J Appl Phycol (2015) 27:1373–1384
DOI 10.1007/s10811-014-0432-1

Taxonomy of *Grateloupia* (Halymeniales, Rhodophyta) by DNA barcode marker analysis and a description of *Pachymeniopsis volvita* sp. nov.

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Received: 24 April 2014 / Revised and accepted: 9 October 2014 / Published online: 23 October 2014

Abstract We applied DNA barcoding analysis to the red alga *Grateloupia* because it is one of the most complicated taxa in the family Halymeniaceae. We used two DNA barcoding markers, the 5′ end of the cytochrome *c* oxidase I gene and the universal plastid amplicon of the 23S rRNA gene, to define levels of genetic diversity and species relationships within *rbc*L phylogeny. We detected nine species of *Grateloupia*, four species of *Pachymeniopsis*, and one *Kintokiocolax* in Korea: *G. angusta*, *G. asiatica*, *G. catenata*, *G. divaricata*, *G. imbricata*, *G. jejuensis*, *G. kurogii*, *G. subpectinata*, *G. turuturu*, *P. elliptica*, *P. gargiuli*, *P. lanceolata*, a new species, and *K. aggregato-cerantha*. The COI-5P barcode successfully differentiated species, with interspecific divergence values ranging between 3.7 and 14 %. Barcoding data provided a rapid and accurate methodology for species-level identification and for taxonomic explorations when used in combination with morphological data. We describe *Pachymeniopsis volvita* sp. nov. based on DNA barcoding analysis. Fourteen species detected in four different subclades with strong bootstrap support within *rbc*L phylogenetic tree.

Keywords DNA barcoding · Genetic diversity · *Grateloupia* · *Pachymeniopsis* · Rhodophyta · Taxonomy

Introduction

The red algal genus *Grateloupia* C. Agardh is one of the most taxonomically complex one in the family Halymeniaceae. The generitype *Grateloupia filicina* (Lamouroux) C. Agardh is characterized by a finely pinnate branching pattern, numerous marginal proliferations, laxly constructed medullary structure, and four-celled carpogonial branches and three-celled auxiliary branches (De Clerck et al. 2005). Recently, the genus *Pachymeniopsis* Yamada was resurrected from the genus *Grateloupia* based on the reproductive anatomy and postfertilization development, including molecular data (Gargiulo et al. 2013; Kim et al. 2014). The generitype *Pachymeniopsis lanceolata* (Okamura) Yamada ex Kawabata has six-celled carpogonial branches, five-celled auxiliary branches, and upwardly and downwardly directed branched nutritive filaments (Gargiulo et al. 2013). Two genera have ecologically important roles in temperate and tropical coastal waters (Kawaguchi et al. 2001). Some of the species have invaded geographic regions beyond their previous ranges and have become serious impacts (Inderjit et al. 2006), e.g., *Grateloupia imbricata* in waters of the Canary Islands (Garcia-Jiménez et al. 2008), *Grateloupia turuturu* in France, and the Gulf of Maine, USA (Simon et al. 2001; Mathieson et al. 2008), and *P. lanceolata* (as *Grateloupia lanceolata*) in California, France, and New Zealand (Miller et al. 2009; Verlaque et al. 2005).

Grateloupia is a source of bioactive materials for foods and medicines. Wang et al. (2007) examined the structure and antiretroviral (HIV-1) activity of native sulfated galactans extracted from *Grateloupia filicina* and *Grateloupia longifolia*. *G. turuturu* may have potential in (i) the production of valuable molecules (R-phycoerythrin), (ii) enzymatic degradation, and (iii) antifouling activity (Denis et al. 2009a; 2010). There is a long history of human seaweed consumption in Asian nations (Dawczynski et al. 2007). *G. turuturu* and *G. asiatica* (as *G. filicina*) are commonly used dietary items (Denis et al. 2009b). However, algal chemical composition varies by species, geography, season, and environmental conditions (Denis et al. 2010); this variability is of relevance with respect to nutritional and pharmacological considerations.

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Approximately 25 *Grateloupia* species occur in the northwestern Pacific water; some of these represent a substantial resource of unexploited red algal materials (Denis et al. 2009a, b, 2010). Several of the congeners have been subjected to morphological, anatomical, geographic, and taxonomic analyses (D'Archino et al. 2007; Kawaguchi 1997; Lin et al. 2008). Nevertheless, the identification of *Grateloupia* species is difficult using only traditional morphological traits because terete to blade-like thalli are highly variable in structure. The taxonomic status of congeners belonging to closely related genera is confused, particularly in East Asia (Wang et al. 2000; Zhao et al. 2012; Faye et al. 2004; Wang et al. 2001). For example, it had been necessary to transfer *G. lanceolata* and *G. elliptica* from the genus *Pachymeniopsis* (Kawaguchi 1997), but Gargiulo et al. (2013) resurrected again the genus *Pachymeniopsis*. The morphology and *rbc*L sequences of *Grateloupia catenata* were reexamined by Wang et al. (2000). *Grateloupia subpectinata* has been resurrected to full specific status; previously, it had been reduced to synonymy under *G. asiatica* and *G. prolongata* (Faye et al. 2004). Wilkes et al. (2005) has stressed the need to establish robust criteria for taxonomic discrimination. Therefore, reliable species identification will be required to take advantage of the bioindustrial potential of *Grateloupia*. DNA barcoding facilitates disentanglement of taxonomic confusion in such large and complex genus.

The DNA barcoding technique uses short, standardized DNA sequences from designated regions of the genome. The sequences function as tags for rapid and accurate species-level identification and biodiversity explorations (Hebert et al. 2003). Two DNA barcode markers, cytochrome *c* oxidase I (COI) and UPA, have been proposed as standard markers for cataloging red algal biodiversity and resolving differences between closely related species (Kucera and Saunders 2012). COI-5P (ca. 650 bp) is a protein-coding gene in the 5′ region of the mitochondrial cytochrome *c* oxidase I gene that has been investigated extensively as a taxonomic tool (Saunders 2005; Le Gall and Saunders 2010). This marker requires specific amplification primers for the various red algal lineages (Saunders 2008). UPA (ca. 400 bp) is the universal plastid amplicon in domain V of the 23S rRNA gene, and it has been amplified from numerous species in algal lineages using a single set of primers (Sherwood and Presting 2007). DNA barcoding has been applied previously to two species of *Grateloupia* (Yang et al. 2013): two congeners had a sequence divergence of 3.7–4.6 % in COI, but among conspecific individuals, sequence divergences were only 0.3 % in *P. elliptica* (as *G. elliptica*) and 1.0 % in *P. lanceolata* (as *G. lanceolata*), respectively (Yang et al. 2013).

The chloroplast-encoded large subunit of the Rubisco gene (*rbc*L) is frequently used for the phylogenetic analysis within the red algae at diverse taxonomic levels (Bellorin et al. 2008; Kim et al. 2008; Geraldino et al. 2009; Kim et al. 2010). Although molecular analyses have been used to clarify the taxonomic status of *Grateloupia* (Faye et al. 2004; D'Archino et al. 2007; Zhao et al. 2012), no DNA barcoding determination of the level of genetic variation has been performed. In addition, *Kintokiocolax aggregato-cerantha* Tanaka & Nozawa, characterized by a small and clustered morphology, was first described as a parasite of *Grateloupia angusta* (Tanaka and Nozawa 1960), but this species has not been confirmed yet by molecular analysis. In the present study, we applied DNA barcoding to *Grateloupia*, *Pachymeniopsis*, and *Kintokiocolax* using COI and UPA markers to define levels of genetic diversity and to discriminate species in Korea. Concurrently, we reviewed the phylogenetic relationships available from the sequence analysis of *rbc*L gene to make recommendations on the direction of the future studies.

Materials and methods

Collections of samples were conducted between 2009 and 2013. Each specimen was prepared as a herbarium voucher, and a small portion was removed for DNA analysis. Portions for molecular work were dried in silica gel and stored at −20 °C. A complete list of specimens is presented in Table 1. Samples used in morphological observations were preserved in 5 % formalin/seawater or pressed on herbarium sheets. Voucher specimens are housed at the herbaria of Jeju National University (JNUB), Jeju, Korea, and the National Institute of Biological Resources (NIBR), Incheon, Korea. Sections were produced by hand or with the aid of a freezing microtome. Sections on slides were stained with 1 % aqueous aniline blue acidified with a drop of 1 % HCl and mounted in 30 % Karo corn syrup. Photomicrographs were captured with a QIMAGING 1,394 camera (QImaging, Canada) attached to a BX50 microscope (Olympus, Japan). All images were imported into the Adobe PhotoShop 5.5 (Adobe Systems Inc., USA) for plate assembly.

Total genomic DNA was extracted following the protocol of a DNeasy Plant Mini Kit (Qiagen, Germany) modified by incubating the disrupted samples with AP1 buffer for a minimum of 1 h at 63 °C. AccuPower PCR Premix (Bioneer, Daejeon, Korea) was used according to the manufacturer's recommendations for all PCR reactions. The PCR procedure for COI-5P followed the outline of Saunders (2005, 2008) except that we used an annealing temperature of 50 °C in reactions that involved primers. The PCR procedure for UPA followed Sherwood and Presting (2007). The PCR procedure for *rbc*L was as follows: initial denaturation at 96 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, an extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min. PCR products were cleaned using an AccuPrep PCR Purification Kit

Table 1 List of samples for molecular analyses generated in this study, with collection information and GenBank accession numbers

Species	Voucher	Collection data		GenBank accession numbers			
		Location	Date	COI	UPA	*rbc*L	References
Grateloupia angusta (Okamura) S. Kawaguchi and H.W. Wang	G006	Udo, Jeju, Korea	22 Jul. 2009	KJ648509	KJ648575		This study
	HAL001	Seongsan, Seogwipo, Korea	08 May 2012	KF475714	KF475743	KF475727	This study
	HAL042	Geomundo, Yeosu, Korea	25 Jul. 2012	KJ648510			This study
	GT116	Marado, Jeju, Korea	12 Jul. 2010	KJ648511			This study
	GT142	Wimi, Seogwipo, Jeju, Korea	15 Jun. 2011	KJ648512			This study
Grateloupia asiatica S. Kawaguchi and H.W. Wang	G105	Nokonosima, Fukuoca, Japan	04 Mar. 2010	KJ648513			This study
	HAL002	Anin, Gangneung, Korea	19 May 2012	KJ648514	KJ648576		This study
	HAL003	Noguri, Namhaedo, Korea	20 May 2012	KF475715	KF475744		This study
	HAL005	Jumunjin, Gangneung, Korea	19 May 2012		KJ648577	KF475728	This study
	HAL028	Hansuri, Hallim, Jeju, Korea	06 Apr. 2012	KJ648515	KJ648578		This study
	HAL055	Gimnyeong, Jeju, Korea	05 Jul. 2012	KJ648516			This study
Grateloupia catenata Yendo	HAL007	Sehaw, Jeju, Korea	06 Jan. 2012	KJ648517	KJ648579	KJ648558	This study
	HAL008	Gimnyeong, Jeju, Korea	18 May 2012	KF475716	KF475737	KF475745	This study
	HAL043	Gimnyeong, Jeju, Korea	05 Jul. 2012		KJ648580		This study
Grateloupia divaricata Okamura	HAL012	Jumunjin, Gangneung, Korea	19 May 2012	KF475717	KF475746		This study
	HAL024	Gyungpodae, Gangneung, Korea	21 Aug. 2011	KJ648518	KJ648581	KJ648559	This study
	HAL044	Anin, Gangneung, Korea	19 May 2012	KJ648519	KJ648582	KJ648560	This study
	HAL045	Sachunjin, Gangneung, Korea	19 May 2012	KJ648520	KJ648583		This study
Grateloupia imbricata Holmes	GT018	Sinyang, Jeju, Korea	27 Jul. 2010	KJ648523			This study
	GT143	Haengwon, Jeju, Korea	05 May 2011	KJ648524			This study
	G008	Udo, Jeju, Korea	19 Aug. 2009	KJ648525	KJ648587		This study
	G125	Ongpo, Hallim, Jeju, Korea	28 May 2010	KJ648526			This study
	HAL016	Namhaedo, Korea	20 May 2012	KJ648527	KJ648588	KJ648562	This study
	HAL017	Sagye, Seogwipo, Korea	24 Mar. 2012	KF475719	KF475748		This study
	HAL047	Ulsan, Korea	20 Jul. 2012	KJ648528	KJ648589		This study
Grateloupia jejuensis S.Y. Kim, E.G. Han and S.M. Boo	G119	Sungsan, Jeju, Korea	05 May 2010				This study
	MI15	Misaki, Japan	30 Apr. 2010				This study
	MI29	Misaki, Japan	30 Apr. 2010				This study
	GT035	Jongdal, Jeju, Korea	27 Jul. 2010	KJ648529			This study
	HAL009	Anin, Gangneung, Korea	19 May 2012	KF475722	KF475751		This study
	HAL010	Namhaedo, Korea	19 May 2012	KJ648530	KJ648590		This study
	HAL011	Gosung, Jeju, Korea	23 Mar. 2012	KJ648531	KJ648591		This study
	HAL041	Sinchon, Jeju, Korea	16 May 2012	KJ648532		KJ648563	This study
Grateloupia kurogii Kawaguchi	G128	Sagye, Jeju, Korea	23 May 2010	KJ648533	KJ648592	KJ648564	This study
	GT005	Pyosun, Seogwipo, Korea	04 Feb. 2010	KJ648534			This study
	GT042	Udo, Jeju, Korea	28 Feb. 2010	KJ648535			This study
	GT104	Marado, Seogwipo, Korea	13 Jul. 2010	KJ648536			This study
	GT148	Sinsan, Seogwipo, Korea	04 May 2011	KJ648537			This study
	GT149	Oewol, Jeju, Korea	27 Apr. 2011	KJ648538			This study
	HAL049	Daesambudo, Yeosu, Korea	25 Jul. 2012	KF475720	KF475749	KF475736	This study
Grateloupia subpectinata Holmes	GT050	Jongdal, Jeju, Korea	04 Apr. 2010	KJ648540	KJ648598		This study
	GT255	Geumneung, Jeju, Korea	15 Jan. 2010				This study
	HAL004	Jongdal, Jeju, Korea	25 Mar. 2012	KJ648541	KJ648599	KJ648566	This study
	HAL006	Sagye, Jeju, Korea	24 Mar. 2012	KF543067	KF543073		This study
	HAL029	Pyeongdae, Jeju, Korea	01 Jun. 2012		KJ648600	KJ648567	This study
	HAL050	Busan, Korea	20 Jul. 2012	KJ648542			This study
Grateloupia turuturu Yamada	G020	Daejung, Seogwipo, Korea	28 Nov. 2011	KF475725	KF475754		This study

Table 1 (continued)

Species	Voucher	Collection data		GenBank accession numbers			
		Location	Date	COI	UPA	*rbc*L	References
	G047	Hamo, Seogwipo, Korea	16 Jan. 2010	KJ648543			This study
	G048	Bomok, Seogwipo, Korea	17 Jan. 2010	KJ648544	KJ648601		This study
	HAL032	Sinheung, Jeju, Korea	21 Mar. 2012		KJ648602	KJ648568	This study
	HAL051	Busan, Korea	20 Jul. 2012		KJ648603	KJ648569	This study
Kintokiocolax aggregato-cerantha Tak. Tanaka and Y. Nozawa	HAL057	Oedo, Jeju, Korea	06 Aug. 2012	KF475726	KF475733	KF475755	This study
Pachymeniopsis elliptica (Holmes) Yamada	G014	Udo, Jeju, Korea	08 Nov. 2011		KJ648584		This study
	HAL013	Jookbyeon, Uljin, Korea	28 Apr. 2012	KF475718	KF475747		This study
	HAL014	Gosung, Jeju, Korea	23 Mar 2012		KJ648585		This study
	HAL015	Siheung, Seogwipo, Korea	23 Sep. 2011	KJ648521	KJ648586		This study
	HAL046	Cheongsando, Wando, Korea	27 Jul 2012	KJ648522		KJ648561	This study
	G112	Jongdal, Jeju, Korea	29 May 2010	JX475019			Yang et al. 2013
	GM03	Geomundo, Yeosu, Korea	12 Jun. 2010	JX475014			Yang et al. 2013
	HAL013	Jookbyeon, Uljin, Korea	28 Apr. 2012	JX475020			Yang et al. 2013
	HAL014	Gosung, Jeju, Korea	23 Mar. 2012	JX475018			Yang et al. 2013
	G122	Jongdal, Jeju, Korea	29 May 2010	JX475021			Yang et al. 2013
	G014	Udo, Jeju, Korea	08 Nov. 2009	JX475023			Yang et al. 2013
	MI44	Misaki, Japan	30 Apr. 2010	JX475017			Yang et al. 2013
Pachymeniopsis garguili S. Y. Kim, A. Manghisi, M. Morabito and S. M. Boo	G045	Sinyang, Jeju, Korea	18 Jan. 2010	KJ648551			This study
	GT102	Udo, Jeju, Korea	28 Feb. 2010	KJ648552	KJ648604		This study
	GT103	Jongdal, Jeju, Korea	04 Apr. 2010	KJ648553			This study
	HAL019	Namhaedo, Korea	20 May 2012	KF475723	KF475752		This study
	HAL020	Sinchon, Jeju, Korea	16 May 2012	KJ648554	KJ648605		This study
	HAL027	Geumneung, Jeju, Korea	08 May 2012	KJ648555	KJ648606	KJ648574	This study
	HAL031	Namhaedo, Korea	20 May 2012	KJ648556			This study
	HAL048	Sinchon, Jeju, Korea	16 May 2012	KJ648557			This study
Pachymeniopsis lanceolata (K. Okamura) Y. Yamada and S. Kawabata	HAL021	Guryongpo, Pohang, Korea	29 Apr. 2012	KF475721	KF475750		This study
	HAL022	Namhaedo, Korea	20 May 2012	KJ648539	KJ648593	KJ648565	This study
	HAL023	Sinheung, Jeju, Korea	21 Mar. 2012		KJ648594		This study
	HAL025	Taeheung, Seogwipo, Korea	27 Mar. 2012		KJ648595		This study
	HAL033	Namhaedo, Korea	20 May 2012		KJ648596		This study
	HAL036	Namhaedo, Korea	19 May 2012		KJ648597		This study
	G022	Ongpo, Hallim, Jeju, Korea	26 Nov. 2009	JX475004			Yang et al. 2013
	G106	Fukuoca, Japan	30 Apr. 2010	JX475000			Yang et al. 2013
	G040	Hamo, Jeju, Korea	16 Jan. 2010	JX474988			Yang et al. 2013
	MI06	Misaki, Japan	30 Apr. 2010	JX475007			Yang et al. 2013
	EN06	Enoshima, Japan	29 Apr. 2010	JX474996			Yang et al. 2013
	HAL021	Guryongpo, Pohang, Korea	29 Apr. 2012	JX474993			Yang et al. 2013
	HAL023	Sinheung, Jeju, Korea	21 Mar. 2012	JX474994			Yang et al. 2013
	HAL025	Taeheung, Jeju, Korea	27 Mar. 2012	JX474990			Yang et al. 2013
	HAL033	Noguri, Namhaedo, Korea	20 May 2012	JX474991			Yang et al. 2013
	HAL035	Oedo, Namhaedo, Korea	19 May 2012	JX474992			Yang et al. 2013
	HAL036	Oedo, Namhaedo, Korea	19 May 2012	JX474989			Yang et al. 2013
Pachymeniopsis volvita sp. nov.	HAL056	Oedo, Jeju, Korea	06 Aug. 2012	KF475724	KF475753	KF475734	This study
	HAL069	Gapado, Seogwipo, Korea	26 Mar. 2013	KJ648545			This study

Table 1 (continued)

Species	Collection data				GenBank accession numbers		
	Voucher	Location	Date	COI	UPA	*rbc*L	References
	HAL070	Gapado, Seogwipo, Korea	26 Mar. 2013	KJ648546		KJ648570	This study
	HAL071	Mureung, Seogwipo, Korea	15 Mar. 2013	KJ648547		KJ648571	This study
	HAL072	Mureung, Seogwipo, Korea	15 Mar. 2013	KJ648548			This study
	HAL073	Siheung, Jeju, Korea	19 Mar. 2013	KJ648549		KJ648572	This study
	HAL075	Seopsum, Seogwipo, Korea	05 Apr. 2013	KJ648550		KJ648573	This study

(Bioneer, Korea). Sequencing was performed by a commercial contractor (Macrogen, Seoul, Korea).

Sequences obtained from COI, UPA, and *rbc*L genes were edited using Chromas version 1.45 software (Technelysium Pty Ltd, Australia); multiple sequence alignments were made in BioEdit (Hall 1999) and aligned visually. None of the alignments posed a problem since no gaps were observed. The COI-5P alignment consisted of 86 isolates and 661 nucleotides. The UPA alignment consisted of 59 isolates and 386 nucleotides. The *rbc*L alignment consisted of 69 isolates and 1,440 nucleotides. We assessed the level of variation in the sequences of the three genes; uncorrected (*p*) pair-wise genetic distances were estimated with the MEGA 4.0 software (Tamura et al. 2007). The clustering tree for COI was constructed with the MEGA 4.0 software using a neighbor-joining (NJ) algorithm based on the Kimura-2-parameter (K2P) distance method.

We used the *rbc*L dataset to evaluate the phylogenetic relationships of newly acquired genetic species groups, including the three outgroups *Polyopes affinis*, *Polyopes constrictus*, and *Polyopes lancifolius*. Maximum likelihood (ML) analysis was performed using the RAxML version 7.2.6 software (Stamatakis 2006) using the GTR+Γ evolutionary 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. We performed 1,000 replications using the same software and settings to generate bootstrap values for the phylogeny.

Results

Molecular analyses

We report here on a total of nine species of *Grateloupia*, four species of *Pachymeniopsis*, and one *Kintokiocolax* based on the molecular analyses (Fig. 1); the taxa are listed in the Table 1. Among 101 collections, we amplified COI-5P sequences of 60 specimens selected, and neighbor-joining tree is depicted in Fig. 2. Genetic variation within groups ranged from 0 to 1.6 %, with a mean of 0.41 %. Interspecific genetic variation ranged from 3.7 to 14 %, with a mean of 9.3 %. Interspecific genetic variation between *Pachymeniopsis volvita* sp. nov. and other taxa ranged from 7.2–7.5 % (with *P. elliptica*) to 9.8–10 % (with HQ422590 *G. filicina*). Interspecific divergence between *P. gargiuli* and *P. lanceolata*, which are sister taxa, ranged from 3.7 to 4.4 %. *G. jejuensis* from Japan was 0.2 % divergent from six conspecific specimens collected in Korea. Intraspecific divergence reached 0.7 % among Korean specimens of *G. divaricata*. Japanese specimens of *G. asiatica* were 0.7 % divergent from Korean conspecifics. Seven samples of *G. imbricata* from Korea formed a clade with *G. hawaiiana* (HQ422635); interspecific genetic variation in this clade reached 0.5 %. Hawaiian samples identified as *G. catenata* were genetically linked to Korean collections of the same species; maximum sequence divergence in this group reached 1.6 %.

We sequenced UPA marker in 46 samples, and neighbor-joining analyses revealed few differences in the resolution of genetic species groups by comparison with COI-5P and *rbc*L (tree not shown). In particular, intraspecific and interspecific variability were overlapped in UPA analysis. Intraspecific divergences ranged from 0 to 0.9 %, with a mean of 0.28 %. A high intraspecific divergence was 0.9 % between specimens of *G. catenata* collected in Korea and Hawaii. We measured intraspecific variation value of 0.6 % in Korean *G. divaricata* and *P. lanceolata*. Intraspecific divergences in *G. subpectinata* and *G. imbricata* reached 0.3 %. Interspecific variation ranged from 0.6 to 4.6 %, with a mean of 2.6 %. We detected an interspecific divergence of 0.6 % between *G. angusta* and *G. kurogii*.

We successfully sequenced *rbc*L gene in 30 specimens, and maximum likelihood estimation for this gene is represented in Fig. 3. Intraspecific divergences among our specimens ranged from 0 to 0.9 %, with a mean of 0.22 %. Interspecific divergences ranged from 1.0 to 9.9 %, with a mean of 6.44 %. The highest intraspecific divergence (0.9 %) was detected among collections of *G. subpectinata* from Korea, Japan, and France (as *G. filicina* var. *luxurians*). We found nine species of *Grateloupia*, four species of *Pachymeniopsis*, and one of *Kintokiocolax* in four different subclades of the *rbc*L phylogenetic tree with strong bootstrap values. ML analysis produced an unknown *Pachymeniopsis* species, *P. volvita* sp. nov. We measured an interspecific divergence of 1.0–1.2 % between

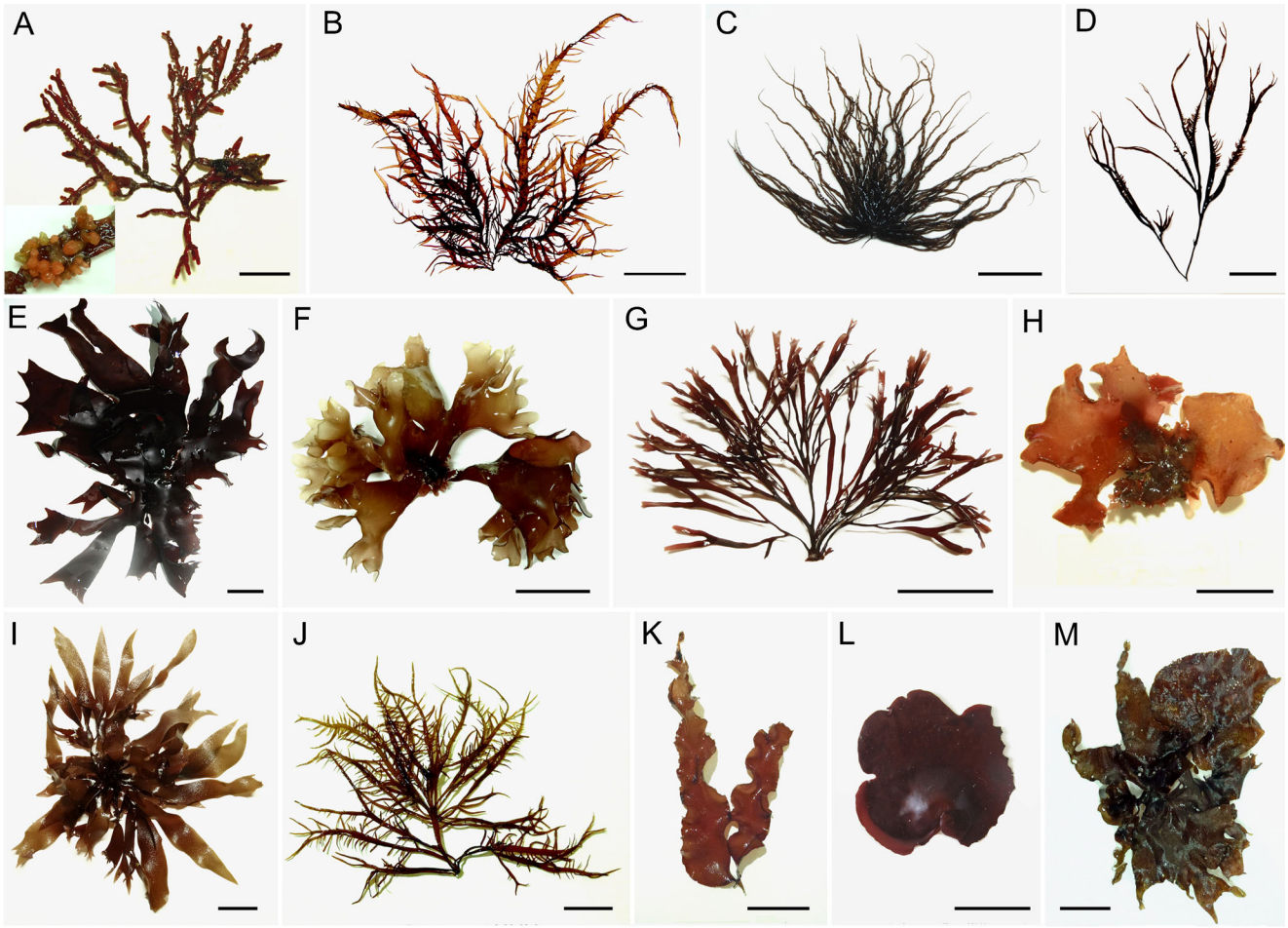

Fig. 1 Nine species of *Grateloupia*, four of *Pachymeniopsis*, and one of *Kintokiocolax* used in this study. **a** *Grateloupia angusta* with the parasite, *Kintokiocolax* (enlargement), Jeju, Korea (5 May 2012). **b** *G. asiatica*, Gangneung, Korea (19 May 2012). **c** *G. catenata*, Jeju, Korea (1 June 2012). **d** *G. divaricata*, Gangneung, Korea (19 May 2012). **e** *P. elliptica*, Uljin, Korea (28 April 2012). **f** *G. imbricata*, Namhaedo, Korea (20 May 2012). **g** *G. jejuensis*, Namhaedo, Korea (19 May 2012). **h** *G. kurogii*, Daesambudo, Korea (25 July 2012). **i** *P. lanceolata*, Namhaedo, Korea (19 May 2012). **j** *G. subpectinata*, Pohang, Korea (21 July 2012). **k** *G. turuturu*, Ulsan, Korea (20 July 2012). **l** *Pachymeniopsis volvita* sp. nov., Jeju, Korea (22 May 2013). **m** *P. gargiuli*, Jeju, Korea (16 May 2012). *Scale bars*: **a**, **c**, **e**, **g**, **i**, **j**=3 cm; **b**, **d**, **k**=5 cm; **f**, **h**, **l**=2 cm

P. gargiuli and *P. lanceolata* (100 % bootstrap value) and 1.9–2.3 % between the sequences of *P. volvita* sp. nov. and *P. elliptica* (100 % bootstrap value). Intraspecific divergence among specimens of *P. volvita* sp. nov. reached 0.2 %. *G. imbricata* specimens from Korea and Japan were sister to *G. crispate* from Japan (0.2–0.3 % interspecific divergences). *Kintokiocolax aggregato-cerantha* fell in the *Grateloupia* clade and grouped with *G. cornea* and *G. chiangii* from Japan (2.8–3.6 % divergences; low bootstrap value: 52 %) in the ML analysis.

Taxonomic results

Pachymeniopsis volvita M.Y. Yang et M.S. Kim *sp. nov.* *(Fig. 4)*

Description Thallus solitary, up to 6 cm tall, flattened, circle to peltate or reniform, dark brown, fleshy coriaceous and slippery in texture, lacking a stipe, arising from single discoid holdfast 0.7 cm in diameter, rolling toward the ventral surface when detached from the substratum; margins entire or irregularly curved, sometimes crenulate. Cortex composed of six to nine layers of outer moniliform cells and one or two layers of polygonal shaped inner cells; medulla refractive filaments with granular protoplasts. Tetrasporophytes and carposporophytes isomorphic. Tetrasporangia forming from the cortical cell layer, cruciately divided when mature, 43–52 μm long, 13–16 μm wide. Carposporangial ampullae not observed, auxiliary cell ampullae abundant; first-order ampullar filaments initiating from subbasal inner cortical cell, more than 13 cells long and curved, grows apically; the sixth cell of the primary filament enlarged, a function as the auxiliary cell producing a simple second-order filament. Mature cystocarp 135–220 μm in diameter; carposporangia rounded, 10–17 μm in diameter.

Holotype JN-HAL073 (tetrasporophyte, Fig. 4a) collected from Siheung-ri, Jeju Province, Korea (33° 28′ 30″ N, 126°

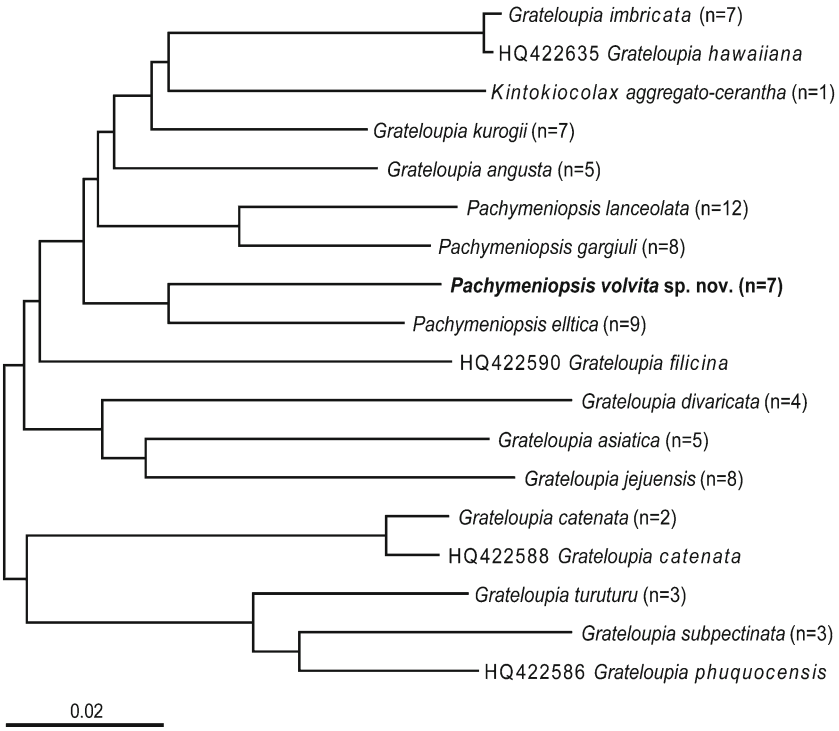

Fig. 2 Unrooted neighbor-joining phylogram for COI-5P alignment. Number of collections per species indicated by "n=." *Scale* indicates substitutions per site

54′ 41″ E) on 30 August 2013, deposited in JNUB (Herbarium of the Department of Biology, Jeju National University, Korea).

Isotypes JN-HAL085~087 (JNUB), NIBRAL0000138753~55 (NIBR).

Etymology The specific epithet (*volvita*) was chosen to describe the ventrally oriented thallus rolling of specimens following detachment from the substratum.

Habitat Pachymeniopsis volvita was collected at 10–15 m depth where plants grew on rocky substrata or conch shells. Reproductive thalli were collected mostly during summer. A hitherto endemic in Jeju Island, Korea.

Other specimens examined JN-HAL082, vegetative thallus (Gapado, Jeju on 4 Mar. 2013); JN-HAL080, vegetative thallus (Taeheung, Jeju on 5 Mar. 2013); JN-HAL071, vegetative thallus (Mureung, Jeju on 15 Mar. 2013); JN-HAL073, vegetative thallus (Siheung, Jeju on19 Mar. 2012); JN-HAL083, tetrasporophyte (Gangjeong, Jeju on 3 Jun. 2013); JN-HAL075, tetrasporophyte (Seopsum, Jeju on 4 May 2013); JN-HAL056, female gametophyte (Oedo, Jeju on 6 Aug. 2012).

Morphology Thallus is solitary, flattened, circle to peltate or reniform, and up to 6 cm in length (Fig. 4a). Blades are dark brown in color, fleshy coriaceous, and slippery in texture. Margins are entire or irregularly curved, sometimes crenulate, rolling toward the ventral surface when detached from the substratum (Fig. 4b). Thallus is arising from single discoid holdfast, 0.7 cm in diameter and lack stipe (Fig. 4c). In the cross section, thallus is 660–775 μm in thickness; it comprises a compact cellular cortex and a filamentous medulla. The cortex comprises six to nine layers of outer moniliform cells measuring 2–6×4–12 μm and one or two layers of polygonal shaped inner cells (Fig. 4d). The medulla comprises stellate cells and refractive filamentous cell, sometimes with granular protoplasts, 400–454 μm in diameter (Fig. 4e).

Tetrasporangia occur scattered over the entire thallus and form from cortical cells. Mature tetrasporangia are narrowly ellipsoidal and cruciately divided, measuring 43–52 μm in length and 13–16 μm in width (Fig. 4f).

Gonimoblasts are scattered over the thallus and are embedded between the cortex and medulla of fertile blades. Carpogonial ampullae were not observed in the specimens examined; auxiliary cell ampullae were abundant in all female plants. Initials of the first-order ampullar filaments are cut off from the subbasal inner cortical cells. The first-order filament is more than 13 cells long and curved and grows apically by transverse divisions (Fig. 4g). The sixth cell of the primary filament is enlarged and a function as the auxiliary cell, and produces a simple second-order filament toward the thallus surface. Mature cystocarps are 135–220 μm in diameter (Fig. 4h). Carposporangia are rounded and 10–17 μm in diameter (Fig. 4h). Spermatangial thalli were not found in our collections.

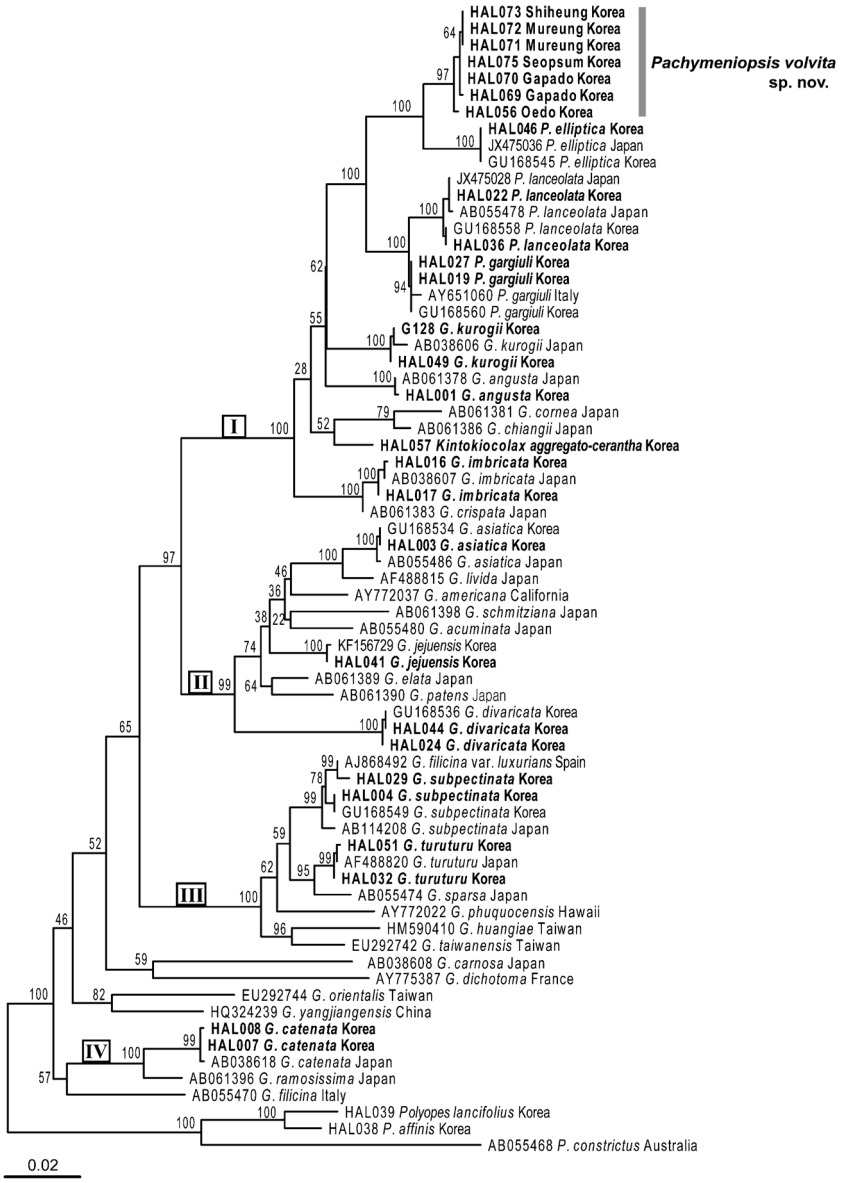

Fig. 3 Maximum likelihood phylogenetic tree derived from *rbc*L analyses. Bootstrap values for ML are indicated above the branches. *Scale* indicates substitutions per site

Discussion

The difficulties encountered in attempting to identify red macroalgae may be greatly mitigated by procedures of molecular-assisted alpha taxonomy (Saunders 2008), such as DNA barcoding. DNA barcoding has advantages in the ease of sequencing and aligning the relatively short fragments to provide of additional evidence by complementing morphological traits (Hebert et al. 2003; Saunders 2005). To determine the level of genetic diversity among *Grateloupia* species in Korea, we performed analyses of DNA barcoding: 661 bp of COI-5P (60 specimens) and 386 bp of UPA (46 specimens). Yang et al. (2013) had previously barcoded two *Grateloupia* species. COI and UPA have been officially accepted as DNA barcodes for marine red algal taxa (Kucera and Saunders 2012). Our analysis allowed us to clearly discriminate nine species of *Grateloupia*, four species of *Pachymeniopsis*, and one *Kintokiocolax* in Korea. COI provided valuable data and may be a more useful barcode than UPA for accurate identification of *Grateloupia* species. According to Hebert et al. (2003), COI-5P is highly variable, especially in the third codon position, which makes it an effective element for discriminating between even closely related species. Although a total of 14 species examined were relatively variable in morphology, intraspecific divergence in COI was 1.6 %, and interspecific divergences ranged from 3.7 to 14 %. This clear barcode gap between intraspecific and interspecific divergences in *Grateloupia* and *Pachymeniopsis* is similar to patterns in other red algal taxa (Kucera and Saunders 2012).

An intraspecific divergence of >2 % is generally regarded as adequate for the discrimination of red algal species (Le Gall and Saunders 2010). As an alternative, Hebert et al. (2004)

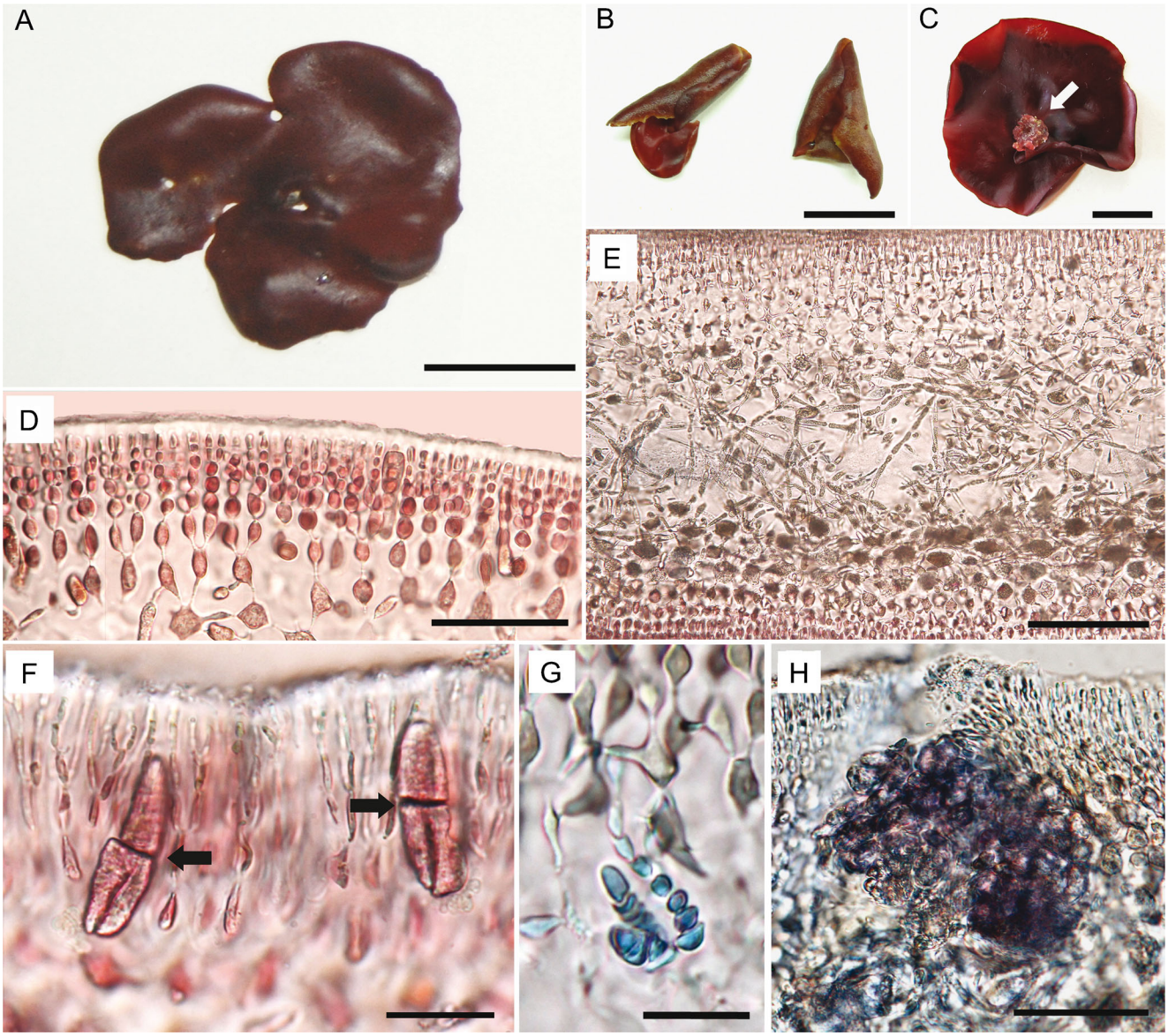

Fig. 4 *Pachymeniopsis volvita* sp. nov. **a** Holotype (JN-HAL073), a tetrasporophyte collected from Siheung, Jeju, Korea, on 30 August 2013. **b** Specimens showing to be rolled toward ventral when detached from substrate. **c** Ventral surface with single holdfast. **d** Transverse section of the thallus showing cortex. **e** Transverse section of the thallus showing medulla with granular protoplasts. **f** Transverse section of the thallus showing tetrasporangia cruciately divided (*arrows*). **g** Formation of auxiliary cell ampulla. **h** Mature cystocarp. *Scale bars*: **a**, **b**=2 cm; **c**=1 cm; **d**=50 μm; **e**=200 μm; **f**, **g**=30 μm; **h**=100 μm

proposed a standard sequence threshold divergence for species discrimination that is tenfold greater than the mean intraspecific variation within the group. In *Pachymeniopsis volvita* sp. nov., we examined 0.66 % intraspecific divergence. Hence, the standard sequence threshold divergence for species discrimination would be 6.6 %. We detected a minimum interspecific divergence of 7.6 % (based on COI gene) in *P. gargiuli* and *P. volvita* sp. nov. Therefore, COI barcode is now available for the identification of *Pachymeniopsis* species. Kucera and Saunders (2012) compared molecular markers for use as species identification tools among members of the Bangiales. They demonstrated that COI-5P had the highest level of variability and therefore the greatest taxonomic resolving power. However, they found that significant effort was required to develop appropriate primers. They also identified an intron (4–5 kb) in COI-5P of the Bangiales. We did not detect introns in our study of *Grateloupia* and were able to demonstrate the highest levels of intraspecific and interspecific divergence in this barcode. Levels of intraspecific and intraspecific variation overlapped in UPA (unlike COI-5P), indicating reduced taxonomic resolving power for this second barcode. Hence, UPA is unsuitable as a marker for species identification in the genus *Grateloupia*, as in the Bangiales (Kucera and Saunders 2012).

We also confirmed the presence of nine species of *Grateloupia*, four species of *Pachymeniopsis*, and one of *Kintokiocolax* in Korea using *rbc*L phylogeny. The interspecific divergence among these species ranged from 1.1 to 9.9 %; 14 species were detected in four subclades. Subclade I included eight species: *P. lanceolata*, *P. elliptica*, *P. gargiuli*, *P. volvita* sp. nov. *G. kurogii*, *G. angusta*, *G. imbricata*, and *Kintokiocolax aggregato-cerantha*. Yang et al. (2013) recently reassessed the relationships between *P. elliptica* and *P. lanceolata* (as *Grateloupia*); they used molecular identification to more accurately define genetic diversity in these morphologically confused taxa, which share blade-like thalli with leathery textures (Kawaguchi 1997; Lee and Lee 1993). In our study, COI, UPA, and *rbc*L analyses detected a cryptic species, *Pachymeniopsis volvita* sp. nov. with morphology very similar to young specimens of *G. kurogii*, which have circular blades with refractive cells (Kawaguchi 1990), but the interspecific divergence of two species is 3.8–4.2 % (Table 2). *Rbc*L gene sequence of *P. volvita* sp. nov. diverged from that of *P. elliptica* by 1.9–2.3 %. *G. imbricata* Holmes was first described in Japan and recently introduced into the Canary Islands (Garcia-Jiménez et al. 2008). In our COI and UPA analyses, *G. hawaiiana* from Hawaii (HQ422635) fell among specimens of *G. imbricata* from Korea (Sherwood et al. 2010). *G. angusta* was first described by Harvey as a variety of *Gymnogongrus ligulatus* Harvey from specimens collected at Shimoda, Japan; many nomenclatural changes have been made since that time. Finally, Wang et al. (2001) transferred *Cryptonemia angusta* Okamura into *Grateloupia angusta* based on the texture of blades and the positions of reproductive structures. We found that *Kintokiocolax aggregato-cerantha* is genetically grouped with *G. chiangii* and *G. cornea*, with 2.9–3.7 % interspecific divergences.

Our subclade II included *G. jejuensis*, *G. asiatica*, and *G. divaricata*. Kim et al. (2013) reported *G. jejuensis* as a new species and described diagnostic characteristics, including a caespitose and flattened habit with discoid holdfasts, cartilaginous texture, and a blunt or bifid axis. We found that *G. jejuensis* differed by 27–29 bp (2.28–2.45 %) from *G. elata* in *rbc*L gene and by 63 bp (5.48 %) from *G. cornea*. *G. asiatica* was proposed as a new species from the northwestern Pacific by Kawaguchi and Wang (Kawaguchi et al. 2001). *G. asiatica* may be distinguished by its compressed to narrowly flattened axes with numerous pinnate proliferations. *G. divaricata* was first recognized as *Carpopeltis divaricata* by Okamura (1934) and characterized by compressed thalli with cuneate bases, repeatedly dichotomously branching in one plane, and rounded axils that result in a flabellate shape (Kawaguchi 1989).

Our subclade III included *G. subpectinata* and *G. turuturu*. Faye et al. (2004) reinstated *G. subpectinata* based on *rbc*L sequences and morphological features, including fleshy texture, thick axes, long proliferations, and oblong auxiliary cells.

Table 2 A comparison of taxonomic characteristics among closely related species with *Pachymeniopsis volvita* sp. nov

	P. volvita	*P. elliptica*	*P. lanceolata*	*P. gargiuli*	*Grateloupia kurogii*
Habit	Circle to peltate or reniforme blade, up to 6 cm	Circle to elliptical blade, up to 30 cm	Narrowly lanceolate to obong blade, up to 60 cm	Lanceolate to linear blade, up to 50 cm	Circle to elliptical blade, 20–30 cm
Texture	Fleshy coriaceous and slippery	Leathery	Gelatinous to tough or somewhat leathery	Membranaceous and lubricous	Lubricuous when young, becomes firmer with age
Blade thickness	660–775 μm	1,000 μm	400–1,000 μm	140–170 μm	(200) 300–450 μm
Thickness of cortex	6–9 cells thick	20 cells thick	10–20 cells thick	5–7 cells thick	7–8 cells thick
Medullary structure	Refractive filamentous with stellate or gangloinic cells	Densely filamentous with stellate cells	Filamentous at first rather lax, becomes dense with age	Filamentous with mucilaginous material	Filamentous with large stellate cells and long refractive arms
Tetrasporangia	13–16×43–52 μm	18–22×48–55 μm	28×36–57 μm	–	18–25×35–45 μm
Mature cystocarp	135–220 μm in diameter	300 μm in diameter	300–400 μm in diameter	320–380 μm in diameter	250–300 μm in diameter
Geographical distributions	Korea	Asia	Asia, Europe, North America	Korea and Italy	Asia
References	This study	Kawaguchi 1997	Kawaguchi 1997	Kim et al. 2014	Kawaguchi 1990

Their sequences for *G. subpectinata* from Japan (AB114208) diverged by 0.6–0.9 % from those of three conspecific specimens from Korea. Our *rbc*L analyses also showed that *G. subpectinata* is genetically grouped with *G. luxurians* from France and Australia (AJ868492 and AY435175). *G. luxurians*, however, is currently regarded as a taxonomic synonym of *G. subpectinata* Holmes (Wilkes et al. 2005; De Clerck et al. 2005). *G. turuturu* Yamada was originally described from Hokkaido, Japan, and is distinguished by the anticlinal arrangement of medullary filaments, a thin cortex of roundish cells and an abrupt transition between cortex and medulla (Gavio and Fredericq 2002). *G. turuturu* has been the focus of much research as it has invaded many parts of the world (Verlaque et al. 2005; Saunders and Withall 2006).

Our subclade IV included *G. catenata*, which was first described by Yendo from Hokkaido, in northern Japan. It has subsequently been reduced to synonymy of several other taxa (Sheng et al. 2012). Wang et al. (2000) clarified the taxonomic status of western Pacific *G. catenata*, which has a hollow axis, numerous short proliferations, and a tendency for reproductive structures to be restricted to the proliferations. In our COI analyses, Korean specimens of *G. catenata* fell among Hawaiian specimens, with a 1.6 % intraspecific divergence value. UPA sequences also demonstrated a similar topology in samples from Hawaii (Sherwood et al. 2010).

In conclusion, we have demonstrated for the first time that COI and UPA barcoding markers are effective for species identification in the genus *Grateloupia* and *Pachymeniopsis*, but COI gene had the greatest utility at the species level. Within- and between-species divergence values were lower for UPA than for COI-5P. UPA may lack resolving power among closely related species, which could lead to underestimations of species diversity (Clarkston and Saunders 2010). COI-5P and *rbc*L markers in combination provide a powerful tool for species identification, discovery of cryptic species, and phylogenetic reconstruction (Kucera and Saunders 2012). We confirmed the presence of a cryptic species: *Pachymeniopsis volvita* sp. nov.

Acknowledgments We thank Mr. J.C. Kang for collecting samples from the subtidal and other members of the molecular phylogeny of marine algae laboratory at Jeju National University. This study was supported by a grant from the National Institute of Biological Resources (NIBR), funded by the Ministry of Environment (MOE) of the Republic of Korea (NIBR No. 2013-02-001 for collecting samples and NIBR No. 2012-02-042 for molecular phylogeny of Korean major taxa).

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