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Phylogenetic position of *Petrospongium rugosum* (Ectocarpales, Phaeophyceae): insights from the protein-coding plastid *rbc*L and *psa*A gene sequences[1]

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Abstract — The spongy, crustose brown alga *Petrospongium rugosum* (Okamura) Setchell *et* Gardner occurs in Korea, Japan, Australia, New Zealand, and along the Pacific coast of North America. Although the species has been classified in the Chordariaceae of the Ectocarpales *sensu lato* or the family Leathesiaceae of the Chordariales *sensu stricto*, the relationship of the species to other brown algal lineages is less studied in terms of the plastid ultrastructure and molecular phylogeny. We examined the morphology of *P. rugosum* and also determined protein-coding *psa*A and *rbc*L sequences from four samples of the species from different locations, comparing them with homologous positions of newly sequenced putative relatives (*Leathesia difformis* and *Spermatochnus paradoxus*) and with published sequences of other brown algae. The species occurs in the upper intertidal zone on the Korean south coast from November to June. Thalli are markedly rugose and are comprised of haplostichous filaments, arranged into cortical and medullary layers. Unilocular sporangia arise laterally on the lower cells of cortical layers. A large pedunculate pyrenoid, with a cap, is present in the parietal discoid plastids. The specimens from four different locations were almost identical in *rbc*L and *psa*A sequences, and were monophyletic. All phylogenetic analyses of both genes reveal that *P. rugosum* is clearly separated from *Leathesia* and other members of the Chordariaceae. The sister relationship of the species to *Ectocarpus* was not supported by bootstrap or Bayesian analyses.

***Petrospongium rugosum* / brown algae / molecular phylogeny / *psa*A / *rbc*L / pyrenoid / taxonomy / ultrastructure**

Résumé — Position phylogénétique de *Petrospongium rugosum* (Ectocarpales, Phaeophyceae) : contributions des séquences des gènes plastidiaux *rbc*L et *psa*A. L'algue brune crustacée et spongieuse *Petrospongium rugosum* (Okamura) Setchell et Gardner est présente en Corée, au Japon, en Australie, en Nouvelle-Zélande et le long de la côte Pacifique de l'Amérique du Nord. Bien que cette espèce ait été placée dans les Chordariaceae, au sein des Ectocarpales *sensu lato*, ou dans la famille des Leathesiaceae, dans les Chordariales *sensu stricto*, ses relations avec les autres lignées d'algues brunes a été peu étudiée en termes d'ultrastructure plastidiale et de phylogénie moléculaire. Nous avons examiné la morphologie de *P. rugosum* et déterminé les séquences des gènes *psa*A et *rbc*L à partir de quatre récoltes provenant de différentes localités. Nous avons comparé les séquences avec celles, homologues, d'espèces supposées proches et nouvellement

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séquencées (*Leathesia difformis* et *Spermatochnus paradoxus*), ainsi qu'avec des séquences publiées d'autres algues brunes. L'espèce croît dans la zone interdidale supérieure des côtes du sud de la Corée, de novembre à juin. Les thalles sont nettement rugueux et formés de filaments haplostiques, disposés en couches corticales et médullaires. Les sporocystes uniloculaires sont produits latéralement à partir des cellules inférieures des couches corticales. Un gros pyrénoïde pédonculé, recouvert d'un sac pyrénoïdien, est présent dans les plastes pariétaux discoïdes. Les séquences *rbc*L et *psa*A des spécimens provenant des quatre localités différentes sont presque identiques ; ces spécimens forment un groupe monophylétique. Toutes les analyses phylogénétiques des deux gènes montrent que *P. rugosum* est nettement séparé de *Leathesia* et des autres membres des Chordariaceae. *Petrospongium* est taxon frère d'*Ectocarpus* mais cette relation n'est soutenue ni par les valeurs de bootstrap ni par les analyses bayésiennes.

***Petrospongium rugosum* / algues brunes / phylogénie moléculaire / *psa*A / *rbc*L / pyrénoïde / taxinomie / ultrastructure**

INTRODUCTION

Petrospongium Nägeli *ex* Kützing is a curious, brown to dark colored, carnose, brown algal genus that includes two species: *P. berkeleyi* (Greville *in* Berkeley) Nägeli *ex* Kützing (1858) and *P. rugosum* (Okamura) Setchell *et* Gardner. Both are summer annuals and occur exclusively in the North Atlantic, Mediterranean, and Pacific. The genus is typified by *P. berkeleyi,* which occurs along the European coast, and is characterized by a spongy, crustose thallus, with determinate, branched cortical and medullary filaments with hyphae, and phaeophycean hairs produced from medullary cells. Plurilocular and unilocular sporangia arise laterally on the lower cells of determinate cortical filament (Setchell & Gardner, 1924; Womersley, 1987). *Petrospongium rugosum* is the vicariant species of the genus from the Pacific Ocean. It was described as *Cylindrocarpus rugosa*, based on specimens from Chiba, Japan (Okamura, 1903, 1907), but was transferred to the genus *Petrospongium* by Setchell & Gardner (1924) based on its differentiated tissues with cortical and medullary filaments and difform unilocular sporangia. *Petrospongium rugosum* is distinguished from *P. berkeleyi* by its markedly wrinkled thallus surface, with cortical filaments about twice the diameter of those in the latter species (Okamura, 1903; Setchell & Gardner, 1924; Inagaki, 1958). *Petrospongium rugosum* occurs in Korea, Japan, Australia, New Zealand, and on the west coast of North America (Okamura, 1903; Setchell & Gardner, 1924; MacLennan, 1956; Kang, 1966; Adams, 1994). It is annual in winter to early summer and occurs at the high tide mark. The crustose thalli of *P. rugosum* are the sporophyte stage, which produces swimming spores with flagella. This phase alternates with a microscopic, filamentous gametophyte that produces gametes (Tatewaki *in* Hori, 1993).

Petrospongium was classified in the family Corynophleaceae by Oltmanns (1922), but recently has been included in the Leathesiaceae (Womersley, 1987). However, recent works on nuclear ribosomal DNA (de Reviers & Rousseau, 1999; Rousseau & de Reviers, 1999; Rousseau *et al.*, 2000, 2001) and plastid *rbc*L sequences (Siemer *et al*., 1998; Draisma *et al.*, 2001; Peters & Ramírez, 2001) have amended the order Ectocarpales by including the Chordariales, Dictyosiphonales, Punctariales, and Scytosiphonales and excluding

the Ralfsiales and taxa with stellate plastids. In the new system, most of the previous chordarialean and dictyosiphonalean families are placed within the Chordariaceae *s.l.* (Peters & Ramírez, 2001): *Petrospongium* and other members of the family Leathesiaceae are forced into the Chordariaceae *s.l.* However, the Chordariaceae is a current focus of research due to of the inclusion of previously recognized families, and the phylogenetic relationship of *Petrospongium* in the new system remains unknown.

The present study investigated the phylogenetic relationships of *Petrospongium rugosum* by analyzing the protein-coding plastid *rbc*L and *psa*A genes. Both genes have proved useful in other brown algal phylogeny reconstructions and give high resolution (Cho *et al*., 2004). In addition to the molecular analyses of *P. rugosum*, we studied the morphology of plastids in the vegetative cells of the species.

MATERIALS AND METHODS

Morphology

Thalli of *Petrospongium rugosum* were collected in the high tidal zone at several places in Korea. The thalli were prepared for electron microscopy using the following protocol. Tissue slices measuring approximately 1 × 1 mm were prepared with a razor. They were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 2% NaCl and 0.1% $CaCl_2$ for 3 h at 4°C, and then postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h. The apical parts were rinsed with cold distilled water, cut, and transferred into test tubes at 4°C. They were dehydrated in a graded series of ethanol and propylene oxide at 4°C, and infiltrated gradually with Spurr's epoxy resin (Spurr, 1969). After polymerizing the resin at 70°C for 24 h, serial sections (120-200 sections per sample) were cut with a diamond knife on an Ultracut E ultramicrotome (Reichert-Jung, Germany) and mounted on Formvar-coated slot grids. Thin sections were stained with Reynolds' lead citrate (Reynolds, 1963) and uranyl acetate, and examined with a JEM-1010 TEM (JEOL, Tokyo, Japan). Voucher specimens are deposited in the herbarium of Chungnam National University (CNUK), Daejon, Korea.

Analyses of the *rbc*L and *psa*A regions

We analyzed the *rbc*L and *psa*A regions from four samples of *Petrospongium rugosum*. Both genes were also newly sequenced for two putative relatives, *Leathesia difformis* and *Spermatochnus paradoxus*: the former was collected in Korea and the latter sourced from the Sammlung von Algenkulturen der Universität Göttingen (SAG) in Germany. The specimens included in the present study and the corresponding GenBank accession numbers are listed in Table 1. Previously published *rbc*L data from 52 taxa and *psa*A data from 16 taxa from GenBank were included to construct the phylogeny (Figs 7 and 8).

Table 1. Material information of *Petrospongium rugosum* and relatives.

Species	*Collection site, date and voucher no.*	*GenBank accession number*	
		*psa*A	*rbc*L
Petrospongium rugosum (Okamura) Setchell *et* Gardner	Chaguido, Jejudo, Korea; 17 Apr. 2003; CNUK PE141	AY996367	AY996361
	Haegeumgang, Geojedo, Korea; 23 Mar. 2004; CNUK PE236	AY996368	AY996362
	Sangjokam, Goseong, Korea; 9 Feb. 2004; CNUK PE163	AY996369	AY996363
	Suryeomri, Gyeongju, Korea; 18 Dec. 2002; CNUK PE277	AY996370	AY996364
Leathesia difformis (L.) Areschoug	Wuelpo, Pohang, Korea; 3 Jun. 2002; CNUK PE302	AY996371	AY996365
Spermatochnus paradoxus (Roth) Kützing	Culture Collection of Algae; SAG 10.82	AY996372	AY996366

Total DNA was extracted from approximately 0.01 g of dried thalli ground in liquid nitrogen using a DNeasy Plant Mini Kit (Qiagen Gmbh, Hilden, Germany), according to the manufacturer's instructions, and then dissolved in 150 µL of distilled water. Extracted DNA was stored at –20 °C and used to amplify the *rbc*L and *psa*A regions.

The *rbc*L region was amplified and sequenced using the method of Kogame *et al*. (1999) and Yoon & Boo (1999). Primers PRB-F0, F2, F3, R1A, R2, R3A, RS1, and RS2 have been used for most brown algae. The same DNA aliquot was used to amplify the *psa*A region, and the amplification and sequencing reactions for this region were the same as those used for *rbc*L. The *psa*A region was amplified and sequenced using primers psaA130F, psaA870F, psaA970R and psaA1760R (Yoon *et al*., 2002). The polymerase chain reaction (PCR) products were purified using a High Pure™ PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. The sequences of the forward and reverse strands were determined for all taxa using an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) at the Center for Research, Chungnam National University, Daejon, Korea. The electropherogram output for each sample was edited using the program Sequence Navigator v. 1.0.1 (Applied Biosystems).

All the sequences of the *rbc*L gene from 58 taxa, including 52 published sequences of brown algae, were collated using the multisequence editing program, SeqPup (Gilbert, 1995), and aligned by eye to compare our sequences with those published previously (Draisma *et al*., 2001; Cho *et al.,* 2003). The undefined sequences at the 5' and 3' ends of the *rbc*L data set were coded as missing data. The *psa*A sequences from 22 taxa, including 16 published sequences of brown algae, were also aligned by eye. Both the *rbc*L and *psa*A sequence data sets were combined for phylogenetic analysis.

Phylogenetic analyses

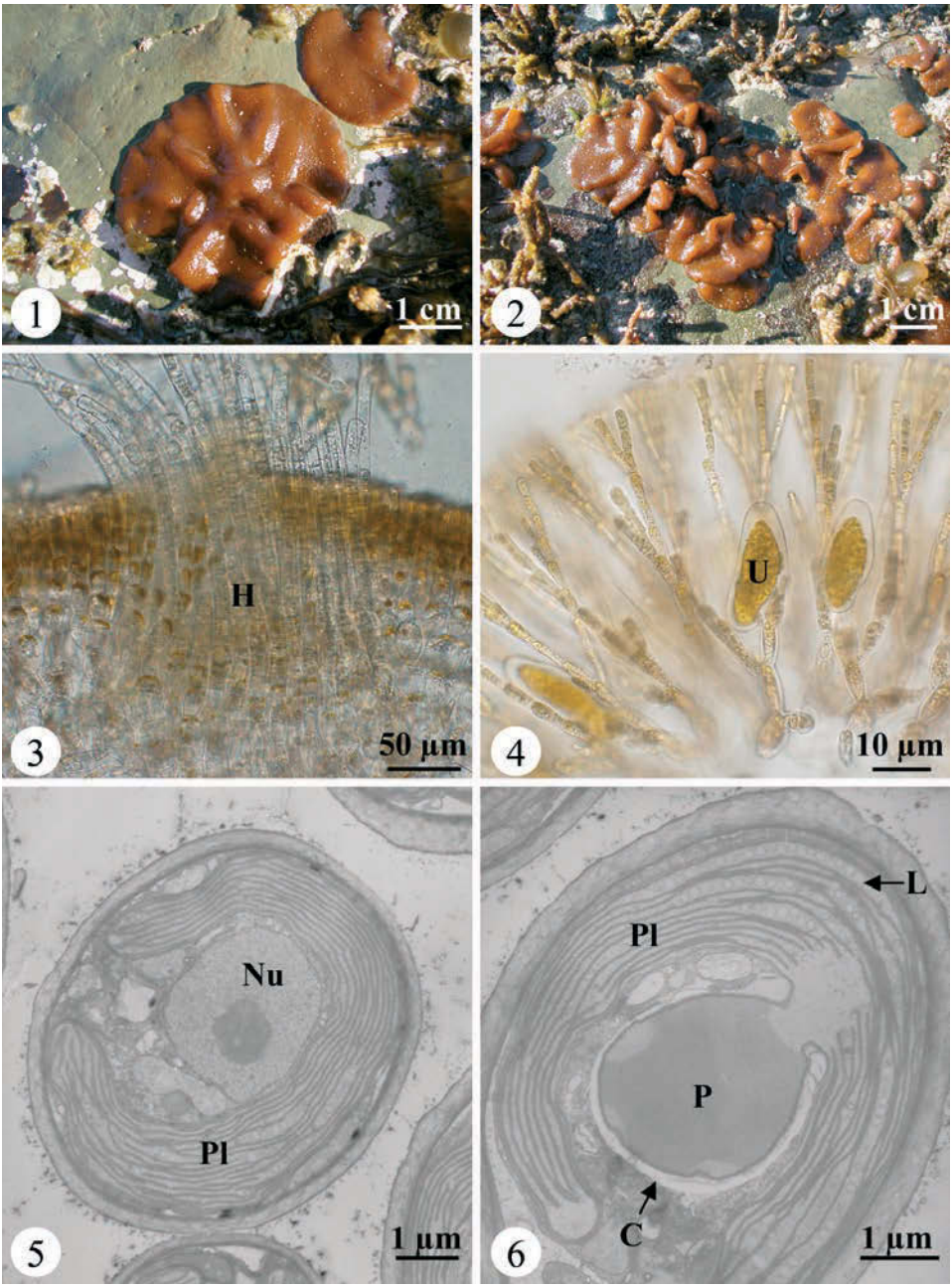
Three data sets were used for the phylogenetic analyses: 58 taxa for *rbc*L, 22 taxa for *psa*A, and 22 taxa for the combined *rbc*L + *psa*A data sets. Since we previously performed the saturation and incongruence length difference tests for both genes (Cho *et al.,* 2004), we did not repeat the tests. Maximum parsimony (MP) trees were constructed for each dataset using PAUP* (Swofford, 2002) using a heuristic search algorithm with the following settings: 100 random sequence-addition replicates, TBR branch swapping, MulTrees, all characters unordered and unweighted, and branches with a maximum length of zero collapsed to yield polytomies. The bootstrap values (BS) for the resulting nodes were assessed using bootstrapping with 1,000 replicates. For Bayesian analyses (BA), we performed a likelihood ratio test using Modeltest 3.06 (Posada & Crandall, 1998) to determine the best available model for the individual and combined data sets. For all three data sets, the best model was a general time reversible (GTR) model with gamma correction for among-site variation (Γ) and invariant sites (I). Bayesian phylogenetic analyses were performed using MrBayes 3.0 (Huelsenbeck & Ronquist, 2001). Each analysis was initiated from a random starting tree and the program was set to run four Markov chain Monte Carlo iterations simultaneously for 2,000,000 generations with trees sampled every 100th generation. The likelihood scores stabilized at approximately 300,000 generations, so the first 3000 trees were burned. For comparison with bootstrapping, we considered nodes with Bayesian probabilities (BP) greater than 0.9 (*i.e.*, the node appears in more than 90% of the sampled trees) as being well.

RESULTS

Morphology of *Petrospongium rugosum*

Thalli of *P. rugosum* are common from November to June on rocks in the upper intertidal zone exposed to surf on the southern coast of Korea. The carnose thalli are flat when young but become markedly rugose when mature (Figs 1 and 2). They grow up to 20 cm in diameter and 0.5- 2 mm thick. The thalli are haplostichous and consist of branching filaments; the lower parts of these form a colourless medulla of loosely compacted large swollen cells and multicelluar rhizoids arising perpendicular to the filaments. The middle to upper part of branching filaments forms a compact, pigmented, cortical layer of elongate-ovoid to cylindrical cells. The terminal cells of the filaments are round and pigmented. Phaeophycean hairs are hyaline and arise from the cortical cells (Fig. 3). Unilocular zoidangia are narrowly ellipsoidal to irregular in form, and arise laterally on the lower cells of cortical filaments (Fig. 4).

The thalli contain plastids in cortical and terminal cells. The plastids are parietal, discoid, and are one or two per cell. The ultrastructure of the vegetative cells is typical for brown algal cells, with a large nucleus in the centre (Fig. 5). A large pedunculate pyrenoid with a cap is found in each plastid (Fig. 6).



Figs 1-6. *Petrospongium rugosum* (Okamura) Setchell *et* Gardner. **1**. Flat crustose thallus in the field. **2**. Rugose crustose thallus in the field. **3**. Anatomy of the thallus showing hairs (H) originating from cells of the cortical layer. **4**. Unilocular zoidangium (U) attached to the basal cells of branched filaments. **5**. Ultrastructure of a vegetative cell showing the nucleus (Nu) and a plastid (Pl). **6**. Ultrastructure of a vegetative cell showing a large peduculate pyronoid (P) with a cap (C) and lipid granules (L) between the thylakoids.

Molecular phylogeny

The *rbc*L sequences analyzed here were 1467 nucleotides (nt) long. The 58 aligned *rbc*L sequences had 500 (34.1%) variable positions and 377 (25.7%) parsimoniously informative sites. The sequences were identical for the specimens from Haegeumgang and Sangjokam on the south coast and Suryeomri on the southeast coast, but they differed by six bases from the specimen from Chaguido in Jeju. The spacer region is 179 nt and identical among the four specimens. Nevertheless, *P. rugosum* differed by 90-92 nt from *Ectocarpus* species and by 99-104 nt from *Leathesia difformis*.

The *rbc*L tree (Fig. 7) showed that the Ectocarpales, comprising the Acinetosporaceae, Adenocystaceae, Chordariaceae, Ectocarpaceae, and Scytosiphonaceae, was strongly monophyletic (100% BP for BA and 87% BS for MP). The Acinetosporaceae, Adenocystaceae, Ectocarpaceae, and Scytosiphonaceae were strongly monophyletic, respectively. The Chordariaceae was monophyetic but not well supported (98% BP for BA and 56% BS for MP). *Petrospongium rugosum* was intermediate between the Ectocarpaceae and Adenocystaceae, but the position was not supported.

The sequences determined for the *psa*A region totaled 1488 bases. For the 22 aligned sequences of the *psa*A gene, 482 (32.4%) bases were variable and 318 (21.4%) were parsimoniously informative. The *psa*A sequences were the same for the specimens from Suryeomri and Sangjokam. However, they differed by three nt from the specimen from Haegeumgang and by eight nt from the specimen from Chaguido. *Petrospongium rugosum* differed by 118-121 nt from *Ectocarpus* sp. and by 140 nt from *Leathesia difformis*.

The *psa*A tree (Fig. 8) was similar to the *rbc*L tree in having the monophyletic Ectocarpales. However, the *psa*A tree differed from the *rbc*L tree in having the *Petrospongium/Ectocarpus* clade, which was supported by weak bootstrap values (77% BP for BA and 52% BS for MP).

Although the number of taxa was reduced, the phylogenies of the *rbc*L + *psa*A data set were congruent to those of individual data sets in the phylogenetic separation of *P. rugosum* from the Chordariaceae (Fig. 9). Most clades made in the phylogenetic tree received higher bootstrap values and Bayesian probabilities than the *rbc*L and *psa*A data sets. On the other hand, *Spermatochnus paradoxus* and *Leathesia difformis*, newly analyzed in the present study, were well resolved in the Chordariaceae clade in all analyses of *rbc*L, *psa*A, and *rbc*L + *psa*A data.

DISCUSSION

Our collections of *P. rugosum* from Korea correspond in habitat and the structure of the cortex, medulla, and unilocular sporangia to the descriptions of Okamura (1903, 1907, as *Cylindrocarpus rugosus*) and Setchell & Gardner (1924). The thalli are spongy, crustose, rugose, and consist of haplostichous filaments, phaeophycean hairs scattered or clustered from outer medullary cells, and unilocular sporangia attached at the basal or middle part of branched filaments. An elaborate large pedunculate pyrenoid, with a cap, is present in the parietal discoid plastid, as seen in Hori (1971). Although it usually occurs on the southern coast of Korea from November to June, thalli bearing unilocular zoidangia are

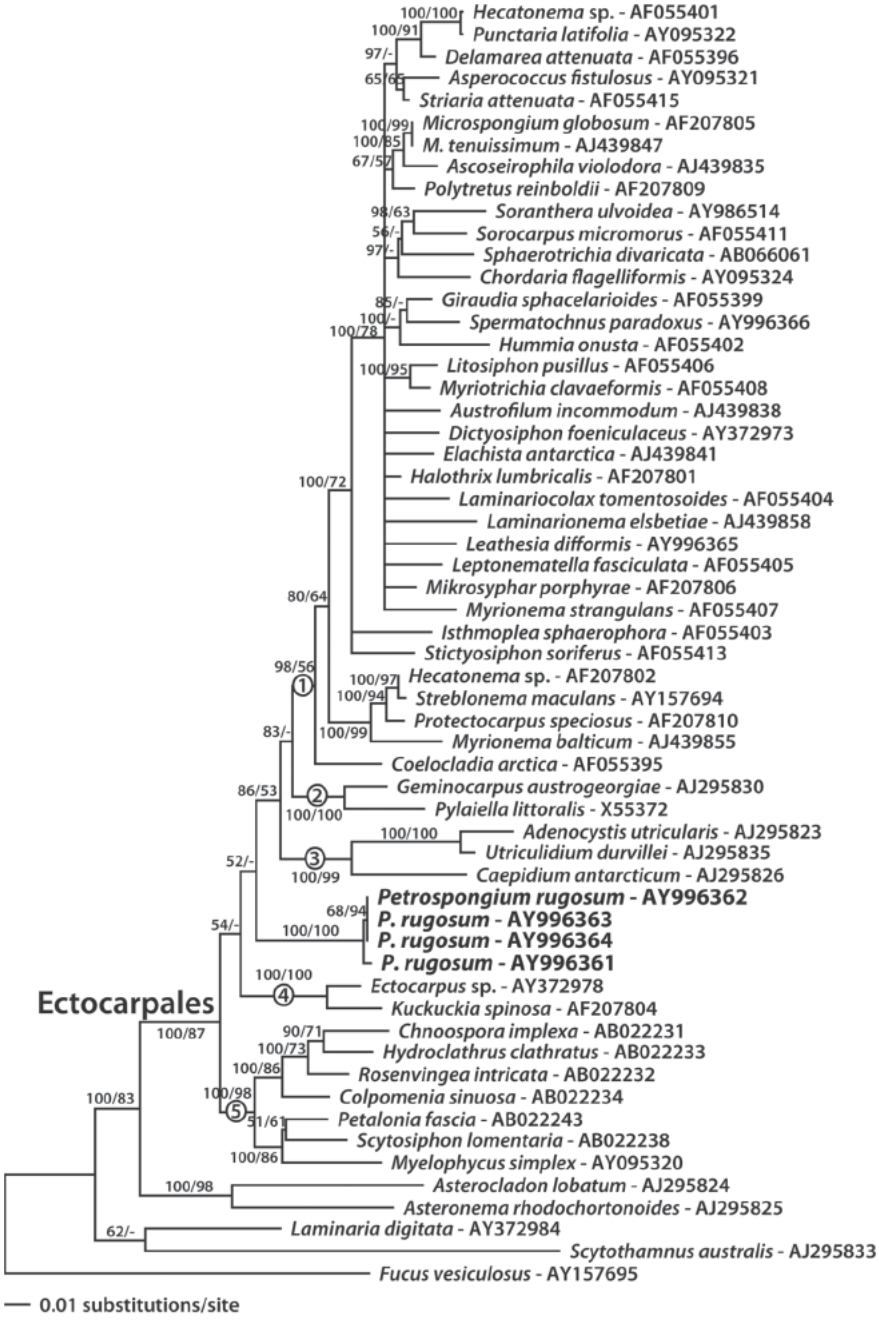


Fig. 7. Bayesian tree for *Petrospongium rugosum* and other ectocarpalean brown algae estimated from *rbc*L sequence data. Numbers above the branches indicate Bayesian posterior probabilities (left) and Maximum Parsimony bootstrap percentages (right). Dashes indicate < 50% bootstrap support. Clade numbers ① - Chordariaceae, ② - Acinetosphoraceae, ③ - Adenocystaceae, ④ - Ectocarpaceae, ⑤ - Scytosiphonaceae.

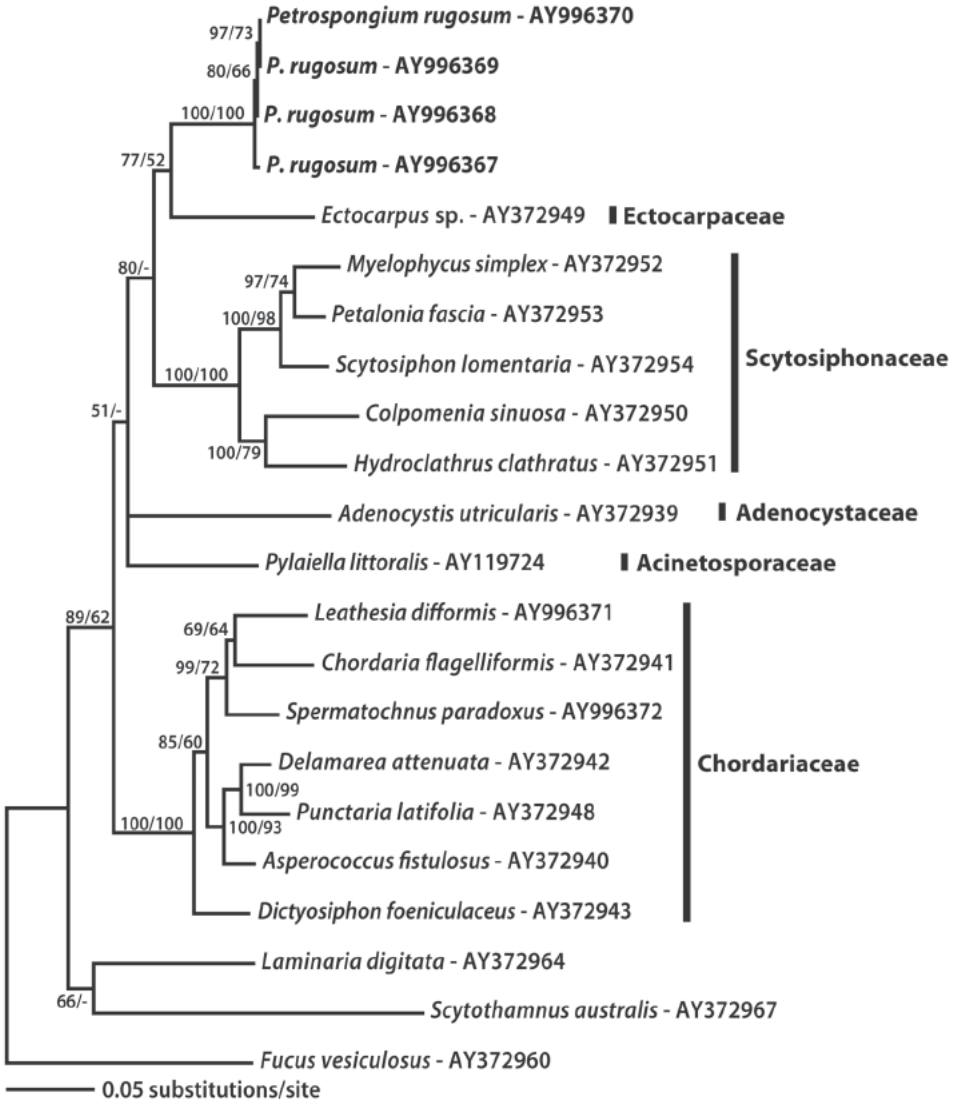


Fig. 8. Bayesian tree for *Petrospongium rugosum* and other ectocarpalean brown algae estimated from *psa*A sequence data. Numbers above the branches as in Fig. 7.

found only from May to June. However, no thalli with plurilocular sporangia were found in our collections.

The specimens of *P. rugosum* from different locations were identical or differed by six nt in *rbc*L and up to eight nt in *psa*A. By comparison, these differences are within the range of seven nt in *rbc*L and 11 nt in *psa*A within each of two *Ishige* species (Cho *et al*., 2004) and less than the 32 nt difference between two species of *Myelophycus* (Cho *et al.*, 2003).

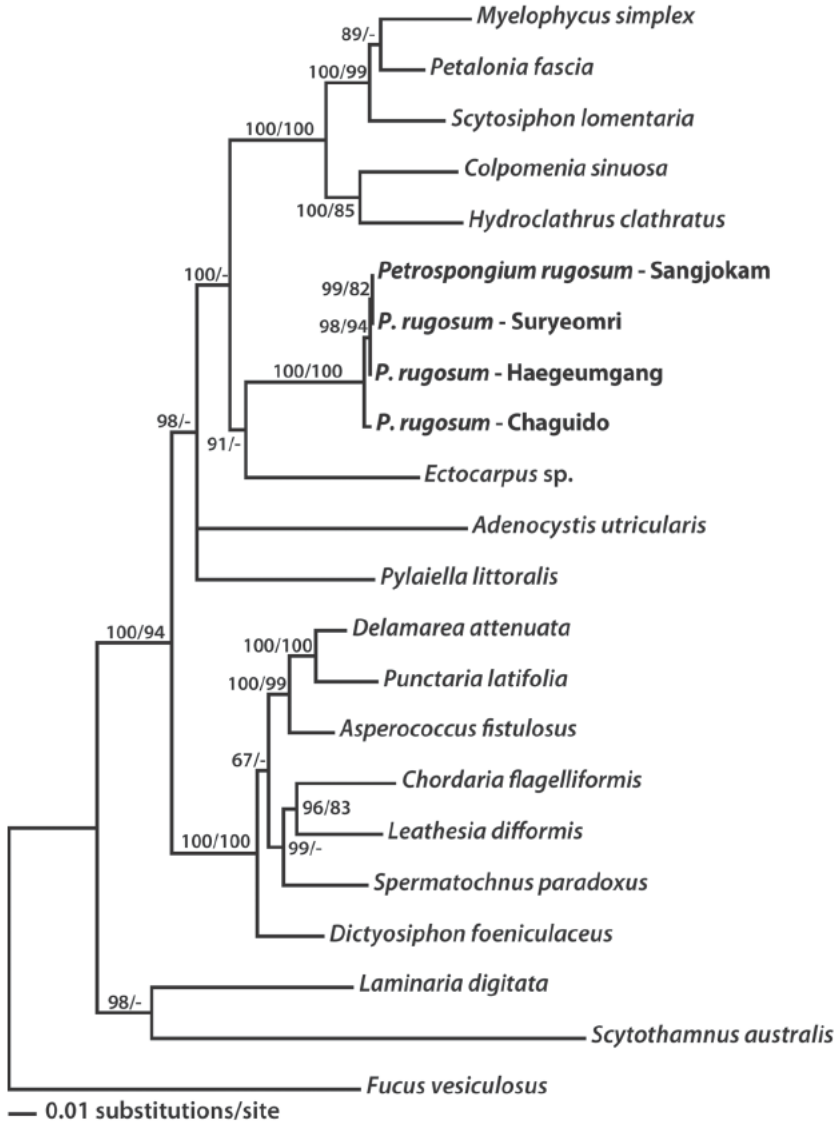


Fig. 9. Bayesian tree for *Petrospongium rugosum* and other ectocarpalean brown algae estimated from the combined *rbc*L + *psa*A sequence data. Numbers above the branches as in Fig. 7.

Petrospongium rugosum forms an independent clade in the Ectocarpales, separating from both the Chordariaceae and Ectocarpaceae in the *rbc*L tree. The position of *P. rugosum* shown in the present study is unlikely to change, even if *Kuckuckia kylinii* could be added to the *psa*A data set, because the sister relationship between *P. rugosum* and *Ectocarpus* sp. is not supported in the *psa*A tree and weakly supported in the *rbc*L + *psa*A tree. According to Peters & Ramírez (2001), the five families of the Ectocarpales are clearly classified based

on two diagnostic characters: plastid morphology and life history. Draisma *et al.* (2003) listed two molecular and five non-molecular characters used to classify and identify brown algal orders and families: the diagnostic sequences of the end of the RuBisCO spacer and the start codon of *rbc*S, and pheromones, life history, plastid type, anatomy of the most complex generation, and the pyrenoid.

Petrospongium rugosum has a heteromorphic life history, with macroscopic spongy, crustose sporophytes alternating with microscopic filamentous gametophytes (Tatewaki *in* Hori, 1993). The crustose sporophytes and filamentous gametophytes are haplostichous. The vegetative cells have one or two parietal discoid plastids that include a large pedunculate pyrenoid with a cap. The end of the RuBisCO spacer has the sequence TT TGA ATA GTG and the start codon of *rbc*S is ATG, although the spacer sequences are identical among specimens of the species from four different sites in Korea. Except for pheromones, which have not been studied for the genus *Petrospongium*, the above-listed characters do not differ from those of the Chordariaceae (Peters & Ramírez, 2001; Draisma *et al*., 2003). However, the spongy, crustose thalli are quite different from those of *Leathesia* and putative relatives, and appear autapomorphic in *Petrospongium*. The gross morphology of the macroscopic generation is generally regarded as an important character for most taxa in brown algae. Both the *rbc*L and *psa*A sequences have been used to infer phylogenetic relationships of brown algae above the genus level, as seen by Peters & Ramírez (2001) and Cho *et al.* (2004). In all the phylogenetic analyses, *P. rugosum* is clearly separated from the Scytosiphonaceae and Acinetosporaceae, as well as the Chordariaceae. However, in the Ectocarpales, *P. rugosum* looks similar to the Ectocarpaceae. Both the sporophyte and gametophyte generations of *Petrospongium* corresponds to haplostichous ramified filaments, like in *Ectocarpus* and *Kuckuckia,* the two genera of the Ectocarpaceae *sensu* Peters and Ramírez (2001). The erect filaments of *Petrospongium* are more developed in the sporophyte than in the gametophyte, similar to what is known in *Ectocarpus siliculosus.* The lower number of plastids per cell is also a common character between *Petrospongium* and *Ectocarpus.* However, the scope of this paper and the lack of complete homologous sequences from *P. berkeleyi* do not permit testing whether the genus *Petrospongium* forms an independent clade that is different from the currently recognized Ectocarpaceae and other families of the Ectocarpales.

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