Phycologia Volume 57 (5), 581–592 Published 23 July 2018

Ecology, cytology and phylogeny of the snow alga *Scotiella cryophila* K-1 (Chlamydomonadales, Chlorophyta) from the Austrian Alps

Daniel Remias[1]*†, Lenka Procházková[2]†, Andreas Holzinger[3] and Linda Nedbalová[2]

[1]*University of Applied Sciences, Campus Wels, Stelzhamerstr. 23, A-4600 Wels, Austria*
[2]*Charles University, Faculty of Science, Department of Ecology, Viničná 7, CZ-128 44 Prague, Czech Republic*
[3]*University of Innsbruck, Department of Botany, Sternwartestr. 15, A-6020 Innsbruck, Austria*

Abstract: Long-lasting, slowly melting snowfields in mountainous regions are frequently populated by specialised microalgae whose diversity is still vastly underestimated. Cysts causing sub-surficial green snow were collected in the Austrian Alps, Tyrol, and morphologically accorded to the snow alga *Scotiella cryophila sensu* Chodat, initially described from Switzerland. The cytology and photobiology of this population were investigated to understand mechanisms of adaptation to the harsh habitat. Cysts of *S. cryophila* K-1 had secondary cell walls with pronounced rib-like surface structures and contained several small spherical plastids. The cytoplasm was dominated by lipid bodies, which developed reddish secondary pigmentation. Partial life cycle observations showed that daughter cells lacked structured cell walls. Cysts performed active photosynthesis at temperature conditions close to the freezing point and were photoinhibited at irradiances greater than 70 μmol m^{-2} s^{-1}. This corresponded exactly to habitat conditions 20 to 40 cm below the snow surface. Phylogenetic analyses using 18S rDNA, *rbc*L and ITS2 rDNA sequences indicated that *S. cryophila* K-1 is related to *Chloromonas*, known to contain several snow algae. The taxon forms an independent lineage and is clearly genetically distinct from the type strain of *Chloromonas rosae* var. *psychrophila* from North America that is supposed to have morphologically identical cysts. For a taxonomic treatment including a species assignment of *S. cryophila* K-1 from Europe within *Chloromonas*, flagellates will have to be cultivated from cysts or from acquired field material for a detailed morphological description. Acquisition and genetic analysis of cysts that resemble *S. cryophila* from America could elucidate their relationship to European samples.

Key Words: Cryoflora, Cryospheric algae, Cysts, Extremophiles, Fluorometry, Ultrastructure

INTRODUCTION

Blooms of snow algae can cause green, orange, yellow or red snow discolourations, depending on prevailing cell pigmentation (Hoham & Duval 2001; Anesio *et al.* 2017). In a global context, snow and glacial algae are considered to play a role in accelerating Arctic snow melt due to albedo reduction driven by their abundant secondary pigments. Thus, they should be incorporated into climate models (Lutz *et al.* 2016; Ganey *et al.* 2017). Members of Chlamydomonadaceae (Chlorophyceae) dominate these extreme habitats, most likely because of (1) morphological and physiological adaptations of their life cycles to low or comparable high temperatures before and after snowmelt, (2) temporary or restricted availability of liquid water, or (3) extreme ultraviolet and visible irradiation at the snow surface (Komárek & Nedbalová 2007; Lukeš *et al.* 2014; Cvetkovska *et al.* 2017).

We investigated a snow alga that morphologically matches the previously described *Scotiella cryophila* Chodat (Chlorophyceae, Chodat 1922). This species is not known to cause striking monospecific blooms at the snow surface, unlike many other snow algae. The alga investigated here was collected in the Kühtai region (K-1) of the Austrian Alps and characterised by its cytology, photobiology and phylogeny. It was initially described from Switzerland (Chodat 1922), and scattered cells were further reported from the Giant Mountains in the Czech Republic (Nedbalová *et al.* 2008), the High Tatra Mountains in Slovakia (Kol 1968), Scotland (Light & Belcher 1968), Greenland (Kol 1968) and North America (Arizona, New York and Vermont; Hoham *et al.* 2002). This taxon is recognized by having oblong, immotile, fusiform cells with undulating or alternating surface ribs, several disc-like plastids and occasional reddish pigmented 'droplets' close to the cell poles. Species of *Scotiella* were initially considered to develop daughter cells with the same ribbed wall morphology as the mother cells. Later, it was shown that daughter cells of several species of '*Scotiella*' inhabiting snow lack any cell wall ornamentation, and the stage of *Scotiella* was recognized either as a vegetative cyst or a generative zygote (Hoham & Mullet 1977). Thus, these taxa were transferred to the genus *Chloromonas* (e.g. Hoham & Mullet 1978). Using a strain isolated from snow in the White Mountains, Arizona, *Chloromonas rosae* var. *psychrophila* Hoham, Bonome, Martin, & Leebens-Mack was proposed as a synonym of *S. cryophila* (Hoham *et al.* 2002). However, it is unknown if the type strain of *C. rosae* var. *psychrophila* (consisting of flagellates) is genetically identical to the cysts that caused the field bloom. Furthermore, it is not clear if snow algae with identical cyst

* Corresponding author (daniel.remias@fh-wels.at).
† DR and LP contributed equally to this work.
DOI: 10.2216/18-45.1

morphology from elsewhere belong to the same species or not.

The major aim of this study was to determine if the cyst population of the Kühtai region 1 (K-1) of *Scotiella cryophila* from the European Alps was morphologically and phylogenetically related to other similar extremophilic algae living in snow. Because harvested cells dominated subsurficial layers, covered by a white snow pack, we wanted to determine whether the photobiology was adapted to low irradiances. This is ecologically significant, since light penetration through snow decreases logarithmically with depth (Gorton *et al*. 2001). Finally, in order to find the correct phylogenetic position, a combination of the three molecular markers (18S rDNA, *rbc*L and ITS2) was applied. This revealed that the type strain of *Chloromonas rosae* var. *psychrophila* (UTEX SNO47) from North America is not related to the blooms of *S. cryophila* K-1 in alpine snow in Europe. Since *Scotiella* is invalid for species where daughter cells have smooth walls instead of ribbed ones, and the epithet *cryophila* already exists in *Chloromonas* (Hoham & Mullet 1977), a *nomen novum* for this taxon will be necessary once flagellates can be described.

MATERIAL AND METHODS

The green cryoflora was collected 5 June 2009 (sample GK02) and 30 May 2017 (sample WP125) in Austria (Tyrol, district Imst at Kühtai Valley, between Schwarzmoos and Gossenkölle Lake). Global positioning system locations and altitude were 47°13.748′N, 11°00.704′E, 2432 m (GK02) and 47°13.754′N, 11°00.718′E, 2435 m (WP125), respectively. First, surface snow was removed, and then cells at depths of about 20–30 cm were harvested into buckets with a stainless steel shovel. Snow was gently melted at 4°C in the dark. Some of the cells were intentionally kept at these conditions in the meltwater and additionally illuminated with approximately 40 µmol photons m^{-2} s^{-1} for several weeks to follow the further development of the cysts. Electrical conductivity and pH of the meltwater were measured immediately after melting with a conductometer from WTW Instruments (Weilheim, Germany). Light conditions in the snow were acquired with a PMA2100 logger equipped with a photosynthetic active radiation (PAR) sensor PMA2131 (Solar Light, Glenside, Pennsylvania USA). Light microscopy (LM) and cell size measurements were performed with a Zeiss Axiovert 200M, photomicrographs taken with an Axiocam MRc5 (Zeiss, Oberkochen, Germany). The protocols described in Procházková *et al*. (2018a) were used to determine cell concentration per meltwater volume and to study cell wall structure by scanning electron microscopy (SEM). The ultrastructure of the cells was investigated by transmission electron microscopy (TEM) according to Remias *et al*. (2010). Permanently cryopreserved cysts of *Scotiella cryophila* K-1 collected 5 June 2009 (field sample GK02) were deposited at the Culture Collection of Algae of Charles University in Prague (CAUP), Czech Republic.

For evaluation of the light-dependent plastid photon flux rates, *in vivo* chlorophyll fluorescence measurements were performed with a PAM 2500 in a 0.6 ml suspension cuvette KS-2500 (Walz GmbH, Effeltrich, Germany) at 1°C. To measure the relative electron transport rate (rETR) and the light saturation point I_k, cells from sample WP125 were exposed to photon flux densities (PFD) of 5, 9, 34, 67, 104, 201 µmol photons m^{-2} s^{-1} for 30 seconds each. Four independent replicates were measured. For further details see Procházková *et al*. (2018a).

For genetic analyses, the type strain of *Chloromonas rosae* var. *psychrophila* SNO47 was acquired from UTEX (Houston, Texas USA).

Total genomic DNA was extracted according to Procházková *et al*. (2018a). The 18S small subunit ribosomal RNA gene (18S rDNA), internal transcribed spacer regions 1 and 2 (ITS1, ITS2 rDNA) and ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L) gene regions were amplified from DNA isolates by polymerase chain reaction using existing primers (Table S1). Amplification and sequencing reactions for these markers were identical to those described by Procházková *et al*. (2018a). The obtained sequences were submitted to the National Center for Biotechnology Information (NCBI) Nucleotide sequence database (accession numbers Table S2). The methods of annotation and prediction of the secondary structure of the nuclear rDNA ITS2 region were the same as those described by Procházková *et al*. (2018a). The ITS2 sequences of *Scotiella cryophila* K-1 (this study) and *Chloromonas nivalis* Gassan-B (Matsuzaki *et al*. 2015) were aligned using the sequence-structure analysis in 4SALE (Seibel *et al*. 2006, 2008) in order to find compensatory base changes (CBCs). Manual validation and correction of alignment were included. The secondary structure of nuclear rDNA ITS2 was drawn using VARNA version 3.9 (Darty *et al*. 2009). 18S rDNA alignment contained 41 sequences (1668 bp); *rbc*L matrix consisted of 38 sequences (1128 bp); members of the clade of *Reinhardtii*, the clade of *Oogamochlamys* and the clade of *Carteria sensu* Pröschold *et al*. (2001) were selected as the outgroup. The best-fit nucleotide substitution model was estimated by jModeltest 2.0.1 (Posada 2008). Based on the Akaike Information Criterion, the 'TIM1 + I + G' model was selected for 18rDNA. Three partitions were set for *rbc*L gene sequences and the following substitution models were applied: 'TIM1 + I + G' (first codon position), 'GTR + I + G' (second codon position) and 'TIM3 + I + G' (third codon position). The 18S rDNA and *rbc*L phylogenetic trees were inferred by Bayesian inference (BI) and maximum likelihood (ML) according to Nedbalová *et al*. (2017), with the minor modification that Markov Chain Monte Carlo runs were carried out for three million generations in BI. Convergence of the two cold chains was checked by the average standard deviation of split frequencies (0.000738 and 0.000943 for 18S rDNA and *rbc*L dataset, respectively). Bootstrap analyses and Bayesian posterior probabilities were performed as described by Nedbalová *et al*. (2017).

For evaluating the additional growth of *Scotiella cryophila* K-1 during cyst maturation, length, width and the length to width ratio determined directly after the harvest (GK02, WP125) were compared with those kept for 3 months in laboratory conditions (GK02 old, WP125 old). Statistical

analyses followed Procházková *et al.* (2018a), and the Mann–Whitney test was used for testing the hypothesis that the median of two groups was identical.

RESULTS

Habitat description

The sampling location in the Austrian Alps (47°13.748′N, 11°00.704′E) was flat and slightly southwest orientated. The snowfield was exposed above timberline, the underground was scree, and some larger granite rocks were not snow covered (Fig. S1). In June 2009, the snow surface was red and dominated by spherical cysts of *Chlamydomonas nivalis* (Bauer) Wille to approximately 10 cm depth. After removing about 20 cm of snow, a band of green snow dominated by oblong fusiform cysts was found and harvested (GK02). Eight years later, in late May 2017, the surface at practically the same location appeared uncoloured; however, a green band at 20 to 30 cm depth with the same species (WP125) was found and harvested (Fig. S2). Further algae that co-occurred in 2017 were scattered cells of *C. nivalis*, *Chloromonas brevispina* (Fritsch) Hoham, Roemer & Mullet and an undetermined unicellular biflagellate chrysophyte. The water content of this snow layer was 49.2 ± 3.2%. The meltwater had an electrical conductivity of 7.0 μS cm^{-1} and a pH of 5.5 in 2009, and 9.0 μS cm^{-1} and pH of 5.1 in 2017. At noon, PAR 20 cm below the snow surface accounted for 2% of surface amounts during sunny weather (40 vs 2062 μmol m^{-2} s^{-1}) and 1.4% in cloudy conditions (12 vs 836 μmol m^{-2} s^{-1}). In 2017, the concentration at the margin of the bloom was 1325 cells ml^{-1} snow meltwater.

Morphological description

The elongate cysts had a fusiform shape (Fig. 1), were 33.3 ± 3.0 μm long (mean ± *s*; n = 92) and 10.5 ± 2.0 μm wide, with a length to width ratio from 2.1 to 4.7 (3.3 ± 0.5; WP125 and GK02 averaged together; sizes of each sample shown separately in Figs S3–S5). Many cells were in the process of shedding a smooth outer cell wall (Fig. 2), leaving a secondary wall with straight to undulate ribs (Figs 3, 4), which sometimes alternated toward the cell poles. The cytoplasm of young cysts was dominated by a parietal chloroplast, which was obviously rearranged during maturation to irregular band-like sections, and finally into small spherical plastids (Fig. 5). During sampling, only a few cells contained reddish lipid globules; however, after aging for 3 months at low irradiance in laboratory conditions, virtually all cells developed peripheral secondary pigmentation (Fig. S6). Under the same conditions, many cells underwent two cell divisions. First, the protoplast contracted by becoming rounder (Fig. 6), then it cleaved into four, smooth-walled, oblong daughter cells (Fig. 7). Concurrently, the cysts grew slightly (to 36.1 ± 2.6 μm long, 12.5 ± 1.4 μm wide; sample WP125). Mature cysts (3 months after harvest) were significantly wider and had a lower length to width ratio than cysts when harvested (both $P < 0.01$); whereas, the influence of ageing on cyst lengths was not significant (Figs S3–S5). Motile flagellates were never observed.

The arrangement of the characteristic cell wall surface ribs, which were sometimes difficult to observe by LM, was depicted by SEM (Figs 8–11): some ribs reached from one pole nearly to the antapex (Fig. 8), sometimes fusing or diverging (Fig. 9); moreover, an isolated short rib could be present (Fig. 10). The most prominent rib could have two lateral secondary ribs (Fig. 11). In median cross section, usually six to ten ribs were present. TEM revealed that cyst cell walls had up to three layers (Fig. 12) and some prominent wall ribs were secondarily ribbed (Fig. 13). Mature cysts were covered by an electron-dense innermost cell wall, peripheral lipid bodies surrounding the nucleus and several roundish plastids (Figs 14–16).

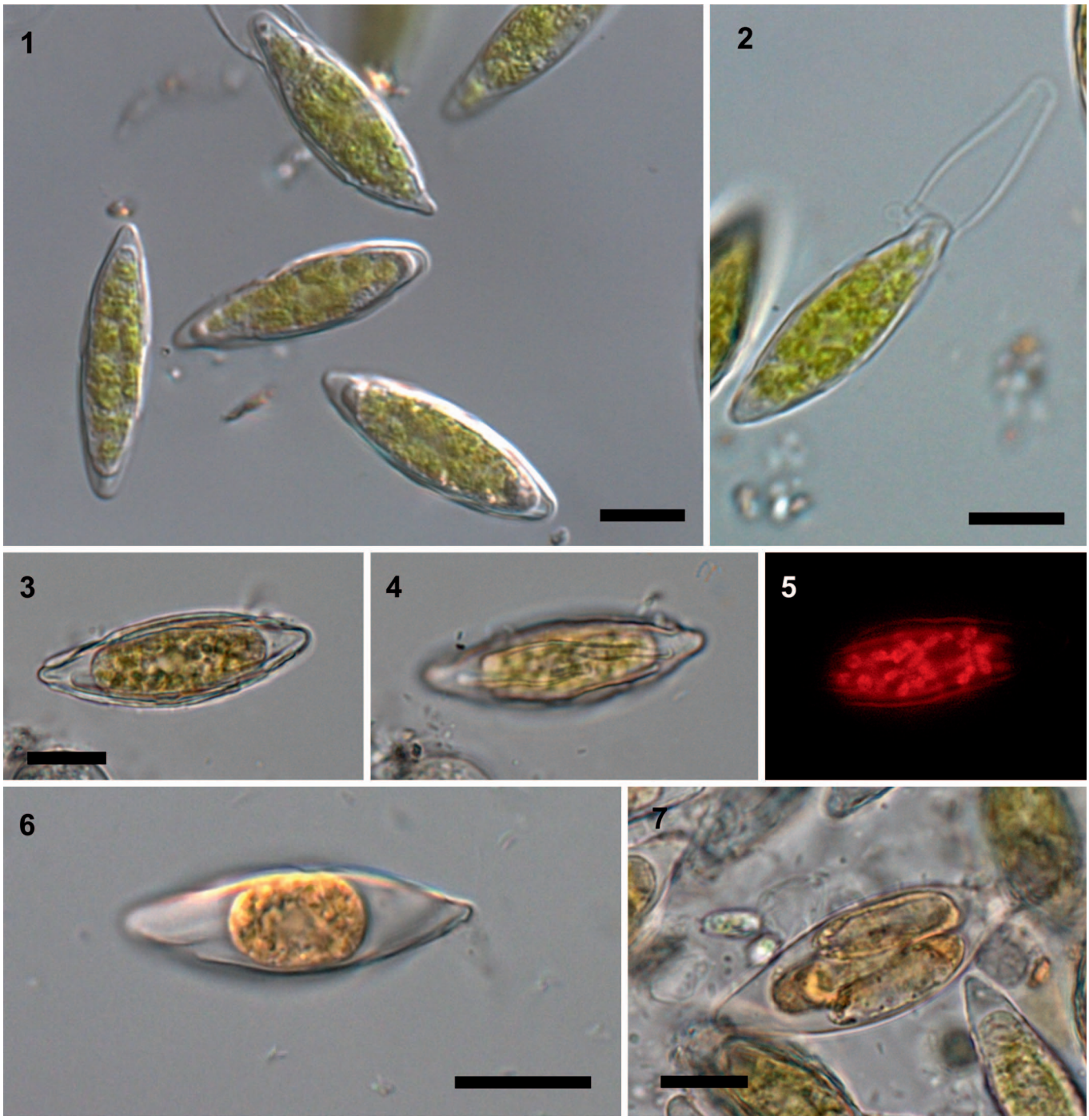
Photosynthesis

The light-dependent relative electron transport rates revealed the extent of adaptation of photosystem II to the low-light habitat conditions. *Scotiella cryophila* K-1 (sample WP125) exhibited an alpha (slope of the light-limited region of the photosynthesis-irradiance curve) of 0.24 ± 0.01, a relative ETR_{max} of 3.9 ± 0.2 and an I_k value of 27 ± 2 μmol photons m^{-2} s^{-1} (Fig. 17). Photoinhibition occurred above approximately 70 μmol photons m^{-2} s^{-1}.

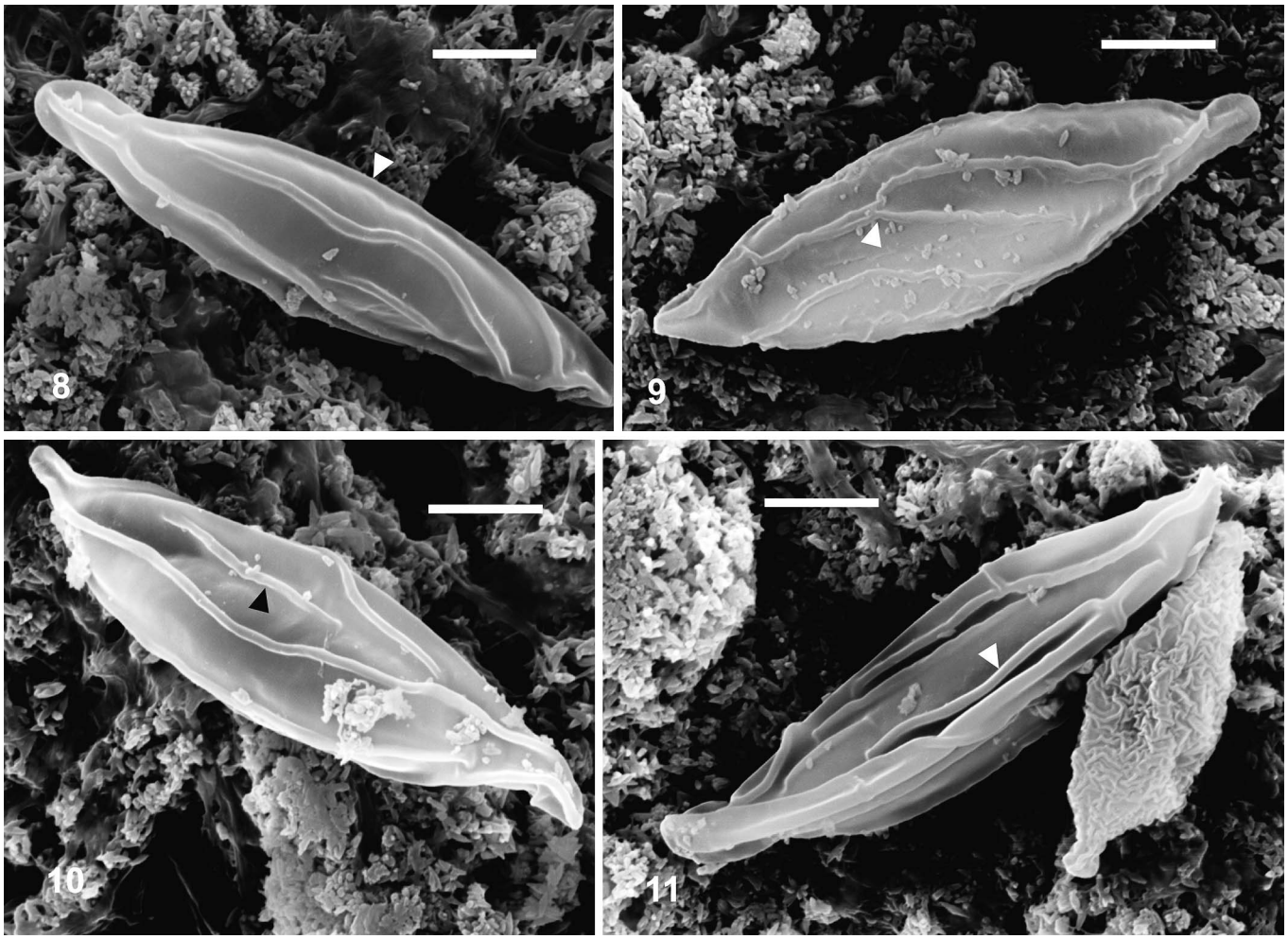
Molecular phylogeny

With the NCBI basic local alignment search tool server, the closest hit for the ITS2 variable marker of *Scotiella cryophila* K-1 was *Chloromonas nivalis* Gassan-B (LC012714, Matsuzaki *et al.* 2015), a snow alga sampled from Japanese mountains. Still, these two field samples were independent taxa because several CBCs in helixes II–III of the ITS2 rDNA secondary structure were present (Fig. 18). For ITS1 of *S. cryophila* K-1 (MG253843), no hits with more than 90% identity were found. In contrast, UTEX SNO47, the type strain of *Chloromonas rosae* var. *psychrophila*, turned out to be genetically identical (ITS1) and nearly identical (ITS2), except for one nucleotide change in the middle part of helix I, to *Chloromonas reticulata* CCCryo 213-05 (= UTEX 1970 = SAG 29.83) (MG253846 vs HQ404885).

In 18S and *rbc*L phylogenies, *Scotiella cryophila* K-1 was placed in a well-supported clade 'B' consisting of several species of *Chloromonas* causing snow discolouration (Figs 19, 20). Its closest relatives were *Chloromonas polyptera* (Fritsch) Hoham, Mullet & Roemer from Antarctica, *Chloromonas nivalis* (Chodat) Hoham & Mullet from the Austrian Alps, *Chloromonas nivalis* subsp. *tatrae* (Kol) Procházková, Remias, Řezanka & Nedbalová from the High Tatras in Slovakia, *Chloromonas nivalis* Gassan-B, uncultured *Chloromonas* sp. TA8 (both from Japan) and an uncultured eukaryote from a periglacial environment at Mount Kilimanjaro in Tanzania. Strain UTEX SNO47 was in the clade 'A1' *sensu* Hoham *et al.* (2002; corresponding to the clade of '*Chloromonas reticulata*' *sensu* Pröschold *et al.* 2001), which was confirmed by 18S rDNA and *rbc*L phylogenetic trees (Figs 19, 20). For *rbc*L, the sequence of strain UTEX SNO47 was identical to Hoham *et al.* (2002 comparison of MG253847 and AF517073). For 18S rDNA, the new sequence of this strain (MG253845) was identical to Hoham *et al.* (2002) except for a single nucleotide gap in the initial sequencing (AF517093), which was not shared in the whole 18S rDNA alignment.



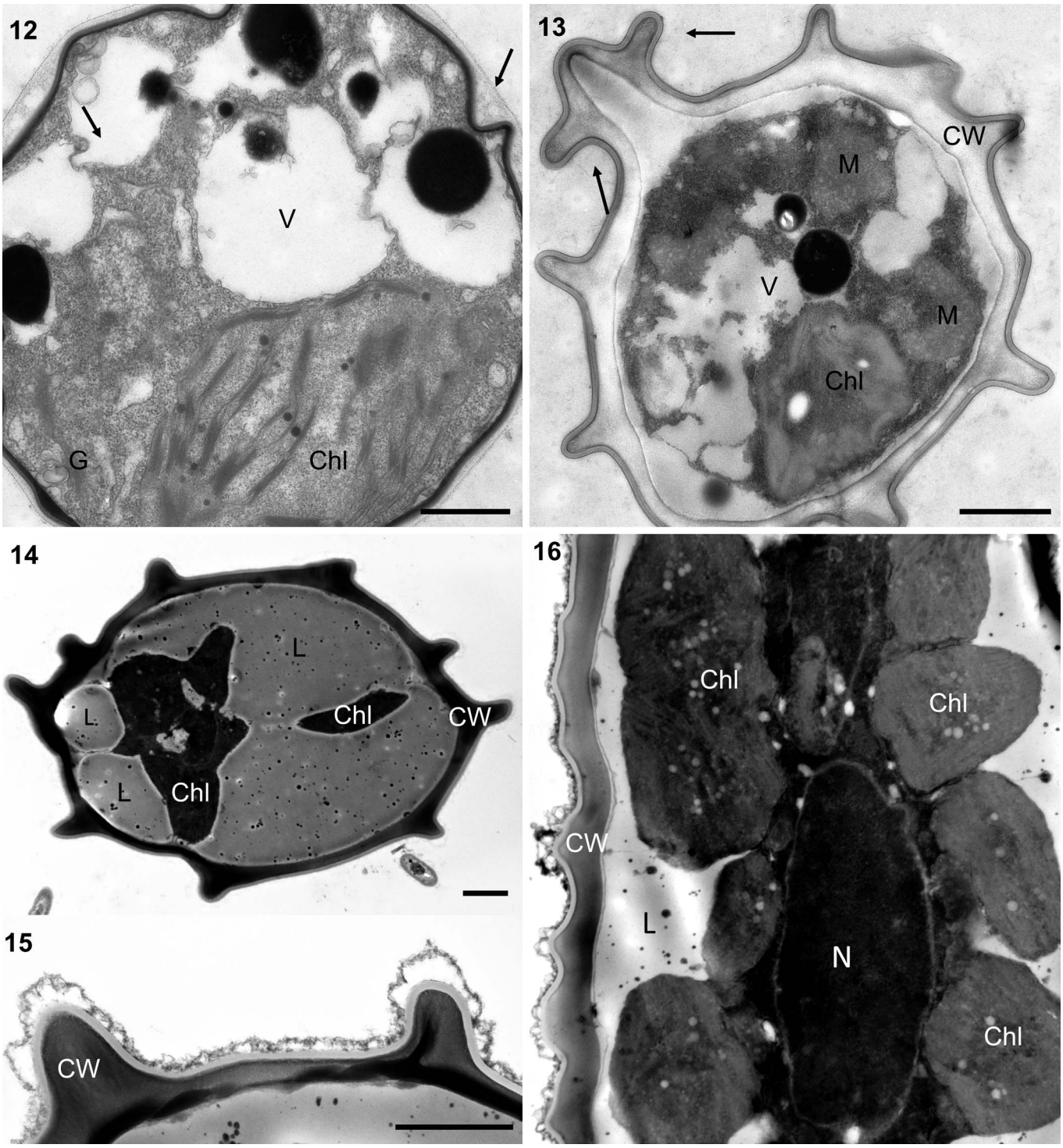
Figs 1–7. Light micrographs of cysts of *Scotiella cryophila* K-1 (sample GK02, if not stated). Scale bars = 10 μm.
Figs 1–2. Young cysts.
Fig. 1. Overview with a group of typical immotile cells with inconspicuous secondary structured cell walls.
Fig. 2. Primary cell wall is shed.
Figs 3–5. A mature cell (WP125) at bright field and fluorescence mode.
Fig. 3. Cell focused at median plane.
Fig. 4. Cell focused on top revealing characteristic longitudinal cell wall ribs.
Fig. 5. Chlorophyll-autofluorescence showing spherical plastids.
Figs 6–7. Old cysts 3 months after sampling.
Fig. 6. Contracted protoplast likely prior to cell division. Note the orange colour caused by secondary carotenoids (WP125).
Fig. 7. Four smooth-walled, oblong daughter cells inside mother cell, three of them visible.



Figs 8–11. Scanning electron micrographs of mature cysts of *Scotiella cryophila* K-1 (WP125). Scale bar = 5 μm.
Fig. 8. A cell wall rib reaching from pole nearly to antapex (arrowhead).
Fig. 9. A bifurcation of one flange into two independent ones is shown (arrowhead).
Fig. 10. An isolated short rib (arrowhead).
Fig. 11. Note the main rib on top, which is composed of at least two twisted ribs (arrowhead); the most prominent rib can have two lateral secondary ribs.

DISCUSSION

Scotiella cryophila sensu Chodat is a rather neglected snow alga, probably because it has been reported less frequently than other cryoflora such as *Chlamydomonas nivalis* (Remias *et al*. 2005) or *Chloromonas brevispina* (Hoham *et al*. 1979). In this study, the sampled snow field consisted of a virtually pure population of cysts. Scattered cysts of *S. cryophila* were found in many snow fields from other regions (Stein & Brooke 1964; Kol 1970; Whitford & Kim 1971). *Scotiella cryophila* cysts associated with *Chloromonas rosae* var. *psychrophila* dominate the green snow packs at Whiteface Mountain, Adirondacks, New York (Hoham *et al*. 2008). The fact that after 8 years the population was found again at more or less the same spot in the Tyrol Alps indicates a local occurrence (site fidelity) and implies that the mature cysts rest directly on the dry rock ground after snow melt, where they may accumulate over years and develop a kind of 'seed bank' for germination during suitable spring conditions. The cell stages present during harvest exhibited the typical morphology of immotile stages of *Chloromonas*, which dominate snow pack for most of the season. Different from many snow algae of the genus *Chloromonas*, *S. cryophila* K-1 likely produces asexual cysts directly out of biflagellate swarmers. This was reported for field material of *C. rosae* var. *psychrophila* by Hoham *et al*. (2002). *Scotiella cryophila* K-1 is distinguished from its close relative *Chloromonas nivalis* in having significantly elongated cysts with length to width ratios from 2.1 to 4.7 (this study) vs 1.4 to 2.1 for more ellipsoidal to ovoid cysts of the latter species (Procházková *et al*. 2018a). Consequently, *S. cryophila* K-1 should not be confused with *C. nivalis*. Also, the other species of cryoflora of this genus have shorter cysts of broad-ellipsoidal shape (e.g. length to width ratios of 1.1–1.8 for *Chloromonas polyptera*; unpublished data, D.R.), or 1.3–1.8 for *Chloromonas nivalis* subsp. *tatrae*, Procházková *et al*. 2018a). Furthermore, the cell wall ribs of other species are more pronounced and clearly visible with LM. The cysts of *S. cryophila* K-1 and associated cryoflora species of *Chloromonas* share morphological and cytological traits: flagellar loss, contractive vacuoles and possibly an eyespot, shedding a non-



Figs 12–16. TEM micrographs of *Scotiella cryophila* K-1. Abbreviations: Chl, chloroplast; CW, cell wall; G, Golgi stacks; M, mitochondrion; N, nucleus; L, lipid; V, vacuole. Scale bar: 1 μm.

Figs 12–13. Transverse section of young cysts (GK02).

Fig. 12. Single compact chloroplast and large vacuoles, primary wall still present (arrows).

Fig. 13. Young electron translucent cell wall ribs and one rib secondary ribbed (arrows).

Figs 14–16. Cross sections of mature cysts with fully developed cell wall ribs (WP125).

Fig. 14. Cytoplasm occupied by several large peripheral lipid bodies.

Fig. 15. Detail of the bi-layered secondary cell wall, the inner layer is electron dense.

Fig. 16. Longitudinal section of a cyst showing a detailed view of the cytoplasm containing several roundish plastids close to the nucleus.

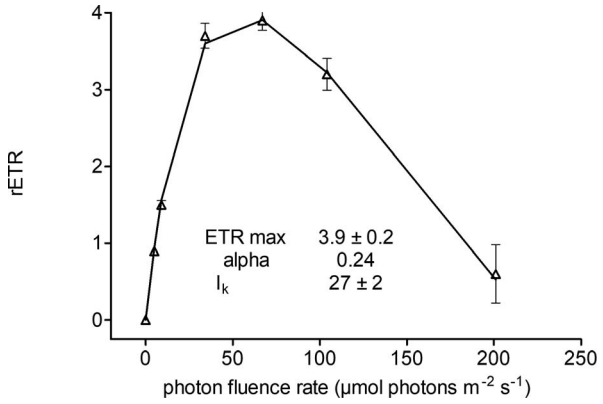


Fig. 17. Effect of increasing photon fluence rates (*x*-axis) on the relative electron transport rate (rETR; *y*-axis) of chloroplasts in non-motile stages of *Scotiella cryophila* K-1 (sample WP125). Values are means of four replicate measurements (± *s*). The data points were fitted to the photoinhibition model of Walsby (1997).

ribbed primary cell wall during maturation, releasing a structured secondary cell wall, having small spherical or discoid plastids instead of a compact chloroplast and accumulation of secondary reddish pigments.

Fluorometric measurement demonstrated that the photosystem II of green cysts of *Scotiella cryophila* K-1 is adapted to low light conditions. The light compensation point could not be acquired by pulse amplitude modulation but since I_k had occurred at a PFD of 27 µmol m^{-2} s^{-1} and photoinhibition started from about 70 µmol photons m^{-2} s^{-1}, it is obvious that these stages must have a positive photosynthetic balance at PFDs between 12 and 40 µmol m^{-2} s^{-1} PAR, which occurred 20 cm below the surface. Hoham *et al.* (2006) reported that slightly higher irradiation of 95 and 115 µmol m^{-2} s^{-1} were optimal for sexual reproduction of the snow algae *Chloromonas tughillensis* and *Chloromonas chenangoensis*, respectively. Furthermore, *Chloromonas rosae* var. *psychrophila* from North America grew at low irradiances (Hoham *et al.* 1998), thus with the same light conditions as the cysts studied here. However, once the cysts reach the snow surface due to ongoing melting, they would be subject to severe light stress if they did not rearrange their photosynthetic apparatus to high irradiance. Alternatively, cells should produce protective carotenoids like astaxanthin. Light adaptations must have taken place in the course of cyst maturation, as indicated by observation of surficial populations of *S. cryophila* from snow fields partly shaded by a

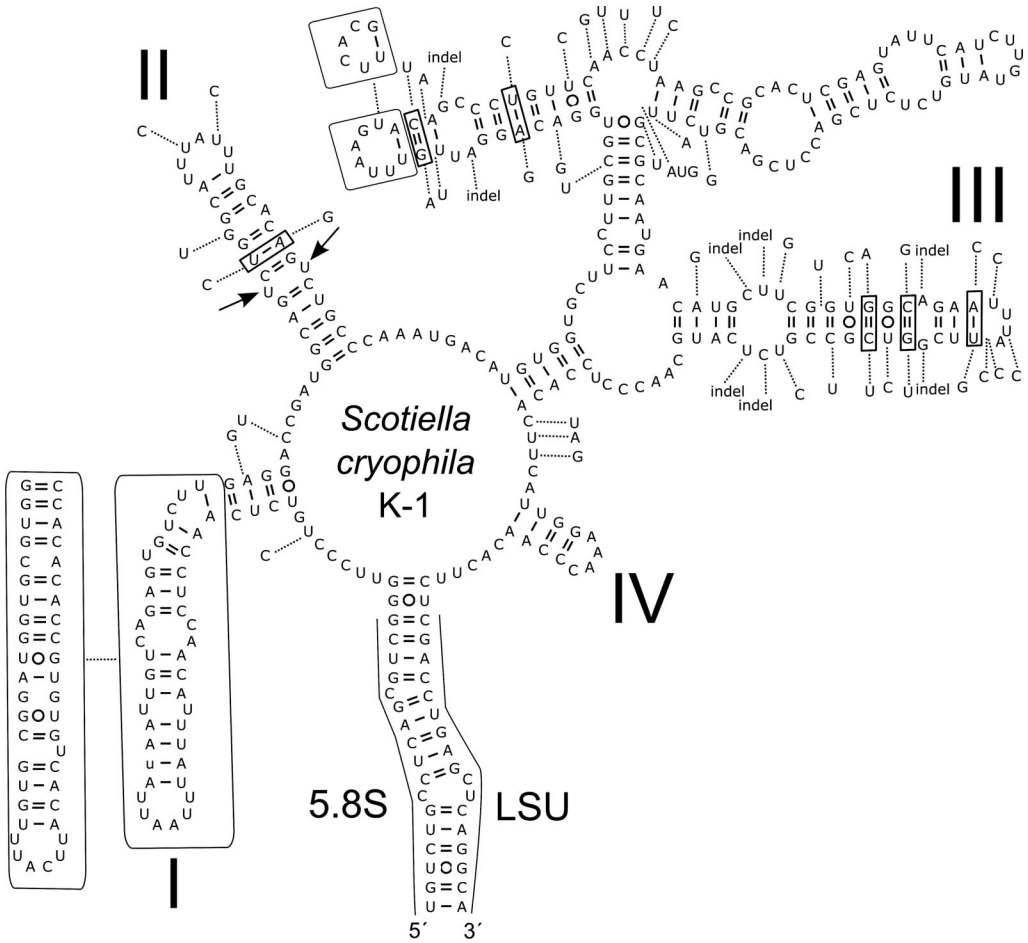


Fig. 18. Comparison of the secondary structure of ITS2 rDNA transcripts between *Scotiella cryophila* K-1 from the Austrian Alps (accession number MG253843, this study) and *Chloromonas nivalis* Gassan-B from Japan (accession number LC012758, Matsuzaki *et al.* 2015). Helices are labelled with Latin numbers. Nucleotide differences of the second species are outside the structure and linked by dotted lines. Compensatory base changes between both algae are indicated by rectangles. Note the U–U mismatch in helix II (arrows).

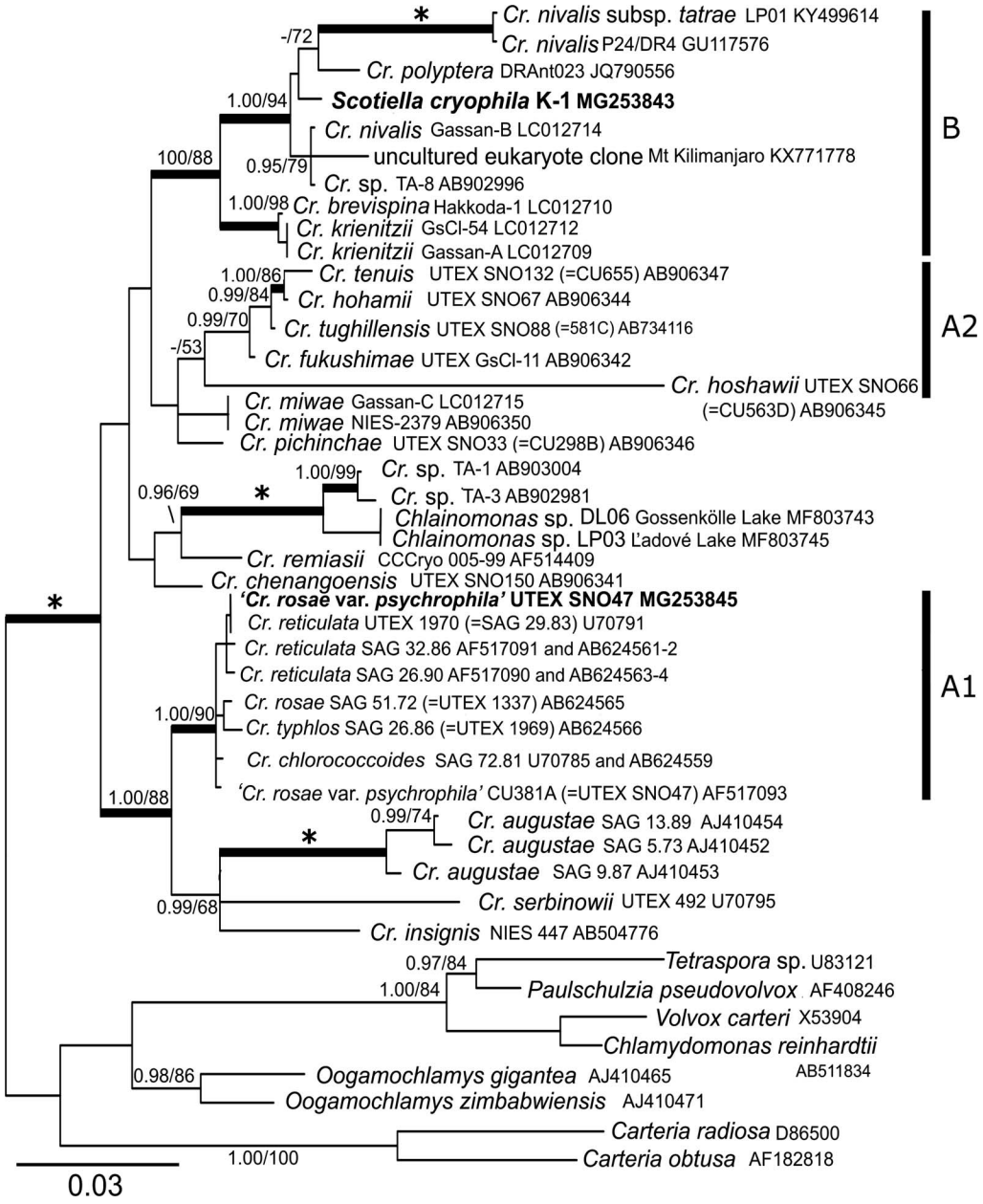


Fig. 19. 18S ribosomal DNA gene-based Bayesian phylogenetic tree of *Chloromonas* focusing on snow-inhabiting species and selected mesophilic relatives. *Cr.* = *Chloromonas*. The labelled clades 'A1' and 'A2' correspond to Hoham *et al.* (2002), clade 'B' is according to Matsuzaki *et al.* (2015). Posterior probabilities (0.95 or more) and bootstrap values from maximum likelihood analyses (50% or more) are shown. Full statistical support (1.00/100) is marked with an asterisk. Thick branches represent nodes receiving the highest posterior probability support (1.00). Newly obtained sequences are in bold. Accession numbers, strain or field sample codes are indicated after each species name.

sparse canopy (Nedbalová *et al.* 2008). The light regime (open exposures) of the Austrian site of cysts that resemble *S. cryophila* is very different from locations of *C. rosae* var. *psychrophila* in North America, which were usually associated with coniferous trees that shade the snow banks (Hoham *et al.* 2008). Nonetheless, microhabitat light conditions seem to be similar, since *S. cryophila* K-1 caused green snow at least 20 cm below the snow surface.

Phylogenetically, all species of *Chloromonas* investigated to date that thrive exclusively in snow are members of the closely related clades 'A2' (Hoham *et al.* 2002) and 'B' (Matsuzaki *et al.* 2015). The type strain of *Chloromonas rosae* var. *psychrophila* (UTEX SNO47) was closely related to *C. rosae* SAG 26.90 (Hoham *et al.* 2002). Additionally, Matsuzaki *et al.* (2012) assigned the latter strain to *Chloromonas reticulata* based on multigene analysis and observations with light and electron microscopy. The absence of any CBCs in the ITS2 secondary structure between UTEX SNO47 and SAG 26.90 indicates that UTEX SNO47 belongs to *C. reticulata*. Generally, a phylogeny based solely on the conservative 18S rDNA marker does not provide sufficient resolution to discriminate between inde-

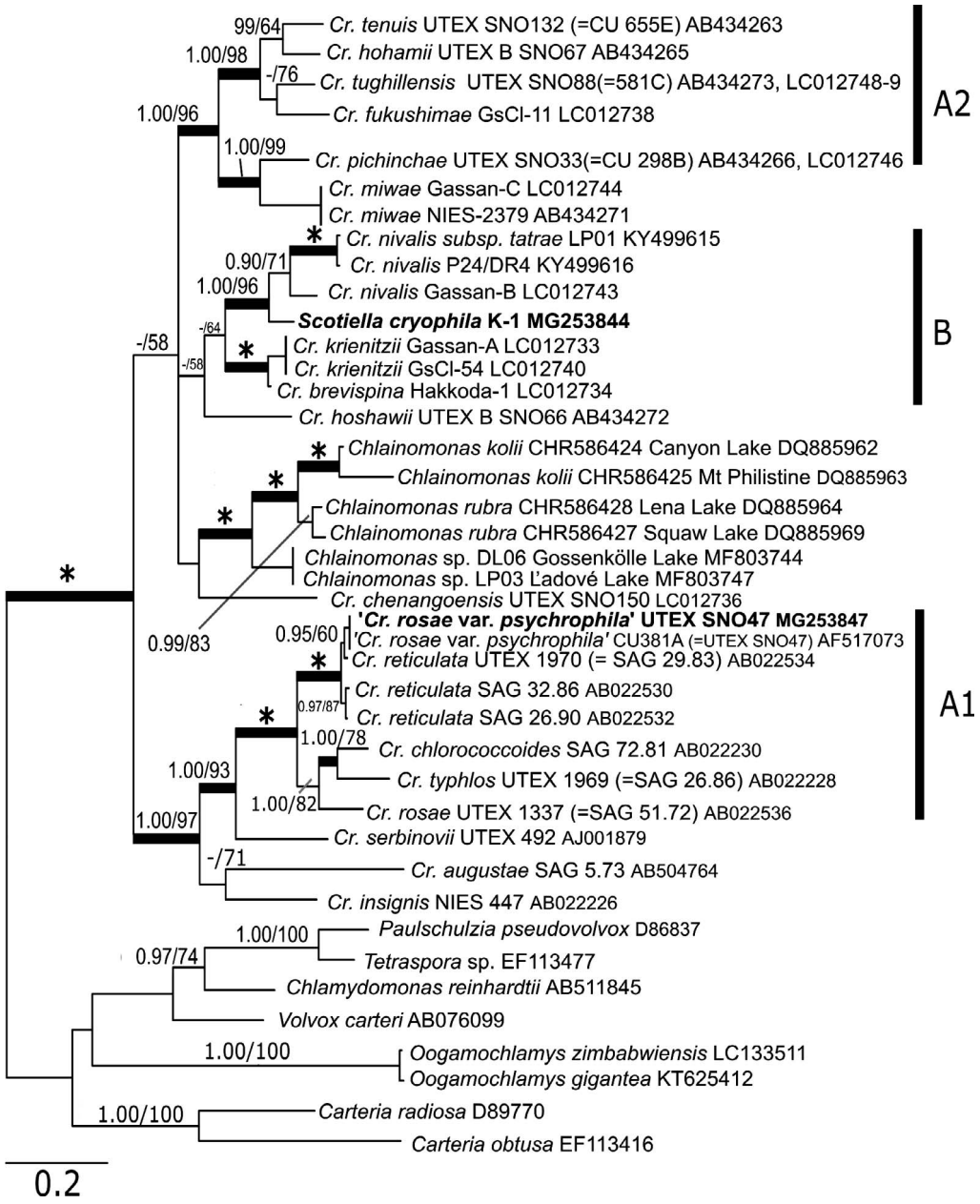


Fig. 20. *rbc*L gene-based Bayesian phylogenetic tree of the genus *Chloromonas* including snow-inhabiting species and mesophilic relatives. *Cr.* = *Chloromonas*. The labelled clades 'A1' and 'A2' correspond to Hoham *et al*. (2002), clade 'B' is according to Matsuzaki *et al*. (2015). Posterior probabilities (0.95 or more) and bootstrap values from maximum likelihood analyses (50% or more) are shown. Full statistical support (1.00/100) is marked with an asterisk. Thick branches represent nodes receiving the highest posterior probability support (1.00). Newly obtained sequences are in bold. Accession numbers, strain or field sample codes are indicated after each species name.

pendent species, as indicated by low bootstrap values of some branches (only posterior probabilities > 95% shown in our phylogenetic trees). For example, *C. polyptera*, *C. nivalis* and *S. cryophila* K-1 seemingly form a monophyletic clade where any subclades are less supported. Nevertheless, an 18S rDNA based phylogeny of snow algae may at least reveal the taxonomic context between related species. The *rbc*L marker verified the topology of the clades. Finally, with the help of the variable ITS2 rDNA marker, lineages of closely related species (e.g. *S. cryophila* K-1 and *C. nivalis* Gassan-B) were successfully resolved.

At the sampling site of *Scotiella cryophila* K-1 in the Tyrolean Alps, the slightly acidic pH and very low electrical conductivity values of meltwater were similar to rainwater and typical of other snow fields in the same region (Remias *et al*. 2010). North American populations of *Chloromonas rosae* var. *psychrophila* occurred in habitats with pH and conductivity values in the same range (Hoham *et al*. 1989, 2007, 2008). The snow water content was, however, lower than in slush on an almost frozen, high alpine lake about 50 m away from the sampling site, where a different cryoflora of *Chlainomonas*

sp. persisted (Procházková *et al*. 2018b). *Scotiella cryophila* K-1 was found in comparably low concentrations, thus causing no striking snow discolourations, or it was reported as an additional component in blooms dominated by other species above the treeline (Remias *et al*. 2005) or at both forested and non-forested slopes (Nedbalová *et al*. 2008). So far, it has not been reported from polar habitats like the well investigated Arctic Archipelago of Svalbard (Kvíderová 2012). Kol (1959) found *S. cryophila* at Cape York in Greenland and described a new and larger variety, *S. cryophila* var. *groenlandica* Kol, with well-developed, straight ribs from pole to pole and cell sizes of 33–42 × 15–18 μm. However, this latter taxon has neither been observed again, nor is a strain available, thus a molecular proof is not possible. Moreover, the drawings of these cells do not closely resemble *S. cryophila* K-1.

Generally, the average cell sizes of mature cysts may reflect how suitable the habitat conditions were in the course of a season (e.g. nutrient and water availability, freeze–thaw events, light regime). As a consequence, we think that the samples of this study are identical to *Scotiella cryophila* initially described by Chodat (1922), though he gave lower dimensions of size ranges from a comparable habitat in the Swiss Alps (12–30 μm × 6–10 μm). Since we found larger than average sizes for old cysts producing daughter cells, further growth during maturation seems to be possible (observed also for North American samples; R. Hoham, pers. comm.), and maybe the smaller sizes reported by Chodat included flagellate-like younger stages, which we did not find. Such stages likely occur very early in the melting season, when the alpine snow becomes waterlogged at the end of March (results found by DR, data not shown). While the putatively sensitive flagellates enable a migration within a waterlogged snow pack to a certain extent, the immotile stages can be regarded as robust. This reflects their thick walled structures, and the carotenoids accumulated in cytoplasmic lipid bodies are powerful antioxidants and absorbers of excessive ultraviolet (UV) and PAR (Remias 2012). Moreover, incorporated secondary metabolites, which were probably reflected by the electron-dense innermost wall layer (Figs 14–15), could also protect against harmful UV irradiation. *S. cryophila* K-1 shares these strategies with other snow algae, e.g. *Chloromonas brevispina* (Hoham *et al*. 1979), *C. nivalis* (Remias *et al*. 2010) and *C. polyptera* (Remias *et al*. 2013).

The morphology of swarmers and the complete life cycle of *Scotiella cryophila* K-1 from Europe remain unknown. Interestingly, morphologically identical cysts in field material from North America (Hoham *et al*. 2002), addressed as *Chloromonas rosae* var. *psychrophila*, had multiple plastids and sizes comparable with those of this study (28–31 μm × 10–18 μm). Conductivity, pH values and light regime of the North American and the Austrian microhabitats of cysts of *S. cryophila* were similar but it remains unresolved if these field cysts from America were genetically identical to *S. cryophila* K-1 from Europe. Accordingly, we found scattered cells of *S. cryophila* recently in Iceland (Snæfjellsjökul, July 2017, DR, pers. obs.), the cell sizes were again in the range of the Austrian and North American samples.

A question that remains is why vegetative cells of '*Chloromonas rosae* var. *psychrophila*' and cysts of *Scotiella cryophila* K-1 are placed in different parts of the phylogenetic tree. An explanation could be that cysts similar to *S. cryophila* K-1 are produced by different species worldwide. This phenomenon was reported by Matsuzaki *et al*. (2015) for *C. nivalis*. Also, for *C. reticulata* (e.g. SAG 29.83, SAG 32.86, SAG 26.90) the morphology of stages of '*Scotiella*' is still unknown. Moreover, no molecular proof was given that the type strain UTEX SNO47 of '*C. rosae* var. *psychrophila*' (or any other strains in the UTEX collection deposited under this taxon) and cysts resembling *S. cryophila* from the Americas are genetically identical, which should be revealed by molecular protocols.

In summary, we characterised the morphology, physiology and phylogenetic position of a snow alga causing monospecific green blooms in the Austrian Alps that most likely represent the previously described *Scotiella cryophila* (Chodat 1922). Other physiological aspects, such as the occurrence of antifreeze agents, accumulation of soluble carbohydrates (osmolytes), presence of ice binding proteins (Raymond 2014) or the fatty acid composition (e.g. Procházková *et al*. 2018a), have not yet been studied.

The biodiversity of cryoflora is still not thoroughly explored. Molecular and life cycle studies including both field blooms and strains isolated from snow are needed to answer general ecological questions. Do a few globally distributed species of Chlamydomonadaceae dominate melting snow fields, or are local taxa with distinct distribution but similar morphology of immotile stages more common? Long distance dispersal strategies of snow algae have hardly been investigated. New techniques like high-throughput-sequencing of several molecular markers (e.g. 18S, ITS2) will be a support in establishing biogeography of snow algae (Lutz *et al*. 2016). In addition, molecular re-investigations of strains from North American sites, which were designated as *Chloromonas rosae* var. *psychrophila*, and field cysts, which are morphologically identical to *Scotiella cryophila* Chodat, are needed. Resampling of the type locality of this latter taxon in western Switzerland could be useful. Similarly, a phylogenetic position of *S. cryophila* var. *groenlandica* is lacking. Mechanisms of physiologic adaptation remain unexplained, such as drastic cellular changes like photo acclimation to low light below the snow surface and high-light exposure at the surface in the course of a few weeks. Finally, what are the mechanisms that cause cyst germination?

ACKNOWLEDGEMENTS

The authors acknowledge grants from the Austrian Science Fund (FWF): P 29959 to DR, I 1951-B16 to AH, Czech Science Foundation (GACR) project 18-02634S to LP and LN. We would like to thank Sabrina Obwegeser (University of Innsbruck) for help in TEM sectioning and image generation (sample GK02). We express sincere thanks to Birgit Sattler (University of Innsbruck, Institute of Ecology, Austria) for providing access to the limnologic field station at Kühtai.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found online at http://dx.doi.org/10.2216/18-45.1.s1.

REFERENCES

Anesio A.M., Lutz S., Chrismas N.A.M. & Benning L.G. 2017. The microbiome of glaciers and ice sheets. *NPJ Biofilms and Microbiomes* 3: 1–11.

Chodat R. 1922. Matériaux pour I'histoire des algues de la Suisse. *Bulletin de la Société Botanique de Genève* 13: 66–114.

Cvetkovska M., Hüner N.P.A. & Smith D.R. 2017. Chilling out: the evolution and diversification of psychrophilic algae with a focus on Chlamydomonadales. *Polar Biology* 40: 1169–1184.

Darty K., Denise A. & Ponty Y. 2009. VARNA: Interactive drawing and editing of the RNA secondary structure. *Bioinformatics* 25: 1974–1975.

Ganey G.Q., Loso M.G., Burgess A.B. & Dial R.J. 2017. The role of microbes in snowmelt and radiative forcing on an Alaskan icefield. *Nature Geoscience* 10: 754–759.

Gorton H.L., Williams W.E. & Vogelmann T.C. 2001. The light environment and cellular optics of the snow alga *Chlamydomonas nivalis* (Bauer) Wille. *Photochemistry and Photobiology* 73: 611–620.

Hoham R.W. & Duval B. 2001. Microbial ecology of snow and freshwater ice with emphasis on snow algae. In: *Snow Ecology: an interdisciplinary examination of snow-covered ecosystems* (Ed. by H.G. Jones, J.W. Pomeroy, D.A. Walker & R.W. Hoham), pp. 168–228. Cambridge University Press, Cambridge, UK.

Hoham R.W. & Mullet J.E. 1977. The life history and ecology of the snow alga *Chloromonas cryophila* sp. nov. (Chlorophyta, Volvocales). *Phycologia* 16: 53–68.

Hoham R.W. & Mullet J.E. 1978. *Chloromonas nivalis* (Chod.) Hoh. & Mull. Comb. Nov., and additional comments on the snow alga, *Scotiella*. *Phycologia* 17: 106–107.

Hoham R.W., Roemer S.C. & Mullet J.E. 1979. The life history and ecology of the snow alga *Chloromonas brevispina* comb. nov. (Chlorophyta, Volvocales). *Phycologia* 18: 55–70.

Hoham R.W., Yatsko C.P., Germain L. & Jones H.G. 1989. Recent discoveries of snow algae in Upstate New York and Quebec Province and preliminary reports on related snow chemistry. In: *Proceedings of the 46th annual eastern snow conference*; 1989 June 8–9; Quebec City (Ed. by J.E. Lewis), pp. 196–200.

Hoham R.W., Schlag E.M., Kang J.Y., Hasselwander A.J., Behrstock A.F., Blackburn I.R., Johnson R.C. & Roemer S.C. 1998. The effects of irradiance levels and spectral composition on mating strategies in the snow alga, *Chloromonas* sp. -D, from the Tughill Plateau, New York State. *Hydrological Processes* 12: 1627–1639.

Hoham R.W., Bonome T.A., Martin C.W. & Leebens-Mack J.H. 2002. A combined 18S rDNA and rbcL phylogenetic analysis of *Chloromonas* and *Chlamydomonas* (Chlorophyceae, Volvocales) emphasizing snow and other cold-temperature habitats. *Journal of Phycology* 38: 1051–1064.

Hoham R.W., Berman J.D., Rogers H.S., Felio J.H., Ryba J.B. & Miller P.R. 2006. Two new species of green snow algae from Upstate New York, *Chloromonas chenangoensis* sp. nov. and *Chloromonas tughillensis* sp. nov. (Volvocales, Chlorophyceae) and the effects of light on their life cycle development. *Phycologia* 45: 319–330.

Hoham R.W., Filbin R.W., Frey F.M., Pusack T.J., Ryba J.B., McDermott P.D. & Fields R.A. 2007. The optimum pH of the green snow algae, *Chloromonas tughillensis* and *Chloromonas chenangoensis*, from Upstate New York. *Arctic, Antarctic, and Alpine Research* 39: 65–73.

Hoham R.W., McCay T.S., Poirier M.B. & Bell T. 2008. Balsam fir leaf litter extract stimulates growth of the green snow alga *Chloromonas rosae* var. *psychrophila* (Chlorophyta, Volvocales) from Whiteface Mountain, New York. *Nova Hedwigia* 86: 133–140.

Kol E. 1959. The red snow of Greenland I. West Greenland. *Acta Botanica Academiae Scientiarum Hungaricae* 5: 57–70.

Kol E. 1968. Kryobiologie. Biologie und Limnologie des Schnees und Eises. I. Kryovegetation In: *Die Binnengewässe* (Ed. by H.J. Elster & W. Ohle), Band XXIV. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart, Germany. 216 pp.

Kol E. 1970. Vom roten Schnee der Tiroler Alpen. *Annales Historico-Naturales Musei Nationalis Hungarici* 62: 129–136.

Komárek J. & Nedbalová L. 2007. Green cryosestic algae. In: *Algae and cyanobacteria in extreme environments* (Ed. by J. Seckbach), pp. 321–342. Springer, Dordrecht, Netherlands.

Kvíderová J. 2012. Research on cryosestic communities in Svalbard: the snow algae of temporary snowfields in Petuniabukta, Central Svalbard. *Czech Polar Reports* 2: 8–19.

Light J.J. & Belcher J.H. 1968. A snow microflora in the Cairngorm Mountains, Scotland. *British Phycological Bulletin* 3: 471–473.

Lukeš M., Procházková L., Shmidt V., Nedbalová L. & Kaftan D. 2014. Temperature dependence of photosynthesis and thylakoid lipid composition in the red snow alga *Chlamydomonas* cf. *nivalis* (Chlorophyceae). *FEMS Microbiology Ecology* 89: 303–315.

Lutz S., Anesio A.M., Raiswell R., Edwards A., Newton R.J., Gill F. & Benning L.G. 2016. The biogeography of red snow microbiomes and their role in melting arctic glaciers. *Nature Communications* 7: 11968.

Matsuzaki R., Hara Y. & Nozaki H. 2012. A taxonomic revision of *Chloromonas reticulata* (Volvocales, Chlorophyceae), the type species of the genus *Chloromonas*, based on multigene phylogeny and comparative light and electron microscopy. *Phycologia* 51: 74–85.

Matsuzaki R., Kawai-Toyooka H., Hara Y. & Nozaki H. 2015. Revisiting the taxonomic significance of aplanozygote morphologies of two cosmopolitan snow species of the genus *Chloromonas* (Volvocales, Chlorophyceae). *Phycologia* 54: 491–502.

Nedbalová L., Kociánová M. & Lukavský J. 2008. Ecology of snow algae in the Giant Mts. *Opera Corcontica* 45: 59–68.

Nedbalová L., Mihál M., Kvíderová J., Procházková L., Řezanka T. & Elster J. 2017. Identity, ecology and ecophysiology of planktic green algae dominating in ice-covered lakes on James Ross Island (northeastern Antarctic Peninsula). *Extremophiles* 21: 187–200.

Posada D. 2008. jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution* 25: 1253–1256.

Procházková L., Remias D., Řezanka T. & Nedbalová L. 2018a. *Chloromonas nivalis* subsp. *tatrae*, subsp. nov. (Chlamydomonadales, Chlorophyta): re–examination of a snow alga from the High Tatra Mountains (Slovakia). *Fottea* 18: 1–18.

Procházková L., Remias D., Holzinger A., Řezanka T. & Nedbalová L. 2018b. Ecophysiological and morphological comparison of two populations of *Chlainomonas* sp. (Chlorophyta) causing red snow on ice-covered lakes in the High Tatras and Austrian Alps. *European Journal of Phycology* 53: 230–243.

Pröschold T., Marin B., Schlösser U.G. & Melkonian M. 2001. Molecular phylogeny and taxonomic revision of (Chlorophyta). I. Emendation of *Chlamydomonas* Ehrenberg and *Chloromonas* Gobi, and description of *Oogamochlamys* gen. nov. and *Lobochlamys* gen. nov. *Protist* 152: 265–300.

Raymond J.A. 2014. The ice-binding proteins of a snow alga, *Chloromonas brevispina*: probable acquisition by horizontal gene transfer. *Extremophiles* 18: 987–994.

Remias D. 2012. Cell structure and physiology of alpine snow and ice algae. In: *Plants in alpine regions. Cell physiology of adaption and survival strategies* (Ed. by C. Lütz), pp. 175–186. Springer, Vienna.

Remias D., Lütz-Meindl U. & Lütz C. 2005. Photosynthesis, pigments and ultrastructure of the alpine snow alga *Chlamydomonas nivalis*. *European Journal of Phycology* 40: 259–268.

Remias D., Karsten U., Lütz C. & Leya T. 2010. Physiological and morphological processes in the Alpine snow alga *Chloromonas nivalis* (Chlorophyceae) during cyst formation. *Protoplasma* 243: 73–86.

Remias D., Wastian H., Lütz C. & Leya T. 2013. Insights into the biology and phylogeny of *Chloromonas polyptera* (Chlorophyta), an alga causing orange snow in Maritime Antarctica. *Antarctic Science* 25: 648–656.

Seibel P.N., Müller T., Dandekar T., Schultz J. & Wolf M. 2006. 4SALE – a tool for synchronous RNA sequence and

secondary structure alignment and editing. *BMC Bioinformatics* 7: 498.

Seibel P.N., Müller T., Dandekar T. & Wolf M. 2008. Synchronous visual analysis and editing of RNA sequence and secondary structure alignments using 4SALE. *BMC Research Notes* 1: 91.

Stein J.R. & Brooke R.C. 1964. Red snow from Mt. Seymour, British Columbia. *Canadian Journal of Botany* 42: 1183–1188.

Walsby A.E. 1997. Modelling the daily integral of photosynthesis by phytoplankton: its dependence on the mean depth of the population. *Hydrobiologia* 349: 65–74.

Whitford L.A. & Kim Y.C. 1971. Algae from alpine areas in Rocky Mountain National Park, Colorado. *The American Midland Naturalist* 85: 425–430.

Received 26 April 2018; accepted 27 April 2018