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Morphology and phylogenetic position of a freshwater *Prasiola* species (Prasiolales, Chlorophyta) in Korea

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The genus of leafy green algae, *Prasiola* Meneghini, includes marine, terrestrial, and freshwater species. A total of 11 species and one variety have been identified in China, Korea, and Japan. In Korea, *Prasiola formosana* var. *coreana* has been reported in Muncheon, North Korea, while a different type of *Prasiola* species has been reported in South Korea. The South Korean species has been found growing along a small stream originating from Chodanggul Cave, a limestone cave in Samcheok, Gangwon Province. Here, we revised the morphological characteristics of the South Korean *Prasiola* species and analyzed plastid *rbc*L, *psa*B, and *tuf*A genes to clarify its identity. Although the external and anatomical morphologies varied among individuals, our results were very similar to previous reports. Plastid three genes sequences of the South Korean specimens were identical to those of *P. japonica* collected from Japan as well as to published sequences of *P. yunnanica* from China. A short *rbc*L-3P sequence (196 bp) from *P. formosana* var. *coreana*, which was identified in the type specimen, was also identical to a sequence from *P. japonica*. These *Prasiola* species and variety from Korea, Japan, and China are all distributed in areas characterized by limestone bedrock. Based on morphological, phylogenetic, and distributional features, the South Korean *Prasiola* species is regarded herein as *P. japonica*. Here, we also propose to synonymize *P. formosana* var. *coreana* and *P. yunnanica* with *P. japonica*.

Key Words: morphology; *Prasiola japonica*; *P. formosana* var. *coreana*; *P. yunnanica*; *psa*B; *rbc*L; *tuf*A

INTRODUCTION

The genus of leafy green algae, *Prasiola* Meneghini includes marine, terrestrial (mostly supralittoral), and freshwater species characterized by monostromatic blades generally expanding above and narrowing to a short stipitate region at the base (Rindi et al. 2004, 2007, Moniz et al. 2012*a*, 2012*b*, Guiry and Guiry 2015). A total of 36 species have been reported in this genus, at least 14 of which are freshwater organisms (Moniz et al. 2012*b*, Guiry and Guiry 2015).

In east Asia, 11 species and one variety have been reported as freshwater or subaerial *Prasiola* species: *P. crispa* (Lightfoot) Kützing, *P. elongata* Hu, *P. fluviatilis* (Sommerfelt) Areschoug ex Lagerstedt, *P. formosana* Okada, *P. formosana* var. *coreana* Okada, *P. hubeica* Bi L.-J., *P. japonica* Yatabe, *P. lanpingensis* C. Qian et R. -N. Wang, *P. sinica* C. -C. Jao, *P. subareolata* Skuja, *P. tibetica* C. -C. Jao, and *P. yunnanica* C. -C. Jao. All of these species, except one variety, have been reported in China (including Taiwan), and only one freshwater species, *P. japonica* has been reported in Japan (Yatabe 1891). In Korea, two *Prasiola* species, *P.*

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formosana var. *coreana* and *Prasiola* sp., have been identified. *Prasiola formosana* var. *coreana* Okada was firstly reported from Muncheon in North Korea (Okada 1939). This variety is distinguished from *P. formosana* on the basis of the thickness of the thallus, the dimension of cells in cross-sections, and the outward form of the frond (Okada 1936, 1939). Okada (1939) pointed out the similarity of this variety to *P. japonica*, which is commonly distributed in Japan, in terms of the shape of cells in cross-sections and the external appearance of the frond. In South Korea, a different type of *Prasiola* species was reported by Park et al. (1970). This species was found growing along a small stream originating from Chodanggul, a limestone cave in Samcheok, Gangwon Province. This species was later identified as *P. japonica* by Chung (1993).

Molecular phylogenetic studies and DNA barcode marker analyses in *Prasiola* have primarily been conducted in marine species from Europe, North America, Tasmania, New Zealand, and the Antarctic (Sherwood et al. 2000, Rindi et al. 2004, 2007, Saunders and Kucera 2010, Heesch et al. 2012, Moniz et al. 2012*a*, 2012*b*, 2014). In contrast, the molecular phylogenies of Asian freshwater species have rarely been reported. Only Naw and Hara (2002) reported on the molecular phylogenetic position of a freshwater *Prasiola* species collected from Myanmar. These authors analyzed the 18S rRNA gene from *Prasiola* sp. as well as from *P. japonica* collected in Japan. Moniz et al. (2012*b*, 2014) generated plastid *rbc*L, *psa*B, and *tuf*A genes of *P. yunnanica* collected from China to determine the phylogenetic position of this species. However, no Korean species have been examined in molecular phylogenetic analyses.

Therefore, the objective of the present study was to assess the molecular phylogenetic identity of the South Korean *Prasiola* species and to compare it with Japanese *P. japonica* and published representative species. To this end, we observed the morphological features and analyzed the plastid *rbc*L, *psa*B, and *tuf*A genes sequences of the South Korean *Prasiola* species.

MATERIALS AND METHODS

Sampling

Thalli of *Prasiola* species were collected from the stream originating from Chodanggul Cave in Samcheok for both morphological observation and molecular analyses. We also collected *P. japonica* samples from Kyushu and Shizuoka in Japan for molecular analysis. Fresh material was used for surface observations using a microscope. Photographs were taken with a digital camera (C-4040 zoom; Olympus, Tokyo, Japan) attached to a light microscope (BX50; Olympus). All voucher specimens analyzed in this study have been deposited in the Plant Collections Storage of the National Institute of Biological Resources (NIBR) in Incheon, Korea (Table 1).

Plastid *rbc*L, *psa*B, and *tuf*A genes analyses

Total genomic DNA was extracted from silica gel-preserved dry materials. Approximately 0.05 g dried thallus was ground using TissueLyser (Qiagen, Austin, TX, USA) with two tungsten carbide beads (3 mm; Qiagen) at 25/s frequencies for 2 min. Ground powder was used for the DNA extraction procedure using a NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany), following the manufacturer's protocol. Plastid *rbc*L regions were amplified using the PF2 / PR2 primer pair (Rindi et al. 2004) for fresh materials and nine newly designed primer sets for the type specimen of *P. formosana* var. *coreana* (Table 2, Fig. 1). Polymerase chain reaction (PCR) amplifications were conducted using AccuPower PCR premix (Bioneer, Daejeon, Korea) and a HotStarTaq Plus Master Mix Kit

Table 1. Specimen information of *Prasiola* species used in this study

Species	Collection site and date	Voucher No.	GenBank accession No.		
			*rbc*L	*psa*B	*tuf*A
Prasiola sp.	Sohancheon, Samcheok, Korea; Oct 26, 2011	NIBRCL0000100964-8	KR261677	KR261678	KR261679
P. japonica	Oonogawa, Dakedashi, Kyushu, Japan; Nov 29, 2011	NIBRCL0000101130	KR261680	KR261681	KR261682
	Shibagawa, Shizuoka, Japan; Nov 28, 2011	NIBRCL0000101128-9	KR261683	KR261684	KR261685
P. formosana var. *coreana*	Jiseonri, Muncheongun, Hamgyeongnamdo; Sep 13, 1938	SAP021454 (type)	KR261686	-	-

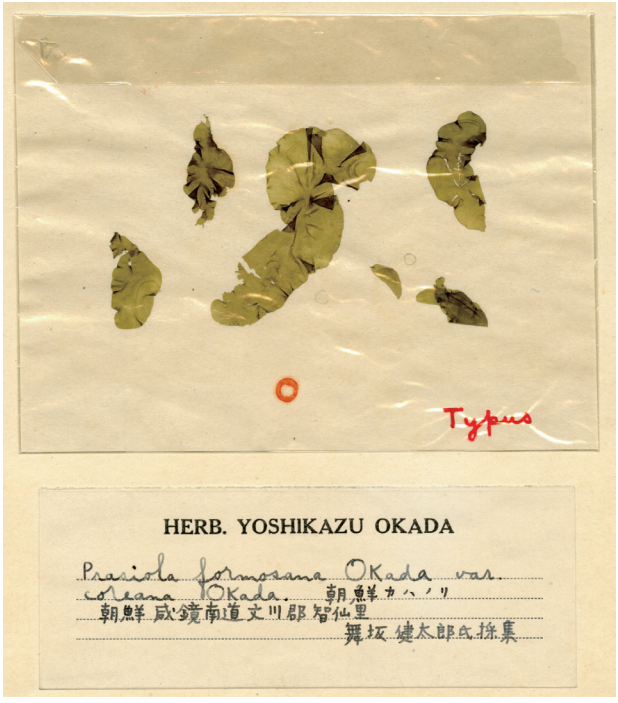

Fig. 1. Type specimen of *Prasiola formosana* var. *coreana* deposited at SAP, Hokkaido University, Japan.

(for herbarium samples; Qiagen) on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The amplification cycle consisted of an initial denaturation step at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 47 or 50°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. The *psa*B and *tuf*A genes were amplified using the Pp1F / Pp4R (Novis et al. 2010) and tufGF4 / tufAR (Saunders and Kucera 2010) primer pairs respectively, as recommended by Moniz et al. (2012*b*, 2014) for *Prasiola* specifically. PCR amplification parameters for the *psa*B and *tuf*A regions were the same as those for *rbc*L, except that the annealing temperature was 58 or 60°C.

The sequencing reactions were performed using ABI-PRISM BigDye Terminator Cycle Sequencing Kits, and the florescent signals were detected using an ABI PRISM 3730XL Analyzer (Applied Biosystems). The sequences were verified using Chromas 1.45 (McCarthy 1996) and aligned visually using previously published data (Sherwood et al. 2000, Rindi et al. 2004, 2007, Saunders and Kucera 2010, Heesch et al. 2012, Moniz et al. 2012*a*, 2012*b*, 2014).

Maximum likelihood (ML) analysis was performed using RAxML 7.2.8 (Stamatakis 2006) under the GTR + Γ model. We used 200 independent tree inferences using the default option of automatically optimized subtree pruning and regrafting (SPR) rearrangements and 25 "distinct rate categories" options to identify the best tree. Bootstrap analysis was conducted for 1,000 replications.

Table 2. Primers designed for PCR amplification of a short fragment of *rbc*L

Name	Sequence (5'→3')	Size	Tm (°C)	Position
PrbcL1F	AGTTCCGGCTGAAGAATGT	19	58	111-130
PrbcL1R	ATCAAGAGGATATGCTAC	18	53	271-288
PrbcL2F	CAACTGTGTGGACTGATGGA	20	60	170-189
PrbcL2R	TCTTCCAGACGTAAAGCAC	19	58	362-380
PrbcL3F	GTTATGATATTGAACCAG	18	51	221-238
PrbcL4F	ACATCAATTGTAGGAAACG	19	53	322-340
PrbcL4R	CTAATAAACCACGACCGTAT	20	56	462-481
PrbcL5F	TTCAAGTTGAGCGTGATAAA	20	54	434-453
PrbcL5R	CGCATAAATGGTTGTGAG	18	55	591-608
PrbcL6F	TACGTGGTGGATTAGACT	18	55	548-565
PrbcL6R	CATACCAAGGTCTTTTGC	18	55	739-756
PrbcL7F	TATCTAAATGCTACTGCTGC	20	56	685-704
PrbcL7R	CGGTCAATTACAGCGTGC	18	60	861-878
PrbcL8F	ATTACTTCACATTCACCGTG	20	56	837-856
PrbcL8R	AGGAAGTGATACCCAATCTT	20	56	1067-1086
PrbcL9F	ACTTTAGGATTCGTTGATT	19	51	994-1012
PrbcL9R	CCAAGGGTGTCCAAGAGT	18	60	1186-1203

PCR, polymerase chain reaction; Tm, melting temperature.

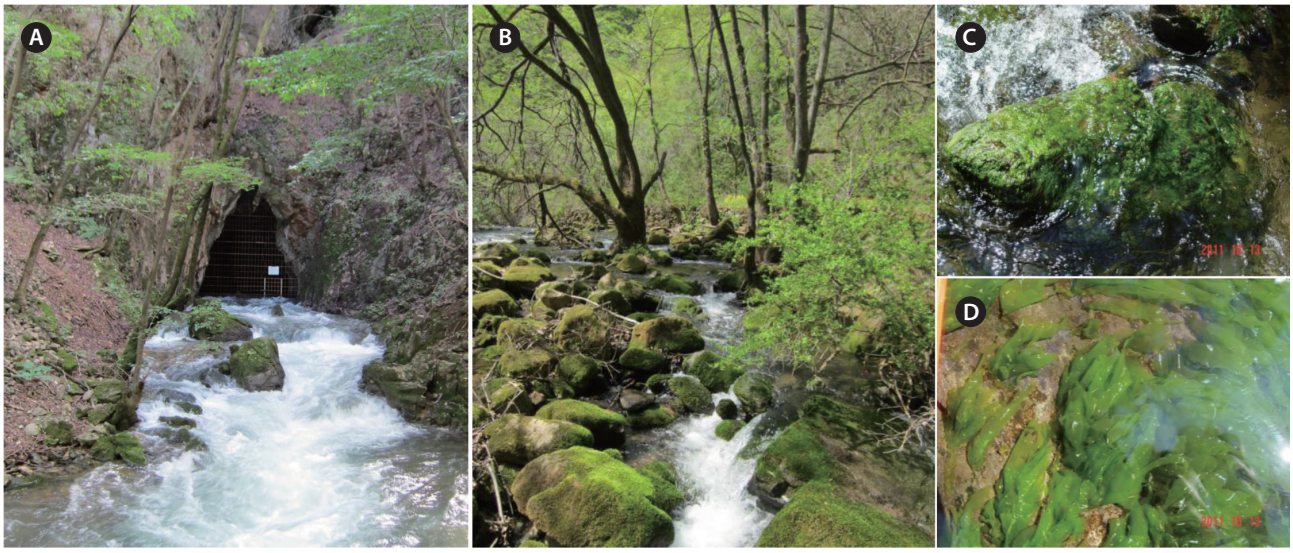

Fig. 2. Habitats of *Prasiola* species in Korea. (A) Limestone cave from which the Sohancheon stream originates. (B) Middle part of the Sohancheon stream where *Prasiola* species occurs. (C & D) Growth habitats on the submerged limestone rocks.

Fig. 3. Herbarium specimens of South Korean *Prasiola* species collected in October 2011.

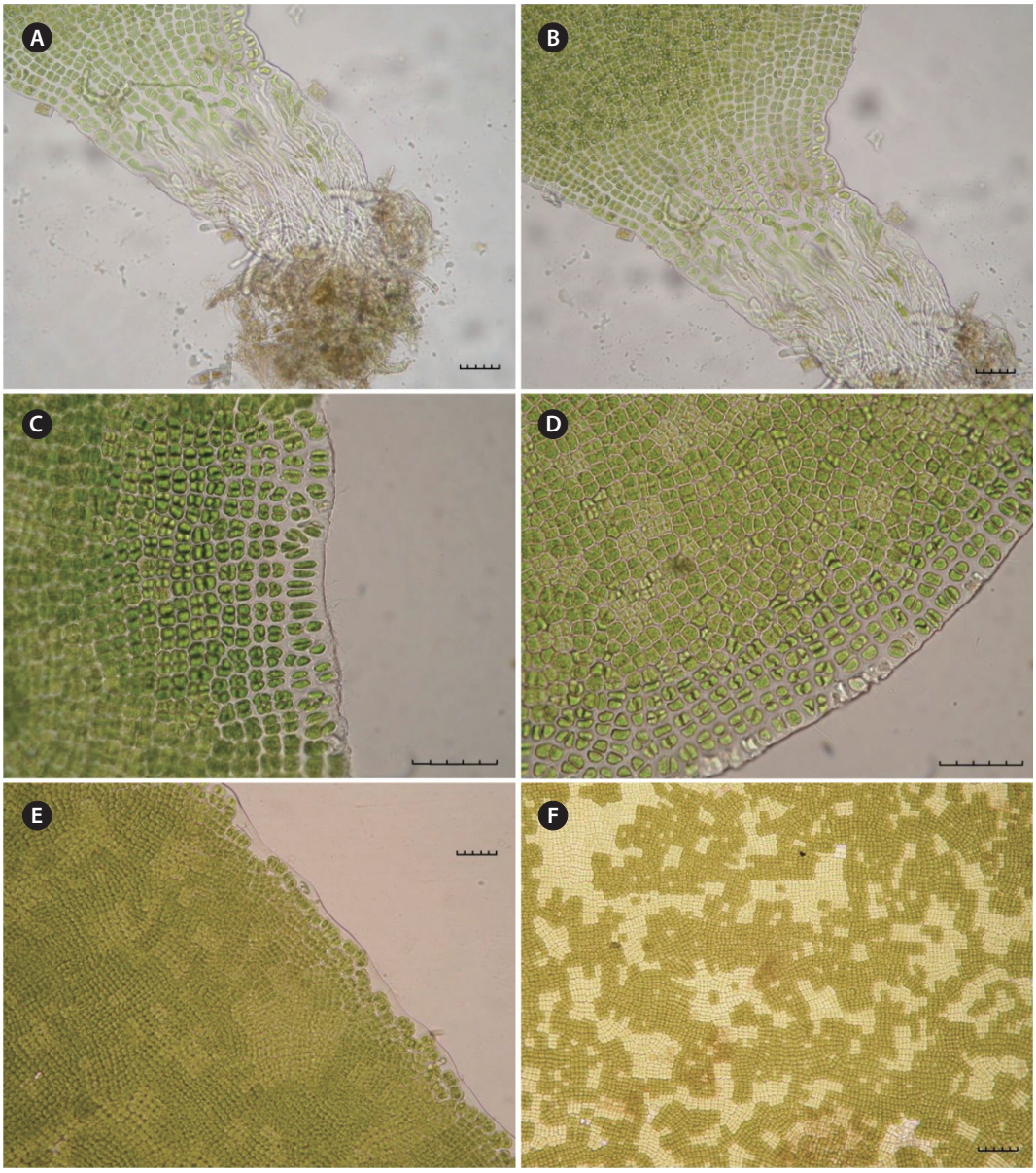

Fig. 4. Morphology of South Korean *Prasiola* species. (A-D) Surface view of young thallus collected in April 2012. (A) Surface view of rhizoid. (B) Thallus showing rhizoidal part and multiseriate base. (C & D) Surface view of the blade margin. (E & F) Surface view of thallus showing male and female gametangial areas (mosaic, Nov 2011). Scale bars represent: A-F, 50 μm.

RESULTS

Habitat and morphology

The South Korean *Prasiola* species grows on rocks along the Sohancheon stream, which is located 6.37 km from the East Sea. This species primarily grows along the central part of the stream, which shallows, exposed to light, and has a fast stream velocity (Fig. 2).

The thallus is light green, leafy, linear, lanceolate or ovate, 1-7 cm long, 0.5-3.5 cm wide (Fig. 3), and attached to the surface of rocks with a small mass of fibrous cells comprising the rhizoid (Fig. 4A & B). Vegetative cells are single-layered, round-square, and arranged in two or four cells together. At the margin of the thallus, cells are elliptical or lanceolate, and the space between cells gets wider than the inner part (Fig. 4C & D). Gametangial cells arise from vegetative cells and are arranged as an irregular mosaic from early winter to spring (Fig. 4E & F).

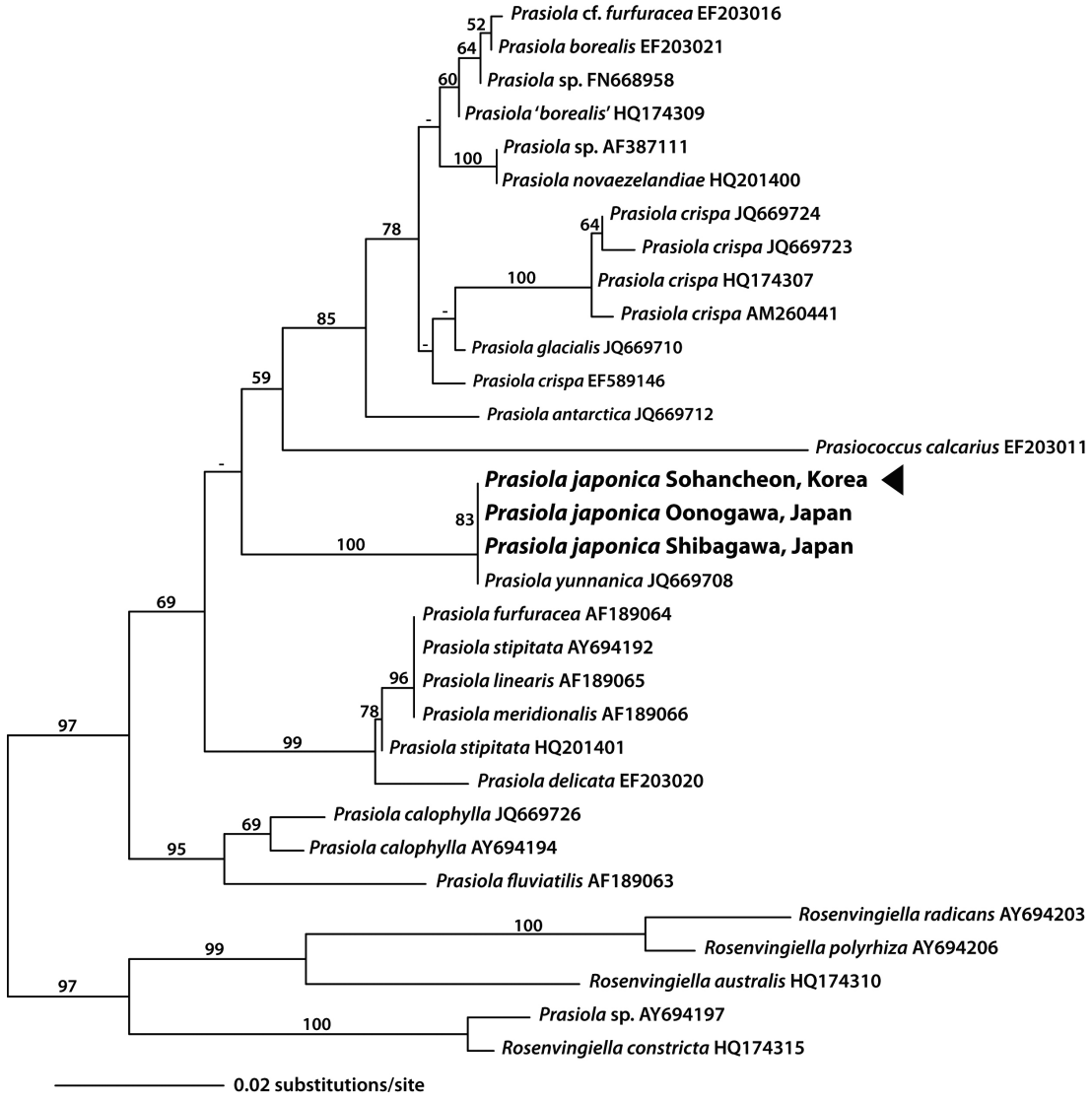

Fig. 5. Phylogenetic position of South Korean *Prasiola* species (arrowhead) inferred from maximum likelihood analysis of the *rbc*L gene.

Plastid *rbc*L, *psa*B, and *tuf*A data

In the present study, we obtained 1,094 bp of three new *rbc*L sequences from a South Korean *Prasiola* species and two Japanese *P. japonica*. The plastid *rbc*L sequences of the Korean *Prasiola* species were identical to those of Japanese *P. japonica* and Chinese *P. yunnanica* (JQ669708), with the exception of one undesignated base (N) in the sequences by Moniz et al. (2012*b*). Pairwise distances of *rbc*L sequences ranged from 0 to 6.5% (average 3.5%) in the genus *Prasiola*. Based on the ML tree of *rbc*L, the South Korean *Prasiola* species was included in a monophyletic clade with *P. japonica* and *P. yunnanica* with a 100% bootstrap value (Fig. 5). The sister relationship of this clade was not clear in *rbc*L gene tree.

We also attempted to amplify the *rbc*L region from the type specimen (SAP021454) of *Prasiola formosana* var. *coreana* and succeeded in obtaining 196 bp of *rbc*L-3P using the PrbcL9F / 9R primer set. This short fragment was also identical to those of the South Korean *Prasiola* species and Japanese *P. japonica*. Although the resolutions for several species were low when the phylogenetic tree was generated using the 196 bp of *rbc*L-3P, the Asian *Prasiola* specimens were clustered as a strong monophyletic group (figure is not shown). Only 75 bp from these short sequences overlapped in comparison with sequences from *P. yunnanica*, but these sequences were identical among all species. Pairwise distances of these short *rbc*L-

3P sequences ranged from 0 to 4.6% (average 2.2%) in the genus *Prasiola*.

A total of 1,292 bp of *psa*B and 813 bp of *tuf*A genes sequences were generated from one specimen of South Korean *Prasiola* species and two specimens of Japanese *P. japonica*. These sequences were identical to published data for *P. yunnanica* (JQ669686 for *psa*B and KF993445 for *tuf*A), except for one undesignated base (N) in the sequences of *psa*B by Moniz et al. (2012*b*, 2014).

DISCUSSION

Our morphological observations indicated that the South Korean *Prasiola* species had an external morphology that was mostly lanceolate or ovate, as described by Park et al. (1970), who also reported a similar arrangement of cells and gametangial mosaic pattern to what we found. According to the previous report of the Korean *Prasiola* species, morphological features and cell shape can vary among individuals (Park et al. 1970). Cell shape and arrangement were once regarded as diagnostic characteristics distinguishing Asian freshwater *Prasiola* species (Hu and Wei 2006). Indeed, the presence or absence of a stalk and disc-like holdfast can be used to distinguish several species. However, some characteristics, such as cell shape and arrangement, are quite variable not only at the species level, but also among individuals.

Park et al. (1970) noted that *Prasiola* species from Sohancheon is lanceolate and ovate, which is similar to all morphological types of *P. japonica*, *P. formosana*, and *P. formosana* var. *coreana*. When Okada (1939) first described *P. formosana* var. *coreana*, he also noted that this new species resembled *P. japonica* in the shape of cells in cross section and the external appearances of the thallus. Jao (1947) documented *P. yunnanica* as a new species based on morphological differences from *P. japonica*, noting that the thickness and shape of the thallus and the length and form of cells in sectional views of this Chinese species were "quite dissimilar" from the Japanese species. Therefore, it can be quite difficult to distinguish Asian freshwater species using only external morphology and anatomical features.

Three plastid genes, *rbc*L, *psa*B, and *tuf*A genes of the South Korean *Prasiola* species were identical to those of Japanese *P. japonica* and Chinese *P. yunnanica*, the latter of which was published by Moniz et al. (2012*b*, 2014). The phylogenetic trees for the South Korean species were also similar to those published previously (Moniz et al. 2012*b*). Moniz et al. (2012*b*) noted that the unrelated lineage of *P. yunnanica* was particularly interesting; however, because they only analyzed one Asian species, their results are not necessarily applicable to other Asian freshwater species. The present study is the first report of *rbc*L, *psa*B, and *tuf*A genes of *Prasiola* species from South Korea and *P. japonica* from Japan, as well as of partial *rbc*L sequences of *P. formosana* var. *coreana*.

Even though the type specimen is most important when delineating species, amplifying an adequate length of the gene from old specimens can be quite challenging due to DNA degradation. Our partial *rbc*L sequences of the type specimen of *P. formosana* var. *coreana* (SAP021454) included the 3′ portion (*rbc*L-3P), which is one of the best barcode markers for green macroalgae (Saunders and Kucera 2010). In addition, small-sized "mini-barcodes" are considered adequate to represent a sufficient number of signals for species identification. In insects, short DNA barcode sequences of mitochondrial cytochrome oxidase subunit I (less than 150 bp) can provide effective taxonomic sequence tags (Meusnier et al. 2008). Here, the pairwise distance value was moderate in the genus *Prasiola* when only 196 bp of *rbc*L-3P were compared.

The distributions of *Prasiola* species in Korea and Japan are similar. In South Korea, *Prasiola* only grows attached to small limestone rocks along the Sohancheon stream in Samcheok, which is a geologically limestone region (Park et al. 1970). In Japan, *P. japonica* is mostly distributed along pristine streams connected to the Pacific Ocean from central Honshu (Tochigi Prefecture) to Kyushu. Iwamoto (1984) reviewed the literature to clarify the relationship between the distribution of *P. japonica* and the geological features of the region. He reported that *P. japonica* is distributed within geologically distinct regions, such as the areas of Fossa Magna and along the Median Tectonic Line. Although he did not address the relationship between the distribution of *P. japonica* and mineralogically distinct areas, the regions described above are mostly limestone areas. Many recent studies have reported that *P. japonica* grows well within concrete waterways (Ishikawa et al. 2005, 2007, Ishikawa 2009). The type locality of *P. formosana* var. *coreana* is Muncheon, North Korea, which is also a limestone area (Okada 1939). Moreover, *P. yunnanica*, which was also molecularly identical to the Korean *Prasiola* species, has only been reported in Yunnan, another limestone area (although quite distant from the ocean). Additional research is necessary to determine whether the distribution of Asian freshwater *Prasiola* species is related to the geological features of their habitat.

Although Park et al. (1970) concluded that the South Korean *Prasiola* species appeared to be a new species,

they also noted that three eastern Asian species, *Prasiola japonica*, *P. formosana*, and *P. formosana* var. *coreana*, could be regarded as a single species. Here, we conclude that the South Korean *Prasiola* species be considered as *P. japonica* based on its habitat, morphology, and plastid genes phylogenies, and propose to synonymize *P. formosana* var. *coreana* and *P. yunnanica* with *P. japonica.*

Prasiola japonica Yatabe 1891

Type: Unknown. **Syntype localities:** Nikko (Province of Shimotsuke), Kiriu (Kozuke), Shibakawa (Suruga), and Oimura (Mino), March 1891 (Yatabe 1891:188). **Taxonomic (heterotypic) synonyms:** *Prasiola formosana* var. *coreana* Okada 1939 (J. Jpn. Bot. 15:450), *P. yunnanica* C. -C. Jao 1947 (Bot. Bull., Acad. Sin. 1:110).

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