

# GENETIC DIVERSITY AND HAPLOTYPE DISTRIBUTION OF *PACHYMENIOPSIS GARGIULI* SP. NOV. AND *P. LANCEOLATA* (HALYMENIALES, RHODOPHYTA) IN KOREA, WITH NOTES ON THEIR NON-NATIVE DISTRIBUTIONS<sup>1</sup>

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The red alga *Pachymeniopsis lanceolata*, formerly known as *Grateloupia lanceolata*, is a component of the native algal flora of northeast Asia and has been introduced to European and North American waters. It has been confused with a cryptic species collected from Korea and Italy. Our analyses of *rbcL*, *cox3* and ITS from *P. lanceolata* and this cryptic species has revealed two distinct entities, forming a clade, which were clearly separated from its congeners and positioned with other Asian species. Here, we describe the cryptic species as *P. gargiuli* sp. nov., a species that differs from others by molecular sequence and subtle anatomical characters. We hypothesize that *P. gargiuli* may have been recently dispersed by anthropogenic vectors, possibly at or near the same time as was *P. lanceolata*. Our *cox3* data set revealed that one haplotype of *P. gargiuli*, shared between Korea and Italy, and two haplotypes of *P. lanceolata*, commonly occurring in Korea and USA, are invasive haplotypes. This is the first report of the utility of the mitochondrial coding *cox3* sequences in red algae.

**Key index words:** *Grateloupia*; Halymeniaceae; haplotype network; invasive species; *Pachymeniopsis*; phylogeography

*Grateloupia* C. Agardh (Halymeniales, Rhodophyta) sensu lato is a large genus containing ~90 species, many of them poorly understood and in need of taxonomic revision. New molecular and morphological data have shown that these species belong to several genera, including *Pachymeniopsis* Y. Yamada (Gargiulo et al. 2013, Calderon et al. 2014). Interestingly, six species currently recognized as belonging to the genera *Grateloupia* and *Pachymeniopsis* have been introduced from Asia to Australasia, Europe and North America: *G. asiatica* S. Kawaguchi & H. W. Wang, *G. imbricata* Holmes, *G. patens* (Okamura) S. Kawaguchi & H. W. Wang, *G. subpectinata* Holmes, *G. taiwanensis* S. M. Lin & H. Y. Liang, *G. turuturu* Yamada and *P. lanceolata* (K. Okamura) Y. Yamada ex S. Kawabata (as *G. lanceolata* [Okamura] S. Kawaguchi; (Verlaque 2001, Gavio and Fredericq 2002, Marston and Villalard-Bohnsack 2002, Verlaque et al. 2005, Saunders and Withall 2006, D'Archino et al. 2007, García-Jiménez et al. 2008, Cerere et al. 2011, De Priest and López-Bautista 2012, Nelson et al. 2013). The exponential increase in global shipping and export of aquaculture products has provided numerous opportunities for dispersing species from ocean to ocean (Carlton and Geller 1993, Mineur et al. 2007, Miller et al. 2011), but little is known about the population structure of these species within their native ranges.

*Pachymeniopsis lanceolata* is common in Korea, Japan, and China (Yoshida 1998, Xia 2004, Boo and Ko 2012). The species was originally described as *Aeodes lanceolata* Okamura based on material collected

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in Japan (Okamura 1934) and later became the generitype of Kawabata's (1954) new genus *Pachymeniopsis*, which was described on the basis of its vegetative anatomy, carpogonial branch ampullae, the lack of pericarps surrounding the cystocarps, and the disposition of reproductive structures. Kawaguchi (1997) merged *Pachymeniopsis* with *Grateloupi*, making the combination *G. lanceolata*. Gargiulo et al. (2013) reassessed the correlation of the reproductive anatomy of female gametophytes and the development of carposporophytes with the *rbcl* tree, focusing on species that occur in the Mediterranean Sea, and resurrected the genus *Pachymeniopsis* and its generitype, *P. lanceolata*. This species has been the focus of interest because of its introduction to the Mediterranean Sea (Verlaque 2001, Verlaque et al. 2005), the Canary Islands (García-Jiménez et al. 2008), and California, USA (Miller et al. 2009).

De Masi and Gargiulo (1982) reported "*Grateloupi doryphora*" from the Strait of Messina, Italy. A recent analysis (Wilkes et al. 2006) has revealed that the specimen was neither *G. doryphora* (Montagne) M.A. Howe nor *G. turuturu* Yamada, but instead, a distinct species (herein designated *Pachymeniopsis gargiuli* sp. nov.) closely related to *P. lanceolata* (Gargiulo et al. 2013). Lee et al. (2009) reported a specimen from Uhyoung, Jeju Island, Korea with an *rbcl* sequence that formed a monophyletic clade with *P. lanceolata*, which has a similar morphology, and tentatively identified it as *P. lanceolata*, noting that it diverged by 1% from other *P. lanceolata* *rbcl* sequences. This specimen, and one collected at Onphyeong, Jeju Island (Yang et al. 2013) proved to have the same sequence as the Italian specimen.

The aims of the present study were: (i) to describe *P. gargiuli* sp. nov. based on specimens from Korea, Japan, and Italy, (ii) to resolve the phylogenetic position of this new species within the Halymeniaceae, (iii) to confirm the introductions of *P. gargiuli* outside of Asia, and (iv) to assess the genetic diversity of *P. gargiuli* and *P. lanceolata* in Korea. We used three DNA markers: plastid *rbcl*, mitochondrial *cox3* and nuclear ribosomal DNA internal transcribed spacer (ITS). Identification of halymeniacean algae has routinely involved the use of *rbcl* sequences (e.g., Wang et al. 2001, Boo et al. 2013). We are the first to employ mitochondrial *cox3* to reveal the population structure of red algal species, although it has been used frequently for brown algae (e.g., Voisin et al. 2005, Uwai et al. 2006, Lee et al. 2012). ITS were analyzed to see if the phylogeny based on this nuclear marker was congruent with those from organelle genes and to test for hybridization between species.

#### MATERIALS AND METHODS

A total of 205 specimens of *P. lanceolata* and 51 of *P. gargiuli* were collected from Korea, China, Japan, Italy, and USA (Table 1). Information about these specimens and the Gen-

Bank accession number for each haplotype are listed in Table S1 (Supporting Information). However, ITS sequences were not uploaded because of less than 200 bp according to the rule of GenBank. Anatomical observations were performed on free-hand sections from thalli at different degrees of development and at different levels (basal, middle, and subapical) and stained with a 1% aniline blue solution. Microphotographs were obtained using a Diaplan Leica microscope equipped with a Leica DFC 500 camera (Leica Microsystems, Milan, Italy). Voucher specimens are housed in the herbarium of Chungnam National University (CNUK), Daejeon, Korea and in the Phycological Lab Herbarium (PhL) of the University of Messina, Italy.

DNA extraction, PCR amplification, and sequencing were performed as described in Boo et al. (2009). Two primer pairs, *rbcl*LF762-R1442 and *rbcl*LF145-*rbcl*LR898 (Kim et al. 2010a,b), were used for the amplification of *rbcl*. For *cox3*, newly designed primers F290 (5'-ATG TAT ATG CAT GCT TAT RWM AAT GG-3') and R962 (5'-ACC TCC TCA YCA ATA AAT RGA-3') were used. ITS regions were sequenced with TW81 and AB28 primer pair (Goff et al. 1994). To determine if hybridization occurred between *P. lanceolata* and *P. gargiuli*, we analyzed 129 bp of the parentally inherited gene region, ITS1, from 44 randomly selected specimens from Korea.

Electropherograms were edited with Chromas v. 1.45 and all sequences were aligned manually using Se-Al v. 2.0a11 (Rambaut 2002). A maximum likelihood (ML) tree was constructed using 53 *rbcl* sequences containing 14 sequences (eight new, six previously published) of *P. lanceolata* and *P. gargiuli*, 35 other halymeniacean species, and four outgroups. ML analyses were conducted using RAxML v. 7.2.8 (Stamatakis 2006) under the GTR +  $\Gamma$  evolutionary model. We used 200 independent tree inferences using the default option of automatically optimized SPR rearrangements and 25 "distinct rate categories" options to identify the best tree. Bootstrap values were determined with 1,000 replications. The Bayesian analysis was conducted with MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003) using the same evolution model as used in the ML analysis. The GTR +  $\Gamma$  model parameters were unfixed and three million metropolis-coupled Markov Chain Monte Carlo (MC<sup>3</sup>) simulations were completed with following: two independent runs from different random start points, one cold chain and three heated chains for each run, and tree sampling at every 100th generation. The burn-in point of the run was identified by the average standard deviation of split frequencies (less than 0.01) between the two independent MC<sup>3</sup> chains. Sampled trees after the burn-in point were used only for the calculation of the Bayesian posterior probability of each node.

To assess the genetic diversity within *P. lanceolata* and *P. gargiuli*, we used 184 *rbcl* (617 bp) sequences, including 30 previously published from GenBank and 249 *cox3* (556 bp) sequences. The minimum spanning network to analyze relationships and geographical distributions of each haplotype was constructed using Arlequin v. 3.5 (Excoffier and Lischer 2010). Haplotype diversities (*h*) and nucleotide diversities ( $\pi$ ) were measured using DnaSP v. 5.10.01 (Librado and Rozas 2009).

#### RESULTS

*Phylogenetic relationships.* In the *rbcl* tree, *P. lanceolata* and *P. gargiuli* were distinct and strongly monophyletic (100% for ML), with *P. elliptica* from Korea and Japan as the nearest sister species (Fig. 1). The combined clade of *P. elliptica* and *P. lanceolata*/*P. gargiuli* clustered with *Grateloupi angusta* (Okamura)

S. Kawaguchi & H. W. Wang, *G. chiangii* S. Kawaguchi & H. W. Wang, *G. cornea* Okamura, *G. imbricata*, and *G. kurogii* S. Kawaguchi, all from Asia. In the *cox3*

tree, the sister relationship of *P. lanceolata* and *P. gargiuli* (tree not shown) was the same as that in the *rbcL* tree (Fig. 1). *P. lanceolata* comprised two

TABLE 1. Sampling location and haplotype information for *Pachymeniopsis lanceolata* and *P. gargiuli*.

Species	Region	Locality	N	rbcL	cox3	ITS	
<i>Pachymeniopsis lanceolata</i>	Korea East	Gijang	29	R1, R4	C1, C2, C3, C4, C8, C9	I2, I3	
		Kyeongju	3	R3	C1		
		Donghae	15	R1, R4	C1, C7, C8, C9	I1, I2	
		Kangneung	20/1 <sup>a</sup>	R1	C1		
		Yangyang	14	R1	C1		
		Sockcho	2/3 <sup>a</sup>	R1, R2	C1		
		Gosung	3 <sup>a</sup>	R1, R2			
		West Jeju	Taeam	23	R4	C8	
			Yongwundong	1	R4	C9	
			Ongpo	1 <sup>b</sup>	R4		
			Sasudong	1	R4	C9	
			Chujado	1	R1		
			Hamduck	1		C9	
			Hangwon	5	R4	C9	I3
			Jondal	1 <sup>b</sup>	R4		
	Udo		1 <sup>b</sup>	R4			
	Seokimnyoung		16	R4	C8, C9		
	South	Chaguido	2	R4	C9	I3	
		Hamo	1 <sup>b</sup>	R4			
		Kangjung	1 <sup>b</sup>	R4			
		Jindo	2	R4	C8, C10	I3	
		Segeochado	2 <sup>a</sup>	R1			
		Haenam	4	R4	C10		
		Wando	4	R4	C8, C10		
		Dolsando	3	R4	C10		
		Geojaedo	7	R5	C8		
		Maemuldo	21	R1, R4	C1, C5, C6, C10	I2	
	China	Dalian	1	R4	C10		
		Qingdao	5	R4	C8		
		Rongcheong	1 <sup>c</sup>	R4			
	Japan	Fukuoka	8/2 <sup>d</sup>	R4, R5	C8	I3	
		Hokkaido	1 <sup>d</sup>	R5			
		Kobe	1 <sup>d</sup>	R4			
		Enoshima	3 <sup>b</sup>	R4			
		Misaki	2 <sup>b</sup>	R4			
	USA	Elkhorn Slough, CA	11	R4	C9		
		Newport Bay, CA	1	R4	C8	I5	
		San Francisco, CA	5	R4	C9		
		Thau Lagoon	3 <sup>c</sup>	R4			
	Total		205/27				
	<i>Pachymeniopsis gargiuli</i>	Korea West Jeju	Jindo	12	R7	C11, C12	I6
Uhyoung			4/1 <sup>a</sup>	R6	C14	I5	
Saewha			1	R6	C16	I6	
Onpheyong			1 <sup>b</sup>	R6			
Chaguido			1	R6			
Sakyae			9	R6	C14, C15	I4	
Kangjeong			1	R6			
Japan			Fukuoka	1	R7	C13	I5
			Tsushima	2		C14	
			Reggio Calabria	9/1 <sup>f</sup>	R7	C11	I6
Italy		Messina	12	R7	C11	I6	
		Canary Islands	Las Palmas	1 <sup>g</sup>	R7		
Total			51/4				

Superscript with slash mark indicates previously published data.

<sup>a</sup>Lee et al. 2009.

<sup>b</sup>Yang et al. 2013.

<sup>c</sup>Zhang and Ye unpublished.

<sup>d</sup>Kawaguchi et al. 2002.

<sup>e</sup>Verlaque et al. 2005.

<sup>f</sup>Wilkes et al. 2006.

<sup>g</sup>García-Jiménez et al. 2008.

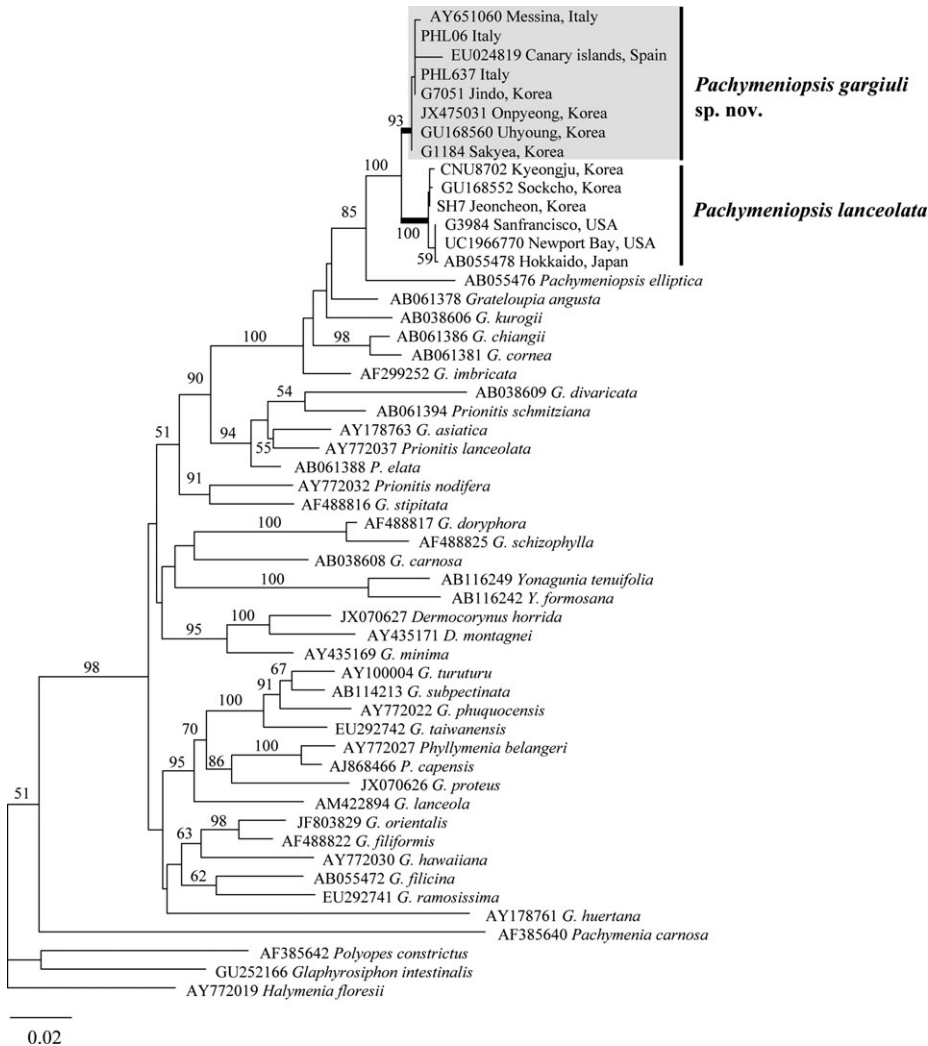


FIG. 1. Maximum likelihood tree of *rbcL* sequences. Bootstrap support values (>50% for ML) are presented near branches.

genealogical groups (I and II), and both of these groups occurred at Gijang, on the southeast coast of Korea. One specimen (DGJ5) from Gijang was included in Group II in the *rbcL* and *cox3* data, while it fell into Group I in the ITS data (Fig. 2), implying genetic connectivity between these two genealogical groups.

*Pachymeniopsis gargiuli* comprised two genealogical groups; a Korean specimen from Sewha was placed in Group I in *rbcL* and *cox3* data sets, but it also occurred in Group II in ITS. A Japanese specimen (Fukuoka) belonged to II in *rbcL* and *cox3*, while it was a member of Group I in ITS (Fig. 2). These results also suggest genetic connectivity between Group I and II in Korean and Japanese populations of *P. gargiuli*. However, there was no evidence of genetic connectivity (hybridization) between *P. gargiuli* and *P. lanceolata*, although both species occurred at Jindo, Korea and Fukuoka, Japan.

*Genetic diversity of P. lanceolata and P. gargiuli.* Haplotype and nucleotide diversities of *rbcL* in Korean

populations of *P. lanceolata*, calculated from 110 *rbcL* sequences, were 0.561 (Hd) and 0.00265 ( $\pi$ ; Table 2). The southern population (South) was high in both diversities (Hd 0.498,  $\pi$  0.00242), and the Jeju population was low (Hd 0.065,  $\pi$  0.00031). For *cox3*, haplotype and genetic diversities of *P. lanceolata* were 0.711 (Hd) and 0.0049 ( $\pi$ ). The South population revealed high genetic diversities (Hd 0.702,  $\pi$  0.0062), but the Jeju population was low in nucleotide diversity ( $\pi$  0.0009), and the eastern (East) population was low in haplotype diversity (Hd 0.177).

Haplotype and nucleotide diversities of *rbcL* in Korean populations of *P. gargiuli* were 0.497 (Hd) and 0.0008 ( $\pi$ ) calculated from 30 sequences. For *cox3*, the haplotype and nucleotide diversities, based on 25 sequences from Korean specimens, were 0.75 and 0.072 (Table 2).

*Distribution of haplotypes of P. lanceolata and P. gargiuli.* The haplotype network of *rbcL* for *P. lanceolata* revealed that five haplotypes fell into two groups, I and II (Fig. 3). Group I, consisting of three haplotypes



TABLE 2. Genetic diversity of *Pachymeniopsis lanceolata* and *P. gargauii*.

Country	<i>rbcL</i>					<i>cox3</i>				
	<i>n</i>	<i>s</i>	<i>h</i>	Hd	$\pi$	<i>n</i>	<i>s</i>	<i>h</i>	Hd	$\pi$
<i>Pachymeniopsis lanceolata</i>										
Asia										
Korea	110	5	4	0.561	0.00265	172	29	11	0.711	0.0049
East	54	5	4	0.497	0.00189	54	6	4	0.177	0.0012
South	23	3	2	0.498	0.00242	41	25	6	0.702	0.0062
Jeju	31	3	2	0.065	0.00031	26	1	2	0.517	0.0009
West	2	0	1			23	0	1	0	0
China	7	0	1			6	0	1		
Japan	16	1	2	0.233	0.00038	7	0	1		
North America										
USA	17	0	1	0	0	16	1	1	0.125	0.002
Europe										
France	3	0	1							
Total	153	6	5	0.506	0.00242	201	29	11	0.72	0.0047
<i>Pachymeniopsis gargauii</i>										
Asia										
Korea	30	1	2	0.497	0.0008	25	25	6	0.75	0.0072
Jeju	18	0	1	0	0	13	3	3	0.615	0.0022
West	12	0	1	0	0	12	22	3	0.318	0.0066
Japan	1	0	1			3	4	2		
Europe										
Italy	3	0	1			20	0	1	0	0
Total	34	1	2	0.513	0.00083	48	26	7	0.567	0.0049

Number of specimens (*n*), number of polymorphic sites (*s*), number of haplotypes (*h*), haplotype diversity (Hd), nucleotide diversity ( $\pi$ ).

and R7 respectively. For *cox3*, *P. gargauii* comprised Groups I and II. Group I consisted of three haplotypes (C11–C13) in Korea (Jindo), Japan (Fukuoka) and Italy (Messina and Reggio Calabria). Group II included three haplotypes (C14–16) in Korea (Jeju) and Japan (Tsushima). Haplotype C11 was shared by 30 specimens from Jindo, Korea and from both sides of the Strait of Messina, Italy.

**Morphology of *P. gargauii*.** Thalli are solitary or gregarious, arising from a discoid holdfast, up to 40 cm long and 5 cm wide, purplish-red to orange-red, with lanceolate to linear, pseudo-dichotomously branched blades; margins are entire with rare proliferations in old plants; blades are membranaceous, lubricious, increasing in thickness in old plants (Fig. 4A).

Thalli are multiaxial, with a narrow cortical zone and a broad filamentous medullary zone (Fig. 4, B and C). The cortical zone is composed of 5–6 layers of cells increasing in size inwardly, with an outer cortex and inner cortex. The outer cortex consists of pseudo-dichotomous anticlinal filaments, composed of 3–4 pigmented spherical cells, united exclusively by primary pit connections (Fig. 4C). In surface view, the outermost cortical cells are round to polygonal, solitary or in pairs (Fig. 4D). The inner cortex consists of pseudo-dichotomous anticlinal filaments, composed of 2–3 lobed to stellate hyaline cells, united by primary and secondary pit connections (Fig. 4B, arrow). The medulla is filled by: (a) primary medullary filaments (i.e., axial filaments) composed of longitudinally elongate cells (Fig. 4B, arrow-head), each with a lateral branch

issuing to produce either cortical filaments or secondary pit-connections with adjacent axial filaments, (b) anticlinal or oblique filaments, produced secondarily as branches from both primary axial filaments and innermost cortical cells (Fig. 4B, double arrow-head), and (c) extracellular mucilaginous material. Darkly staining stellate cells are rare (Fig. 4C, arrow). The medulla is loose in the distal and middle parts of the thallus, with most axial medullary filaments located close to the inner cortex, and dense in the basal parts, with axial medullary filaments thoroughly dispersed (Fig. 4, E and F).

Dioecious gametophytes and tetrasporophytes are isomorphic.

Female reproductive structures are borne in ampullae; the carpogonial branch is 6-celled with a terminal carpogonium; the auxiliary cell is the terminal cell of a 5-celled branch. After fertilization, an extensive carpogonial fusion cell and an extensive auxiliary fusion cell develop, accompanied by both upwardly and downwardly directed branched nutritive filaments (fig. not shown). Details of ampullar structure and postfertilization events are in agreement with Gargiulo et al. (2013). Tetrasporangia arise from inner cortical cells, and are cruciately divided and embedded beneath cortical surface (fig. not shown).

**Morphology of *Pachymeniopsis lanceolata*** (K. Okamura) Y. Yamada ex S. Kawabata (Fig. 5)

**Basionym:** *Aeodes lanceolata* K. Okamura in *Icones of Japanese Algae*. Vol.7, pp.19–48, pl. CCCXI–XV, 1934.

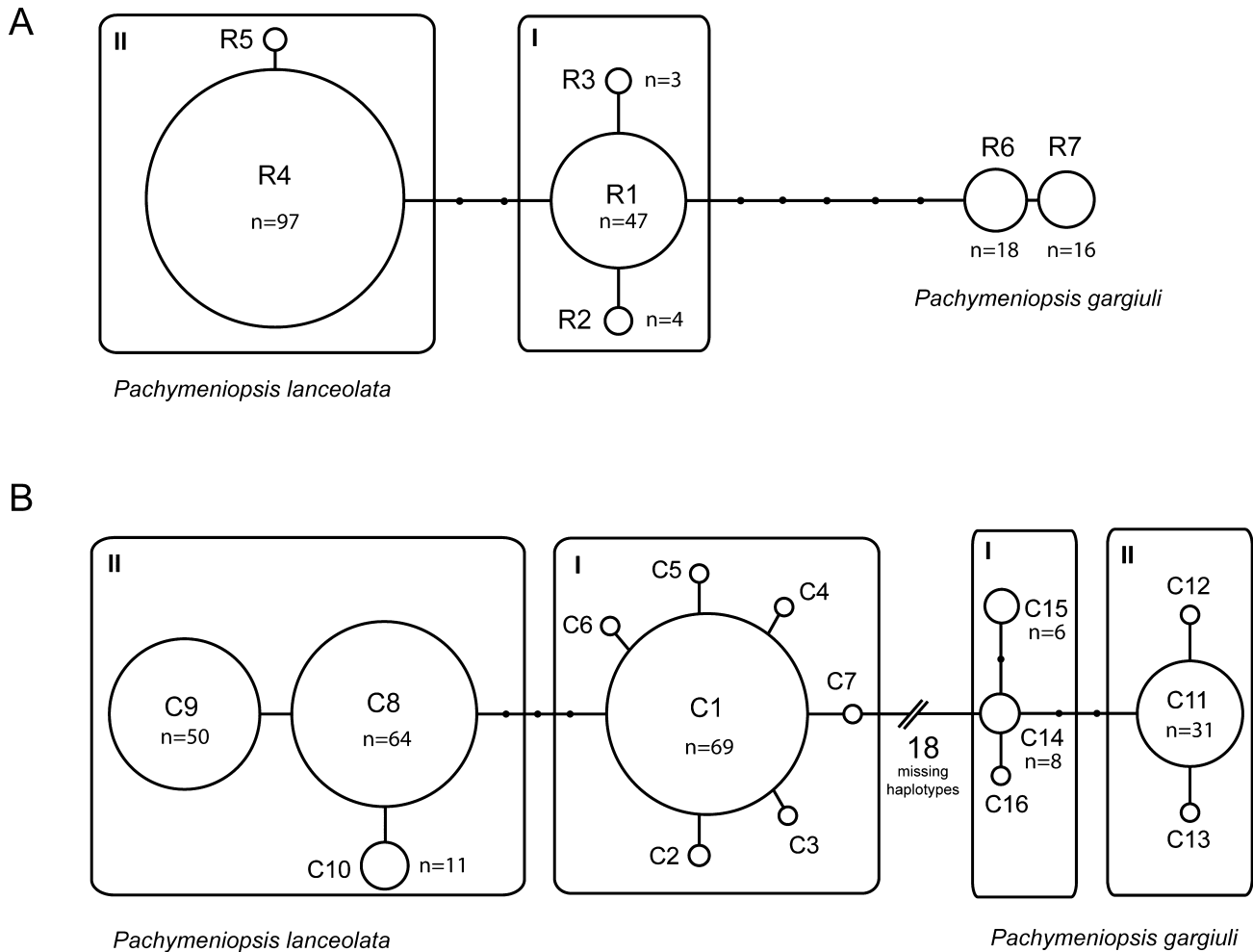


FIG. 3. Minimum spanning networks of *Pachymeniopsis lanceolata* and *P. gargiuli* based on *rbcL* (A) and *cox3* (B). See Table 1 for information about haplotypes.

*Synonym: Grateloupia lanceolata* (K. Okamura) S. Kawaguchi in Phycological Research 1997, 20: 9, figs. 2–28.

Thalli are solitary or clustered, up to 50 cm long and 15 cm wide, arising from a discoid holdfast, purplish-red, with lanceolate to oblong flat blades, pseudo-dichotomously to palmately branched; margins are entire with many proliferations in old plants; blades are membranaceous, lubricious, increasing in thickness in old plants (Fig. 5, A and B).

Thalli are multiaxial with a narrow cortical zone and a broad filamentous medullary zone (Fig. 5C). The cortical zone is composed of 6–7 layers of cells in the upper part of the thallus to 9–10 layers of cells in the lower part. The cortical cells increase in size inwardly, forming an outer cortex and inner cortex (Fig. 5C). The outer cortex consists of pseudo-dichotomous anticlinal filaments, composed of 3–4 compact layers of pigmented spherical cells in the upper part of the thallus to 7–8 layers of cells in the lower parts, united exclusively by primary

pit-connections (Fig. 5C). In surface view, the outermost cortical cells are round to polygonal, solitary or in pairs (Fig. 5D). The inner cortex consists of pseudo-dichotomous anticlinal filaments, composed of 2–3 layers of lobed to stellate hyaline cells, united by primary and secondary pit-connections (Fig. 5C, arrow). The medulla is filled by: (a) primary medullary filaments (i.e., axial filaments) composed of longitudinally elongate cells (Fig. 5C, arrow-head), each with a lateral branch producing either cortical filaments or connecting by secondary pit-connections to adjacent axial filaments, (b) anticlinal or oblique medullary filaments, produced secondarily as branches from both primary axial filaments and innermost cortical cells (Fig. 5C, double arrow-head), and (c) extracellular mucilaginous material. The medulla is moderately dense in the distal and middle part of the thallus and very dense in the basal parts (Fig. 5, E and F).

Diocious gametophytes and tetrasporophytes are isomorphic.

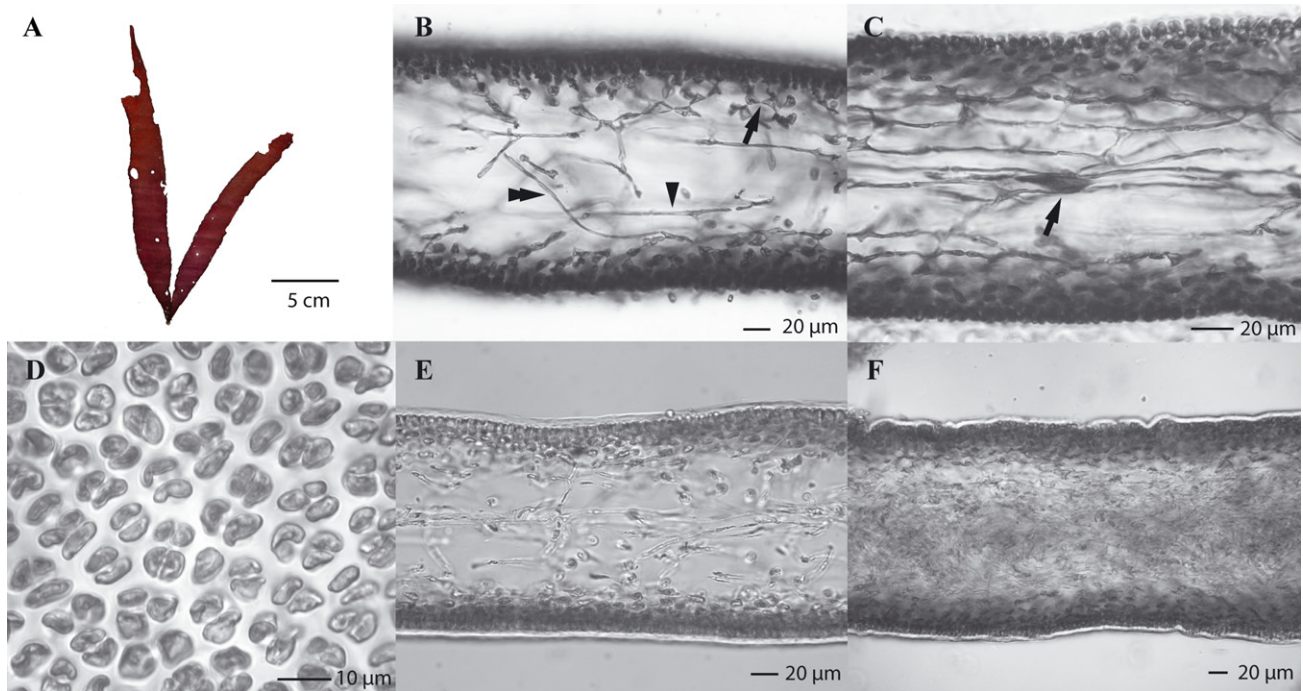


FIG. 4. *Pachymeniopsis gargiuli* S.Y. Kim, A. Manghisi, M. Morabito & S.M. Boo sp. nov. (A) Habit of type specimen (CNU055670) collected from Hoidong, Jindo, Korea (December 17, 2012). (B and C) Longitudinal sections of thalli (voucher PhL00065); (B) Subapical section, showing secondary pit connected inner cortical cells (arrow), primary medullary filament (arrow-head), oblique medullary filament (double arrow-head) produced secondarily as a branch from a primary filament; (C) Darkly staining medullary stellate cell (arrow); (D) Surface view of the cortex (voucher PhL00065). (E and F) Transverse sections of thalli; (E) middle region (Voucher SKKU60); (F) Basal region (Voucher PhL00065).

Description of *Pachymeniopsis gargiuli* S. Y. Kim, A. Manghisi, M. Morabito & S. M. Boo sp. nov. (Fig. 4)

**Description:** Thalli solitary or gregarious, arising from a discoid holdfast, up to 40 cm long and 5 cm wide, purplish-red to orange-red, with lanceolate to linear, pseudo-dichotomously branched blades; margins entire with rare proliferations in old plants; blades membranaceous, lubricious. Axes multiaxial, with a narrow cortical zone and a broad filamentous medullary zone; cortical zone composed of 5–6 layers of cells; outer cortex consisting of pseudo-dichotomous anticlinal filaments; inner cortex composed of 2–3 lobed to stellate hyaline cells. Medulla filled by primary medullary filaments, anticlinal or obliquely arranged, and extracellular mucilaginous material. Carpogonial branch 6-celled; ampullar structure and post-fertilization events in agreement with those in the type of the genus. Tetrasporangia arising from inner cortical cells, cruciately divided, embedded beneath cortical surface.

**Holotype:** CNU055670 deposited in the Herbarium of Chungnam National University, Daejeon, Korea; on rocks in the lower intertidal zone off Hoedong (34°25' 17.17" N, 126°20' 41.23" E), Jindo, Korea; collected December 17, 2012.

**Isotypes:** CNU055671-2 deposited in the herbarium of Chungnam National University, Daejeon, Korea and CNU055673-4 in the Phycological Lab

Herbarium (PhL) of the University of Messina, Italy.

**Etymology:** The specific epithet “*gargiuli*” is chosen to honor Prof. Gaetano Maurizio Gargiulo of the University of Messina, Italy, for his great contributions to the taxonomy of Mediterranean Halymeniaceae and other marine algae.

#### DISCUSSION

Our analyses of mitochondrial *cox3* and nuclear ITS as well as plastid *rbcL* clearly showed that *P. gargiuli* is genetically different from *P. lanceolata*. The pairwise divergence between the two species is 1.1%–1.5% (13–17 bp) in *rbcL*, and these levels of divergence are comparable to those between other species of halymeniacean algae (1.0%–8.8%; Kawaguchi et al. 2002). Similar high divergence values between *P. gargiuli* and *P. lanceolata* were found in the ITS (1.7%–3.3%; 3–6 bp) and in *cox3* (3.6%–4.3%; 20–24 bp). *Pachymeniopsis gargiuli* occurs sympatrically with *P. lanceolata* in Jeju Islands, Korea and Fukuoka, Japan, but our ITS results demonstrate that there is no hybridization between these two species. Although the type locality of *P. lanceolata* was not designated in the protologue (Okamura 1934, Kawaguchi 1997), Yoshida (1998) reported that it likely was Enoshima, Kanagawa



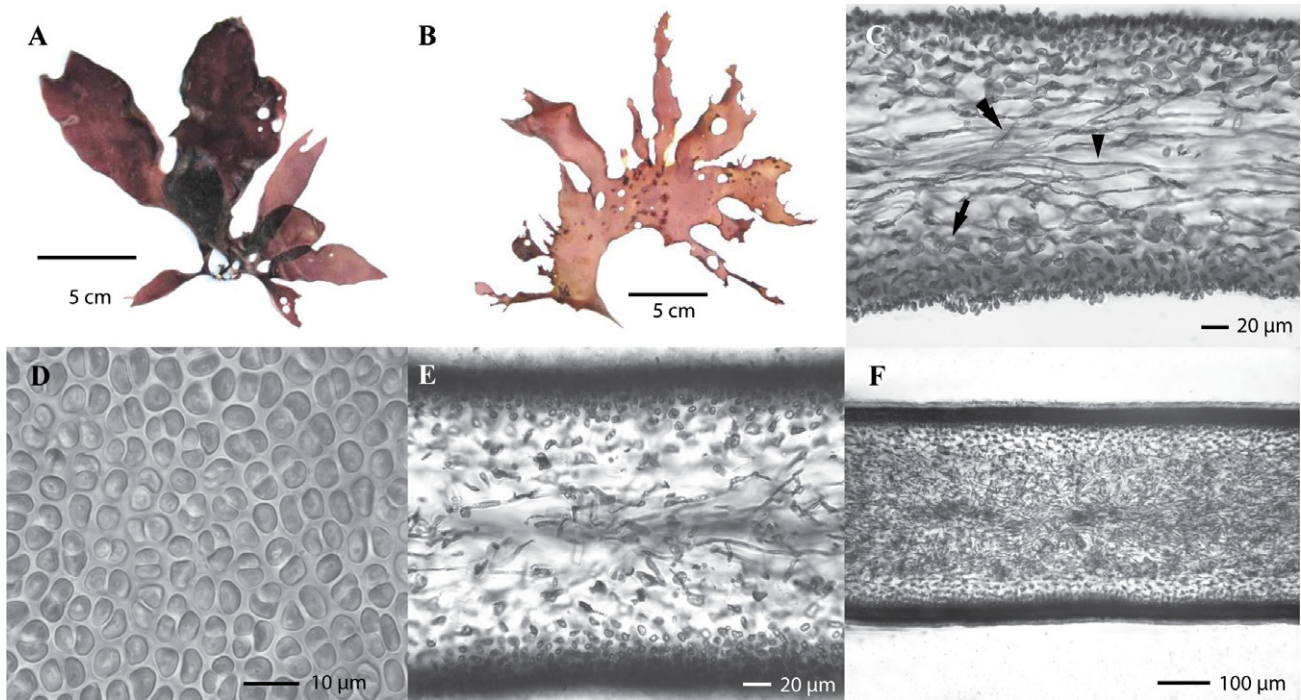


FIG. 5. *Pachymeniopsis lanceolata* (K. Okamura) Y. Yamada ex S. Kawabata. (A) Habit of specimens collected from Hangwon, Jeju, Korea (March 29, 2010). (B) Habit of specimens collected from Uhran, Haenam, Korea (January 21, 2010). (C) Longitudinal sections of thalli (voucher SH179). Subapical section showing secondary pit connected inner cortical cells (arrow), primary medullary filament (arrow-head), oblique medullary filament (double arrow-head) produced secondarily as a branch from a primary filament. (D) Surface view of the cortex (voucher SH132). (E and F) Transverse sections of thalli (voucher SH322); (E) middle region; (F) Basal region.

Prefecture, Japan. Previously published *rbcL* sequences from Enoshima and nearby Misaki, Japan (Kawaguchi et al. 2002, Yang et al. 2013) matched many sequences from Korea as well as from other locations in Japan (Fig. 1).

We confirmed the presence of *P. gargiuli* at three more sites in Korea, at two in Japan, and on both sides of the Strait of Messina, Italy. Its occurrence was previously reported in Jeju, Korea, Italy and the Canary Islands (Wilkes et al. 2006, García-Jiménez et al. 2008, Lee et al. 2009, Yang et al. 2013). Further collections may allow us to expand our knowledge of its distribution in Europe as well as in Korea and Japan. *P. gargiuli* is not easily distinguishable from *P. lanceolata* in the field. It has elongated blades, with a few degrees of pseudo-dichotomous divisions (Fig. 4A) and *Pachymeniopsis*-type carpogonial and auxiliary cell ampullae (Gargiulo et al. 2013). Marginal branching sometimes occurs in older thalli but more commonly, blades bear fewer branches than *P. lanceolata* blades. *P. gargiuli* has a more lax medulla and a thinner cortex than *P. lanceolata*, and most axial medullary filaments are located close to the inner cortex except in the basal parts of blades, where the medulla is denser. Conversely, the medulla of *P. lanceolata* is moderately dense throughout the blade, showing greater density toward the base of the blade. These anatomical differences in different parts of the thallus, although

consistent within species, are subtle. Consequently, the two species can be considered cryptic, definitively identified only by DNA sequence, though trained taxonomists can distinguish them with thin sections.

Phylogenetic analyses of all three gene sequences placed *P. gargiuli* as a nearest sister to *P. lanceolata* in the genus *Pachymeniopsis*. These two species consistently shared clades with other Asian species, confirming that *P. gargiuli* is Asian in origin, as was suggested by Wilkes et al. (2006). Analyses of *rbcL*, *cox3*, and ITS sequences from specimens collected in Korea and Japan demonstrate that the occurrence of *P. gargiuli* in Italy and the Canary Islands (Wilkes et al. 2006, García-Jiménez et al. 2008) is a result of recent anthropogenic introduction, although the timing of the introduction is not clear. It was first reported (as *Grateloupia cuneifolia* J. Agardh ex Kützing) from Italy in 1969 (Giaccone 1969). Wilkes et al. (2006) concluded that Giaccone's specimens were the same as those from the Strait of Messina reported by De Masi and Gargiulo (1982).

After these early reports, the species apparently spread to both coasts of the Strait of Messina, where established populations currently include all reproductive stages. According to DNA data obtained in the present study, Italian specimens have an invasive haplotype from Korea (C11 for *cox3* and R7 for *rbcL*).

The most likely scenario is that the Italian populations were introduced from Korea (and possibly Japan), since the two populations from both sides of the Strait of Messina are genetically identical to each other and to the Korean Jindo specimen.

The most frequent vector of Asian macroalgae to the Mediterranean Sea is the import of oysters for aquaculture (Verlaque et al. 2007). The brackish lagoon of Cape Peloro (Messina, Italy) is a site where adult shellfish (mussels, clams and oysters) imported from farming areas in Spain, France, the Netherlands and various Italian localities are held for a stabulation period in accordance with food quality and safety regulations. Recently, introduced species have been detected in the lagoon (Manghisi et al. 2010, 2011). DNA barcoding demonstrated that oyster importation was the vector for the introduction of the red alga *Agardhiella subulata* (C. Agardh) M. J. Wynne, and that a Dutch aquaculture site was the most likely source (Manghisi et al. 2010). It is possible that Asian *Pachymeniopsis* species arrived in Cape Peloro via other Italian and European farming sites and spread to nearby localities, including the Strait of Messina.

We hypothesize that *P. gargiuli* may have been introduced to European coasts at the same time as *P. lanceolata*. Verlaque et al. (2005) stated that several *Grateloupia* species, including *P. lanceolata* (as *G. lanceolata*), were introduced to the Thau Lagoon during massive importation of Japanese oysters during 1971–1976. According to Miller et al. (2009), *P. lanceolata* was probably introduced to California through the cultivation of Asian oysters in California and Puget Sound, Washington. *P. gargiuli* may have been introduced at the same time as *P. lanceolata*, but overlooked or misidentified on European coasts, the Canary Islands or in California. Thus, the non-native distribution of *P. gargiuli* may be underestimated until more specimens are examined.

Recent studies on the genetic diversity of introduced marine algae have revealed invasive haplotype type(s) and/or donor regions (Provan et al. 2005, Voisin et al. 2005, Uwai et al. 2006, Kim et al. 2010b). Our *rbcL* data set suggests that the invasions of *P. lanceolata* in Europe and North America originated from populations in Korea and/or Japan, as shown in previous studies (e.g., Verlaque et al. 2005, Lee et al. 2009). Our *cox3* data set revealed that the haplotypes C8 and C9 of *P. lanceolata*, shared by populations in Korea and California, USA, are invasive haplotypes. However, because marine algal introductions might have occurred more than once (McIvor et al. 2001, Provan et al. 2005, Voisin et al. 2005), more sampling is necessary to determine whether *P. lanceolata* was introduced via single or multiple events. The haplotype C11 of *P. gargiuli*, shared between Korea and Italy, is an invasive haplotype, and the vector

of this species may be the same as that of *P. lanceolata*.

We found that *cox3* sequences from *P. lanceolata* showed a low number of haplotypes (5%, 10/205 individuals) compared to those in *cox1* reported for *Hypnea asiatica* Geraldino et al. (54%, 15/28; Geraldino et al. 2009), *Gracilaria vermiculophylla* (Ohmi) Papenfuss (16%, 17/106; Kim et al. 2010b), and *Gelidium elegans* Kützinger (13%, 34/272; Kim et al. 2012). Similarly, haplotype diversity ( $Hd = 0.72$ ) for *P. lanceolata* was relatively low, compared to *cox1* in red algae ( $Hd = 0.757$  in *Gracilaria vermiculophylla* [Kim S.Y. et al. 2010];  $Hd = 0.711$  in *Gelidium elegans*, [Kim et al. 2012, ]) or *cox3* in brown algae ( $Hd = 0.781$  in *Ishige okamurae* Yendo [Lee et al. 2012, ]). Although *P. lanceolata* is widely distributed along the Korean and Japanese coastlines (Yoshida 1998, Boo and Ko 2012), with high biomass in the spring on the south coast of Korea (Kang and Kim 2004), the low number of haplotypes may make populations of this species vulnerable to environmental alterations due to global climate change.

In conclusion, we describe a new species, *P. gargiuli*, previously identified as *P. lanceolata*, from specimens collected in Korea, Japan, and Italy. We have demonstrated that *cox3* can be a suitable tool for evaluating the phylogeography and population structure of red algae. Our investigation of genetic diversity in *P. lanceolata* and *P. gargiuli* using *rbcL*, *cox3*, and ITS sequences confirmed the distinctiveness of these two species. Although they are sympatrically distributed in Korea and Japan, there was no evidence of hybridization between the two species. *P. gargiuli* in Italy and probably the Canary Islands was introduced from Korea/Japan. Our phylogeographical analysis of *P. lanceolata* revealed that its introduction to California originated in Jeju and/or the southeastern coasts in Korea or Japan.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

**Table S1.** Information about specimens used in the present study and the GenBank accession number for each haplotype.