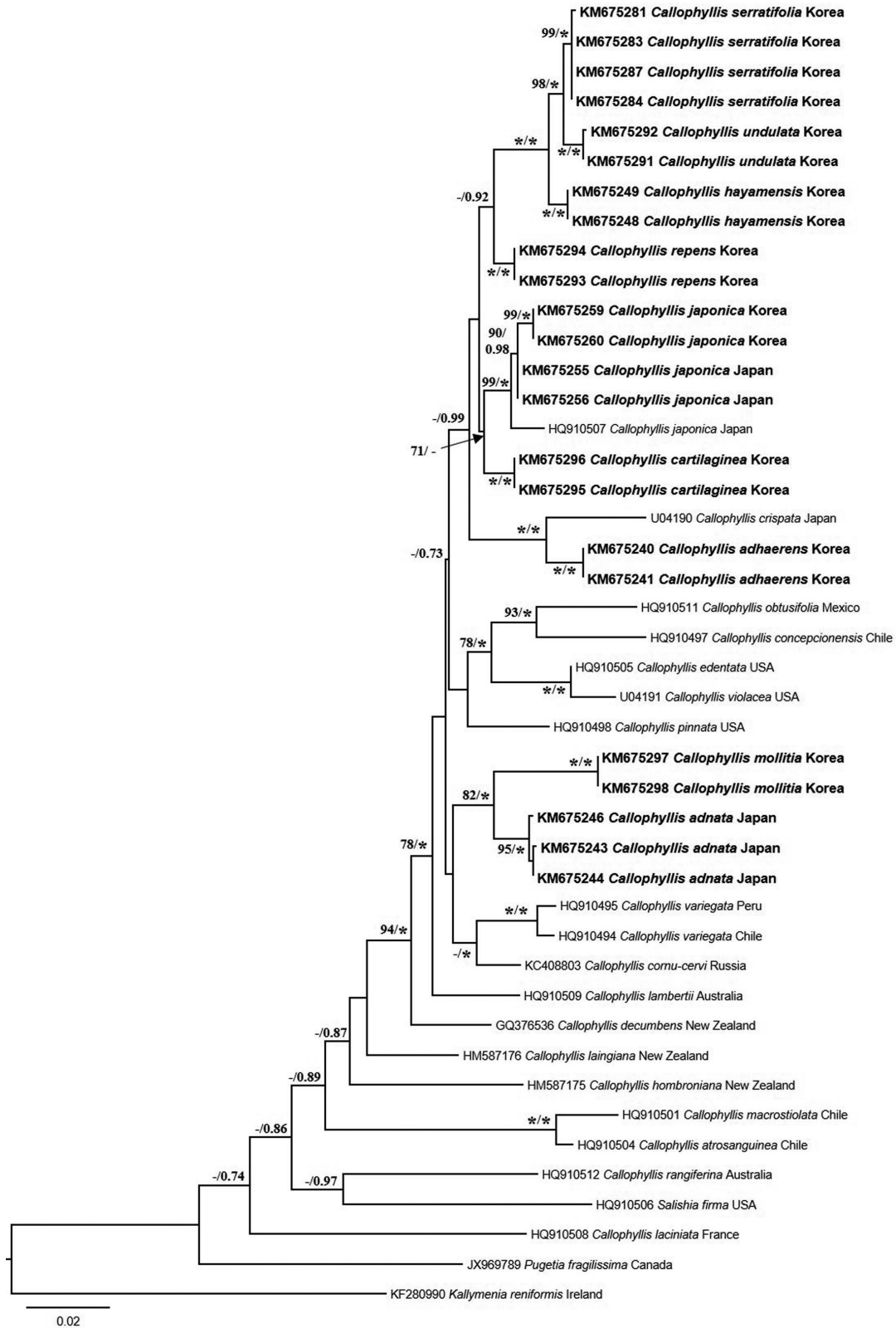


Fig. 1. Phylogenetic tree of the genus *Callophyllis* species (bold is collected in this study and normal is acquired from GenBank) based on *rbcL* sequences inferred from maximum-likelihood analysis. Bootstrap value are shown above the branches: maximum-likelihood (left) and Bayesian posterior probabilities (right). Branches marked with an asterisk received 100% support in both analyses, whereas those lacking values received less than 70% support. Scale bar: substitutions/site.

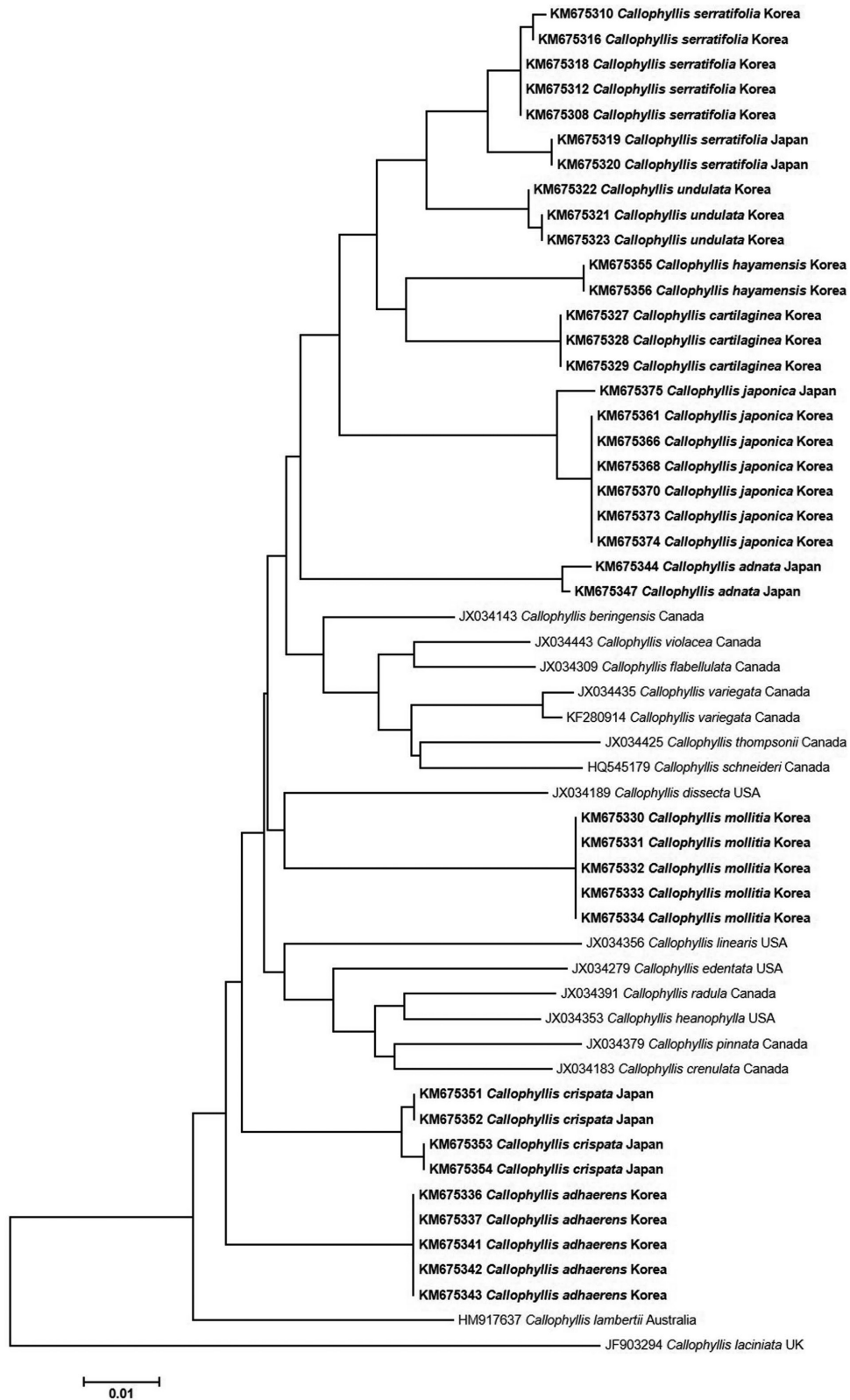


Fig. 2. Unrooted phylogram generated using neighbor-joining analysis from the cytochrome oxidase I (COI) sequences of *Callophyllis* specimens collected in this study (taxa in bold) and acquired from GenBank (taxa not bold). Scale bar: substitutions/site.

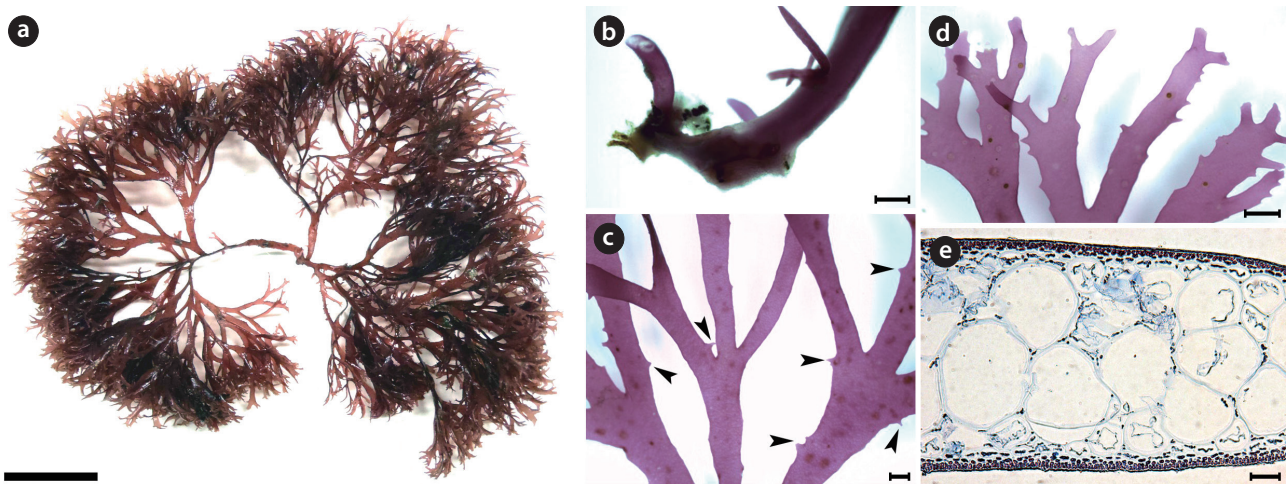


Fig. 3. *Callophyllis cartilaginea* H. W. Lee & M. S. Kim sp. nov. (a) Holotype specimen (JN120606-01, vegetative plant, Jongdal, 06 June 2012). (b) Very short compressed stipe arising from flattened holdfast. (c) Frond trichotomously branched at the middle portion bearing short spinescent protuberances (arrowheads). (d) Frond sub-dichotomously branched at the apical portion and having acute apex. (e) Cross section view showing three celled layer of medulla consisting of isodiametric cells. Scale bars represent 2 cm (a), 500 μ m (b), 1,000 μ m (c, d), and 50 μ m (e).

showed 0.3% intraspecific divergence between the specimens from Enoshima, the type locality, and from Chiba in Japan.

Taxonomic conclusions

Callophyllis cartilaginea H. W. Lee & M. S. Kim sp. nov. (Fig. 3)

Description: Thallus cartilaginous, erect, narrowly flabellate, dark- to brown-red in color, di- or trichotomously branched in a bushy clump, stipitate in slightly compressed, 5-7 cm long, 1.1-1.7 mm wide in the middle, attached to the substratum by a sub-discooidal holdfast; apex acute; margin usually smooth with spinescent protuberances.

Holotype: JN120606-01 (Fig. 3a), vegetative plant, collected at 5–10 m in depth of the subtidal zone on 06 June 2012 and deposited in the Herbarium of Department of Biology, Jeju National University, Korea (JNUB).

Type locality: Jongdal, Jeju, Korea (33°29'34.40" N, 126°55'16.23" E).

Etymology: The specific epithet (*cartilaginea*) was chosen to represent this species, as it has a cartilaginous thallus.

Distribution: Korea (this study).

Selected specimens: JN120606-01-2 (Jongdal, 06 June 2012, vegetative); 120727-45, 60 (Dueokdo, 27 July 2012, vegetative); NIBRAL0000143264, 140423-s01-3 (Jongdal, 23 April 2014, vegetative).

Morphology: Plants are erect, flabellate, cartilaginous,

and dark-red in color (Fig. 3a). Thallus has a short sub-cylindrical stipe arising from a discooidal holdfast, which gets more compressed (Fig. 3b). The thallus is di- or trichotomously branched (Fig. 3c), and is irregularly sub-dichotomously branched at the upper portion (Fig. 3d). The thallus is 5–7 cm long, 310–358 μ m thick, and 1.1–1.7 mm wide, but 2.2–3.4 mm wide at the branching point. The thallus margin is almost smooth, and often small spinescent protuberances laterally (Fig. 3c and 3d). The apex is acute (Fig. 3d). The medulla is made of a two- or three-celled layer: Two large isodiametric cells, 112–146 μ m \times 104–160 μ m in size, and one small oblong cell, 49–61 μ m \times 58–72 μ m in size (Fig. 3e). Outer cortical cells are bead-like and 4.3–5.6 μ m \times 4.5–5.4 μ m in size, whereas inner cells are oblong and 4.1–5.7 μ m \times 6.4–8.5 μ m in size.

Callophyllis mollitia H. W. Lee & M. S. Kim sp. nov. (Fig. 4)

Description: Thallus membranous, tender, flattened, palmate, bright brown-red in color, sub-dichotomously branched, shortly stipitate, 3–5 cm long, 4.3–5.1 mm wide in the middle, attached to the substratum by a discooidal holdfast with several minute radical discs arising from the thallus; apex roundish, margin smooth, cystocarps hemispherical scattered over the entire blades with 2–3 one-sided ostioles; tetrasporangia cruciately divided, scattered irregularly in the cortex.

Holotype: JN120725-07 (Fig. 4a), female gametophyte, collected at 12–15 m in depth of the subtidal zone on 25

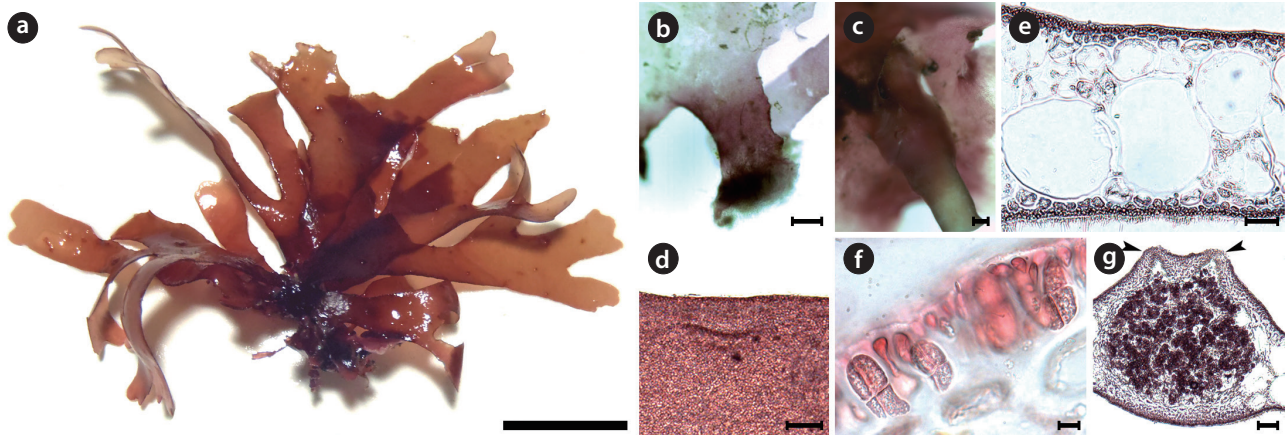


Fig. 4. *Callophyllis mollitia* H. W. Lee & M. S. Kim sp. nov. (a) Holotype specimen (JN120727-07, ♀, Daesambudo, 07 July 2012). (b) The Main radical disc. (c) Minute radical discs borne at the basal portion of frond grasping substratum (articulated coralline algae). (d) Smooth margin of frond. (e) Cross section view showing medulla consisting of irregular size of isodiametric cells. (f) Tetrasterangia cruciately divided scattered in the cortical layer. (g) Cross section view showing cystocarp with two ostioles (arrowheads). Scale bars represent 1 cm (a), 500 μ m (b), 300 μ m (c), 100 μ m (d, g), 50 μ m (e), 10 μ m (f).

July 2012 and deposited in the Herbarium of Department of Biology, Jeju National University, Korea (JNUB).

Isotype: NIBR (NIBRAL0000143265, vegetative) and JNUB (JN120725-08, ♀).

Type locality: Daesambudo, Yeosu, Korea (34°03'07.17" N, 127°22'56.58" E).

Etymology: The specific epithet (*mollitia*) was chosen to represent the tender thallus of this species.

Distribution: Korea (this study).

Selected specimens: JN12sp001-4 (Jongdal, 06 January 2012, ♂); NIBRAL0000143265 (Daesambudo, 25 July 2012, vegetative), 120725-07-8 (Daesambudo, 25 July 2012, ♀); 120130-16-18 (Munseom, 30 January 2013, vegetative).

Morphology: Plant is flattened, palmate, tender, membranous, and bright brown-red in color (Fig. 4a). Its thallus is branched irregularly 2–3 times (Fig. 4a). The thallus has a very short stipe arising from a radical disc (Fig. 4b), and is attached by several minute radical discs borne laterally or ventrally at the basal portion (Fig. 4c). The thallus is 3–5 cm long, 279.8–315.7 μ m thick, 1.9–2.9 mm wide at the basal, 4.3–5.1 mm wide at the middle, and 2.3–2.9 mm at the upper portion. The thallus margin is smooth all over (Fig. 4d). The apex is roundish or bluntly dissected. The medulla is made up of irregular isodiametric or subisodiametric cells (Fig. 4e), the smaller cells are 75–83 μ m \times 80–110 μ m in size, and the larger cells are 141–204 μ m \times 152–198 μ m. The cortex is made up of bead-like cells, 5.3–5.9 μ m \times 4.9–6.1 μ m in size (Fig. 4e). Tetrasterangia are oblong and cruciately divided, and they are located in the cortex, 31.1–34.5 μ m \times 13.0–15.6 μ m in size (Fig. 4f).

Cystocarps are scattered over the entirety of the blades, more protuberant dorsally than ventrally, and they have several ostioles opened dorsally (Figure 4g). The cystocarps are 738.6–784.6 μ m \times 795.3–818.7 μ m.

Callophyllis repens H. W. Lee & M. S. Kim sp. nov. (Fig. 5)

Description: Thallus creeping, flattened, flabellate, slightly cartilaginous, dark red in color, dichotomously branched, 2–5 cm long, 2.1–2.8 wide in the middle, attached to the substratum by a discoidal holdfast with several minute radical discs; apex is acute; margin minutely crenulated, a few globular lateral protuberances, and adherent laterally to neighboring blade margins.

Holotype: JN120717-20 (Fig. 5a), vegetative plant, collected at 25–30 m in depth of the subtidal zone on 17 July 2012 and deposited in the Herbarium of Department of Biology, Jeju National University, Korea (JNUB).

Isotype: NIBR (NIBR0000143263, vegetative).

Type locality: Shinchang, Jeju, Korea (33°21'00.88" N, 126°09'51.26" E).

Etymology: The specific epithet (*repens*) was chosen to represent the creeping thallus of this species.

Distribution: Korea (this study).

Morphology: Plants are flattened, slightly cartilaginous, and dark-red in color (Fig. 5a). The thallus is dichotomously branched in the same plane (Fig. 5a). The thallus arises from a main discoidal holdfast (Fig. 5b) and bears several minute radical discs at the basal portion (Fig. 5c).

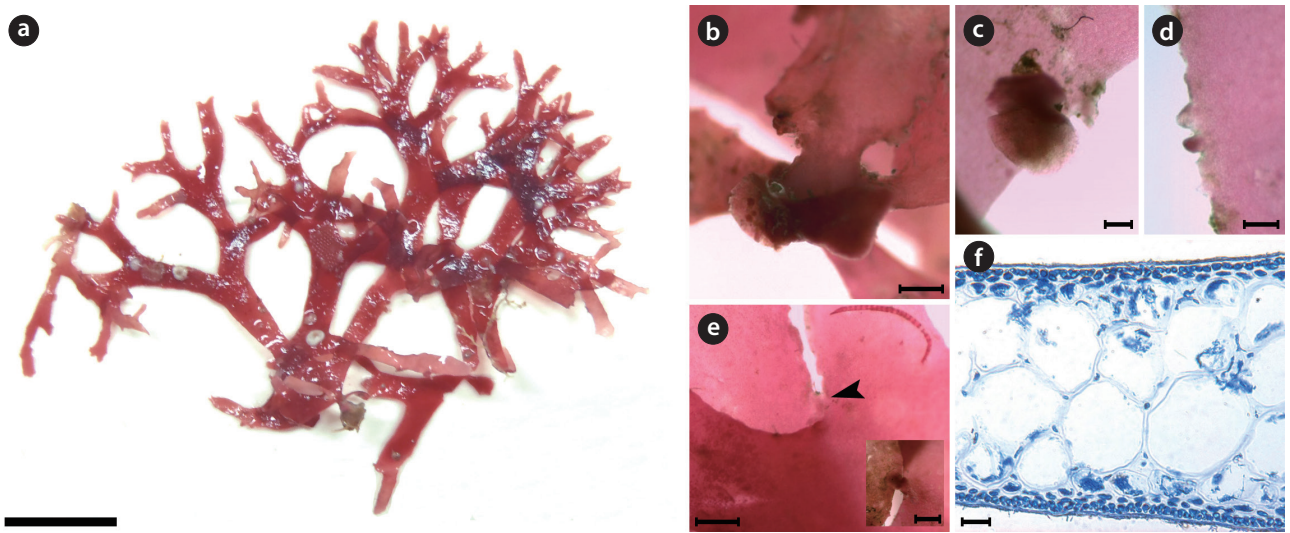


Fig. 5. *Callophyllis repens* H. W. Lee & M. S. Kim sp. no. (a) Holotype specimen (JN120717-20, vegetative plant, Shinchang, 17 July 2012). (b) Main holdfast of the frond. (c) Minute radical disc borne at the basal portion of frond. (d) Globular protuberances borne from minutely crenulate margin of thallus. (e) The adhering point (arrowhead) of frond attached at the margin. (f) Cross section view showing one celled cortical layer and large isodiametric medullary cells surrounded by small cells. Scale bars represent 2 cm (a), 500 μ m (b, e), 200 μ m (c, d), 25 μ m (f).

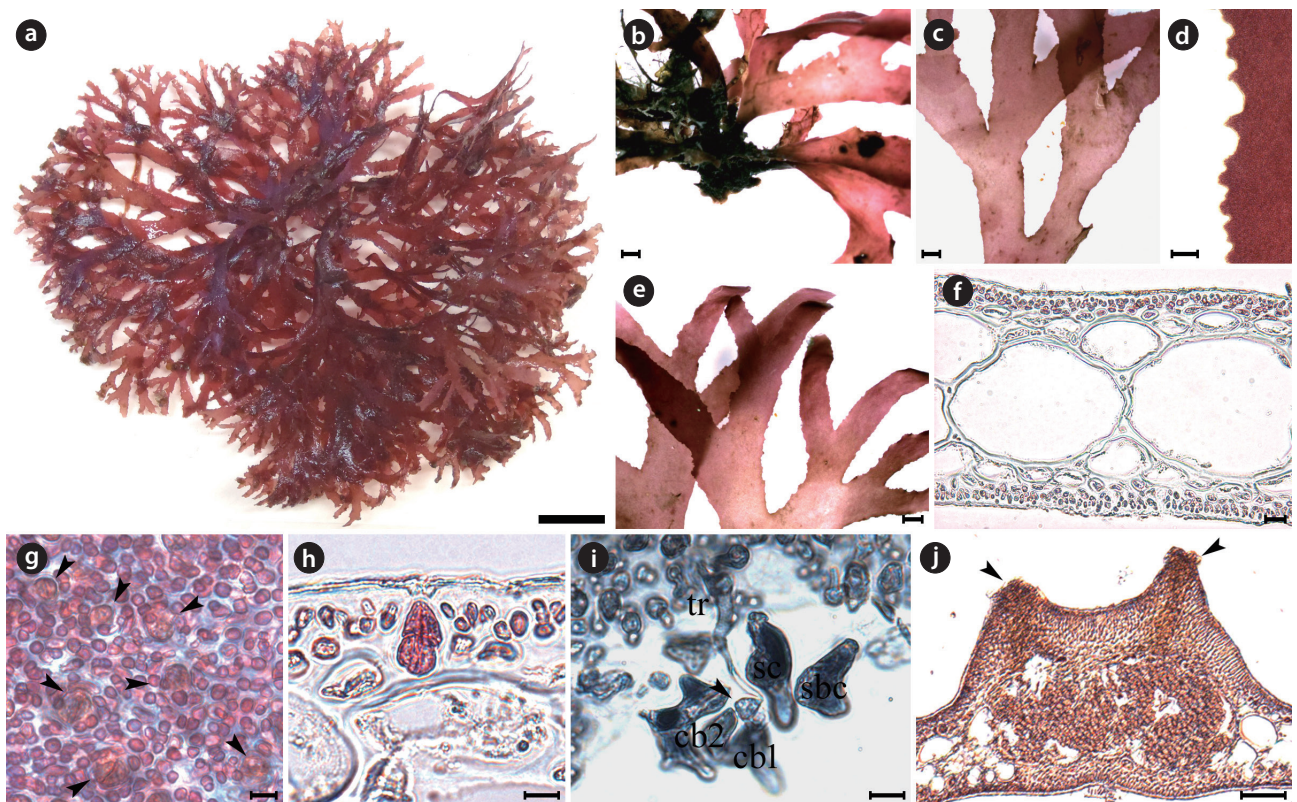


Fig. 6. *Callophyllis serratifolia* H. W. Lee & M. S. Kim sp. nov. (a) Holotype specimen (JN120602-04, vegetative plant, Munseom, 02 June 2012). (b) A discoidal holdfast bearing several fronds. (c) Thallus dichotomously branched. (d) The serration at the margin of frond. (e) Frond having narrowly blunt apex (f) Cross section of frond showing two celled layer of cortex, and large isodiametric cells of central medulla surrounded by small cells. (g) Surface view showing scattered tetrasporangia (arrowheads). (h) Tetrasporangia cruciately divided in the cortical layer. (i) Three celled carpoogonial branch and carpoogonium (arrowhead) borne supporting cell with subsidiary cells. (j) Cross section of cystocarp with two ostioles (arrowheads). Scale bars represent 1 cm (a), 1,000 μ m (b, c, e), 100 μ m (d, j), 20 μ m (f), 10 μ m (g-i).

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The thallus is 2–5 cm long, 200–220 μm thick, and 2.1–2.8 mm wide at the middle portion, getting narrower to the upper portion, 0.9–1.7 mm wide. The apex is acute. The thallus margin is minutely crenulated (Fig. 5d). One blade of the thallus is adhered to another blade at the margin by the lateral protuberances of both blades (Fig. 5e). The medulla is made up of irregular isodiametric or sub-isodiametric cells, 61.8–90.1 μm \times 58.6–84.9 μm in size (Fig. 5f). The cortex is a one- or two-celled layer, 5.3–6.7 μm \times 5.2–6.9 μm in size (Fig. 5f).

***Callophyllis serratifolia* H. W. Lee & M. S. Kim sp. nov. (Fig. 6)**

Description: Thallus flattened, flabellate, slightly cartilaginous, dark red in color, dichotomously branched in a bushy clump, shortly stipitate, 5–7 cm long, 2.3–4.9 mm wide in the middle, attached to the substratum by a discoidal holdfast; apex narrowly blunt; margins serrated; procarpus consists of a supporting cell, 2–4 subsidiary cells, three-celled carpogonial branch; cystocarps spherical and scattered over the upper blades with 2–6 both-sided ostioles; tetrasporangia cruciately divided and scattered irregularly in the cortex.

Holotype: JN120602-04 (Fig. 6a), vegetative plant, collected at 8–15 m in depth of the subtidal zone on 02 June 2012 and deposited in the Herbarium of Department of Biology, Jeju National University, Korea (JNUB).

Isotype: JNUB (JN120602-5, ♀) and NIBR (NIBR0000143261, vegetative).

Type locality: Munseom, Seogwipo, Korea (33°13'40.25" N, 126°34'01.05" E).

Etymology: The specific epithet (*serratifolia*) was chosen to represent the fact that the marginal outline of this species has serrated edges.

Distribution: Korea (Lee 2008) and Japan (this study).

Selected specimens: JN120513-22, 24 (Munseom, 13 May 2012, vegetative); JN120602-04, NIBR0000143261 (Munseom, 02 June 2012, vegetative); JN120602-05 (Munseom, 02 June 2012, ♀); JN120725-49-52 (Geomundo, 28 July 2012, vegetative); JN120727-43 (Dueokdo, 27 July 2012, ♂); 120727-44 (Dueokdo, 27 July 2012, ♀); JN2013-v0285 (Seopseom, 05 April 2013, ♂); 130616-25 (Munseom, 16 July 2013, ♀).

Morphology: Plants are flattened, flabellate, slightly cartilaginous, and dark-red in color (Fig. 6a), arising from a short cylindrical stipe. The thallus grows as a single blade or a small clump with several blades from a discoidal holdfast (Fig. 6b). The thallus is usually 5–7 cm long, occasionally up to 10 cm long, 2.3–4.9 mm wide at

the middle branches, 1.2–2.1 mm wide at the upper part, 320–395 μm thick at the basal, and 193–212 μm thick at the middle. The thallus is dichotomously branched in the same plane (Fig. 6c), and serrated at the margins (Fig. 6d). The apex is narrowly blunt (Fig. 6e). The medulla consists of large sub-isodiametric central cells, 97–102 μm \times 101–127 μm in diameter, surrounded by several small cells, 54–71 μm \times 55–82 μm in diameter (Fig. 6f). The cortex is usually a two-cell layer consisting of cortical cells, 4.6–6.7 μm \times 5.2–7.8 μm in diameter (Fig. 6f). Tetrasporangia are irregularly scattered on the thallus surface (Fig. 6g), and are cruciately divided in the cortical layer, 2.0–2.3 μm \times 9.5–10.9 μm in diameter (Fig. 6h). The procarpus consists of three-celled carpogonial branches arising from a supporting cell with two subsidiary cells (Fig. 6i). Cystocarps are located on the surface of the upper branches, are 890.4–842.4 μm \times 870.6–920.9 μm , and protude toward both sides with both-sided ostioles (Fig. 6j).

***Callophyllis undulata* H. W. Lee & M. S. Kim sp. nov. (Fig. 7)**

Description: Thallus flattened, flabellate, membranous, erect, stipitate, light scarlet to bright red in color, dichotomously branched in a bushy clump, 3–8 cm long, 2.8–9.9 mm wide in the middle, attached to the substratum by a discoidal holdfast; apex roundish; margin pleated.

Holotype: JNHU110612-01 (Fig. 7a), vegetative plant, collected at 25–30 m in depth of the subtidal zone on 12 June 2011 and deposited in the Herbarium of Department of Biology, Jeju National University, Korea (JNUB).

Isotype: JNUB (JNHU110612-2, vegetative) and NIBR (NIBR0000143262, vegetative).

Type locality: Udo Channel, Jeju, Korea (33°29'55.19" N, 126°56'02.53" E).

Etymology: The specific epithet (*undulata*) was chosen to represent the pleated edge of the marginal outline of this species.

Distribution: Korea (this study).

Selected specimens: JNHU110612-01 & 03, NIBR0000143262 (Udo Channel, 12 June 2011, vegetative).

Morphology: Plants are erect, flabellate, scarlet to bright-red in color (Fig. 7a), and have a cylindrical stipe that arises from a discoidal holdfast (Fig. 7b). The thallus is 3–8 cm long, 2.8–9.9 mm wide, and 99.1–190.5 μm thick. The thallus is dichotomously branched (Fig. 7c), pleated, and bluntly serrated at the margin (Fig. 7d). The apex is roundish (Fig. 7e). The medulla is almost entirely a two-celled layer of large sub-diametric cells, 42.2–85.4 μm \times

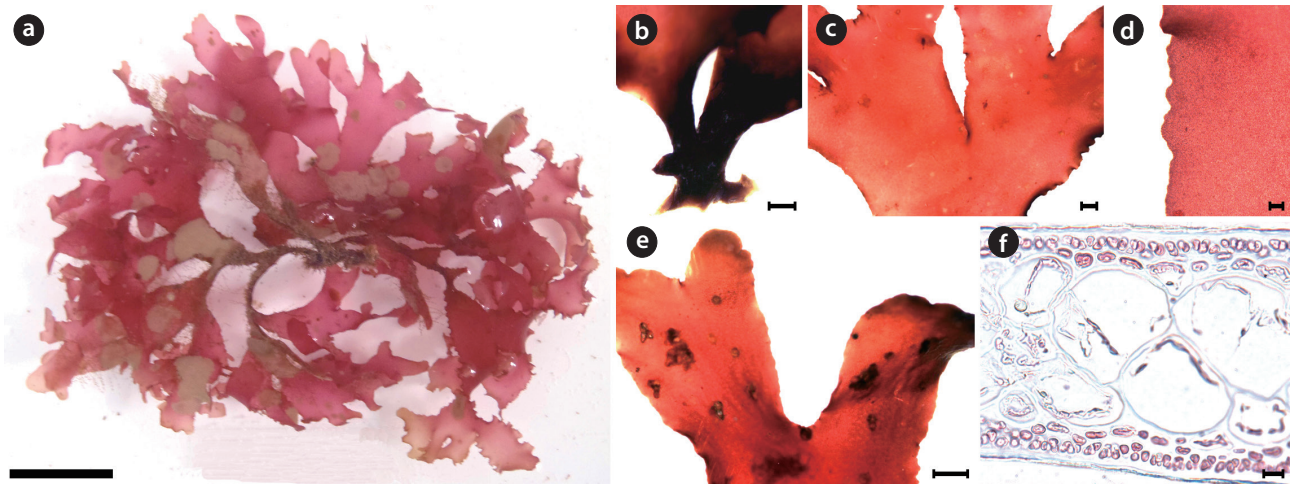


Fig. 7. *Callophyllis undulata* H. W. Lee & M. S. Kim sp. nov. (a) Holotype specimen (JNHU110612-01, vegetative plant, Udo Channel, 02 June 2011). (b) Two stipes arising from a discoidal holdfast. (c) Thallus having pleated margin and dichotomously branched frond. (d) Thallus margin pleated and bluntly serrate. (e) Frond having roundish apex. (f) Cross section showing two celled layers of the cortex consisting of isodiametric cells at the outer layer and oblong cells at the inner and two or three layered isodiametric medullary cells. Scale bars represent 1 cm (a), 1,000 μm (b, c, e), 100 μm (d), 10 μm (f).

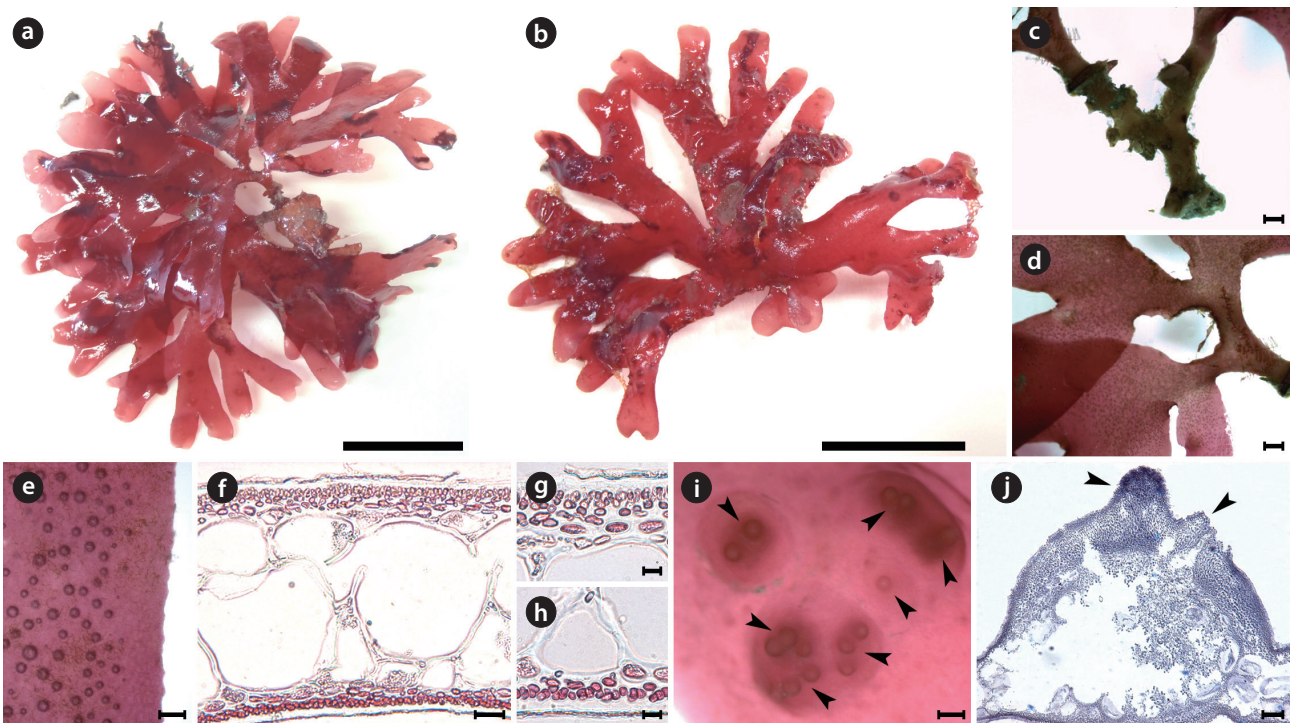


Fig. 8. *Callophyllis hayamensis* Yamada. (a) JN2013-v0454, vegetative plant collected from Sasu-dong on 07 July 2013, showing flattened membranous thallus. (b) JN2013-v0495, female gametophyte collected from Sasu-dong on 12 July 2013. (c) Cylindrical and dichotomously branched stipe arising from a discoidal holdfast. (d) Thallus dichotomously branched at the basal part. (e) Slightly crenulation at the margin of frond. (f) Cross section of the frond showing medulla and cortex. (g) Cross section of the dorsal cortex. (h) Cross section of the ventral cortex. (i) Cystocarp borne at the upper part of thallus having ostioles (arrowheads). (j) Cross section view of cystocarp protuberated dorsally with two ostioles (arrowheads). Scale bars represent 2 cm (a, b), 1,000 μm (c, d), 200 μm (e, i), 25 μm (f), 10 μm (g, h), 100 μm (j).

39.6–86.6 μm in size (Fig. 7f). The cortex is one or two layered: the outer cortical cells are bead-like, 3.5–5.2 $\mu\text{m} \times$ 3.3–5.0 μm in size, and the inner cortical cells are oblong, 2.5–5.2 $\mu\text{m} \times$ 6.4–8.6 μm in size (Fig. 7f).

Callophyllis hayamensis Yamada 1941 (Fig. 8)

Holotype: TNS (Yoshida 1998).

Type locality: Hayama, Kanagawa, Japan.

Distribution: Korea (this study) and Japan (Yoshida 1998).

Selected specimens: JN2013-v0454 (Sasu-dong, 07 July 2013, ♀); JN2013-v0457-460 (Guideok 1-ri, 10 July 2013, vegetative); JN2013-s0222-4, JN2013-v0494, NIBR0000143266 (Sasu-dong, 12 July 2013, ♀)

Morphology: Plant is flattened, flabellate, scarlet to dark-red in color, 5–8 cm long, and 190–206 μm thick (Fig. 8a and 8b). The thallus has a cylindrical stipe arising from a discoidal holdfast and is 3.9–7.3 mm long (Fig. 8c). The thallus is dichotomously branched 2–4 times, and broadens upwardly (Fig. 8d). The width of the thallus is ranged from 6.4–8.1 mm in the middle to 3.5–4.3 mm in the upper. The margin is slightly crenulated (Fig. 8e). The apex of the thallus is obtuse and roundish. The medulla consists of two isodiametric celled layer, which are 62–114 $\mu\text{m} \times$ 46–114 μm in size (Fig. 8f). The outer cortex is dorsiventral, consisting of a two- or three-celled layer in the dorsal cortex and a one-celled layer in the ventral cortex (Fig. 8g and 8h). Cystocarps are scattered over the upper blades (Fig. 8i) and protude more dorsally with several one-sided ostioles (Fig. 8j).

DISCUSSION

In the present study, we recognized a total of ten *Callophyllis* species, including five new cryptic species and one unrecorded species from Korea based on the morphology with molecular analyses of the plastid *rbcL* and mitochondrial COI-5P. We proved five *Callophyllis* species that have been previously recorded in the northwestern Pacific (Yoshida 1998, Xia 2004, Lee and Kang 2001): *Callophyllis adhaerens* Yamada, *C. adnata* Okamura, *C. crispata* Okamura, *C. hayamensis* Yamada, and *C. japonica* Okamura. Two species, *C. adnata* and *C. crispata*, were only collected from Japan in this study, and the distribution of *C. hayamensis* was expanded to Korea. In particular, the diversity of *Callophyllis* from the northwestern Pacific was increased by discovering five new cryptic species: *C. cartilaginea* sp. nov., *C. mollitia* sp. nov., *C. repens* sp. nov.,

C. serratifolia sp. nov., and *C. undulata* sp. nov.

To delimit the species boundaries of the genus *Callophyllis*, morphological features have been compared such as thallus type and size, branching pattern, marginal type, position and size of cystocarps, number and shape of ostioles, and presence of tetrasporangia sori (Sectchell 1923, Dawson 1954, Norris 1957, Abbott and Norris 1966, Womersley and Norris 1971). We compared ten *Callophyllis* species on the basis of the combination of the above morphological features, and consequently defined five new species in this study that could be discriminated from other species (Table 2). *C. cartilaginea* sp. nov. can be mistaken as *C. japonica*, but two species differ in thallus type and the shape of stipe: the former species has a cartilaginous thallus with a short compressed stipe, and the latter has a membranous thallus with apparent cylindrical stipe and spinescent protuberances at the lateral margin (Okamura 1936). *C. mollitia* sp. nov. resembles *C. adnata*, as they both have membranous blades in tender and are attached by several radical discs (Table 2), but the thallus of *C. adnata* is more than twice as wide, with a red and often fading ferruginous color and slightly crenulated margins (Okamura 1932). *C. repens* sp. nov. is similar to *C. mageshimensis* in its creeping habit, attached to the substratum with several radical discs, although *C. mageshimensis* has no adherent branches and an apparently acute apex (Tanaka 1963). *C. serratifolia* sp. nov. is characterized by its narrowly blunt apex, regularly dichotomous branching pattern, and serrated margin without pleat and both-sided cystocarp ostioles, and it is clearly distinguished from any other species distributed in the northwestern Pacific (Table 2). *C. undulata* sp. nov. is similar to *C. hayamensis* on the thallus type and width and apex shape, but *C. undulata* is pleated and bluntly serrated at the margin (Table 2), unlike *C. hayamensis* without pleat margin (Yamada 1941).

Arakaki et al. (2011) described two new species, *C. conceptionensis* and *C. macrostiolata*, which exhibit interspecific divergences of *rbcL* with their close sister taxa, 4.0% with *C. obtusifolia* and 2.5% with *C. atrosanguinea*, respectively. Our interspecific divergences of *rbcL* are from 0.6–0.8% (between *C. serratifolia* sp. nov. and *C. undulata* sp. nov.) to 1.4–1.5% (between *C. japonica* and *C. cartilaginea* sp. nov.), which divergences are insufficient to distinguish the species (Arakaki et al. 2011). Otherwise, the interspecific divergences among our specimens in COI-5P ranged from the minimum 2.8–3.3% (between *C. serratifolia* sp. nov. and *C. undulata* sp. nov.), to the maximum 8.8% (between *C. hayamensis* and *Callophyllis mollitia* sp. nov.). Clarkston and Saunders (2013) defined that

Table 2. A comparison of taxonomic characteristics among the species of *Callophyllis* including five new species

	<i>C. cartilaginea</i> sp. nov.	<i>C. mollitia</i> sp. nov.	<i>C. repens</i> sp. nov.	<i>C. serratifolia</i> sp. nov.	<i>C. undulata</i> sp. nov.
Habitat (depth)	On rocks (5-10 m deep)	On corallines or invertebrates (10-15 m deep)	On rocks or corallines (25-30 m deep)	On rocks (8-15 m deep)	On rocks (25-30 m deep)
Thallus	Erect, cartilaginous, in a bushy clump	Flattened, palmate, membranous in tender	Flattened, slightly cartilaginous, creeping	Flattened, slightly cartilaginous, in a bushy clump	Flattened, membranous, in a bushy clump
Branching	Di- or trichotomous	Sub-dichotomous	Dichotomous	Dichotomous	Dichotomous
Color	Dark- to brown-red	Bright brown-red	Dark red	Dark red	Light scarlet to bright red
Length	5-7 cm long (up to 15 cm long)	3-5 cm long (up to 8 cm long)	2-5 cm long	5-7 cm long (up to 10 cm long)	3-8 cm long
Width (in middle)	1.1-1.7 mm wide	4.3-5.1 mm wide	2.1-2.8 mm wide	2.3-3.2 mm wide	7.6-9.9 mm wide
Apex	Acute	Roundish or bluntly dissected	Acute	Narrowly blunt	Roundish
Margin	Smooth and spinescent protuberances	Smooth	slightly crenulate	Serrate	Pleated and crenulate
Adherence	None	None	lateral margin to margin	None	None
Attachment	A sub-discoïd holdfast	Minute radical discs	A discoïd holdfast and minute radical discs	A discoïd holdfast	A discoïd holdfast
Cystocarp	-	Over the entire blades scatteredly	-	Over the upper blades scatteredly	-
Ostiole	-	2-3 one-sided ostioles	-	2-6 one or both-sided ostioles	-
Reference	This study	This study	This study	This study	This study
<i>C. adhaerens</i> Yamada					
Habitat (depth)	On rocks or other algae (10-15 m deep)	Not determined	On rocks in subtidal	<i>C. hayamensis</i> Yamada (20-25 m deep)	<i>C. japonica</i> Okamura
Thallus	Flattened, membranous	Flattened, softly membranous	Erect, membranous	Stipe erect and blade flattened, membranous	Erect, membranous, in a bushy clump
Branching	Pinnately dichotomous	Dichotomous in short interval	Di- or polychotomous	Dichotomous	Irregularly di- to subdichotomous
Color	Bright red	Red and often fading ferruginous	Blooded or rosy	Scarlet to dark red	Dark- to brown-red
Length	3-7 cm long	6-10 cm long	10-20 cm long	4-7 cm long	7-10 cm long (up to 20cm long)
Width (in middle)	1.8-2.3 mm wide	10-20 mm wide	20-30 mm wide	6.4-8.1 mm wide	2.5-9.0 mm wide
Apex	Obtuse	Obtuse and expanded	Roundish	Roundish obtuse	Acute
Margin	Smooth and small protuberance	Entirely or more/less crenulate	Naked in sterile, minutely curved in fertile	Slightly crenulate	Spinescent protuberance and some branchlets
Adherence	To each other or to other things	None	None	None	None
Attachment	A discoïd holdfast	Minute radical discs	A discoïd holdfast	A discoïd holdfast	A discoïd holdfast
Cystocarp	Along the margin	Over the upper blades scatteredly	At the margin or on the surface	Over the upper blades scatteredly	On center of branchlets along the margin
Ostiole	-	1-2 ostioles	1 ostiole	2-6 one-sided ostioles	2-4 one-sided ostioles
Reference	Yamada (1932)	Okamura (1932)	Okamura (1896, 1916)	Yamada (1941)	Okamura (1936)

COI-5P shows a proper divergence to distinguish *Callophyllis* species efficiently, which 2.6–10.45% between collections from the northeastern Pacific. Although our *rbcL* sequences of *Callophyllis* exhibit relatively less interspecific divergences than 2.0%, our five new species are sufficiently supported by the complement of COI-5P analysis and morphological observations to recognize as a cryptic new species. Clarkston and Saunders (2013) emphasized the conjunction of key characteristics based on the morphology typically used for species delimitation and molecular data to identify many *Callophyllis* species exhibiting the high morphological variability (Abbott and Norris 1966). The morphological species boundaries for our taxonomic conclusion regarding *Callophyllis* species from the northwestern Pacific is guaranteed as a reliable criterion by conjunction with molecular evidence.

Abbott and Norris (1966) suggested that the number of carpogonial branches per supporting cell appear to differentiate two evolutionary lineages within *Callophyllis*. Clarkston and Saunders (2013) emphasized that mono- vs. polycarpogony has potential for the accurate definition of species entities and diversity, and that it is necessary to find a diagnosis that could be present in all of the life history of *Callophyllis* species. The mono- or polycarpogony of many *Callophyllis* species remain unrevealed as of yet, except several species from the Pacific coast of both America and Australia, including the generitype *C. variegata* (Norris 1957, Abbott and Norris 1966, Womersley and Norris 1971, Arakaki et al. 2011, Clarkston and Saunders 2013). The mono- or polycarpogony of *Callophyllis* species from the northwestern Pacific are not defined in detail in the present study. Further study of the female reproductive structures of *Callophyllis* should be carried out preferentially to find the diagnosis of *Callophyllis* species and to demonstrate the phylogenetic relationship of two evolutionary lineages. Up to now, several geographically small-scale studies of *Callophyllis* have been conducted in the southeastern Pacific (Arakaki et al. 2011, Lin et al. 2012) and the northeastern Pacific (Harper and Saunders 2002, Clarkston and Saunders 2013), whereas there has been no study in western Pacific region. Like the concept that geographically small-scale studies are a necessary for the first step towards a global assessment of *Callophyllis* (Abbott and Norris 1966), we expect that the present study will provide a basis for determining species diversity and understanding the phylogeographic relationships of *Callophyllis* from the Pacific Ocean.

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