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Morphological and phylogenetic data confirm the identity of *Prasiola fluviatilis* (Prasiolales, Trebouxiophyceae) from glacier streams in the Tianshan Mountains, China

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ABSTRACT

The green alga *Prasiola fluviatilis* (Sommerfelt) Areschoug ex Lagerstedt occurs in cold lotic environments. The species has a mainly circumarctic distribution, but has also been reported from glacier areas in lower latitude regions in both hemispheres. It was reported from China on a single occasion in the first half of the 20th century, but without description, illustrations or voucher specimens the identity of this record cannot be verified. Here we confirm the presence of *P. fluviatilis* in the Tianshan Mountains, Xinjiang Province, China based on morphological features, habitat characteristics, and plastid *rbcl* and *tufA* gene sequences. The biogeographic distribution of *P. fluviatilis* and its phylogenetic relationship with other terrestrial and freshwater *Prasiola* species are discussed.

RÉSUMÉ

Des données morphologiques et phylogénétiques confirment l'identité de Prasiola fluviatilis (Prasiolales, Trebouxiophyceae) dans les cours d'eau glaciaires des montagnes Tianshan, en Chine.

L'algue verte *Prasiola fluviatilis* (Sommerfelt) Areschoug ex Lagerstedt est présente dans les milieux lotiques froids. L'espèce a une distribution principalement circumarctique, mais a également été signalée dans les zones glaciaires des régions de basse latitude dans les deux hémisphères. Elle a été rapportée en Chine à une seule occasion dans la première moitié du 20^e siècle, mais sans description, illustration ou spécimen de référence, l'identité de cette observation ne peut être vérifiée. Nous confirmons ici la présence de *P. fluviatilis* dans les monts Tianshan, dans la province du Xinjiang, en Chine, sur la base des caractéristiques morphologiques, les caractéristiques de l'habitat et les séquences des gènes du plaste *rbcl* et *tufA*. La distribution biogéographique de *P. fluviatilis* et sa relation phylogénétique avec d'autres espèces de *Prasiola* terrestres et d'eau douce sont discutées.

KEY WORDS

Biogeography,
China,
Trebouxiophyceae,
phylogeny,
Prasiolales,
Prasiola fluviatilis.

MOTS CLÉS

Biogéographie,
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phylogénie,
Prasiolales,
Prasiola fluviatilis.

INTRODUCTION

The green algal genus *Prasiola* Meneghini (1838) is characterized by monostromatic laminar thalli, and vegetative cells with stellate or lobed chloroplasts containing a single pyrenoid. It is the most species-rich genus in the trebouxiophycean order Prasiolales Schaffner, including about 35 species distributed in marine, freshwater and terrestrial habitats, mainly in temperate to arctic regions with some species also occurring in lower latitude regions (Børgesen 1946; Waern 1952; Ettl & Gärtner 1995; Kováčik & Pereira 2001; Naw & Hara 2002; Rindi & Guiry 2004; Rodríguez & Jiménez 2005; Rodríguez *et al.* 2007; Guiry & Guiry 2020).

Traditionally, the taxonomy of the genus has relied on a number of diagnostic morphological characters to delineate species, including shape and size of the blade, presence/absence of a holdfast, structure of the stipe, cell size, life history, and type of habitat (Knebel 1935; Printz 1964; Kornmann & Sahling 1974; Ettl & Gärtner 1995; John 2002; Rindi 2007). However, many *Prasiola* species exhibit extensive morphological plasticity under different environmental conditions or developmental stages (Neustupa 1998; Rindi *et al.* 1999, 2007; Kováčik & Pereira 2001; Rindi 2010). In addition, cryptic species diversity has been demonstrated in the genus (Moniz *et al.* 2012b). This combination of plasticity and cryptic diversity makes species identification difficult based on morphology alone. It has therefore been recommended that a combination of morphological, ecological and molecular data should be used for reliable species circumscription and identification in *Prasiola* (Rindi *et al.* 2007; Heesch *et al.* 2012; Heesch *et al.* 2016; Klochkova *et al.* 2017). Molecular phylogenetic studies and DNA taxonomy in *Prasiola* has been facilitated by an increasing amount of available DNA sequence data (Sherwood *et al.* 2000; Naw & Hara 2002; Rindi *et al.* 2004, 2007; Saunders & Kucera 2010; Heesch *et al.* 2012; Moniz *et al.* 2012a, b; Kim *et al.* 2015; Klochkova *et al.* 2017).

Species of *Prasiola* occur in various environments, including fast flowing freshwater streams, humid rocks, barks or soil, and high intertidal and adjacent splash zone habitats, often associated with high inputs of organic nitrogen (Rindi *et al.* 1999; Rindi & Guiry 2004; Sutherland *et al.* 2016). In addition, some species engage in symbiotic relationships with lichens (Garrido-Benavent *et al.* 2017a, b). Although several species can be categorized as strictly freshwater/terrestrial or marine, some species tolerate a wide range of salinities and may occur in both environments (Rindi 2007). About 14 species of *Prasiola* occur in freshwater or terrestrial environments. Remarkably, eleven of these have been described from Asia (Guiry & Guiry 2020). Unfortunately, there are relatively few molecular phylogenetic data of Asian freshwater species compared to other regions (Naw & Hara 2002; Moniz *et al.* 2012b, 2014; Kim *et al.* 2015).

Prasiola fluviatilis (Sommerfelt) Areschoug ex Lagerstedt (1869) was originally described by Sommerfelt (1828) from running water in the mountains of Lerdalselfven, Norway. The linear thallus shape separated it from the other freshwater

Prasiola species (Lagerstedt 1869; Knebel 1935; Hamilton & Edlund 1994). The species has a mainly circumarctic distribution, but has also been reported from cold lotic environments in lower latitude regions in both hemispheres. More specifically, it has been recorded from Europe (Rejment-Grochowska 1952), North America (Setchell & Gardner 1903; Taylor 1928; Kobayasi 1967), the Arctic region (Hamilton & Edlund, 1994; Sherwood *et al.* 2000; Matuła *et al.* 2007), Asia (Borge 1934; Barinova *et al.* 2015; Barinova & Niyatbecove 2018), sub-Antarctica (Eaton 2012), and South America (McClintic *et al.* 2003). In Asia the species has so far only been observed in the Tianshan Mountains, Xinjiang Province, China (Borge 1934), and in neighboring Tajikistan (Barinova *et al.* 2015; Barinova & Niyatbekov 2018). However, no descriptions or illustrations were provided by Borge, nor are there any historical voucher specimens available for China. Globally, the only molecular data of *P. fluviatilis* are available from material of Canada (Sherwood *et al.* 2000) and Svalbard, Norway (Heesch *et al.* 2016).

In this study, specimens of *Prasiola* were collected in the Tianshan Mountains from similar habitats where *P. fluviatilis* was originally found by Borge (1934). Detailed morphological features, habitat characteristics, and plastid *rbcL* and *tufA* gene sequences are presented and confirm that the geographical range of *P. fluviatilis* extends into China.

MATERIAL AND METHODS

COLLECTION OF SAMPLES AND MORPHOLOGICAL OBSERVATION

Samples were collected in August 2017 from Tianshan Mountains, Xinjiang Province, China (43°6'6"N, 86°50'36"E). Populations of *P. fluviatilis* were found tightly adherent to rocks and stones in streams (3570 m a.s.l.) under a glacier (Fig. 1) with water temperature 0.4°C, pH 7.5, and slow velocity. Specimens were preserved in 4% formalin for morphological observations, and dried in silica gel for DNA extraction. Morphological observations were performed using a Zeiss Imager A2 light microscope, and photographs were taken with an Axiocam 506 color mounted on the microscope. The samples (XJ20170806-1, XJ20170806-2, XJ20170806-3, XJ20170806-4) are deposited in the herbarium of the Laboratory of Algae and Environment, Biology Department, Shanghai Normal University (SHTU), Shanghai, China.

DNA EXTRACTION, PCR AMPLIFICATION AND AUTOMATED SEQUENCING

The silica dried specimens were ground using liquid nitrogen, followed by DNA extraction using a NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany), following the manufacturer's protocol. We amplified and sequenced the plastid *rbcL* (1036 bases) and *tufA* (821 bases) genes from two specimens. Polymerase chain reactions (PCR) were performed using published primers: the amplification of the *rbcL* gene was carried out using primers PF2 and PR2 (Rindi *et al.* 2004), and that of the *tufA* gene was carried out using primers *tufGF4* and



FIG. 1. — Habitat of *Prasiola fluviatilis* (Sommerfelt) Areschoug ex Lagerstedt: **A**, streams under a glacier; **B**, population of *P. fluviatilis*.

TABLE 1. — Morphological data of *Prasiola fluviatilis* (Sommerfelt) Areschoug ex Lagerstedt from different sites. Note: the data of *P. fluviatilis* from Bolivia was measured from the figures in McClintic *et al.* (2003).

Site	Size of frond (cm)	Thickness (µm)	Cell size (µm)	Reference
Tianshan Mountains, China	0.2-1.4 × 1.0-8.0	–	3.7-12.1	This paper
Ellesmere Island, Canada	0.02-0.3 × 4.5-9.5	2.0-8.8	3.2-7.6 × 3.0-12.6	Hamilton (1994)
Svalbard, Norway	Up to 1.5 × 0.2	15.0-18.0	2.5-10	Heesch (2016)
Europe	Up to 11.0 × 2.0	8.0-19.0	–	Knebel (1935)
Bolivia	1.0	–	5.0-8.8	McClintic <i>et al.</i> (2003)

*tufA*R (Saunders & Kucera 2010). PCR's were conducted in 50 µL volumes containing 1.0 µL genomic DNA, 1.0 µL of each primer (10 mM), 25 µL *2xEasyTaq*® PCR SuperMix (all from TransGen Biotech, China), and 22 µL ddH₂O, as described by Rindi *et al.* (2004). The PCR reaction profile of both genes was as follows: initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min, and the final extension at 72°C for 10 min. The PCR products were sent to BGI Tech Corporation (Shanghai, China) for sequencing in an ABI 3730XL sequencer. Forward and reverse sequences were assembled using SeqMan, and submitted to GenBank (accession no. [MT846163](#) for *rbcL* and [MT846164](#) for *tufA*).

SEQUENCE ALIGNMENT AND PHYLOGENETIC RECONSTRUCTION

An initial identification of *rbcL* and *tufA* sequence was performed using a similarity search (megablast). All *rbcL* and *tufA* sequences of *Prasiola* in GenBank were exhaustively searched. Very short sequences (< 200 bp) were excluded from the alignment. Based on the result of Neighbor-joining phylogenetic analyses, a selection of sequences was made to be retained by keeping one or a few sequences per species and excluding identical or near identical sequences. The final sequences of the genera *Prasiola*, *Prasiolopsis* Vischer and *Rosenvingiella* Silva (the latter two were used as outgroup) (listed in Appendices 1; 2) were aligned using ClustalW (Thompson *et al.* 1997) in BioEdit v.7.0.9 (Hall 1999). Sequence alignments are available to download from the open-access repository Zenodo: <https://doi.org/10.5281/zenodo.4271930>. Uncorrected pairwise (p) distances were calculated using MEGA 6.0 (Appendix 3). The *rbcL* alignment included 41 *Prasiola*, *Prasiolopsis* and *Rosenvingiella* sequences, to yield a final alignment of 896 bases. The *tufA* gene alignment included 39 *Prasiola*, *Prasiolopsis* and *Rosenvingiella* sequences, to yield a final alignment of 531 bases. The optimal substitution model for each marker was generated using Modeltest 3.7 with related results listed in Appendix 4 (Posada & Buckley 2004). Maximum likelihood (ML) phylogenetic trees were generated using PHYML v.3.0 using default settings and 1000 bootstrap replicates (Felsen-

stein 1981; Guindon & Gascuel 2003). Bayesian inference (BI) analyses were performed using MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). A Markov chain Monte Carlo (MCMC) algorithm running three hot Markov chains and one cold Markov chain was used to estimate the posterior probability of phylogenetic trees. The Markov chains were started from a random tree and run for 2 000 000 generations, sampling every 1000 generations for a total of 2000 samples for each run. Figtree v.1.4.2 was used to edit all resulting phylogenetic trees.

RESULT

MORPHOLOGY

Thalli occurred in tufts of at least seven ribbon-like monostromatic blades, 1.0-8.0 cm long and 0.2-1.4 cm wide (Fig. 2A, B; Table 1). Vegetative cells were quadrat, rectangular or elliptical in surface view, and arranged in many longitudinal rows (Fig. 2C, D). Cells in the lower part of the blade were elongated, 6.3-13.2 µm long and 2.0-5.3 µm wide (Fig. 2E, F), and produced rhizoids that attached to the substrate. Cells in the upper portion of the thallus were 3.7-12.1 µm in diameter, round to square in shape, and arranged more or less in groups of four cells; clear areolae and thickened longitudinal lines were present between these groups (Fig. 2G). Uniseriate filaments were also observed (Fig. 2H). Reproduction was not observed.

PHYLOGENETIC ANALYSES

RbcL and *tufA* sequences from the different specimens were found to be identical, so only one new sequence of each marker was included in the analyses.

ML and BI analyses of the *rbcL* alignment yielded similar tree topologies. Information on estimated substitution models and base frequencies is provided in Appendix 4. The *rbcL* analysis (Fig. 3) showed that our sequences formed a clade with *P. fluviatilis* sequences from Canada and Norway with maximal statistical support (ML bootstrap values / BI posterior probabilities: 100/1.00). This clade was sister to *P. calophylla* (Carmichael ex Greville) Kützing (63/0.90). The pairwise distance between our strain and the *P. fluviatilis* sequences from Canada and Norway was both 0.003, corresponding to three base pair differences.

Similarly to the *rbcL* analyses, our *tufA* sequences of *P. fluviatilis* were sister to *P. calophylla* (78/0.99) (Fig. 4). Since no other *tufA* sequences of *P. fluviatilis* are available on GenBank, no further conclusions could be made based on the *tufA* analyses.

DISCUSSION

Prasiola fluviatilis, a species that has been recorded mainly from Arctic regions of Europe and North America, has also been reported from cold lotic environments in other regions, including China and Tajikistan (Borge 1934; Barinova *et al.*

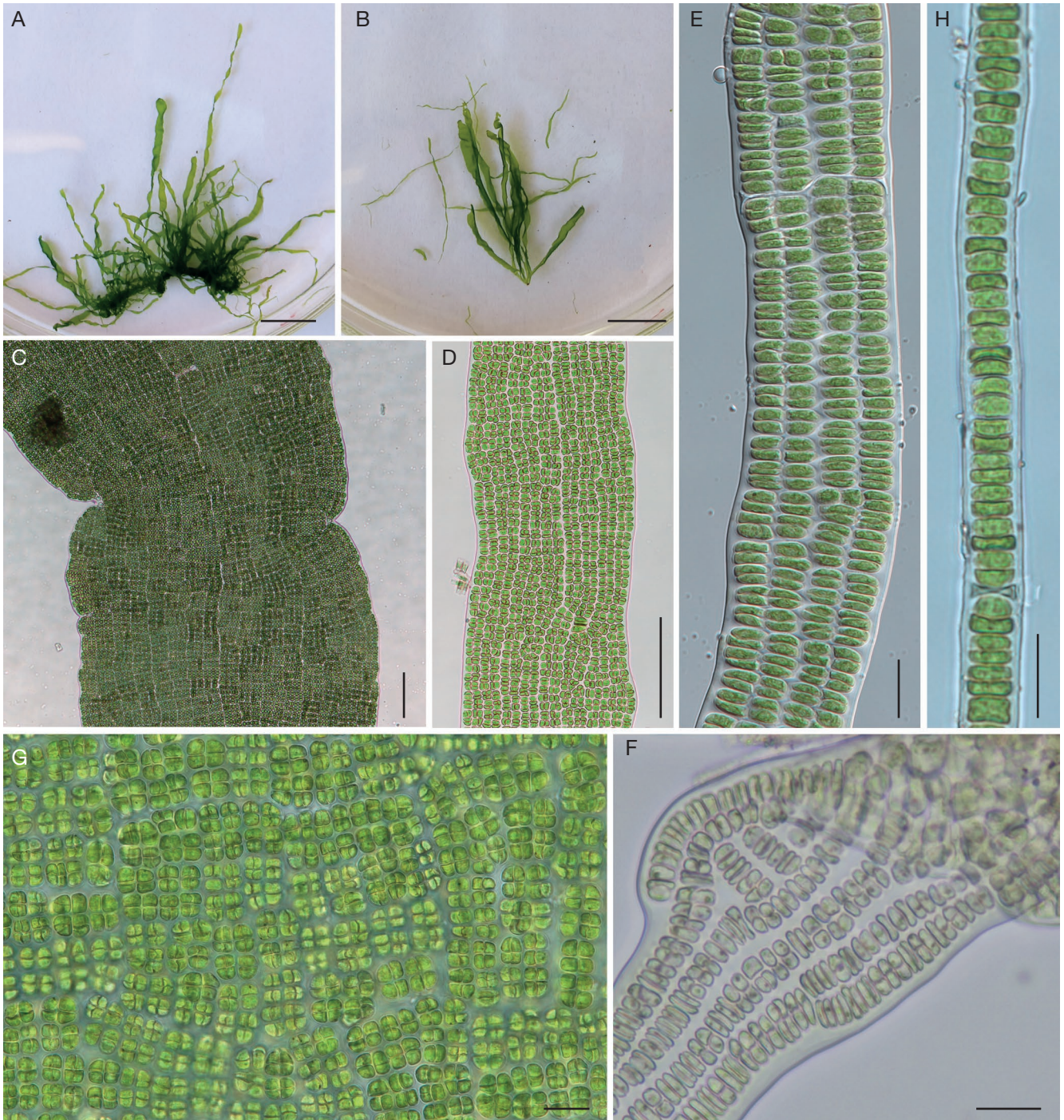
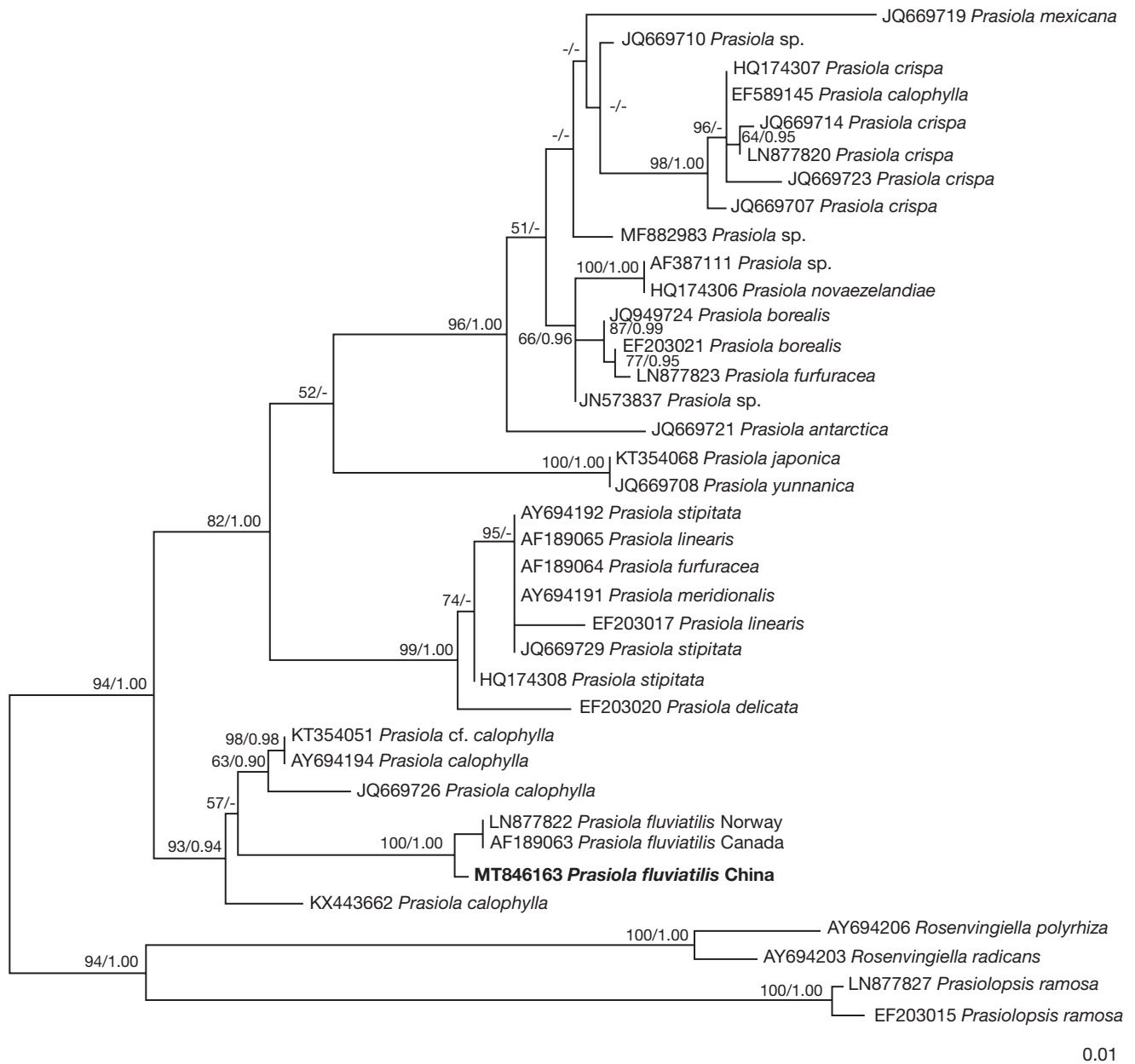


FIG. 2.— Morphology of *Prasiola fluviatilis* (Sommerfelt) Areschoug ex Lagerstedt: **A, B**, thalli; **C, D**, vegetative cells; **E, F**, cells in the lower part of the blade; **G**, cells in the upper portion of the thallus; **H**, uniseriate branches. Scale bars: A, B, 1 cm; C, D, 100 μ m; E-H, 20 μ m.

2015; Barinova & Niyatbecove 2018). However, no morphological information was provided for these Asian records. In addition, no molecular data were available to date for Asian representatives of this species, and as a result their identity as well as their extended geographical range in Asia remained uncertain. We found *P. fluviatilis* in the same region where it was originally collected by Borge (1934), and confirmed its identity based on habitat, morphological, and plastid gene data.

We applied two markers in our phylogenetic analyses, *rbcL* and *tufA*. Both are frequently used for phylogenetic inference, and species delimitation and identification in the genus (Kim *et al.* 2015; Heesch *et al.* 2016; Sutherland *et al.* 2016; Klochkova *et al.* 2017). *TufA* has been proposed as a good candidate marker for DNA barcoding in freshwater and marine green algae because of its high universality, relatively low contamination rate, apparent lack of introns, and high



0.01

FIG. 3.— Phylogenetic position of Chinese *Prasiola fluviatilis* (Sommerfelt) Areschoug ex Lagerstedt inferred from maximum likelihood analysis of the *rbcL* gene. Bootstrap values and Bayesian posterior probabilities are indicated at the branches. Only bootstrap values above 50% and posterior probabilities above 0.9 are shown.

species discrimination power (Hall *et al.* 2010; Saunders & Kucera 2010; Moniz *et al.* 2014). This gene has great potential as a marker for phylogenetic inference at low taxonomic levels in the Trebouxiophyceae, and was able to separate *Prasiola* species for which *SSU* and *rbcL* sequences were identical or nearly identical (Moniz *et al.* 2014).

Our *rbcL* and *tufA* phylogenies both confirmed a sister relationship between *P. fluviatilis* and *P. calophylla* (Carmichael ex Greville) Kützinger, and the two species were separated by relatively long and well supported branches in both analyses (Figs 3; 4). *P. calophylla* is a terrestrial species with linear blades, which is tolerant of sea spray but never grows submerged in

seawater (Rindi 2007; Rindi *et al.* 2007; Moniz *et al.* 2012a, b; Kim *et al.* 2015; Heesch *et al.* 2016). Our specimens of *P. fluviatilis* were found in streams under a glacier which formed only seasonally running cold water. This situation is similar to environments in Svalbard and Ellesmere Island, the only two localities where the presence of *P. fluviatilis* has been confirmed by sequence data. Other freshwater *Prasiola* species, such as *P. mexicana* Agardh, *P. nevadensis* Setchell & Gardner, *P. elongata* Hu, *P. japonica* Yatabe, *P. formosana* Okada, *P. tibetica* Jao, and *P. yunnanica* Jao are exclusively freshwater organisms, which occur completely submerged in permanent streams (Setchell & Gardner 1920; Jao 1947; Printz 1964;

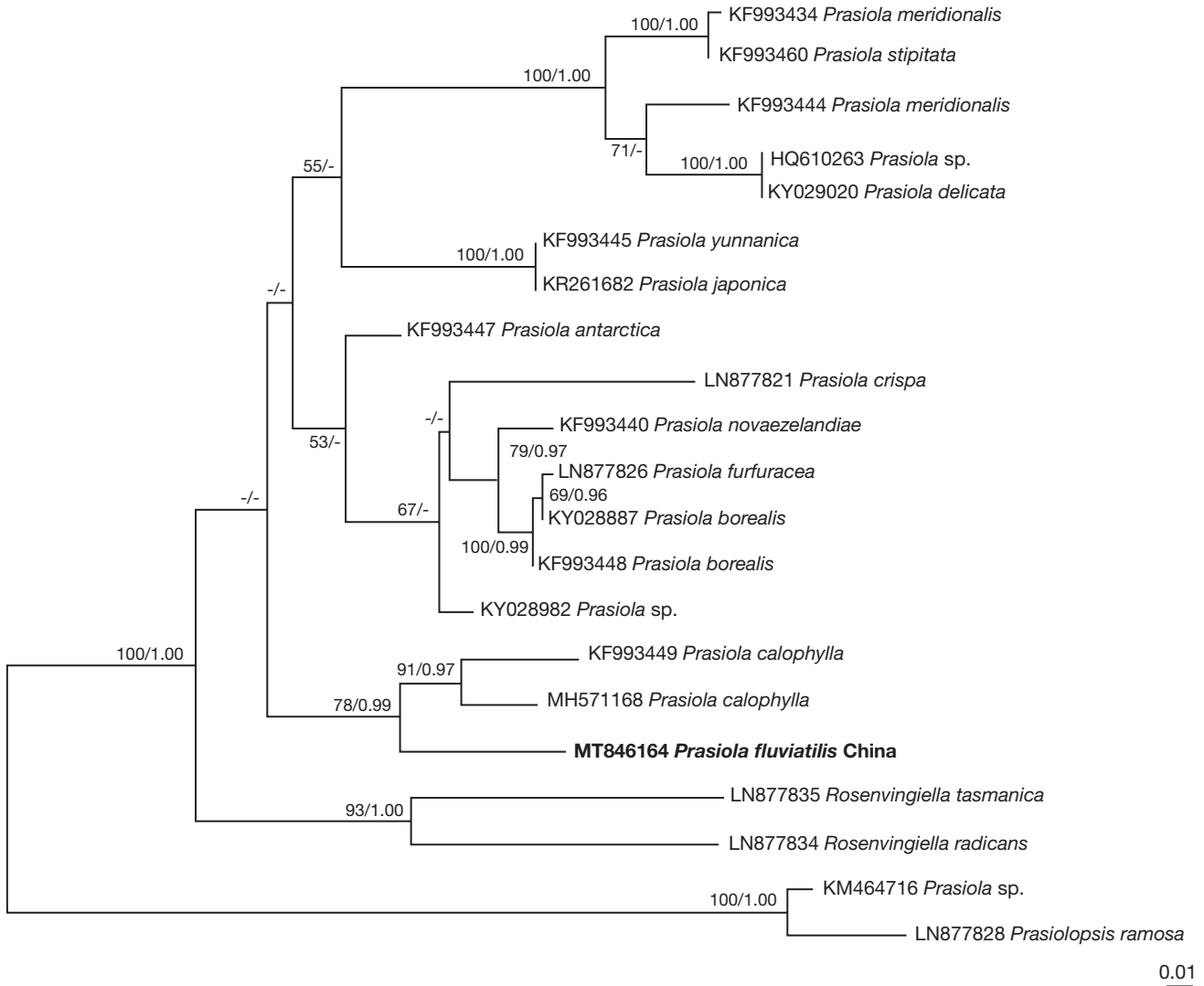


FIG. 4. — Phylogenetic position of Chinese *Prasiola fluviatilis* (Sommerfelt) Areschoug ex Lagerstedt inferred from maximum likelihood analysis and Bayesian analyses of the *tufA* gene. Bootstrap values and Bayesian posterior probabilities are indicated at the branches. Only bootstrap values above 50% and posterior probabilities above 0.9 are shown.

Vazquez & Uriza 2003; Hu & Wei 2006). Our phylogenetic analyses suggest that linear thallus shape, as well as habitat type in *P. calophylla* and *P. fluviatilis*, may be phylogenetically conserved, consistent with the idea of Rindi *et al.* (2007). Phylogenetic analysis shows that *P. yunnanica* (a species that was proposed as a synonym of *P. japonica* and *P. formosana* var. *coreana* Okada based on a plastid gene phylogeny and habitat characteristics by Kim *et al.* (2015)) is unrelated to *P. fluviatilis*. Sequence data for other Asian freshwater *Prasiola* species are lacking. Globally, 14 freshwater *Prasiola* species are accepted taxonomically, but molecular data of only four species, *P. japonica*, *P. fluviatilis*, *P. glacialis* Moniz, Rindi, Novis, Broady & Guiry and *P. mexicana*, are available. More data from different species and different strains within species are needed to unravel the evolutionary history of freshwater and terrestrial *Prasiola* species.

The *rbcL* sequence of our strain only showed 3 bp differences from those from Svalbard and Canada, and the latter two

sequences are identical (Hamilton & Edlund 1994; Heesch *et al.* 2016). The habitats of these three collection sites are very similar. Although *P. fluviatilis* is morphologically well defined by its narrow ribbon-like blades, which sets it apart from all other freshwater species, it remains to be confirmed based on DNA sequence data if strains from other regions actually belong to the same species. Based on our observations and previous descriptions of *P. fluviatilis*, thallus and cell sizes can vary substantially among individuals from different sites (Knebel 1935; Hamilton & Edlund 1994; McClintic *et al.* 2003; Heesch *et al.* 2016) (Table 1). In general, species of *Prasiola* show extensive morphological plasticity, as has been shown in marine species (Rindi *et al.* 2004, 2007; Rindi 2010), and this is likely also true for freshwater and terrestrial *Prasiola* species (Rindi *et al.* 2004; Kim *et al.* 2015). *P. velutina* (Lynbye) Trevisan shares a similar habitat with *P. fluviatilis* and also has a circumarctic distribution. Although the species has uniseriate filaments as dominant growth habit (Lokhorst &

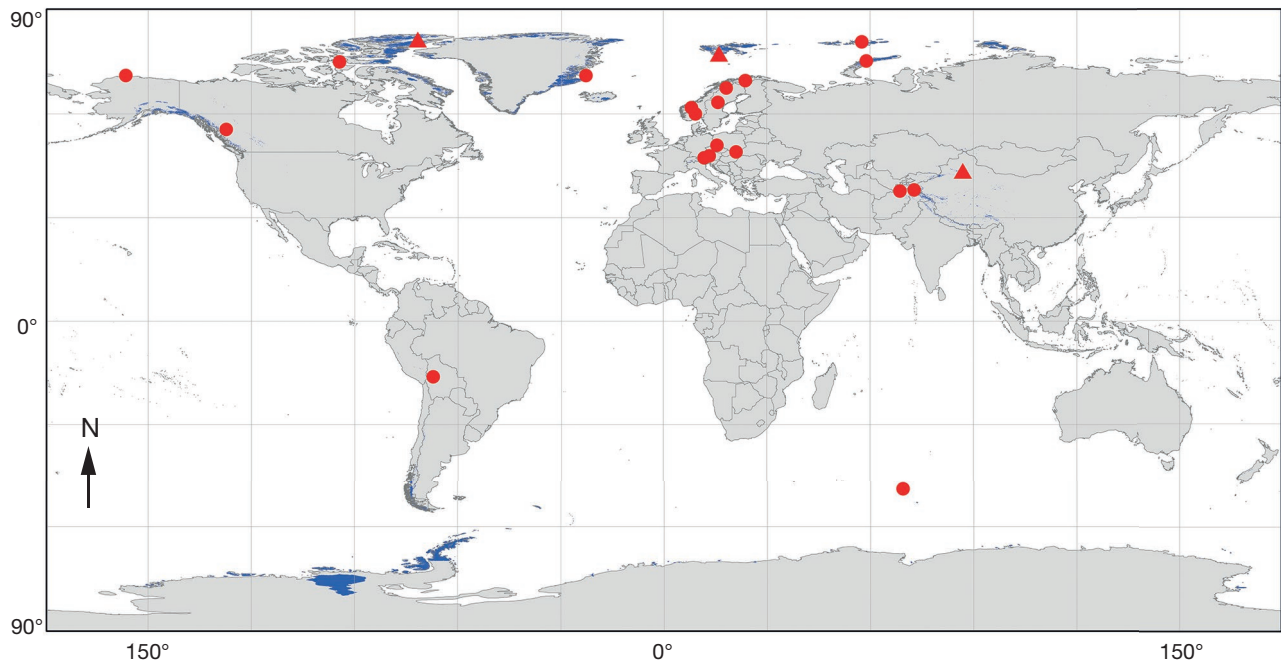


FIG. 5.— Global distribution of *Prasiola fluviatilis* (Sommerfelt) Areschoug ex Lagerstedt (blue: glacier area [GLIMS and NSIDC 2005, updated 2019]; ●, occurrence records based on morphology; ▲, occurrence records based on morphology and molecular evidence)

Star 1988), the morphological distinctions between these two species are not clear (Kobayasi 1967; Hamilton & Edlund 1994), raising the question whether the two may be conspecific. More generally, additional sequence data will be needed to investigate if *P. fluviatilis* is a single, widely distributed species or comprised of several species.

P. stipitata Suhr ex Jessen and *P. meridionalis* Setchell & Gardner, widely distributed in marine habitats of both hemispheres, are expected to be present whenever environmental conditions are suitable (Rindi *et al.* 2004, 2007; Heesch *et al.* 2012, 2016). Some other species, such as *P. calophylla* and those endemic freshwater species reported in China, appear to have more limited distributions. The different dispersal ability among species of *Prasiola* may thus be related to the type of habitat occupied, with terrestrial species generally having wider geographical ranges compared to aquatic species, especially submerged freshwater species (Heesch *et al.* 2016). *P. fluviatilis*, however, does not seem to perfectly conform to this view of restricted distribution. *P. fluviatilis* has been recorded from Polar regions to lower latitudes in both hemispheres, where it is mostly located in very specialized glacier areas (Fig. 5). It was reported as a stenothermic cold water species, and we found that it disappeared in the section of the stream where the water temperature was more than 5°C. *P. fluviatilis* thus apparently occupies a narrow ecological niche, while it shows a wide, though disjunct, geographical distribution. We acknowledge that these hypotheses are still speculative and more morphological, ecological and phylogenetic research of *Prasiola* is needed to understand the patterns and drivers of geographical distributions of species in various environments.

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APPENDICES

APPENDIX 1. — Relevant *rbcL* sequence information downloaded from GenBank in this study.

species	rbcL	country	continent	citation
<i>Prasiola antarctica</i> Kützing	JQ669721	Palmer Station	Europe	Moniz <i>et al.</i> (2012b)
<i>P. borealis</i> M.Reed	EF203021	Canada	North America	Rindi <i>et al.</i> (2007)
<i>P. borealis</i>	JF949724	Australia	Oceania	Moniz <i>et al.</i> (2012a)
<i>P. calophylla</i> (Carmichael ex Greville) Kützing	AY694194	Ireland	Europe	Rindi <i>et al.</i> (2004)
<i>P. calophylla</i>	EF589145	New Zealand	Oceania	Novis (2007)
<i>P. calophylla</i>	JQ669726	Ireland	Europe	Moniz <i>et al.</i> (2012)
<i>P. calophylla</i>	KX443662	Austria	Europe	Hartmann <i>et al.</i> (2016)
<i>P. cf. calophylla</i>	KT354051	Japan	Asia	Sutherland <i>et al.</i> (2016)
<i>P. crispa</i> (Lightfoot) Kützing	HQ174307	Balleny Islands	Antarctica	Heesch <i>et al.</i> (2012)
<i>P. crispa</i>	JQ669723	King George Isl	Antarctica	Moniz <i>et al.</i> (2012b)
<i>P. crispa</i>	JQ669707	Marshall Val.	Antarctica	Moniz <i>et al.</i> (2012)
<i>P. crispa</i>	LN877820	Norway	Europe	Heesch <i>et al.</i> (2016)
<i>P. crispa</i>	JQ669714	Norway	Europe	Moniz <i>et al.</i> (2012)
<i>P. delicata</i> Setchell & N.L.Gardner	EF203020	Canada	North America	Rindi <i>et al.</i> (2007)
<i>P. delicata</i>	KT354050	Japan	Asia	Sutherland <i>et al.</i> (2016)
<i>P. fluviatilis</i> (Sommerfelt) Areschoug ex Lagerstedt	AF189063	Canada	North America	Sherwood <i>et al.</i> (2000)
<i>P. fluviatilis</i>	LN877822	Norway	Europe	Heesch <i>et al.</i> (2016)
<i>P. furfuracea</i> (Mertens ex Hornemann) Trevisan	AF189064	Scotland	Europe	Sherwood <i>et al.</i> (2000)
<i>P. furfuracea</i>	LN877823	Norway	Europe	Heesch <i>et al.</i> (2016)
<i>P. japonica</i> Yatabe	KT354068	Nepal	Asia	Sutherland <i>et al.</i> (2016)
<i>P. linearis</i> Jao	AF189065	Canada	North America	Sherwood <i>et al.</i> (2000)
<i>P. linearis</i>	EF203017	United States	North America	Rindi <i>et al.</i> (2007)
<i>P. meridionalis</i> Setchell & Gardner	AY694191	United States	North America	Rindi <i>et al.</i> (2004)
<i>P. mexicana</i> Agardh	JQ669719	Mexico	North America	Moniz <i>et al.</i> (2012)
<i>P. novaezelandiae</i> S.Heesch & W.A.Nelson	HQ174306	New Zealand	Oceania	Heesch <i>et al.</i> (2012)
<i>P. stipitata</i> Suhr ex Jessen	JQ669729	Canada	North America	Moniz <i>et al.</i> (2012)
<i>P. stipitata</i>	HQ174308	New Zealand	Oceania	Heesch <i>et al.</i> (2012)
<i>P. sp.</i>	JQ669710	Garwood Val.	Antarctica	Moniz <i>et al.</i> (2012)
<i>P. sp.</i>	JN573837	Chile	South America	Thüs <i>et al.</i> (2011)
<i>P. sp.</i>	AY694197	England	Europe	Rindi <i>et al.</i> (2004)
<i>P. sp.</i>	MF882983	King George Island	Antarctica	Garrido-Benavent <i>et al.</i> (2017a)
<i>P. sp.</i>	AF387111	New Zealand	Oceania	Woolcott & King (2001)
<i>P. yunnanica</i> Jao	JQ669708	China	Asia	Moniz <i>et al.</i> (2012)

APPENDIX 2. — Relevant *tufA* sequence information downloaded from GenBank in this study.

species	tufA	country	continent	citation
<i>Prasiola antarctica</i> Kützing	KF993447	Palmer Station	Antarctica	Moniz <i>et al.</i> (2014)
<i>P. borealis</i> M.Reed	KF993448	Canada	North America	Moniz <i>et al.</i> (2014)
<i>P. borealis</i>	KY028887	United States	North America	Garrido-Benavent <i>et al.</i> (2016)
<i>P. calophylla</i> (Carmichael ex Greville) Kützing	KF993455	Ireland	Europe	Moniz <i>et al.</i> (2014)
<i>P. calophylla</i>	KF993449	Ireland	Europe	Moniz <i>et al.</i> (2014)
<i>P. calophylla</i>	MH571168	United States	North America	Bringloe & Saunders (2019)
<i>P. crispa</i> (Lightfoot) Kützing	LN877821	Norway	Europe	Heesch <i>et al.</i> (2016)
<i>P. delicata</i> Setchell & N.L.Gardner	KY029020	United States	North America	Garrido-Benavent <i>et al.</i> (2017a)
<i>P. furfuracea</i> (Mertens ex Hornemann) Trevisan	LN877826	Norway	Europe	Heesch <i>et al.</i> (2016)
<i>P. japonica</i> Yatabe	KR261682	Japan	Asia	Kim <i>et al.</i> (2015)
<i>P. meridionalis</i> Setchell & Gardner	KF993444	United States	North America	Moniz <i>et al.</i> (2014)
<i>P. meridionalis</i>	KF993434	United States	North America	Moniz <i>et al.</i> (2014)
<i>P. novaezelandiae</i> S.Heesch & W.A.Nelson	KF993440	New Zealand	Oceania	Moniz <i>et al.</i> (2014)
<i>P. stipitata</i> Suhr ex Jessen	KF993460	Canada	North America	Moniz <i>et al.</i> (2014)
<i>P. sp.</i>	HQ610263	Canada	North America	Saunders & Kucera (2010)
<i>P. sp.</i>	KY028982	Greenwich Island	Antarctica	Garrido-Benavent <i>et al.</i> (2017a)
<i>P. yunnanica</i> Jao	KF993445	China	Asia	Moniz <i>et al.</i> (2014)

APPENDIX 3. — Uncorrected pairwise distances between *rbcl* sequences of the taxa in this study.

Taxon	No. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
MT846163	1														
<i>Prasiola fluviatilis</i>															
AF189063	2	0.003													
<i>P. fluviatilis</i>															
LN877822	3	0.003	0.000												
<i>P. fluviatilis</i>															
JQ669721	4	0.049	0.049	0.049											
<i>P. antarctica</i>															
EF203021	5	0.042	0.042	0.042	0.016										
<i>P. borealis</i>															
JQ669726	6	0.015	0.018	0.018	0.039	0.036									
<i>P. calophylla</i>															
HQ174307	7	0.049	0.049	0.049	0.024	0.015	0.043								
<i>P. crispera</i>															
EF203020	8	0.039	0.041	0.041	0.035	0.031	0.027	0.038							
<i>P. delicata</i>															
AF189064	9	0.032	0.035	0.035	0.038	0.031	0.023	0.041	0.014						
<i>P. furfuracea</i>															
KT354068	10	0.031	0.032	0.032	0.032	0.034	0.024	0.041	0.034	0.028					
<i>P. japonica</i>															
AY694191	11	0.032	0.035	0.035	0.038	0.031	0.023	0.041	0.014	0.000	0.028				
<i>P. meridionalis</i>															
JQ669719	12	0.054	0.054	0.054	0.030	0.023	0.046	0.024	0.038	0.043	0.041	0.043			
<i>P. mexicana</i>															
HQ174306	13	0.049	0.049	0.049	0.023	0.009	0.043	0.018	0.038	0.038	0.038	0.038	0.024		
<i>P. novaezealandiae</i>															
AF189065	14	0.032	0.035	0.035	0.038	0.031	0.023	0.041	0.014	0.000	0.028	0.000	0.043	0.038	
<i>P. linearis</i>															
JQ669729	15	0.032	0.035	0.035	0.038	0.031	0.023	0.041	0.014	0.000	0.028	0.000	0.043	0.038	0.000
<i>P. stipitata</i>															
JQ669708	16	0.031	0.032	0.032	0.032	0.034	0.024	0.041	0.034	0.028	0.000	0.028	0.041	0.038	0.028
<i>P. yunnanica</i>															

APPENDIX 4. — Substitution models inferred for each gene sequence alignment based on Modeltest 3.7 analysis.

Molecular marker	Model selected	Base frequency	Rate matrix
<i>rbcl</i>	TrN + I + G	freqA = 0.3001	R(a) [A-C] = 1.0000
	-lnL = 2430.4280	freqC = 0.1576	R(b) [A-G] = 5.8833
	K = 7	freqG = 0.2107	R(c) [A-T] = 1.0000
	(I) = 0.6195	freqT = 0.3316	R(d) [C-G] = 1.0000
	(G) = 0.5950	–	R(e) [C-T] = 7.9502
	–	–	R(f) [G-T] = 1.0000
<i>tufA</i>	GTR+I+G	freqA = 0.3704	R(a) [A-C] = 2.8417
	-lnL = 2203.2332	freqC = 0.1241	R(b) [A-G] = 3.7130
	K = 10	freqG = 0.2166	R(c) [A-T] = 0.3758
	(I) = 0.4296	freqT = 0.2889	R(d) [C-G] = 0.6766
	(G) = 0.5266	–	R(e) [C-T] = 7.9474
	–	–	R(f) [G-T] = 1.0000