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DNA analysis for section identification of individual *Pinus* pollen grains from Belukha glacier, Altai Mountains, Russia

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Abstract
Pollen taxon in sediment samples can be identified by analyzing pollen morphology. Identification of related species based on pollen morphology is difficult and is limited primarily to genus or family. Because pollen grains of various ages are preserved at below 0 °C in glaciers and thus are more likely to remain intact or to suffer little DNA fragmentation, genetic information from such pollen grains should enable identification of plant taxa below the genus level. However, no published studies have attempted detailed identification using DNA sequences obtained from pollen found in glaciers. As a preliminary step, this study attempted to analyze the DNA of *Pinus* pollen grains extracted from surface snow collected from the Belukha glacier in the Altai Mountains of Russia in the summer of 2003. A 150-bp *rpoB* fragment from the chloroplast genome in each *Pinus* pollen grain was amplified by polymerase chain reaction, and DNA products were sequenced to identify them at the section level. A total of 105 pollen grains were used for the test, and sequences were obtained from eight grains. From the sequences obtained, the pollen grains were identified as belonging to the section *Quinquefoliae*. Trees of the extant species *Pinus sibirica* in the section *Quinquefoliae* are currently found surrounding the glacier. The consistency of results for this section suggests that the pollen in the glacier originated from the same *Pinus* trees as those found in the immediate surroundings.

Keywords: pollen, DNA, glacier, *Pinus*, Altai

1. Introduction

Fossil pollen analysis has been performed to reveal the historical composition of past vegetation and the nature of past climates and environments by revealing the plant taxon to which a pollen specimen belongs. Modern pollen analysis

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focuses on morphological characteristics of the pollen wall. Identification of related species based on morphology is difficult and is limited primarily to plant genus or family. Plant species belonging to the same genus are often distributed in different vegetation zones, so identification at the species level is therefore extremely useful in paleoenvironmental studies. However, to identify pollen at the species level, a new technique has been required.

DNA analysis of pollen grains is a possible technique for identifying pollen at the species level. DNA analysis of individual pollen grains in sediments such as peat and lacustrine deposits has been demonstrated (Suyama *et al* 1996, 2003, Parducci *et al* 2005); however, the success rate of these DNA studies has ranged from 0 to 3.2%. The reasons for this low figure include the rarity of suitable, well-preserved samples as well as the difficulty of polymerase chain reaction (PCR) amplification of a minute amount of DNA from a pollen grain. Identification of pollen from sediment samples using DNA analysis is currently a relatively undeveloped approach.

Incidentally, pollen is regularly found in mid- and low-latitude glaciers because most glaciers are located within a few tens of kilometers of pollen sources (e.g., Haeberli *et al* 1983, Liu *et al* 1998, Reese and Liu 2002, Reese *et al* 2003, Nakazawa *et al* 2004, 2005, Santibáñez *et al* 2008). Pollen grains found in glaciers are typically well preserved, often having unbroken walls and intact cytoplasm (Nakazawa *et al* 2011, 2012). Moreover, pollen DNA should be better preserved in glaciers than in other kinds of sediments because of the lower temperatures of glaciers ($\leq 0\,^{\circ}\mathrm{C}$). Genetic information should therefore be obtainable from glacial pollen specimens. In addition, samples from glaciers can be expected to be useful in a broad range of research applications based on DNA studies, in contrast to the example of the 0% success rate that the pollen from the lacustrine sediment of Lake Baikal has provided (Suyama *et al* 2003).

Obtaining genetic information from individual pollen grains deposited in glaciers may enable identification of pollen species. Such identification would enable reconstruction of the details of past vegetation, as well as of past climate and environments through ice core studies. Also possible would be investigation of wind systems that affect pollen dispersion. Moreover, obtaining genetic information by high-resolution means may lead to the development of a new field in ice core studies that examines the interaction between genetic diversity and climate change by additionally examining intraspecific variation in the same pollen species. To date, there has been no published study examining the DNA of such glacier-preserved pollen grains.

To investigate the potential of DNA analysis of single pollen grains from glaciers, this study used nucleic acid staining to examine the DNA from *Pinus* pollen grains collected from the Belukha glacier in the Russian Altai Mountains. This study also attempted DNA analysis of single *Pinus* pollen grains to identify them at the section level, because pollen morphology in the genus *Pinus* cannot be used to identify *Pinus* pollen grains at the section level.

The focus on *Pinus* pollen grains for these studies has the following advantages: (1) *Pinus* pollen is known to be the dominant pollen type in the Belukha samples from our previous study (Nakazawa *et al* 2005, 2011) and is easy to collect; (2) previous DNA analysis of pollen grains in sediment samples (Suyama *et al* 2003, Parducci *et al* 2005) used *Pinus* pollen grains, and the methods in those studies provide a useful basis for method development in this study; (3) ecology of *Pinus* trees, as well as that of *Betula* trees, in the Russian Altai region, which has continued to be affected by forest fires and climate change (Eichler *et al* 2011). Therefore, *Pinus* pollen may provide valuable information on population genetics that can be used in future studies. *Pinus* pollen is hence suitable for these initial attempts at DNA analysis.

2. Study area and methods

2.1. Study area and pollen samples

The Belukha glacier (49°49′N, 86°34′E; 4110 m a.s.l.) is located on the western side of Mt. Belukha (4500 m a.s.l.) in the Russian Altai Mountains and is situated in the border region between Russia, Mongolia, China, and Kazakhstan (figure 1). In the summer of 2003, drilling of a 171 m long core and observations of a 4 m deep pit were performed on the plateau of the glacier (4100 m a.s.l.) (Fujita *et al* 2004, Takeuchi *et al* 2004). Snow and ice samples from the pit and ice core were collected at 0.4–0.5 m and 24.4–24.5 m depths, respectively. *Pinus* pollen grains were extracted from the melted samples. The *Pinus* pollen concentration in each sample was 45 600 grains l^{-1} in the pit and 5500 grains l^{-1} in the core (Nakazawa *et al* 2011, Okamoto *et al* 2011). In addition Eichler *et al* (2011), reported that the total pollen concentration in a 139 m ice core retrieved from the glacier saddle between Mt. Belukha and West Belukha Peak in 2001 (4062 m a.s.l., figure 1) ranged from 2000 to 30 000 grains l^{-1}. Our samples were dated summer 2003 and summer 1965 by counting the seasonal distribution of pollen, and the dating was validated by the 1963 tritium peak (Nakazawa *et al* 2011, Okamoto *et al* 2011). The snow pit and ice core samples were kept in a frozen state until analyzed.

The major types of vegetation surrounding the Belukha glacier are tundra, steppe, and boreal forest. The tree line is approximately 2400 m a.s.l., with tundra predominating above this point. With the dominant species *Pinus sibirica*, *Abies sibirica*, and *Larix sibirica*, the boreal forests form a dense belt between approximately 1000 and 2000 m a.s.l. in the region north of the Belukha glacier. *Picea obovata* coexists in these forests, but only where soil moisture is sufficient, specifically in the western part of the southern Altai. *P. sylvestris* and *Betula* also form boreal forests in the region, usually below the stands of *P. sibirica*, *A. sibirica*, and *L. sibirica* (Luchik 1970, Blyakharchuk *et al* 2007, Eichler *et al* 2011).

2.2. Staining DNA in a pollen grain

To examine whether DNA was present, *Pinus* pollen grains from the 2003 pit layer and 1965 ice core layer of Belukha glacier were stained with SYBR Gold (Invitrogen, Carlsbad,

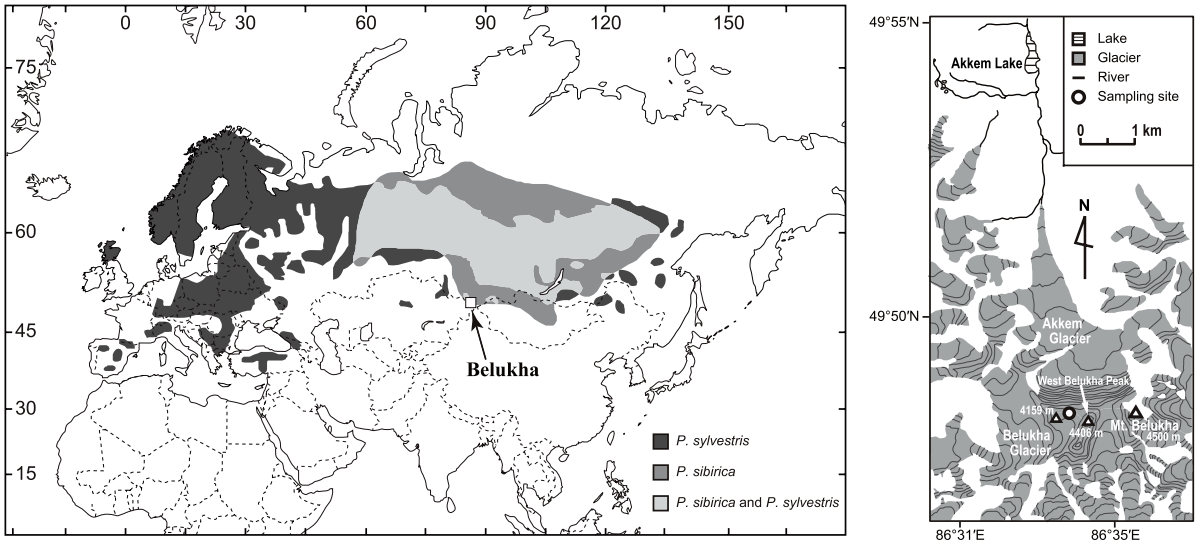

Figure 1. Location of the Belukha glacier in Russia's Altai Republic, and extant distributions of two *Pinus* species (*P. sibirica* and *P. sylvestris*) found surrounding the glacier. The sampling site used in this study was located on the western side of Mt. Belukha. The distribution map was compiled based on the maps of Farjon (2005).

CA). Melted snow and ice samples were first filtered through a hydrophilic PTFE membrane filter with a pore size of 10 μm. Next, the pollen grains on the filter were washed with a few milliliters of sterile water, and the filter was then placed on a glass slide. Pollen grains that showed no structural damage were selected from the filter using a micromanipulator (MM-88, Narishige, Tokyo, Japan) under a microscope and transferred onto a glass slide. The pollen grain was crushed with a 0.5–10 μl pipette tip to stain the DNA within the protoplasm. Then, 1 μl of 4× SYBR Gold solution was dropped onto the pollen grain. The pollen was examined for the presence of DNA under an epifluorescence microscope.

2.3. DNA extraction from a single pollen grain

Single *Pinus* pollen grains from the 2003 pit layer were chosen, and DNA was extracted by using a modified version of the extraction method described by Parducci *et al* (2005) and Suyama (2011). Each pollen grain was collected in the same manner as described for the pollen staining, except that it was transferred to a sterile Petri dish. The pollen grain was washed repeatedly in 15 drops of sterile water aligned on the dish. The washed grain was then transferred to the inner side of the lid of a DNA-free PCR tube containing 0.5 μl of water. The grain was crushed directly in the lid of the tube using a sterile plastic pipette tip and spun down for collection at the bottom of the tube. For each grain, contamination by exogenous DNA was monitored using a PCR blank that included all PCR reagents and 0.5 μl of the last drop of water used for washing the grain. One microliter of extraction buffer containing 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 400 mM NaCl, 0.3% SDS, and 200 μg ml^{-1} Proteinase K was added to the tube. The mixture was incubated at 54 °C for 1 h, then at 95 °C for 10 min, and was used as a template. The 1965 pollen samples were not subjected to PCR because the samples from the ice core are very limited in number and will be used in future work to identify them at the species level.

2.4. PCR amplification and DNA sequencing

PCR amplification was performed using a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA) under the following conditions: initial activation at 95 °C for 10 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 60 s, and extension at 72 °C for 30 s, followed by final incubation at 72 °C for 7 min. The volume of the reaction mixture was 10 μl, containing 1.5 μl of extracted pollen DNA, 0.5 μM of each primer and 5 μl of 2× Ampdirect Plus (Shimadzu Biotech, Kyoto, Japan), and 0.25 U of BIOTAQ DNA polymerase (BioLine, London, UK).

A fragment of the *rpoB* region of chloroplast DNA from nucleotide position 26015–26163 of *P. thunbergii* (accession number D17510) was amplified. The forward primer was 5′-ATGGATCAATCCGACAAAAA-3′ and the reverse primer was 5′-TTCACGTGGTTGGAAGAAAG-3′. Amplification of long fragments from a single ancient pollen grain in sediment samples has previously been described as difficult because of DNA fragmentation and degradation (Pääbo 1989, Suyama *et al* 1996). In addition, because short fragments (<200 bp) amplify more efficiently than longer ones (Parducci *et al* 2005), a ∼150 bp fragment was chosen in this study, although our sample was not ancient. Amplified PCR products were then sequenced using a BigDye Terminator v.3.1 sequencing kit (Applied Biosystems) and an ABI 3130xl genetic analyzer (Applied Biosystems).

3. Results and discussion

3.1. Staining DNA in a single pollen grain

DNA in the *Pinus* pollen grains from Belukha glacier was observed by staining. In figure 2, a generative cell is visible

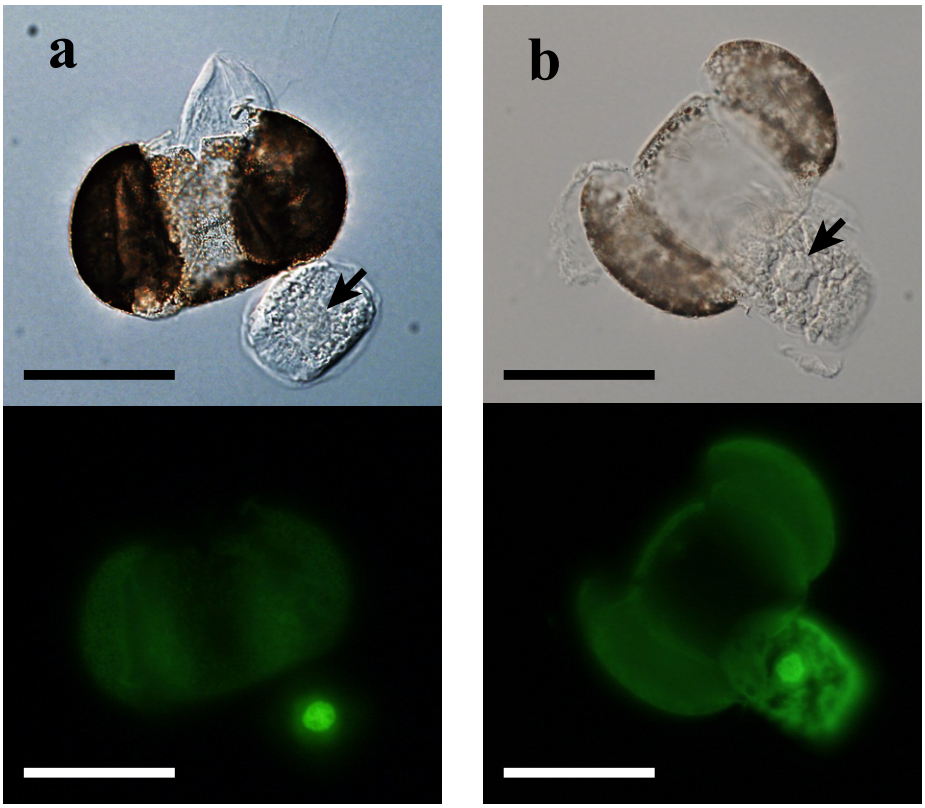

Figure 2. Optical micrographs and epifluorescence micrographs of *Pinus* pollen grains from the Belukha glacier stained with SYBR Gold. Pollen grains obtained from the 2003 pit layer (a) and 1965 ice core layer (b) are shown. The scale bar represents 50 μm. The arrow in each optical micrograph indicates a generative cell.

in both optical micrographs. Moreover, a circular spot of green fluorescence, indicating the generative nucleus, is clearly visible in both images. SYBR Gold is a nucleic acid stain for both RNA and DNA. However, it is well known that RNA, unlike DNA, is very unstable. Therefore, the fluorescence should indicate whether DNA was present in each generative nucleus. Previous studies by Kawamuro *et al* (1995) and Suyama *et al* (1996) examined the DNA preservation in pollen grains from peat deposits. In those studies, however, generative nuclei were indistinguishable, although some fragments of DNA were found in a pollen grain. In contrast, the present results indicate that the pollen DNA is well preserved, regardless of the length of time that pollen grains remain in the glacier, although there may be some degree of fragmentation and degradation of the DNA. These results demonstrate the feasibility of this type of DNA analysis for pollen identification.

Golenberg (1991) and Yang (1997) mentioned that a sample's original condition and its environment during early storage seem to have the most significant effect on DNA preservation. Ultraviolet damage of the pollen grains seems to be confined to only the supraglacial environment. It is well known that snow is a good light insulator and that downward flux of solar radiation with snow cover decreases exponentially with depth. For example, using the extinction coefficient of 45 m^{-1} (Fukami *et al* 1985) for granular snow, which has a relatively small coefficient for different snow types, 1.1% and 1.7×10^{-8}% of solar radiation penetrates through 0.10 m and 0.50 m depths, respectively. Therefore, once the pollen grains are contained by snow, the efficiency of DNA analysis should not differ significantly. The present study provides evidence that pollen grains deposited in glaciers contain DNA which is expected to persist even in older glacier ice because it is preserved at temperatures $\leq$0 °C.

3.2. Amplification DNA in a single pollen grain by PCR

In this study, 105 pollen grains were analyzed, and a total of eight sequences were amplified. Previous analysis of DNA from pollen in sediments used samples collected from peat or lacustrine deposits, with success rates ranging from 0 to 3.2%, independent of sample age and amplification length (table 1). In contrast, the success rate for sequence amplification in this study is 7.6%. Since our samples were younger than those used in the previous studies, however, we cannot make a simple comparison of the success rates between the present study and past studies. Further investigation of older pollen from glaciers is necessary. Nonetheless, the present result demonstrates that DNA from pollen grains in glaciers can be amplified by PCR.

To obtain better success rate for DNA analysis, there is room for further improvement of the current method. Here we used a DNA extraction method with which was based on earlier studies with very low success rates. Because the

Table 1. Success rates of PCR amplification of DNA fragments of various lengths for each sample age in previous studies.

Sample type	Site	Sample age (BP)	Length of fragment (bp)	Success rate (%)	Sequences/no. of samples	References
Lacustrine	Central Sweden	100	220	1.4	7/500	Parducci *et al* (2005)
Lacustrine	Central Sweden	100	140	2.3	7/301	Suyama *et al* (2003)
Lacustrine	Central Sweden	100	105	1.7	4/234	Parducci *et al* (2005)
Lacustrine and swamp	Lake Baikal	>1560	452 or 613	0.0	0/351	Suyama *et al* (2003)
Lacustrine	Central Sweden	9500	140	1.3	4/301	Suyama *et al* (2003)
Lacustrine	Central Sweden	10 000	220	1.3	4/301	Parducci *et al* (2005)
Lacustrine	Central Sweden	10 000	105	1.2	5/408	Parducci *et al* (2005)
Peat	Japan	15 000	220	3.2	4/125	Suyama *et al* (1996)
Snow	Russia	1	150	7.6	8/105	This study

inner wall of a pollen grain (intine) is made of cellulose, the combined application of cellulase and proteinase K may be effective for the extraction. Also, multiplex PCR offers another means of improvement because multiple loci can be amplified simultaneously in a single reaction with an improved probability that at least one locus can be amplified. In addition, different whole genome amplification (WGA) techniques have recently been developed to specifically increase the quantity of DNA obtained from samples with limited DNA content. These techniques also enable amplification of longer fragments and generation of DNA specimens from single pollen grains that can be analyzed by multiple molecular techniques.

3.3. Identification of Pinus pollen grains from the Belukha glacier

We attempted section identification of *Pinus* pollen grains from the Belukha glacier using sequence data obtained by PCR. *Pinus* is a taxon with approximately 111 recognized species in two subgenera, four sections, and 17 subsections. Identification of *Pinus* pollen at a lower taxonomic level has been difficult to date, although some *Pinus* pollen grains are sometimes distinguished as haploxylon type or diploxylon type on the basis of vesicle morphology and other characters. We collected sequence data containing the *rpoB* region from GenBank (table 2), and the collected data were sequences for 89 *Pinus* species derived from all four taxonomic sections. Classification for the genus refers to the study by Gernandt *et al* (2005). Their classification based on chloroplast DNA phylogeny was a modification of (1) the influential classification of Little and Critchfield (1969), which was based primarily on morphology and data from interspecific crosses, and (2) the classification of Price *et al* (1998), which incorporated more recently described species. In general, chloroplast DNA has a low rate of nucleotide substitution, on the order of 10^{-9} per site per year (Wolfe *et al* 1987). Therefore, few mutations are expected within a short period of time such as during the Holocene, the epoch generally covered by ice cores from mid- and low-latitude glaciers. The aligned sequences, except for the primer regions were 112 bp in length and contained 19 variable nucleotide sites of which 7 were parsimony-informative (table 2). Taxon identification was made based on the sequence of the parsimony-informative characters (table 3), and the 8 pollen grains all showed the same sequence, being identified as belonging to subsections *Gerardianae* or *Strobus* in section *Quinquefoliae*.

The 8 pollen grains were estimated to have originated from the periphery of Belukha glacier. The subsections *Gerardianae* and *Strobus* contain a total of 24 pine species. These members are found in East Asia and the Himalayas for subsection *Gerardianae* and in North America and Eurasia for subsection *Strobus* (Gernandt *et al* 2005). *P. sibirica*, which belongs to subsection *Strobus* in section *Quinquefoliae*, is an extant species currently distributed around the glacier (figure 1). In addition, *P. sibirica* is the only member of the subsections found near the glacier. Therefore, the consistency of the section suggests that the pollen grains in the glacier originated from *P. sibirica* trees found in the immediate surroundings.

The PCR method used can be improved for more detailed identification. The obtained sequences provided only limited information for identification, due to their short length, while longer fragments are likely more difficult to amplify. Multiplex PCR or WGA methods should be effective for identifying pollen grains at a lower taxonomic level. Sequence data obtained from multiple loci by these methods may provide sufficient information for further detailed identification.

4. Conclusion

This report describes an initial attempt to analyze DNA contained in pollen grains from a glacier. The fluorescent staining of pollen grains from the 2003 pit layer and 1965 ice core layer clearly demonstrated the persistence of DNA in the generative nucleus, and disappearance of DNA over time was seldom observed. PCR amplifications showed that this DNA was not significantly degraded and was suitable for amplification. The results indicate that pollen grains have been preserved under conditions favorable for the preservation of DNA. Future analysis of pollen DNA from the Belukha ice core is expected to be successful.

The success rate of DNA amplifications in this study exceeded that of previous studies. However, the samples were younger than those used in previous studies. Therefore, further investigation using older samples is necessary in order

Table 2. Data for *Pinus* species, GenBank numbers, and nucleotide sequences used to identify the taxonomic section to which pollen samples belong. Dashes represent alignment gaps, and dots represent identical symbols.

Subgenus	Section	Subsection	Species	Accession no.	1 21 41 61 81 101
Pinus	*Trifoliae*	*Australes*	*attenuata*	FJ899569	AGACTGGTATGTATATTCAG TCTTCACTATATCCCATAGA ATTCTACCCGTATCCAGATA TACTTCCAATTTGAAAAAAA AAA_____GGATAAGGAAGA GGTACTTTGATA
			caribaea	JN854222	···A·· ···· ···· ···· ···_____···· ····
			cubensis	JN854214	···A·· ···· ···· ···· ···_____···· ····
			echinata	JN854204	···A·· ···· ···· ···· ···_____···· ····
			elliottii	JN854202	···A·· ···· ···· ···· ···_____···· ····
			glabra	JN854199	···· ···· ···· ···· ···_____···· ····
			greggii	JN854198	···A·· ···· ···· ···· ···_____···· ····
			lawsonii	JN854188	···A·· ···· ···· ···· ···_____···· ····
			leiophylla	JN854187	···A·· ···· ···· ···· ···_____···· ····
				JN854218	···A·· ···· ···· ···· ···_____···· ····
				FJ899575	···A·· ···· ···· ···· ···_____···· ····
			lumholtzii	JN854186	···A·· ···· ···· ···· ···_____···· ····
			muricata	JN854180	···· ···· ···· ···· ···_____···· ····
			occidentalis	JN854177	···A·· ···· ···· ···· ···_____···· ····
			palustris	JN854176	···A·· ···· ···· ···· ···_____···· ····
			patula	JN854175	···A·· ···· ···· ···· ···_____···· ····
			pringlei	JN854189	···A·· ···· ···· ···· ···A____···· ····
			pungens	JN854167	···A·· ···· ···· ···· ···_A___···· ····
			radiata	JN854165	···· ···· ···· ···· ···A____···· ····
			rigida	JN854163	···A·· ···· ···· ···· ···_____···· ····
			serotina	JN854160	···A·· ···· ···· ···· ···_____···· ····
			taeda	FJ899561	···A·· ···· ···· ···· ···AA___···· ····
		Ponderosae	*coulteri*	JN854215	···· ···· ···· ···· ···AAAAA···· ····
			devoniana	JN854208	···· ···· ···· ···· ···AA___···· ····
			douglasiana	JN854205	···· ···· ···· ···· ···_____···· ····
			hartwegii	JN854196	···· ···· ···· ···· ···_____···· ····
				JN854206	···· ···· ···· ···· ···AAA__···· ····
			jeffreyi	JN854193	···· ···· ···· ···· ···AAAA_···· ····
			montezumae	JN854183	···· ···· ···· ···· ···_____···· ····
			ponderosa	FJ899555	···· ···· ···· ···· ···AA___···· ····
				JN854171	···· ···· ···· ···· ···A____···· ····
			pseudostrobus	JN854169	···· ···· ···· ···· ···A____···· ····
				JN854178	···· ···· ···· ···· ···_____···· ····
			sabiniana	JN854161	···· ···· ···· ···· ···AAAAA···· ····
			torreyana	FJ899563	···· ···· ···· ···· ···A____···· ····
				FJ899564	···· ···· ···· ···· ···A____···· ····
		Contortae	*banksiana*	FJ899571	···· ···· ···· ···C··· ···_____···· ····
			clausa	JN854217	···· ···· ···· ···C··· ···_____···· ····
			contorta	EU998740	···· ···· ···· ···C··· ···_____···· ····
	Pinus	*Pinus*	*densata*	JN854209	···· ···· ···· ···__··· ···A____···· ····
			densiflora	JN854210	···· ···· ···· ···AA··· ···A____···· ····
			hwangshanensis	JN854194	···· ···· ···· ···__··· ···AA___···· ····
			kesiya	JN854191	···· ···· ···· ···__··· ···AA___···· ····
			massoniana	JN854185	···· ···· ···· ···A··· ···AA___···· ····
			merkusii	FJ899579	···· ···· ···· ···AAA··· ···AA___···· ····
			mugo	JN854181	···· ···· ···· ···A··· ···_____···· ····
			nigra	JN854179	···· ···· ···· ···AAA··· ···_____···· ····
			resinosa	FJ899556	···· ···· ···· ···A··· ···AA___···· ····
			sylvestris	JN854158	···· ···· ···· ···A··· ···A____···· ····
			taiwanensis	JN854157	···· ···· ···· ···__··· ···A____···· ····
			thunbergii	D17510	···· ···· ···· ···AA··· ···AA___···· ····
				FJ899562	···· ···· ···· ···__··· ···AAAAA···· ····
			tropicalis	JN854156	···· ···· ···· ···AA··· ···_____···· ····
			yunnanensis	JN854151	···· ···· ···· ···__··· ···_____···· ····
		Pinaster	*brutia*	JN854224	···· ···· ···· ···AAA··· ···_____···· ····
			canariensis	FJ899572	···· ···· ···· ···AAA··· ···AAA__···· ····
			halepensis	JN854197	···· ···· ···· ···AAA··· ···AAA__···· ····
			heldreichii	JN854195	···· ···· ···· ···AA··· ···A____···· ····
			pinaster	FJ899583	···· ···· ···· ···CAA··· ···AAA__···· ····
			pinea	JN854173	···· ···· ···· ·A··· ···AAA··· _______···· ····
			roxburghii	JN854162	···· ···· ···· ···· ···AAA··· _______···· ····
Strobus	*Quinquefoliae*	*Strobus*	*albicaulis*	FJ899566	···A·· ·G·· ···· ···· ···A··· ···_____···· ····
			armandii	FJ899568	···A·· ·G·· ···· ···· ···A··· ···_____···· ····
			ayacahuite	FJ899570	···A·· ·G·· ···· ···· ···A··· ···A____···· ····
			cembra	FJ899574	···A·· ·G·· ···· ···· ···A··· ···AA___···· ····
			chiapensis	JN854219	···A·· ·G·· ···· ···· ···A··· ···_____···· ····
			dalatensis	JN854211	···A·· ·G·· ···· ···· ···A··· ···_____···· ····
			fenzeliana	JN854212	···A·· ·G·· ···· ···· ···A··· ···_____···· ····
			flexilis	FJ899576	···A·· ·G·· ···· ···· ···A··· ···A____···· ····
			koraiensis	AY228468	···A·· ·G·· ···· ···· ···A··· ···AA___···· ····
			lambertiana	FJ899577	···A·· ·G·· ···· ···· ···A··· ···_____···· ····
				EU998743	···A·· ·G·· ···· ···· ···A··· ···AA___···· ····
			monticola	FJ899580	···A·· ·G·· ···· ···· ···A··· ···_____···· ····
			morrisonicola	JN854182	···A·· ·G·· ···· ···· ···A··· ···AA___···· ····
			parviflora	FJ899581	···A·· ·G·· ···· ···· ···A··· ···_____···· ····
			peuce	FJ899582	·····G··A·· ·G·· ···· ···· ···A··· ···_____···· ····
			pumila	JN854168	···A·· ·G·· ···· ···· ···A··· ···A____···· ····
			sibirica	FJ899558	···A·· ·G·· ···· ···· ···A··· ···A____···· ····
			strobus	FJ899560	···A·· ·G·· ···· ···· ···A··· ···AA___···· ····
			wallichiana	JN854154	···A·· ·G·· ···· ···· ···A··· ···_____···· ····
		Krempfianae	*krempfii*	EU998742	·····_____· ·G·· ···· ···· ···A··· ·_____···· ····
		Gerardianae	*gerardiana*	EU998741	···A·· ·G·· ···· ···· ···A··· ·_____···· ····
			squamata	FJ899559	···A·· ·G·· ···· ···· ···A··· ·_____···· ····
	Parrya	*Cembroides*	*cembroides*	JN854220	·····G····· ·G·· ···· ···· ···A··· ···_____···· ····
			culminicola	JN854213	·····G····· ·G·· ···· ···· ···A··· ···A____···· ····
			discolor	JN854207	·····G····· ·G·· ···· ···· ···A··· ···_____···· ····
			edulis	JN854203	·····G····· ·G·· ···· ···· ···A··· ···A____···· ····
			johannis	JN854192	·····G····· ·G·· ···· ···· ···A··· ···A____···· ····
			maximartinezii	JN854184	·····G····· ·G·· ···· ···· ···A··· ···_____···· ····
			monophylla	EU998745	·····G····· ·G·· ···· ···· ···A··· ···_____···· ····
			pinceana	JN854174	·····G····· ·G·· ···· ···· ···A··· ···_____···· ····
			quadrifolia	JN854166	·····G····· ·G·· ···· ···· ···A··· ···AA___···· ····
			remota	JN854164	·····G····· ·G·· ···· ···· ···A··· ···_____···· ····
			rzedowskii	FJ899557	·····G····· ·G·· ···· ···· ···A··· ···_____···· ····
		Balfourianae	*aristata*	FJ899567	·····G····· ·G·· ···· ···· ···A··· ·_____···· ····
			longaeva	EU998744	·····G····· ·G·· ···· ···· ···A··· ·_____···· ····
		Nelsoniae	*nelsonii*	EU998746	·····G····· ·G·· ···· ·····T····· ···A··· ·_____···· ····

Table 3. Sequence variation of parsimony-informative characters in subsections of *Pinus* and *Pinus* pollen from the Belukha glacier. The slash means 'or'. Dashes represent alignment gaps. The nucleotide position number in the table heading indicates the order of nucleotides from the 5′ end of the target region, except for the primer regions.

Subgenus	Section	Subsection	15	18	22	71	72	73	75
Pinus	*Trifoliae*	*Australes*	A	M	C	T	T	G	A
		Ponderosae	A	C	C	T	T	G	A
		Contortae	A	C	C	T	T	G	C
	Pinus	*Pinus*	A	C	C	W/-	A/-	A/-	A
		Pinaster	A	C	M	H	A	A	A
Strobus	*Quinquefoliae*	*Strobus*	R	A	G	T	A	G	A
		Krempfianae	—	—	G	T	A	G	A
		Gerardianae	A	A	G	T	A	G	A
	Parrya	*Cembroides*	G	C	G	T	A	G	A
		Balfourianae	G	C	G	T	A	G	A
		Nelsoniae	G	C	G	T	A	G	A
	Belukha		A	A	G	T	A	G	A

to identify which factor has a greater effect on preservation state: temperature or sample age.

Obtained sequences identified pollen grains as belonging to section *Quinquefoliae*, which includes *P. sibirica*, an extant species found surrounding the glacier. These findings suggest that the source of the pollen found in the glacier was extant *P. sibirica*. Multiplex PCR or WGA methods should improve the ability to obtain sequences and facilitate more detailed taxonomic identification.

The rarity of suitable, well-preserved pollen samples in sediments has so far limited the broad utility of DNA studies for taxonomic identification of pollen. However, due to low-temperature conditions, pollen grains in glaciers are less affected by diagenesis, and their DNA is therefore more likely to be preserved. Accordingly, pollen samples from glaciers should have broad utility for studies on taxonomy, past vegetation, and population genetics, as well as climate and environment.

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