Phylogeny and biogeography of an uncultured clade of snow chytrids

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Summary
Numerous studies have shown that snow can contain a diverse array of algae known as ‘snow algae’. Some reports also indicate that parasites of algae (e.g. chytrids) are also found in snow, but efforts to phylogenetically identify ‘snow chytrids’ have not been successful. We used culture-independent molecular approaches to phylogenetically identify chytrids that are common in long-lived snowpacks of Colorado and Europe. The most remarkable finding of the present study was the discovery of a new clade of chytrids that has representatives in snowpacks of Colorado and Switzerland and cold sites in Nepal and France, but no representatives from warmer ecosystems. This new clade (‘Snow Clade 1’ or SC1) is as deeply divergent as its sister clade, the Lobulomycetales, and phylotypes of SC1 show significant ($P < 0.003$) genetic-isolation by geographic distance patterns, perhaps indicating a long evolutionary history in the cryosphere. In addition to SC1, other snow chytrids were phylogenetically shown to be in the order Rhizophydiales, a group with known algal parasites and saprotrophs. We suggest that these newly discovered snow chytrids are important components of snow ecosystems where they contribute to snow food-web dynamics and the release of nutrients due to their parasitic and saprotrophic activities.

Introduction
Approximately 17% of the world’s population relies on snow melt as a source of freshwater for both agriculture and human consumption (Barnett et al., 2005; Bales et al., 2006). Atmospheric deposition of nutrients can alter biogeochemical processes of high-elevation water systems (Ballantyne et al., 2010), affect water quality from snowmelt (Brooks and Williams, 1999; Mladenov et al., 2011) and could potentially increase the activity of microorganisms within the snowpack. Therefore, identifying the key members of snow microbial communities of high-elevation ecosystems is needed in order to understand nutrient release within the snowpack and its influence on downstream water quality (Mladenov et al., 2012). However, studies of the identities and dynamics of non-algal microbial communities in snow are in their infancy, especially with regard to their influence on nutrient turnover and food-web dynamics within the snow.

Most previous work on snow microbiology has focused on bacteria (Segawa et al., 2005; Liu et al., 2009) and snow algae that are common inhabitants of late melting snowpacks worldwide (Hoham and Duval, 2001; Remias et al., 2010); but little attention has been paid to the other eukaryotic organisms that inhabit snow (Hoham and Duval, 2001). There are several older reports of the presence of zoosporic fungi (chytrids) in high-elevation snowpacks of the Colorado Front Range (Stein and Amundsen, 1967) and Europe (Kol, 1968). Some chytrids are known parasites of algae and have been shown to affect rates of turnover and alter food-web dynamics in aquatic ecosystems (Canter and Lund, 1948; Ibelings et al., 2004; Kagami et al., 2007; Grami et al., 2011; Sime-Ngando et al., 2011). Therefore, the activity of chytrids in high-elevation snowpacks could lead to increased nutrient release from snow, but to date their phylogenetic identity has not been determined. Therefore, the present study was undertaken to determine if ‘snow chytrids’ could be identified using molecular, culture-independent approaches. Such methods have been used successfully to discover major, previously unidentified fungal lineages in the cryosphere (Schadt et al., 2003; Porter et al., 2008); groups that were only much later obtained in pure culture (Rosling et al., 2011). The present study is a first step in efforts to characterize the diversity and function of chytrids in high-elevation snowpacks.

Microbial diversity within the snow may also be contributing to the unexpectedly high diversity of chytrids and other microbes in alpine soils. Recent work in the Colorado Rockies has shown that soils that lie beneath persistent snow beds (‘snow bed’ soils) in high-elevation...
catchments contain very diverse microbial communities that are capable of rapid cycling of carbon and nitrogen (Ley et al., 2004; Freeman et al., 2009a; King et al., 2010). These snow bed soils are covered with snow for up to 10 months of the year, and are dominated by many groups of previously undescribed chytrids (Freeman et al., 2009b). It is possible that some of the chytrids found in high-elevation soils are actually snow chytrids that only spend part of their life cycle in the soil. These putative snow chytrids may swim up into the snowpack to feed on snow algae (and/or aeolian deposited pollen) and then settle back into the soil during the final stages of snow melt. However, given that no snow chytrids have ever been cultured, almost nothing is known about the their phylogeny or function.

The present study was part of a larger project to describe the diversity and function of the microbial community of high-elevation snowpacks of the Front Range of Colorado and to compare them with snowpack organisms worldwide. Our results revealed that chytrids are among the dominant eukaryotic organisms inhabiting some high-elevation snowpacks and that they constitute several new clades that are currently uncultured and likely restricted to snow and snow-covered environments worldwide.

**Results**

Long-read sequences (~1750 bp) that could be unambiguously identified as chytrids were found on most dates that snow samples were taken in 2011 and 2012 (Table 1). On some dates chytrids dominated the clone libraries. For example, on 15 August 2011, chytrids constituted 75% of surface (0–2 cm) snow sequences and 27% of sequences from deeper depths (Table 1).

Phylogenetic analyses demonstrated that most of the chytrids within high-elevation snowpacks are unrelated to known or cultured chytrids (Figs 1 and 3) and in several cases form large clades that have not been previously recognized. The largest of these groups forms a deeply divergent clade sharing a common ancestor with the order Lobulomycetales (Fig. 1). A phylogenetic comparison with all of the closest relatives (from the SILVA 108 SSU database and GenBank) of these snow sequences revealed that this newly discovered clade contains only sequences from cold environments across the Earth. That is, the only other sequences that fell into this clade were from high-elevation soils in Nepal and Colorado (Freeman et al., 2009b), a mountain lake in France (Lefèvre et al., 2008) and high-elevation snow samples from Switzerland (M. Yuhana, unpublished, GenBank numbers AJ867629 and AJ867630), in addition to snow and ice samples from the present study (Fig. 1). Given the apparent global distribution of this clade in high-elevation ecosystems (Fig. 1) we conducted a phylo-geographic analysis of their distribution using previously described methods (Darcy et al., 2011; Schmidt et al., 2011). These analyses reveal a clear pattern of genetic isolation by geographic distance (Fig. 2, Mantel Test \( P < 0.003 \)).

The other snow chytrids from the present study fell into the order Rhizophydiales (Fig. 3). Unlike ‘Snow Clade 1’ (SC1) described above, these chytrids were more closely related to known chytrids but still formed several new sub-clades within the Rhizophydiales, including ‘Snow Clade 2’ (Fig. 3).

In order to quantify possible chytrid food sources in the snowpack, both algae and pollen were counted in some of the same samples in which snow chytrids were discovered. These counts revealed very high densities of both snow algae and pollen in the snow (Fig. 4) with significantly higher concentrations of algae and pollen in the top 2 cm of the snowpack (\( P < 0.05 \)).

**Discussion**

Numerous studies have reported chytrids from cold ecosystems such as high-arctic tundra soils (Booth and

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**Table 1.** Sampling dates, site descriptions and percent of chytrid sequences in eukaryotic clone libraries of the new sequences used in the present study.

<table>
<thead>
<tr>
<th>Date</th>
<th>Location</th>
<th>Elevation (m)</th>
<th>Depth (cm)</th>
<th>% Chytrids</th>
<th>Coordinates</th>
</tr>
</thead>
</table>
| 26 July 2011 | Treeline snow     | 3384          | 0–2        | 25         | 40°02'56.64"N   
|            |                   |               |            |            | -105°34'53.30"W  |
| 15 Aug. 2011 | Talus snow        | 3742          | 0–2        | 75         | 40°03'27.25"N   
|            |                   |               |            |            | -105°37'16.76"W  |
| 12 July 2012 | Talus snow        | 3742          | 4–11       | 26         | 40°02'59.61"N   
|            | Glacier snow/ice  | 3792          | 0–2        | 30         | -105°38'27.91"W  |
| 23 July 2012 | Glacier snow/ice  | 3795          | 0–2        | 13         | 40°02'59.61"N   
|            | Glacier l snow/ice| 3795          | 0–2        | 13         | -105°38'27.91"W  |
| 22 Oct. 2009 | Glacier snow/ice  | 3795          | 0–2        | 33         | 28°38'46.86"N   
|            | Nepal Snowbed soil| 5516          | 0–4        | 80         | 83°56'24.10"E    |

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Barrett, 1976; Barr, 1980) and high-mountain lakes (Koob, 1966). More recent studies of extreme plant-free soils of Antarctica, Colorado and Nepal revealed that chytrids dominate fungal communities in wetter soils and soils under persistent snowpacks ('snow bed soils') (Bridge and Newsham, 2009; Freeman et al., 2009b; Schmidt et al., 2012). These surprising observations led us to hypothesize that some chytrids from polar and alpine regions may spend at least part of their life cycle in the snow. This hypothesis is supported by microscopic observations of chytrid zoospores and sporangia associated with snow algae communities (Stein and Amundsen, 1967; Kol, 1968; C.S. Naff, unpubl. data, 2011) and the phylogenetic results of the present study. We found several unique clades of chytrids (Figs 1 and 3) that were detected not only in surface layers of the snow (0–2 cm deep) but also deeper layers (4–11 cm) in the same samples that contained high populations of snow algae and an abundance of pollen (Fig. 4). Sequences falling into ‘Snow Clade 1’ (SC1) were also found in a culture-independent study of high-elevation snow samples from Switzerland (M. Yuhana, unpubl. data, Fig. 1), further supporting the hypothesis that there exists a unique clade of snow chytrids.

SC1 also encompasses some chytrid sequences that are not exclusively found in snow (Fig. 1) but could have originated from snow. These non-snow sequences were obtained from culture-independent studies of soils that are covered by deep snowpacks for most of the year ('snow bed soils'), and a mountain lake in France that is fed by snow melt (Fig. 1). In addition, the soil sequences encompassed by this clade are from soils (Freeman et al., 2009b) under the Colorado snow banks studied in the present study or from high-elevation snow-covered soils (> 5500 m above sea level) studied by Schmidt and colleagues (2011) in Nepal. These high-elevation soil chytrids were previously referred to as the ‘Unknown 2’ clade by Schmidt and colleagues (2012), and based on their proximity to deep snow banks they could easily be snow chytrids. Likewise, the only other sequences from the SILVA 108 SSU database or GenBank that fall into SC1 (Fig. 1) correspond exactly to...
‘Novel Clade 1’ from culture-independent studies of a snow-fed mountain lake (Lefèvre et al., 2008; Jobard et al., 2012). Thus all of the sequences in Fig. 1 could have originated in snow, but more work must be done to verify this hypothesis. A phylotype (MPE2-22) whose closest relative is a member of SC1 (GQ995409) was also recently sequenced from a ‘moss pilliar’ in an Antarctic lake (Nakai et al., 2012).

Regardless of the actual ecological niches (snow/ice vs near-snow environments) of the members of SC1, it is still quite remarkable that all of the currently known sequences in this clade are from cold environments. This observation demonstrates that we are only just beginning to scratch the surface of the hidden biodiversity of the terrestrial cryosphere (Costello and Schmidt, 2006; Fell et al., 2006; Dennis et al., 2012; Schmidt et al., 2009; Jumpponen et al., 2012; Lynch et al., 2012). In addition, the presence of this new clade only in cold environments should inform future attempts (cf. Carreiro and Koske, 1992) to isolate chytrids from this group, but to date no organisms from this group have been isolated in pure culture.

All of the other snow chytrids found in the present study fall into the chytrid order Rhizophydiales. Phylotypes in the Rhizophydiales were found at the highest elevation Snow/Ice site in Colorado (Arikaree Glacier), the lower elevation snow sites at treeline, and in soils from beneath talus snow beds (Fig. 3). Some of these novel phylotypes were previously detected in high-elevation talus soils and were called unknown clades C15 through C19 by Freeman and colleagues (2009b). Phylogenetic relatives of clades C15 and C19 have recently been isolated from high-elevation (> 5000 m) sites in the Himalayas (Freeman et al., 2009b; Schmidt et al., 2011), but no work has been done to determine if these Himalayan fungi originated in the snow. Indeed, very little is known about the ecology of the Rhizophydiales (Gleason et al., 2010), but some members of this group from warmer areas can utilize pollen (Rhizophydiuim sphero-otheca) and algae (Rhizophydiales fragilaria) for growth (Powell, 1993; Letcher et al., 2008; Letcher and Powell, 2012) and therefore we are attempting to isolate these unknown clades of Rhizophydiales from snow samples by using pollen and snow algae as bait.

Our working hypothesis is that snow chytrids have a complex life cycle that includes an active phase when they feed on abundant algae and/or pollen in the snowpack and a quiescent phase where they lie dormant in the soil awaiting sufficient snow accumulation to become active. This hypothesis is consistent with the fact that phylotypes of some of these chytrids are detected in both snow and soil samples from high elevations and with the fact that the wettest time of year in the high alpine of many mountain ranges is during spring snow melt (Ley et al., 2004; Schmidt et al., 2011). It is also a possibility that these chytrids are not even native to high-elevation sites but rather are being dispersed over long distances (through the atmosphere) to polar and alpine environments as has been shown recently for snow-dwelling bacteria in the genus Polaromonas (Darcy et al., 2011). Polaromonas spp. are ubiquitous in high-elevation snow and ice but show remarkably little genetic diversity even when comparing very geographically distant sites. To determine if this signal of global dispersal is also evident in our genetic data for snow chytrids, we carried out isolation by distance analyses (Darcy et al., 2011; Schmidt et al., 2011). In contrast to results for globally dispersed Polaromonas spp., SC1 shows very strong genetic divergence across all geographic distances (Fig. 2). A similar pattern of extreme genetic isolation has recently been demonstrated for other polar and alpine micro-eukaryotes such as rotifers (Robeson et al., 2011) and algae (De Wever et al., 2009; Schmidt et al., 2011).
We propose two hypotheses that may explain the dispersal pattern of the snow chytrids; First, snow chytrids may be readily dispersed and their survival depends on the ability to adapt to different environmental pressures. Under this hypothesis, the spatial separation of clades within the Snow Chytrids is not due to dispersal limitation, but instead subclades are adapted to specific environmental conditions. Factors such as food sources (type and abundance), microclimate and snowpack depth and duration may be sufficiently different between these sites to permit or bar the presence of specific members of SC1. Further investigation of the microbial communities present at the various sites mentioned in this paper is needed in order to elucidate the variables and to determine if they may be limiting factors for colonization. An alternative hypothesis is that Snow Chytrids are structured by dispersal limitation suggesting that they have been evolving in isolation in each environment. It is possible that members of SC1 are psychrophilic, and are geographically restricted to cold 'islands' in the cryosphere. Dispersal between islands would be difficult for such organisms, since passing through more temperate regions may prove fatal for propagules. Because members of SC1 have yet

Fig. 3. Phylogenetic tree showing the relationship of snow chytrid phylotypes (in the Rhizophydiales) to their closest relatives from GenBank. Asterisks denote nodes with a minimum of 75% bootstrap support and 65% posterior probabilities. ‘Snow Treeline (Colo)’ sequences are from snow at the forest tundra ecotone as described by Brooks and colleagues (1996); ‘Snow/Ice (Colo)’ are from the Arikaree Glacier site (King et al., 2010); ‘Alpine Lake (France)’ are from Lake Pavin in France (Lefèvre et al., 2008); ‘Snowbed Soil (Nepal)’ sequences are from high-elevation soil samples from the Himalayas (Schmidt et al., 2011; 2012).

Fig. 4. The concentration of snow algae and pollen in surface (0–2 cm) and below surface (4–11 cm) snow samples collected on 15 August 2011. There were significantly higher concentrations of algae and pollen within the top 2 cm of snow compared with the concentrations found between 4 and 11 cm (P < 0.05).
to be isolated in culture, it remains to be seen whether they employ the same quiescence strategies as some other chytrids (Gleason et al., 2004; 2010). Dormancy mechanisms are strong enablers of dispersal capacity (Lennon and Jones, 2011), and the ability to form a persistent state may be expected of organisms living in environments with high exposure to ultraviolet radiation, such as snowfields. Common algae living in the same snowfields (e.g. Chlamydomonas nivalis) contain photoprotective pigments (astaxanthin) and encyst to form persistent states (Hoham and Duval, 2001). Since members of the SC1 have been found in the same locations, it is likely that they too have the capability to encyst or otherwise form hardy states.

Finally, it must be noted that all of the clades containing putative snow chytrids show deep phylogenetic divergence, rivaling that of order-level groups such as the Lobulomycetales (Simmons et al., 2009). This divergence is readily apparent in Figs 1–3 despite the fact that no snow chytrids are presently in culture. Culture-independent studies are valid for demonstrating deep levels of phylogenetic structure and in fact have led to important discoveries in fungal phylogenetics (e.g. Porter et al., 2008; Nilsson et al., 2011). The deep level of divergence observed for snow chytrids likely indicates a long history of evolution in the cryosphere, perhaps even during periods of extreme greenhouse events, when chytrids and other micro-eukaryotes may have persisted in high-mountain and high latitude refugia (Stoeck et al., 2007; De Wever et al., 2009). However, much more data are needed from more snow-covered environments to further address questions about the evolution and biogeography of SC1 and other novel groups discovered in the present study.

Conclusions

Overall our results indicate that there are several deeply divergent, novel clades of chytrids present in late-season snowpacks of the Colorado Front Range. From an ecological perspective, these findings are not surprising because it has been known for a long time that microbial life can thrive in any environment where free water is even minimally available. More work is needed to understand the ecological interactions within the snowpack, but just the presence of chytrids in the snow indicates that turnover of algal and other substrates (e.g. pollen) is likely taking place within the snowpack – resulting in the release of nutrients.

From an evolutionary perspective, our results indicate that novel phylotypes of chytrids exist at each of the geographically isolated sites studied. Whether this is a result of genetic drift, selective pressures or a combination of both, remains to be further elucidated. At present it appears that snow chytrids dispersed globally, probably during a colder epoch, and are now continuing to evolve in isolated pockets of the cryosphere. It remains to be seen if these denizens of the cold will survive future increases in global temperatures.

Experimental procedures

Sample collection

Snow samples were collected at sites on Niwot Ridge and in the Green Lakes Valley of the Front Range of Colorado where intensive biogeochemical and microbiological work has been done in the past (Ley et al., 2004; Freeman et al., 2009a; b; King et al., 2010; Mladenov et al., 2012). Most of the present study was done in the vicinity of the talus sites described by Ley and colleagues (2004; 40°03’ 34”N; 105°37’ 0” W; 3739 m) and the high-elevation sites on and next to Arikaree Glacier (King et al., 2010; 40°02’ 57”N; 105°38’ 25” W; 3810 m). These sites are located above the tundra zone (West et al., 1999) and are mostly devoid of vegetation. One sample (26 July 2011) was taken below the tundra near the tree-line site described by Brooks and colleagues (1996). All samples were collected from red and green snow patches during the summer of 2011 and 2012 (on 26 July, 4 and 15 August, and 19 September 2011; and 12 and 23 July 2012). Samples were taken earlier in 2012 than in 2011 because of the unusually early melt out of the snowpack in 2012. Snow samples were taken aseptically from the top 2 cm of snowpack and from 4 to 11 cm below the surface of the snowpack. Snow samples were collected in 50 ml sterile Falcon tubes (BD Biosciences, Bedford, MA, USA), placed on ice in a cooler and returned to the lab where they were frozen at −70°C until utilized.

DNA extraction, PCR amplification and 18S gene clone library construction

A MO BIO Power Water kit with filters (MO BIO Laboratories, Carlsbad, CA, USA) was used to extract DNA from the snow following the manufacturer’s protocol. PCR was carried out using the AmpliTaq Gold 360 Master Mix (Applied Biosystems, Carlsbad, CA, USA) and the 18S primer set 4Fa-short (5’TTCGGGGTATCCTGC-3’) and 1492R (5′-GGTTCAGTTAGACCTT-3’) to amplify small subunit ribosomal DNA. These primers were used by Freeman and colleagues (2009b) who showed that they were not biased towards chytrids compared with other common primers for eukaryotes. Thermal cycling reactions were run for 34 cycles with an annealing temperature of 49°C. DNA amplification was confirmed using gel electrophoresis. Isolated bands of DNA were excised and purified using the QiAquick Gel Extraction Kit (Qiagen, Valencia, CA) and were then ligated into pCR®4-TOPO vectors (TOPO TA Cloning kit, Invitrogen, Carlsbad, CA, USA) and transformed into TOP10 competent Escherichia coli cells following the manufacturers protocol. Successfully transformed cells were grown on Luria Broth agar (MO BIO) with 50 µg ml⁻¹ of ampicillin, and were inoculated into 96-well plates containing 1.5 ml of Luria Broth (MO BIO). They were incubated at 37°C while shaken at

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200 r.p.m. for 16 h and then centrifuged. The pellets were sent to Functional Biosciences (Madison, WI, USA) where the plasmids were bidirectionally sequenced using the primer sets T7 and M13R.

Phylogenetic analyses

The software program Sequencher 4.6 (Gene Codes, Ann Arbor, MI, USA) was used to trim vector from bidirectional sequences and assemble them into contiguous reads. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify the sequences, which were then aligned using SINA (http://www.arb-silva.de/aligner/), and imported into ARB (v 9.6) via a parsimony insertion. Additional related sequences (from both cultured and uncultured chytrids) used in the phylogenetic analysis were selected from the ARB database (v 9.6) and GenBank. The sequence dataset was hand-curated, and a lane mask function was used to filter out alignment columns with less than 40% shared identity. The program Mothur (Schloss et al., 2009) was used to cluster phylotypes sharing 99% sequence identity into operational taxonomic units (OTUs) using the average neighbour algorithm. Representative sequences of approximately 1750 bp were picked from each OTU for use in phylogenetic analyses. First, a rooted tree was created using the maximum likelihood program RaxML (Stamatakis, 2006) with the inverse gamma distribution (INVGAMMA) model, with 1000 bootstrap to provide node support values. Then, confidence levels for each node, in the form of posterior probabilities, were determined using the Baysian inference of the maximum likelihood method. MrBayes (v. 3.1) was run with the following parameters: INVGAMMA model, burn-in of 1250, temperature setting of 0.08, and run to convergence with five million generations (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003).

To test whether the novel clade of chytrids shows significant biogeographic structure, we constructed genetic isolation by geographic distance matrices as previously described (Darcy et al., 2011; Schmidt et al., 2011). Briefly, a matrix of geographic distances between sample locations was computed in R (R Development Core Team, 2012) using the Fields package (Furrer et al., 2012; http://CRAN.R-project.org/package=fields) and a genetic distance matrix for all sequences was created using Mothur (Schloss et al., 2009). To test for a correlation between these matrices, a Mantel test was performed in R using 1000 randomized permutations.

Algal and pollen counts

To quantify possible chytrid food sources in the snow samples, pollen and algae were counted using phase contrast and fluorescent (for chlorophyll autofluorescence) microscopy (400× magnification) and a hemocytometer with a volume of 0.1 μl (Bright Line Counting Chamber, Hauser Scientific, Horsham, PA, USA). Counts were done on surface samples (top 2 cm) and below surface samples (4–11 cm) from the 15 August time point. Statistical analyses comparing surface and subsurface pollen and algal counts were done in R (R Development Core Team, 2012) using a t-Test.

Nucleotide sequence accession numbers

The SSU rRNA sequences generated in this study were submitted to the GenBank database under Accession Numbers KC561936–KC561975.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Full phylogenetic tree showing the relationship between the three clades discussed in this paper (Snow Clade 1, order Lobulomycetales, and order Rhizophydiales) and the out-group (order Chytrediales). *Asterisks denote nodes with minimum of 90% bootstrapping support (RaxML, 1000 bootstraps) and 100% posterior probabilities (Mr Bayes, 5 million generations, INVGAMMA model). #The pound sign denotes nodes with 100% posterior probability support but where bootstrapping support was not available. *Tree tips labelled with a ‘1’ are from this study: ‘Snow’ covering the talus sites described in Freeman and colleagues (2009b), ‘Snow/Ice’ on top of Arkaree Glacier (King et al., 2010), and ‘Snowbed Soils’ from the Himalayan sites described in Schmidt and colleagues (2011; 2012). *Tree tips labelled with a ‘2’ are sequences from ‘Snowbed soils’ in Colorado (Freeman et al., 2009b). *Tree tips labelled with a ‘3’ correspond to sequences obtained from prior studies: Swiss Snow (M. Yuhana, unpublished) and Alpine Lake, France (Lefèvre et al., 2008).