Bipolar dispersal of red-snow algae

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Red-snow algae are red-pigmented unicellular algae that appear seasonally on the surface of thawing snow worldwide. Here, we analyse the distribution patterns of snow algae sampled from glaciers and snow patches in the Arctic and Antarctica based on nuclear ITS2 sequences, which evolve rapidly. The number of phylotypes is limited in both polar regions, and most are specific to either the Arctic or Antarctica. However, the bipolar phylotypes account for the largest share (37.3%) of all sequences, suggesting that red-algal blooms in polar regions may comprise mainly cosmopolitan phylotypes but also include endemic organisms, which are distributed either in the Arctic or Antarctica.
Red snows are a worldwide phenomenon during the melt season and are caused by blooms of red-pigmented green algae (Chlorophyceae) in thawing snow. Historically, they have been recorded in the daily logs of polar and alpine explorers such as Captain John Ross and Charles Darwin (Fig. 1). The red pigments are carotenoids that serve as antioxidants, as an energy sink, and as a light shield for algal cells exposed to the intense radiation, particularly to the photosynthetically active radiation (PAR), on the snow surface. The algae photosynthetically produce organic matter, which can reduce the snow-surface albedo and accelerate the melt rate, and thus algae have an impact on cryospheric environments.

Several taxa of red-snow algae have been recognized in snow fields worldwide, and most have been identified based on microscopic features of the cells. Spherical red-snow cells have often been identified as *Chlamydomonas cf. nivalis* and can be regarded as a cosmopolitan cryophilic species. On the other hand, several studies have used next-generation sequencing technology to elucidate the geographic distribution pattern of red-snow algae based on molecular data. Recently, red-snow algae collected from different regions of the Arctic were reported to be cosmopolitan based on 18S rRNA gene analysis. This gene has frequently been used for establishing algal taxonomy at the species level. However, the 18S rRNA gene resides within a relatively
slow-evolving region, and therefore we do not feel it is very suitable for elucidating the geographic distribution of algae—especially the interpretation of global distribution—because the resolution of any phylogeographical analysis depends on evolutionary rates of focusing genes.

Here, we analyse the geographical distribution of snow algae on red snows collected from the Arctic and Antarctica, using the sequences of the nuclear rDNA internal transcribed spacer 2 (ITS2) region. ITS2 has a high evolutionary rate and is thus suitable for revealing fine-scale genetic structures. We show that some red-snow algal phylotypes have a bipolar distribution, whereas others are present in only limited areas.

**Results**

**Classification of red-snow algae by 18S rRNA gene and ITS2.** We used high-throughput sequencing and single-cell PCR to analyse the geographical distribution of snow algae that cause red snow at polar glacier surfaces and seasonal snow fields on non-glacierised area (omitting mid-latitude mountainous regions). Our analysis focused on unique sequences (phyotypes) of red-snow algae at 24 red-snow sites in the Arctic and Antarctica (Fig. 1 and Supplementary Table 1). In total, 64,047 unique sequences for algal ITS2 were found in all the red-snow samples, and 348 operational taxonomic units (OTUs) were defined with ≥98% nucleotide sequence identity. In the ITS2 sequence, only 38 of 348 OTUs (accounting for 1.2% of the total sequencing reads) were ≥95% similar to the culturable strains in the NCBI-nt database, indicating that the majority of red-snow algae strains for which an OTU was identified have yet to be successfully cultured. Because information on algal ITS2 sequences is currently limited, it is difficult to conduct taxonomic classification of sequences based on general homology-based search approaches such as the BLAST algorithm using only a public DNA sequence database. Therefore, we established an in-house red-snow ITS2 sequence database (http://redsnow18.paleogenome.jp). For this purpose, the 18S-ITS sequences were obtained from red-snow samples via Sanger sequencing; additionally, some sequences were obtained directly from cells that had been morphologically identified as *C. nivalis* from the red-snow samples via single-cell PCR.

We classified the ITS2 sequences at the species level according to the generic species concept based on secondary structural differences in the ITS2 region, which correlate with the delimitation of biological species.

We confirmed that the predicted ITS2 secondary structures of OTUs have four helices, a U–U mismatch in helix II, and a YGGY motif on the 5′ side near the apex of helix III, all of which are common structural hallmarks of ITS2 in eukaryotes. The ITS2 sequences were classified into 22 subgroups belonging to five chlorophycean and six trebouxiophycean groups based on their secondary structures and BLASTN results (Fig. 2a, Supplementary Figs. 1–21). Among those 22 subgroups, sequences belonged to the *Chlamydomonas*-snow group A (6%), *Chlamydomonas*-snow group B (71%), *Raphidonema* group (16%), and *Chloromonadina*-snow group G (6%). The *Chlamydomonas*-snow group included field-collected samples from Svalbard and the European Alps that had been assigned to *C. nivalis* (Supplementary Fig. 22). In addition, several studies have reported *Chloromonas* in the *Chloromonadina*-snow group as identified from glacier snow samples. The aforementioned four groups accounted for 99% of the total number of sequences analysed, and hence they can be considered the major groups present on red snow in the Arctic and Antarctica (Fig. 2b, Supplementary Tables 2–4).

**Endemic distribution of most red-snow algal phylotypes.** The composition of the snow algal communities was not homogenous across the sample sites, and the predominant groups of snow algae also differed between regions although the Shannon-Wiener diversity index did not differ significantly between regions (*P > 0.05; Supplementary Figs. 23 and 24*). The phylogenetic composition of the algal communities differed significantly among regions, and indeed the genetic structure differed between the Arctic and Antarctic samples (Fig. 2b, Supplementary Figs. 25–27, Supplementary Tables 5 and 6). In addition, the snow algal communities could be correlated with geographical distances between the Arctic and Antarctica. This correlation also suggested that the snow algal population could be distinguished based on geographic location (Supplementary Table 7). The genetic differentiation between the Arctic and Antarctica seemed to be related to the ability to migrate rather than the adaptability of their genotypes to various snow environments. These results suggest the existence of a geographic barrier for algal dispersal between the Arctic and Antarctic environments.

We found that most of the algal OTUs are endemic to a particular polar region. In our analysis of the 22 subgroups based on ITS2 secondary structural differences, 15 subgroups were found to be endemic, i.e., were detected in either the Arctic or Antarctica; these 15 subgroups were of low abundance, however, accounting for only 6.0% of the total sequencing reads. In addition, based on the analysis of the 64,047 ITS2 unique sequences, an average of 55.1% of the unique sequences were endemic to a particular region (Antarctica, 77.9%; Svalbard, 49.9%; Greenland, 21.7%; Alaska, 70.8%), accounting for 21.4% of the total sequencing reads (Fig. 3a, Supplementary Tables 8–10). This result suggests that the major unique sequences are endemic based on their distribution, although they apparently do not contribute in a major way to the total sequencing reads. A previous study based on an 18S rRNA gene analysis concluded that cryospheric algae are commonly found in different regions of the Arctic. However, we found that only 1.9% of unique sequences were commonly distributed for the entire Arctic group (distributed across all Arctic regions but absent from Antarctica; Supplementary Table 11). Our results using ITS2 sequences revealed that approximately one-half of the unique sequences were regionally endemic, suggesting limitations to dispersal (Fig. 3a).
predominated on red snows. For the *Raphidonema* group, certain species inhabit soil environments, and the secondary inhabitants of red snows may be a consequence of having been wind-blown from distant snow surfaces\(^1\). A future analysis of the communities in the environment adjoining red snows (e.g., soils) will be important for understanding the causes underlying the global dispersion of the two groups.

**Discussion**

A current hot topic is whether the biogeographical distribution of microorganisms is global or local. The microbial cosmopolitan dispersion hypothesis of Baas Becking (“Everything is everywhere, but the environment selects”)\(^2\) is often invoked to explain the observed patterns of global algae distribution driven by the capacity for widespread dispersal. Our results suggest that a few cosmopolitan species of green algae dominate red-snow habitats, but indeed much endemism was detected. Our findings underscore the importance of understanding the ecology of snow algae as well as improving the population analyses and taxonomic classification methods that utilize environmental samples. Recent work based on globally sampled 16S and ITS sequence data has demonstrated the spatial distribution patterns of cryosphere microorganisms as well as their regional differentiation and adaptability to environments using glacial cyanobacteria samples acquired from both polar and mid-latitude mountain regions\(^3,4\). To understand the mechanism by which snow algae form geographically specific population structures and how they migrate across the global cryosphere, it will be necessary to study samples from glaciers and snowpacks in mid-latitude mountain ranges such as those in Europe, Asia, and the Americas, where red-snow algae also commonly bloom.

**Methods**

**Samples.** We used red-snow samples collected during the melt season from 24 sites on 13 glaciers and seasonal, non-glacier snow fields in the Arctic (including Greenland, Svalbard, and Alaska) and Antarctica (including Livingston Island, the Riiser-Larsen and Yukidori Valley; Supplementary Table 1). Red-snow samples were collected in sterile 50-mL plastic conical tubes or plastic bags (Whirl-pak, Nasco, USA). Samples were kept frozen during transport to the National Institute of Polar Research (Tokyo, Japan) and then stored at \(-80\) °C before use.

**DNA extraction.** Red-snow samples were melted at 4 °C, and 5–10 mL of each sample was centrifuged at 5000 × g for 10 min to obtain a pellet. The pellets from five replicate samples collected at each site were pooled and used for DNA extraction. Genomic DNA was extracted from each pellet using a FastDNA spin kit for soil (Qbiogene, USA) with a Yasui Kikai (Japan) Multi-beads shocker at 2500
rpm for 30 s. All DNA extractions were conducted on a class 100 clean bench (Sanyo, Japan), and subsequent procedures were carried out on another class 100 clean bench.

18S rRNA–ITS2 long-read strategy by Sanger sequencing. We constructed our in-house red-snow ITS2 sequence database in this study. Because information on algae ITS2 sequences is limited in current public DNA sequence databases, it is difficult to conduct taxonomic classification based on general homology-based search approaches. For constructing the in-house database, contiguous 18S–ITS2 sequences were obtained from red-snow samples by Sanger sequencing. The 18S rRNA–ITS2 region sequences were obtained from red-snow samples collected from three glaciers in Alaska (Harding Icefield, Gulkana glacier, Juneau ice field), one in Svalbard (Austre Brenggerbreen), and one in Antarctica (Risser-Larsen). PCR amplification of the eukaryotic 18S rRNA gene to the ITS2 region was performed using high-fidelity PrimeStar polymerase (TaKaRa, Japan) and primers EUK F (AACCTGGTTGATCCTGCCAGT)21 and ITS4 (TCCTCCGCTTATTGATATGC)22. To reduce PCR artifacts, the number of PCR cycles was kept to a minimum. The PCR conditions were as follows: initial denaturation at 94 °C for 3 min, then 12–20 cycles at 98 °C for 10 s, 57 °C for 15 s, and 72 °C for 4 min, with final extension at 72 °C for 7 min. To minimize PCR drift, three independent reactions were conducted. The pooled PCR products were purified using the MinElute PCR Purification kit (Qiagen, Germany) and cloned using the pCR4Blunt-TOPO with a Zero Blunt TOPO PCR Cloning kit for Sequencing (Life Technologies, USA). Escherichia coli HST08 Premium Competent Cells (TaKaRa) were transformed with the cloning vector. We sequenced approximately 1100 clones in total using Big Dye Terminator 3.1 and an ABI 3130xl automatic sequencer.

Fig. 3 Distribution of the endemic and bipolar cosmopolitan snow algae obtained from each region based on ITS2 unique sequences from high-throughput sequencing. OTUs and sequencing read numbers are shown. a Unique sequences. b Sequencing read numbers of the unique sequences. Bars are coloured according to geographical region of the glacier: blue, Antarctica; red, Svalbard; green, Greenland; yellow, Alaska. c Co-occurrence networks for ITS2 unique sequences of snow algae from both polar regions. Unique sequences are coloured according to geographical region of the glacier, as noted above. The arrow points to the cosmopolitan-distributed phylotypes.
Single-cell PCR and Sanger sequencing analysis. To obtain the sequences from algal cells morphologically identified as C. nivalis from the red-snow samples, we carried out a single-cell PCR approach. Single-cell PCR was used to directly obtain 18S rRNA–ITS2 region sequences from snow algal cells contained in the red-snow samples, which were collected from three glaciers in Alaska (Harding Icefield, Gulkana glacier, Juneau ice field) and one in Svalbard (Austre Braugrøbbergen). Single cells of snow algae that carried red pigments were isolated using a CKX1 microscope (Olympus, Japan) and a micromanipulation system (Eppendorf, Germany) on a class 100 clean bench. Single cells were transferred into 0.2-ml PCR tubes (Eppendorf) and washed twice with distilled water that had been sterilized by UV irradiation, and then each algal cell was transferred into a PCR tube containing a sterilized lysis buffer (10 mM Tris–HCl pH 8.0, 0.1 M EDTA, 0.1% Tween 20 in MilliQ water). Tubes containing algal cells were subjected to three consecutive freeze-thaw cycles to lyse the cell wall and then incubated at 55 °C for 2 h. Each lysate was subjected to PCR amplification of the 18S rRNA–ITS2 region using Ex Taq Hot Start Version (Takara) with the primers P1 (ATCTGGTT GATCTGGCAGTT) and ITS4R2 under the following conditions: initial denaturation at 94 °C for 3 min, followed by 45 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 4 min, with final extension at 72°C for 7 min. Sequencing procedures were the same as described in above. We used more than 20 cells in each sample for single-cell PCR, and we successfully determined the sequences from 10 cells in total.

18S rRNA gene and ITS2 region analysis by Illumina sequencing. Partial 18S rRNA gene sequences and ITS2 sequences were amplified using primers TAR euk454FWD1 (CCAGCASCYGCGGTAATTCC) and TAPeukREV3 (GATCCTGCCAGT) and ITS422 under the following conditions: initial denaturation at 94 °C for 3 min, followed by 45 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 4 min, with final extension at 72°C for 7 min. The raw Illumina sequence datasets have been submitted to the NCBI database. Based on the BLASTN search with the top-hit E-value <1e-5, identity >90%, and alignment length >200 bp against (i) the UNITE fungal ITS2 sequence database35, (ii) Viridiplantae ITS2 sequences obtained from NCBI, and (iii) an in-house red-snow ITS2 sequence database. Taxonomic assignments of unique 18S rRNA gene sequences were conducted by a BLASTN search with a top-hit E-value <1e-5, identity >90%, and alignment length >150 bp against the SILVA SSU Ref NR99 sequence database (version 123.36 extended with additional 4 sequences of cryptophytic alga, Chloromonas nivalis (AF514409), Chloromonas tughiilensis (AB006348), Raphidonema nivalis (AF484877), and Raphidonema sempervirens (AF414410)).

Groups based on the secondary structure of the ITS2. For estimating the diversity of microalgae within the red-snow samples, we classified the ITS2 sequences with the MiSeq analysis to exclude the generic species concept based on structural differences in ITS233. Hereafter, we define these unique sequences as “phylootypes” and 98% sequence clusters from unique ITS2 sequences as “OTUs”. The 5.8S-28S rRNA interaction region within the sequence of each OTU (Supplementary Figs. 3–21) was annotated using the web server 2,6,8-interactive domain (http://redsnow18.paleogenome.jp). We merged the forward and reverse MiSeq reads into single reads and used these as input for subsequent analyses. We then discarded the reads that (i) contained ambiguous overhang adaptor sequences attached to their 5′ ends, (ii) contained primer sequences, (iii) were discarded as burn-in. The average standard deviation of split frequencies first 25% of trees were discarded as burn-in. The hierarchical clustering of samples, as by calculating the Bray-Curtis dissimilarity index of unique and OTUs of ITS2 that were not assigned to algae were discarded. The hierarchical clustering of samples, as by calculating the Bray-Curtis dissimilarity index of unique and OTUs of ITS2 that were not assigned to algae were discarded. The hierarchical clustering of samples, as by calculating the Bray-Curtis dissimilarity index of unique and OTUs of ITS2 that were not assigned to algae were discarded. The hierarchical clustering of samples, as by calculating the Bray-Curtis dissimilarity index of unique and OTUs of ITS2 that were not assigned to algae were discarded. The hierarchical clustering of samples, as by calculating the Bray-Curtis dissimilarity index of unique and OTUs of ITS2 that were not assigned to algae were discarded.

Phylogenetic analysis. Representative sequences of the 18S rRNA gene and ITS2 sequences were aligned using MAFFT37. These alignments were carefully inspected by eye, and all ambiguous sites and sequences were manually deleted. Maximum likelihood trees were reconstructed using RAxML v.8.2.1038 with the GTR+Γ model for each of the 18S rDNA and ITS2 datasets. To evaluate the confidence of the internal nodes, the bootstrapped method was completed with 1000 replications using the rapid bootstrap algorithm42. In addition, the Bayesian phylogenetic analysis for the ITS2 dataset was carried out using MrBayes 3.2.643 with the GTR+Γ model. Two runs of four chains of Markov chain Monte Carlo iterations were performed for 1,000,000 generations, and the first 25% of trees were discarded as burn-in. The average standard deviation of split frequencies between the two runs of Markov chain Monte Carlo iterations was below 0.01, indicating convergence.

Data availability. The Illumina sequence datasets have been submitted to the DDBJ Sequence Read Archive under accession number DRA006819. The
nucleotide sequences have been deposited in DDBJ/EMBL/GenBank under the accession numbers LC371160 to LC371143, and LC381735 to LC381756. The in-house red snow ITS2 sequence database and the nucleotide alignments of 98% sequence clustering of unique ITS2 sequences are available from http://redsnow018.paleogenome.jp/. Other relevant data supporting the findings of the study are available in this article and its Supplementary Information files, or from the corresponding author upon request.

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Author contributions
T.S., R.M., H.M., and T.Y. designed the project. T.S. conceived the hypothesis. N.T., T.S., S.S. and F.N. collected snow samples and managed the glacier expedition. A.A. and T.S. supplied the sequences. H.M., R.M., and T.S. analysed the sequencing data. T.S., T.N.,
R.M., H.M., F.N., and T.Y. wrote the manuscript. All authors gave final approval for publication.

**Additional information**

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