

1 **Mitochondrial DNA in Siberian conifers indicates multiple post-glacial**  
2 **colonization centers**

3 **Vladimir L. Semerikov**<sup>1\*</sup> e-mail: semerikov@ipae.uran.ru

4 **Svetlana A. Semerikova**<sup>1</sup> e-mail: s.a.semerikova@ipae.uran.ru

5 **Yuliya A. Putintseva**<sup>2</sup> e-mail: yaputintseva@mail.ru

6 **Natalia V. Oreshkova**<sup>2,3</sup> e-mail: oreshkova@ksc.krasn.ru

7 **Konstantin V. Krutovsky**<sup>2,4,5,6</sup> e-mail: konstantin.krutovsky@forst.uni-goettingen.de

8 <sup>1</sup>Institute of Plant and Animal Ecology, Ural Branch of the Russian Academy of Sciences, 620144

9 Ekaterinburg, Russia

10 <sup>2</sup>Laboratory of Forest Genomics, Genome Research and Education Center, Siberian Federal University,

11 660036 Krasnoyarsk, Russia

12 <sup>3</sup>Laboratory of Forest Genetics and Selection, V. N. Sukachev Institute of Forest, Federal Research

13 Center “Krasnoyarsk Science Center” of the Siberian Branch of the Russian Academy of Sciences,

14 660036 Krasnoyarsk, Russia

15 <sup>4</sup>Laboratory of Population Genetics, N. I. Vavilov Institute of General Genetics, Russian Academy of

16 Sciences, 119333 Moscow, Russia

17 <sup>5</sup>Department of Forest Genetics and Forest Tree Breeding, Georg-August University of Göttingen,

18 37077 Göttingen, Germany

19 <sup>6</sup>Department of Ecosystem Science and Management, Texas A&M University, College Station, TX

20 77843-2138, USA

21 \*Corresponding author

22 **Abstract**

23 The geographic variation of the mitochondrial DNA was studied in Siberian fir using the newly  
24 developed markers and compared with the phylogeographic pattern of another previously studied  
25 Siberian coniferous - Siberian larch. Similar to Siberian larch the distribution of mtDNA haplotypes in  
26 Siberian fir revealed clear differentiation among distinct geographic regions of southern Siberia and the  
27 Urals, likely indicating post-glacial re-colonization from several sources. The northern part of the range  
28 of both species was genetically homogeneous, which is probably due to its recent colonization from one  
29 of the glacial refugia. This conclusion is in agreement with published pollen and macrofossil data in  
30 Siberian fir and with the reconstruction of environmental niches indicating a dramatic reduction of the  
31 range and a likely survival of fir in certain southern areas during the last glacial maximum (LGM) – 21  
32 thousand years ago (kya). Although the modeling of Siberian larch ecological niche reconstructed a shift  
33 of the range to the south at that period, the paleontological data indicated the presence of this species in  
34 most areas of the current range during LGM, that corresponds to the results of previous historical  
35 demography study suggesting the population expansion preceding the LGM.

36 **Key words:** mitochondrial DNA, NGS, phylogeography, *Abies sibirica*, *Larix sibirica*, refugia, pollen  
37 data, macrofossils, environmental niche modelling

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## 42 1. Introduction

43 The response of the boreal forest species to the Pleistocene glacial cycles was different from that of  
44 the temperate forests. During the last glacial maximum (LGM), their ranges were not dramatically  
45 reduced, and they were able to survive near the glacial sheet (Huntley and Birks 1983; Willis and van  
46 Andel 2004). Unlike Europe, Siberia during the Pleistocene was less exposed to the influence of glacial  
47 cover, but the glacial sheet reached the latitude of 60° N by about 250-270 thousand years ago (kya) and  
48 the latitude of 62° N by about 130-190 kya (Volkova et al. 2002). Consequently, the recolonization of  
49 northern Siberia by woody species could occur no earlier than those glacial intervals, but likely earlier  
50 than the LGM. Although the paleontological and genetic data indicate a relatively recent settlement of  
51 the northern part of the range of larch *Larix gmelinii* (Rupr.) Rupr., *L. cajanderi* Mayr (Polezhaeva et al.  
52 2010), *L. sibirica* Ledeb. (Semerikov et al. 2013), common juniper *Juniperus communis* L. (Hantemirova  
53 et al. 2017), wood lemming *Myopus schisticolor* Lilljeborg (Fedorov et al. 2008), and others  
54 (Goropashnaya et al. 2004; Oshida et al. 2005; Zink et al. 2002; Kohli, 2015) the age of those  
55 colonizations is older than the age of LGM, during which the species probably survived in numerous  
56 northern micro-refugia.

57 Siberian fir (*Abies sibirica* Ledeb.) is more demanding for temperature and humidity than other taiga  
58 trees (Krylov et al. 1986), which can lead to a specific reaction of this species to Quaternary climate  
59 fluctuations. Siberian fir fossils related to the Late Pleistocene are rare even in the southern part of the  
60 range, which makes it difficult to determine the location of glacial refugia. Previous range-wide studies  
61 of genetic diversity of Siberian fir were based on allozymes (Semerikova and Semerikov 2006),  
62 chloroplast microsatellite loci (Semerikova and Semerikov 2007), and AFLP (Semerikova and  
63 Semerikov 2011). They revealed several genetically distinct geographic groups, which were probably the  
64 result of post-glacial dispersion out of a few isolated refugia. Such refugia were hypothesized in South

65 Siberia (Altai Mountains, Sayan Mountains, the Baikal Lake area and the South Urals). Northern Siberia  
66 and the Northern Urals were suggested to have been colonized primarily from hypothetical refugia  
67 located in the Baikal Lake area (Semerikova and Semerikov, 2011).

68 A study of the phylogeography of Siberian larch, another representative of the Pinaceae family in the  
69 flora of Siberia and Eastern Europe, which has the range close to that of Siberian fir, using mitochondrial  
70 and chloroplast DNA markers, also revealed a few geographic groups of populations (Semerikov et al.  
71 2013). Similarly, these groups can be regarded as the result of dispersion out of several refugia located  
72 in the Urals and in South Siberia. Possibly, two more refugia existed in more northern areas in the middle  
73 of West Siberia (Semerikov et al. 2013). Based on the similarity in the mitotype distribution in northern  
74 Siberia and the territory in the northern foothills of the Sayan Mountains, it was concluded that the latter  
75 area was the primary source of recolonization of northern Siberia.

76 Comparison of the phylogeography of Siberian fir and Siberian larch helps to identify common  
77 features and differences in the history of the modern populations, to reveal glacial refugia, and time and  
78 direction of re-colonization. For this purpose, this study of the Siberian fir phylogeography was  
79 conducted using the markers of the mitochondrial DNA (mtDNA), which is maternally inherited in the  
80 Pinaceae family and transmitted via seeds, unlike the paternally inherited chloroplast DNA transmitted  
81 via pollen and the biparentally inherited nuclear DNA transmitted by both seeds and pollen (Neale and  
82 Sederoff 1989). Due to this property, the mtDNA markers are especially informative for describing  
83 migrations associated with seed dispersion, including recolonization from glacial refugia. During the  
84 preliminary study, we did not detect any variation in the fragments of the mtDNA amplified using  
85 "universal primers" based on conservative annealing sites in mitochondrial genes of plants (Demesure et  
86 al. 1995; Dumolin-Lapegue et al. 1997), also there were no publicly available mtDNA markers specific  
87 for the Siberian fir, therefore we used NGS data to develop four new mtDNA markers.

88 In addition to the genetic data we analyzed distribution of Siberian fir and Siberian larch during and  
89 after the LGM using available paleontological data and also conducted environmental niche modelling  
90 to reconstruct the expected ranges of these species during the LGM.

91 The main aims of this study were to test the hypotheses regarding the location of glacial refugia, time  
92 and routes of post-glacial migrations of Siberian fir and to compare the observed biogeographic pattern  
93 with one found in other Siberian conifer – *Larix sibirica*.

## 94 2. Materials and methods

### 95 2.1. Development of mtDNA markers

96 To develop mtDNA markers, we searched for polymorphism in the mitochondrial genome  
97 (mitogenome) of Siberian fir. The approach included the following steps:

98 1) Relatively low coverage paired-end (PE) sequencing of the entire Siberian fir genome using  
99 Illumina HiSeq 2000. For this sequencing we used the PE DNA library with the insert size of 200 bp  
100 produced using total DNA isolated with the CTAB method from needles of a single Siberian fir tree  
101 growing in a natural population (56° 39' N 59° 16' E). The library preparation was performed following  
102 a standard Illumina protocol  
103 ([www.bu.edu/iscf/files/2011/05/TruSeq\\_DNA\\_SamplePrep\\_Guide\\_15005180\\_A.pdf](http://www.bu.edu/iscf/files/2011/05/TruSeq_DNA_SamplePrep_Guide_15005180_A.pdf)). For sequencing  
104 we used 2×100 cycles Illumina Kit. In total, 22,821,847 pairs of reads were generated. We used FastQC  
105 and Trimmomatic for quality control and adapter trimming.

106 2) Assembly of contigs using the CLC Assembly Cell software. The expected genome size for *Abies*  
107 *sibirica* is 15.452 Gbp (Ohri and Khoshoo 1986). Because of the low coverage, the genome assembly  
108 was very partial and included only 0.2% of the expected genome size. However, due to the fact that

109 there are multiple copies of **the** mitogenome per nuclear genome in one cell, we were able to identify  
110 several mitochondrial contigs.

111 3) Search for mitochondrial contigs using BLASTn and all plant mitochondrial sequences available  
112 in the NCBI Genbank and other public databases, such as <ftp://plantgenie.org/ConGenIE> and  
113 <https://treegenesdb.org/FTP/Genomes/Pita/mito> for Norway spruce (*Picea abies* (L.) Karst.) and loblolly  
114 pine (*Pinus taeda* L.), respectively. The matching Siberian fir contigs in the BLASTn hits with the  
115 alignment length of more than 100 bp and similarity higher than 90% were selected for further analysis.  
116 In total, 87 contigs with the total length of 958,226 bp were **selected, which represents a significant part**  
117 **of the mitochondrial genome, considering 5.9 Mb of the mitochondrial genome assembled in another**  
118 **conifer *Picea glauca* (Moench) Voss (Jackman et al. 2016).** The selected mtDNA contigs were then used  
119 to design PCR primers and to search for polymorphism by partial amplicon-based resequencing of eight  
120 individuals representing different parts of the Siberian fir range including Altai, Kuznetsk Alatau and the  
121 Sayan Mountains, **the** Lake Baikal Region, the Southern and **the** Northern Urals (Table S1 in the  
122 Supplementary material). The PCR primers (Table 1) were designed using the Primer3 software (Rozen  
123 and Skaletsky 2000).

124 The PCR was performed in a volume of 25 ul, containing about 250 ng of genomic DNA, 1X PCR  
125 buffer (75 mM Tris-HCl, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Tween-20), 2.5 mM MgCl<sub>2</sub>, 200µM of each dNTP,  
126 0.2µM forward and reverse primers, 0.32 units of *Taq* polymerase (SibEnzyme Ltd., Novosibirsk,  
127 Russia). The PCR program consisted of initial denaturation at 94°C for 5 min and 35 cycles of  
128 amplification: 94°C – 30 sec, 60°C – 45 sec, 72°C – 2 min. The final elongation was 7 min at 72°C. The  
129 PCR product was checked using electrophoresis in 1% agarose gel, purified using ExoSAP-IT®  
130 (Affimetrix Inc., Santa Clara, CA, USA) and then sequenced using the BigDye v.3.1. kit and  
131 GeneAnalyser 3130 (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA). The  
132 obtained nucleotide sequences were edited and aligned using CodonCode v. 1.2.4 and BioEdit v. 7.2.5

133 (Hall 1999). Four single nucleotide polymorphisms (SNPs) were detected in four different contigs,  
134 respectively (Table. 2). All four SNPs were biallelic. The identified SNPs were further used as genetic  
135 markers.

## 136 2.2. Genotyping

137 For routine genotyping, the SSCP (single strain conformation polymorphism) method (Fujita and  
138 Silver 1994) was applied with minor modifications. For better SSCP resolution, additional PCR primers  
139 were developed to amplify shorter fragments (less than 250 bp) containing SNPs (Table 1). All four  
140 fragments were amplified in a single 10 µl multiplex reaction. Its composition and PCR conditions were  
141 identical to the described above, except for a 1 min shorter elongation time. The PCR product was further  
142 subjected to digestion with the restriction enzyme *RsaI* in order to obtain shorter fragments containing  
143 polymorphism. As a result, the restriction fragment containing A37 was 95 bp in length, and the  
144 restriction fragment containing marker A126 – 130 bp. The restriction product was 5X diluted with a  
145 loading buffer containing 95% formamide and denatured at 95° C for 3 minutes before electrophoresis  
146 in a 8% polyacrylamide gel and 1X TBE electrode buffer containing 10% glycerol. The gel and buffer  
147 were pre-cooled in a refrigerator to 0-4°C, and the electrophoresis was carried out at 4°C. The electric  
148 power was stabilized at 15 watts, and the electrophoresis was run at 4000 volts × hours in the Model S2  
149 sequencing Gel Electrophoresis System (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham,  
150 MA, USA). The DNA in the gel was visualized after the electrophoresis by silver staining. An example  
151 of an SSCP gel and observed polymorphism is demonstrated in Fig. S1 (Supplementary material).

## 152 2.3. Plant material

153 Samples from 45 populations representing 8 - 24 individuals per population (Fig. 1; Table S1 in the  
154 Supplementary material) used previously for allozyme, chloroplast microsatellite, and AFLP studies

155 (Semerikova and Semerikov 2006, 2007, 2011) were genotyped using the SSCP method to study mtDNA  
156 diversity. In addition, for verification the mtDNA fragments were sequenced in at least one individual  
157 for each detected mitotype in each studied population.

## 158 **2.4. Population genetic differentiation analysis**

159 A hierarchical analysis of molecular variation (AMOVA) within and between populations, and  
160 within and between groups was performed using the Arlequin program v.3.5 (Excoffier et al. 2006). The  
161 statistical significance of the fixation indices was estimated using 1000 permutations. The populations  
162 were grouped based on their clustering into geographic groups using the SAMOVA program (Dupanloup  
163 et al. 2002). The algorithm of the program is aimed at clustering geographically adjacent populations in  
164  $K$  groups, where  $K$  is set a priori, by maximizing differentiation between groups ( $F_{CT}$ ). The analysis was  
165 performed at  $K = 2, 3, 4, 5, 6$ . The  $G_{ST}$  (Nei 1987) and  $N_{ST}$  fixation indices were also calculated based  
166 only on haplotype frequencies or taking into account also the genetic distance between the haplotypes  
167 (Pons and Petit 1996), respectively. The comparison of  $G_{ST}$  with  $N_{ST}$  was carried out using PermutCpSSR  
168 v.1.0 ([http://www6.bordeaux-aquitaine.inra.fr/biogeco/Production-scientifique/Logiciels/Contrib-](http://www6.bordeaux-aquitaine.inra.fr/biogeco/Production-scientifique/Logiciels/Contrib-Permut/Permut)  
169 [Permut/Permut](http://www6.bordeaux-aquitaine.inra.fr/biogeco/Production-scientifique/Logiciels/Contrib-Permut/Permut)) (Burban et al. 1999). If  $N_{ST} > G_{ST}$ , genetically similar haplotypes tend to coexist in the  
170 same population.

## 171 **2.5. Phylogenetic analysis**

172 To investigate the phylogenetic relationships of the identified haplotypes (mitotypes) of *A. sibirica*,  
173 we sequenced polymorphic fragments in one sample in each of the two related species (Semerikova et  
174 al. 2018): *A. nephrolepis* (Trautv. ex Maxim.) Maxim. (Russian Far East) and *A. semenovii* B. Fedtsch.  
175 (Western Tien Shan). The latter was classified (Farjon and Rushforth 1989) as a subspecies of Siberian  
176 fir (*A. sibirica* subsp. *semenovii* (B. Fedtsch.) Farjon), but their species rank was confirmed by molecular



177 data (Semerikova et al. 2012; Semerikova and Semerikov 2016). To infer the relationships of mitotypes,  
178 we used the method of Median-Joining Network, performed with the software NETWORK v. 5.0.0.1  
179 (Bandelt et al., 1999).

## 180 2.6. Environmental niche modelling

181 We used environmental niche modelling to reconstruct putative ranges of Siberian fir and Siberian  
182 larch during the LGM (21 kya). To do so we used data on the current distribution of fir (*A. sibirica*, *A.*  
183 *nephrolepis*, *A. sachalinensis* and *A. semenovii*) and larch (*L. sibirica*, *L. gmelinii*, *L. olgensis*, *L.*  
184 *kamtchatica* and *L. kajanderi*) species, as well as present and past climatic parameter distributions and  
185 the machine learning method based on maximum entropy implemented in the program MAXENT 3.3.3  
186 (Phillips et al. 2006). The fir and larch distribution data were retrieved from the Global Bioinformation  
187 Facility database (<https://www.gbif.org>, accessed on June 17, 2018). The data set was expanded by  
188 adding 170 occurrences of larch from our field records (Polezhaeva et al. 2010; Semerikov et al. 2013)  
189 and fir occurrences from this study and Semerikova et al. (2011). The environmental data describing the  
190 baseline climate (19 BioClim layers for the 1950–2000 period at a spatial resolution of 2.5 arc min), the  
191 LGM climate (BioClim layers derived from the Coupled Model Intercomparison Project Phase 5) were  
192 retrieved from the WorldClim database (Hijmans et al. 2005). To reduce the effect of association between  
193 climate parameters, we computed correlation between all pairs of the 19 parameters for the geographic  
194 points of the species occurrence. For the parameters with correlation 0.8 or more we presented only one  
195 parameter. As a result, we used 9 layers: bio1 - Annual Mean Temperature, bio2 - Mean Diurnal Range,  
196 bio3 - Isothermality, bio4 - Temperature Seasonality, bio5 - Max Temperature of Warmest Month, bio8 -  
197 Mean Temperature of Wettest Quarter, bio12 - Annual Precipitation, bio15 - Precipitation Seasonality,  
198 bio18 - Precipitation of Warmest Quarter. Default settings of MAXENT were used.

## 199 2.7. Paleodata

200 Published paleodata on pollen (Binney et al. 2017) and macrofossils (Binney et al. 2009; Kosintsev  
201 et al. 2012) were used to test the inferences from genetic data on the history of species distribution after  
202 the LGM. We selected data sites in the range of latitude 41°- 75° and longitude 29° - 177°. To reduce the  
203 number of erroneously interpreted cases of fir presence due to redeposition of pollen from older layers  
204 or long-distance pollen dispersion, we selected only cases with the proportion of pollen of the species in  
205 question of above 1% in the database. Since larch pollen has poor preservation and insufficient mobility,  
206 for larch we took into account all the samples where larch pollen was noted. We selected the pollen and  
207 macrofossil data younger than 21500 year old and combined them according to calibrated radiocarbon  
208 age into the following eight categories: 0 - 0.5, 0.5 - 3.5, 3.5 - 6.5, 6.5 - 9.5, 9.5 - 12.5, 12.5 - 15.5, 15.5  
209 - 18.5, and 18.5 - 21.5 kya.

## 210 3. Results

### 211 3.1. New markers revealed a strong spatial structure of the mtDNA variation in *A. sibirica*

212 Sequencing of randomly selected 33 regions of 20 Siberian fir mtDNA contigs in eight trees from  
213 geographically distinct populations with the total length of 49,000 bp identified four biallelic SNPs  
214 (A167, A65, A126 and A37) in four contigs – 167, 65, 126, and 37, respectively (Table 1). The study of  
215 mtDNA variation in 45 populations of Siberian fir revealed three mitotypes different by 1-4 nucleotides:  
216 two of them were relatively frequent – mitotype M1 (GACC haplotype, according to the nucleotide  
217 alleles in A167, A65, A126, and A37 SNPs, respectively) and mitotype M2 (TCAC). M3 (GACA) was  
218 rare and different from M1 by one SNP (Fig. 1). M4 was found only in *A. semenovii* (GCCC) and M5 –  
219 only in *A. nephrolepis* (GCAC). Apart from four SNPs polymorphic in *A. sibirica*, one more mutation  
220 was found specific to mitotype M4 of *A. semenovii* and seven mutations specific to M5 of *A. nephrolepis*

221 (Fig. 1). A closer relationship between *A. sibirica* and *A. semenovii* compared with *A. nephrolepis* is  
222 expected, considering that, based on the previously used mitochondrial DNA fragments, *A. sibirica* and  
223 *A. nephrolepis* have different, albeit closely similar haplotypes, while no differences between *A.*  
224 *semenovii* and *A. sibirica* were found (Semerikova et al. 2018). Most of the studied populations of *A.*  
225 *sibirica* contained only one mitotype (Fig. 1). M2 was fixed in the Baikal region, north-east of the Eastern  
226 Sayan, the Middle and Lower Yenisei, most of West Siberia and the Northern and Subpolar Urals. M1  
227 was fixed or dominant in the mountain ridges of the Western Sayan, Kuznetsk Alatau and the Altai. These  
228 two mitotypes M1 and M2 formed a mosaic structure co-occurring in some populations in the Middle  
229 and Southern Urals, the west of the West Siberia Plain and European Russia.

230 The mitotype M3 was fixed or present as an admixture to M1 in the southernmost populations of the  
231 Altai and Kuznetsk Alatau and completely absent in more northern populations. The phylogenetic  
232 structure was not pronounced, and  $N_{ST}$  was higher than  $G_{ST}$  (0.897 vs. 0.866), but the difference was not  
233 statistically significant ( $P = 0.25$ ). At  $K = 3$ , SAMOVA divided the populations into three geographic  
234 groups according to the areas of the three identified mitotypes. At the same time,  $F_{ST}$  was very high  
235 (0.933, Table 2), which is typical for mtDNA markers in conifers. The highest differentiation, based on  
236 the SAMOVA grouping, was found between populations within the total sample and between groups  
237 (Table 2).

### 238 **3.2. The niche modelling indicates a stronger reduction of the range of *A. sibirica* compared to the** 239 ***Larix* species during the LGM**

240 The computed *Abies* and *Larix* ranges in Northern Eurasia based on the environment niche modeling  
241 for the present day largely coincided with the current ranges of these species (Fig. 2: H and P). The  
242 computed area potentially favorable for fir during the LGM was at the mid-latitudes (south of Moscow's  
243 latitude – about 55 lat.) (Fig. 2, A). It intermittently stretches from west to east and partly overlaps with

244 the most southern parts of the present range of Siberian fir, such as the Southern Urals, Altai, the Sayans  
245 and the south of the Baikal region. This range included also areas where fir species are completely absent  
246 now: the plains of Eastern Europe and Central Kazakhstan.

247 The potential area reconstructed for larch during the LGM (Fig. 2, I) was much wider, and the northern  
248 limit of larch distribution located much further to the north than for fir, reaching the latitude of Surgut  
249 town (about 61 lat.). In the southern and western parts, the range of larch in the LGM period could  
250 probably significantly expand into Eastern Europe, Central Kazakhstan and East Asia.

### 251 **3.3. Peleodata indicates a more limited distribution of fir in the LGM and a later expansion to the** 252 **north after climate improvement in comparison to larch**

253 Pollen and macrofossil records of *Abies* in the time intervals close to the LGM (21.5–18.5 and 18.5–  
254 15.5 kya; Fig. 2, A and B) are extremely rare. The pollen records along the coasts of the Kara and Okhotsk  
255 seas, as well as the macrofossil sample taken near the mouth of the Yenisei River, seem suspicious, as  
256 they are located far beyond the reconstructed range of fir during the LGM and even beyond the modern  
257 range. We believe that the wrong time placement of fir pollen in these records may be due to the re-  
258 deposition of older pollen, and the wrong time placement of the macrofossil may be the result of an error  
259 in determining the radiocarbon age. In the more recent time (12.5–15.5 kya; Fig. 2, C), fir records become  
260 more abundant, however, relatively reliable ones are still limited to southern Siberia and the south of the  
261 Russian Far East. Near the beginning of the Holocene (10–12 kya; Fig. 2, D), fir suddenly becomes  
262 common both in the south of Western Siberia and in the north, where it is noted at several locations.  
263 Then, until about the middle of the Holocene, fir remains abundant in northern Siberia, and 7–9 kya it  
264 was noted in the center of Yakutia (Fig. 2, E). After 4 kya, fir reduces the area in the north (Fig. 2, G and  
265 H). Some pollen deposition corresponding to this time period was found in the north close to the Lena  
266 River delta, but it could be the result of re-deposition of older pollen from the early Pleistocene or an

267 occasional long-distance pollen dispersion. The same trend was observed in the south of the Yamal  
268 Peninsula and north of West Siberia where fir pollen was continuously present in the peat deposits  
269 beginning from 8 kya and disappeared after 5 kya (Panova et al. 2010). It is interesting to note that similar  
270 dynamics of fir was observed in the mountains of southwestern Mongolia, about 600 km beyond the  
271 southern limit of the present range, where, according to radiocarbon dating of fir macrofossils (wood) in  
272 the Holocene peat deposits, fir was present in the middle Holocene and disappeared approximately after  
273 3.5 kya (Dorofeyuk and Tarasov 2000). Similar, fir appeared in peatlands in Northern Kazakhstan (far to  
274 the south of the modern range) in the Holocene optimum and later disappeared (Gorchakovsky 1987).

275 Unlike fir, during the LGM larch is noted in pollen records in northeastern Siberia, and macrofossils  
276 are found in the north of Western Siberia, i. e. much to the north of the area reconstructed by MAXENT  
277 (Fig. 2, I), and 18.3 kya, according to macrofossils – on the coast of the Kara Sea. However, a noticeable  
278 increase in the amount of pollen and macrofossil records throughout the present range is observed only  
279 after 15.5 kya (Fig. 2, K), and especially significantly with the beginning of the Holocene (Fig. 2, L). By  
280 the end of the Holocene, larch retreats to the limits of the modern range.

## 281 **4. Discussion**

### 282 **4.1. NGS facilitates development of mtDNA markers in plants**

283 The maternal inheritance of mtDNA markers makes them very valuable for population and  
284 phylogeographic studies, especially in the Pinaceae family, where chloroplast DNA has paternal  
285 inheritance. MtDNA markers could be useful also in other plants, because plant mitogenomes are much  
286 larger than animal ones ranging from 208 kb in *Brassica hirta* to 11.3 Mb in *Silene conica* (see Liao et  
287 al. 2018 for review). However, until recently, the use of mtDNA was limited, since available markers  
288 were restricted to a few known intron and intergenic spacer regions amplified by the "universal" PCR

289 primers. For many conifers polymorphism in these regions is either absent or not sufficiently informative.  
290 Developing species-specific markers requires *de novo* sequencing of a sufficient part of the mitogenome,  
291 which, prior to the appearance of NGS methods, was a time-consuming task. This is exacerbated by the  
292 low nucleotide variation in plant mtDNA, much lower than the variation in nuclear and chloroplast DNA.  
293 The latter circumstance makes it inefficient to search for variation in plant mitogenomes using the RAD-  
294 seq or other methods that involve sequencing of small fractions of the genome. In this study, we used the  
295 results of the whole-genome-shotgun (WGS) sequencing of the Siberian fir genome to develop species-  
296 specific markers of mtDNA. Since the WGS was based only on one tree, to search for polymorphism we  
297 re-sequenced randomly selected portions of the mitogenome in a few trees using the Sanger method. We  
298 used the same approach recently to develop seven markers for mtDNA in Scots pine (Semerikov et al.  
299 2015, 2018). Unfortunately, this approach implies a large volume of capillary sequencing. The use of  
300 NGS instead of the Sanger method for the sequencing of several trees in the search for polymorphism in  
301 mtDNA of Scots pine and related species proved to be more effective (Donnelly et al. 2017). Thus, at  
302 present the progress of NGS methods makes it relatively inexpensive and very quick to develop mtDNA  
303 markers suitable for population and phylogenetic studies of any plant species.

#### 304 **4.2. Colonization of the northern part of the range of Siberian fir and Siberian larch**

305 The histories of Siberian larch and Siberian fir in the LGM and after it apparently differed significantly  
306 from each other. According to the reconstruction of ecological niches, pollen and macrofossil data,  
307 Siberian larch was present in northern Siberia during the LGM, although treeless landscapes dominated  
308 much of Northern Eurasia (Tarasov et al. 2000; Binney et al. 2017). Siberian larch reached the latitude  
309 of 72° already by 18.37 kya (Kosintsev et al. 2012). Consequently, the colonization of northern Siberia  
310 by larch began before the LGM that agrees with the results of the Bayesian analysis of the historical  
311 demography of Siberian larch based on the chloroplast microsatellite data, which gave the estimate of

312 the age of population expansion that significantly exceeded the age of the LGM and probably  
313 corresponded to the onset of migration from the refugia after one of the intensive Middle-Pleistocene  
314 glaciations (Semerikov et al. 2013). Despite the fact that the structure of the mtDNA variation in Siberian  
315 larch corresponds to the colonization of northern Siberia primarily from a single southern refugium,  
316 heterogeneity of the mtDNA variation in the northern Siberia supported by the spatial analysis of  
317 molecular variation (SAMOVA) suggests post-LGM dispersion out from several secondary refugia  
318 located in the north of Western Siberia (Semerikov et al. 2013).

319 In contrast to larch, Siberian fir had a very limited distribution in Siberia and the Urals during the  
320 LGM, according to both the paleodata and niche modelling. During the LGM, it apparently could exist  
321 only in some mountain and foothill areas of the Southern Urals, the south of Siberia and the Baikal region.  
322 A significant increase in the number of samples containing *Abies* pollen or macrofossils occurred after  
323 15 kya and only in the southern part of the range. However, by the beginning of the Holocene, Siberian  
324 fir had already reached the Kara Sea. The mtDNA data in Siberian fir correspond to the colonization of  
325 the north range from a limited area, because only one of the two most common haplotypes is distributed  
326 in the north of Western Siberia and the north of the Urals. Such a source could be the Baikal region,  
327 where this haplotype is fixed. Data on the allozyme and AFLP variation (Semerikova and Semerikov  
328 2011) are compatible with this hypothesis: based on the allele frequencies, the populations of northern  
329 Siberia and the northern Urals are similar to Baikal populations (Semerikova and Semerikov 2011).  
330 Moreover, higher diversity in populations of southern Siberia and the Baikal region compared to northern  
331 Siberia and the Urals was revealed with chloroplast microsatellites (Semerikova and Semerikov 2007),  
332 which is in agreement with the hypothesis of migrations from southern refugia. The contribution of only  
333 one hypothetical glacial refugium in the recolonization of the North suggested by the mtDNA data  
334 contrasts Siberian fir with Siberian larch, for which the existence of several secondary refugia can be  
335 assumed in the north of the range during the LGM. This circumstance also distinguishes it from boreal

336 conifers in North America, where several refugia were reconstructed for each of the species studied:  
337 balsam fir (Cinget et al. 2015), black spruce (Gérardi et al. 2010; Jaramillo-Correa et al. 2004), tamarack  
338 (Warren et al. 2016), and jack pine (Godbout et al. 2010). This difference is probably related to the dry,  
339 extremely continental climate of Siberia, which **could have been drier** in the late glacial and probably  
340 restricted the fir to the most favorable areas of south Siberia and the Urals, unlike the larch, which  
341 survived close to the present Kara sea, and prevented the fir from crossing the southern, most arid belt  
342 of the West Siberian Plain after warming. As a result, only the populations of the refugium located in the  
343 mountains around the Baikal Lake could do this by spreading along the Angara and Yenisei Rivers.

344 **The phylogeographic pattern of the presence of a common haplotype in the north of Western Siberia**  
345 **and in the Baikal region found in Siberian fir, is not quite common** for the species of the taiga biota of  
346 Northern Eurasia. For instance, the populations of Siberian larch (Semerikov et al. 2013), wood lemming  
347 (Fedorov et al. 2008), flying squirrel (Oshida et al. 2005), and Siberian pine (Dr. D. Shuvaev, pers.  
348 comm.) in the north of Western Siberia **had haplotypes common** with haplotypes in the regions located  
349 west of Lake Baikal. In addition to the peculiarities of the ecological properties of Siberian fir, its unusual  
350 phylogeographic pattern may be a consequence of the random nature of the long-distance seed dispersion,  
351 which undoubtedly plays an important role in the process of colonization.

352 The mitotypes M1 and M3, common in the Altai, the Sayans, and Kuznetsk Alatau **Mountains**, were  
353 absent in northern Siberia, indicating the lack of a significant contribution of populations **of these regions**  
354 to the modern northern populations, which coincides with a similar conclusion about the role of southern  
355 mountain populations of Siberian larch (Semerikov et al. 2013).

#### 356 **4.3. MtDNA data indicate repeated migrations of fir to the Urals**

357 Floristic surveys suggest the Siberian origin of most of the taiga forests species of the Urals  
358 (Krasheninnikov 1937; Hulten 1937; Gorchakovsky 1969). Accordingly, the mitotypes of Siberian fir



359 and Siberian larch in the Urals arose in Siberia or have originated from related mitotypes common in  
360 Siberia. Unlike Siberian larch, which has only one mitotype across the Urals, Siberian fir in the Urals  
361 has two mitotypes, M1 and M2, which are also common in Siberia. It is noteworthy that in the northern  
362 part of the Urals (starting from population #5 to the north) mitotype M2 is almost fixed (Fig. 1). M2 is  
363 also fixed in northern Siberia, which probably indicates that the colonization of the north of the Urals  
364 took place together with the settlement of the north of Siberia by a single migration wave. This  
365 assumption is supported by the results of studies of variation of allozyme and AFLP loci, which make  
366 however the story more complex: populations in the northern Urals genetically are similar both to the  
367 populations of the Baikal region, as well as to the populations of the Urals south (Semerikova and  
368 Semerikov 2006, 2011), suggesting the admixed origin of the populations in the northern Urals. It is  
369 interesting that the fir mitotypes in the south Ural and neighbor areas have a mosaic geographical  
370 distribution, and the majority of the investigated populations contain a single mitotype, which may be  
371 the result of dispersion from multiple local refugia, that suggests that M1 and M2 mitotypes were present  
372 here before LGM. Moreover, they could not come to the Urals together, during the post-LGM  
373 colonization of northern Siberia, since only one of them is present in the center of Western Siberia.  
374 Consequently, the fir migrations to the Urals most likely occurred several times or in different ways, for  
375 example, through more southerly regions, which are now outside the range of fir. For example, according  
376 to the findings of the *Abies* pollen in the peat bog near Lake Karasie, Kokshetau region of Kazakhstan  
377 (Gorchakovsky 1987, and refs. cited therein), the area of fir in the early Holocene could have expanded  
378 southward, capturing Northern Kazakhstan. In addition, the MAXENT reconstruction allows the  
379 presence of Siberian fir in Central Kazakhstan in the LGM (Fig. 2, A) and, accordingly, does not exclude  
380 the possibility of migrations from the Altai to the Urals across this area.

#### 381 4.4. Markers with different inheritance mode reflect different aspects of the colonization

382 The populations in Western Siberia, despite their Baikalian origin suggested by the mtDNA, according  
383 to the allozyme, AFLP and cpDNA data (Semerikova and Semerikov 2006, 2007, 2011) contain an  
384 admixture of genes from the populations of Altai, the Urals and the Sayans. Probably, this discrepancy  
385 of mitochondrial vs. nuclear and chloroplast markers can be explained by the difference in their  
386 inheritance mode and, as a result, the second and third are transferred by both seeds and more mobile  
387 pollen in contrast to the first transferred only by seeds. This feature determines faster homogenization of  
388 the spatial structure for nuclear and chloroplast markers due to the pollen mediated gene flow that more  
389 efficiently connects remote regions.

## 390 **5. Conclusions**

391 The study of mtDNA variation, analysis of paleontological data and environmental niche modelling  
392 shed light on the history of two Siberian conifers in the Late Pleistocene and Holocene and suggested the  
393 most likely scenario of the dynamics of their ranges. The geographical heterogeneity of the mtDNA  
394 variation of Siberian fir and Siberian larch in the southern part of their range is in agreement with their  
395 distribution from several glacial refugia, while the homogeneity of populations in the northern part of the  
396 range indicates that its colonization involved only one of the southern refugia, which, however, was not  
397 the same in these species. In addition, the beginning of the colonization of the northern part of the range  
398 of Siberian larch as indicated by the paleontological data and the results of modeling of ecological niches,  
399 predated the last glacial maximum unlike in Siberian fir. According to the mtDNA data the migrations of  
400 Siberian fir to the Urals probably occurred more than once.

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#### 409 **Compliance with ethical standards**

410 **Conflict of interest** The authors declare that the research was conducted in the absence of any  
411 commercial or financial relationship that could be construed as a potential conflict of interest.

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575

576 **Table 1.** Mitochondrial contigs, mitotypes, GenBank accession numbers, SNPs detected, nucleotide  
577 sequences of primers used for sequencing and SSCP genotyping, and length of the PCR product amplified  
578 in *Abies sibirica* (mitotypes M1, M2 and M3), *A. nephrolepis* (M5) and *A. semenovii* (M4).

Contig number	Mitotype	GenBank accession number	SNP (nucleotide position)	Primer nucleotide sequence	Length of the PCR fragment in <i>A. sibirica</i> , bp
167	M2	MH070276	T (992)	A167F: AGCTGATCCGCTGAATGACT	1339
	M1, M3	MH070277	G (992)	A167R: ACTTCGTCCCTGAAGCAAGA	1339
	M5	MH070278	G (992)		1339
	M4	MH070279	G (992)		1339
				A167NL <sup>a</sup> : AACAAATGGGATTTGGAATGC A167NR <sup>a</sup> : CTCGTCCAATTGATCAAGCA	221
65	M2	MH070284	C (46)	A65F: CCGGAGTGGTTTTGTTGAGT	1113
	M1, M3	MH070285	A (46)	A65R: TATGCCTTCTCGGAAACACC	1113
	M5	MH070286	C (46)		1096
	M4	MH070287	C (46)		1113
				A65NR <sup>a,b</sup> : TGGCCCCCTAATGGTGTTAGA	165
126	M2	MH070272	A (1254)	A126-2F: TGTGGGGATGGATCTCTAGC	1452
	M1, M3	MH070273	C(1254)	A126-2R: AGGGGTGGTGTGGTCAATAA	1452
	M5	MH070274	A(1250)		1448
	M4	MH070275	C(1254)		1452
				A126NL <sup>a</sup> : CTCCTCACCCCTTCGACTCAC A126NR <sup>a</sup> : CCAGAACGGGTGAGTCACTT	182
37	M2, M1	MH070280	C (1191)	A37-1F: GGCGACGAATAAATCAGGAA	1431
	M3	MH070281	A (1191)	A37-1R: TCTTGCTTGTTTTGGTGCTG	1431
	M5	MH070282	C (1191)		1431
	M4	MH070283	C (1191)		1431
				A37NL <sup>a</sup> : CTACAGCGGCACATAGATCG A37NR <sup>a</sup> : GTGGAGAGCTCTGCGCTAAT	250

579 <sup>a</sup>Used for the SSCP genotyping

580 <sup>b</sup>Used in a pair with A65F

581

582 **Table 2.** Hierarchical analysis of the mtDNA genetic variation (AMOVA) based on the SAMOVA  
 583 grouping of the *Abies sibirica* populations into three groups.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	2	422.082	1.244	92.23
Among populations within groups	42	12.793	0.014	1.03
Within populations	648	58.903	0.091	6.74
Total	692	493.778	1.349	
		Fixation indices	<i>P</i> -value	
<i>F<sub>SC</sub></i>		0.133	<0.00001	
<i>F<sub>ST</sub></i>		0.933	<0.00001	
<i>F<sub>CT</sub></i>		0.922	<0.00001	

584

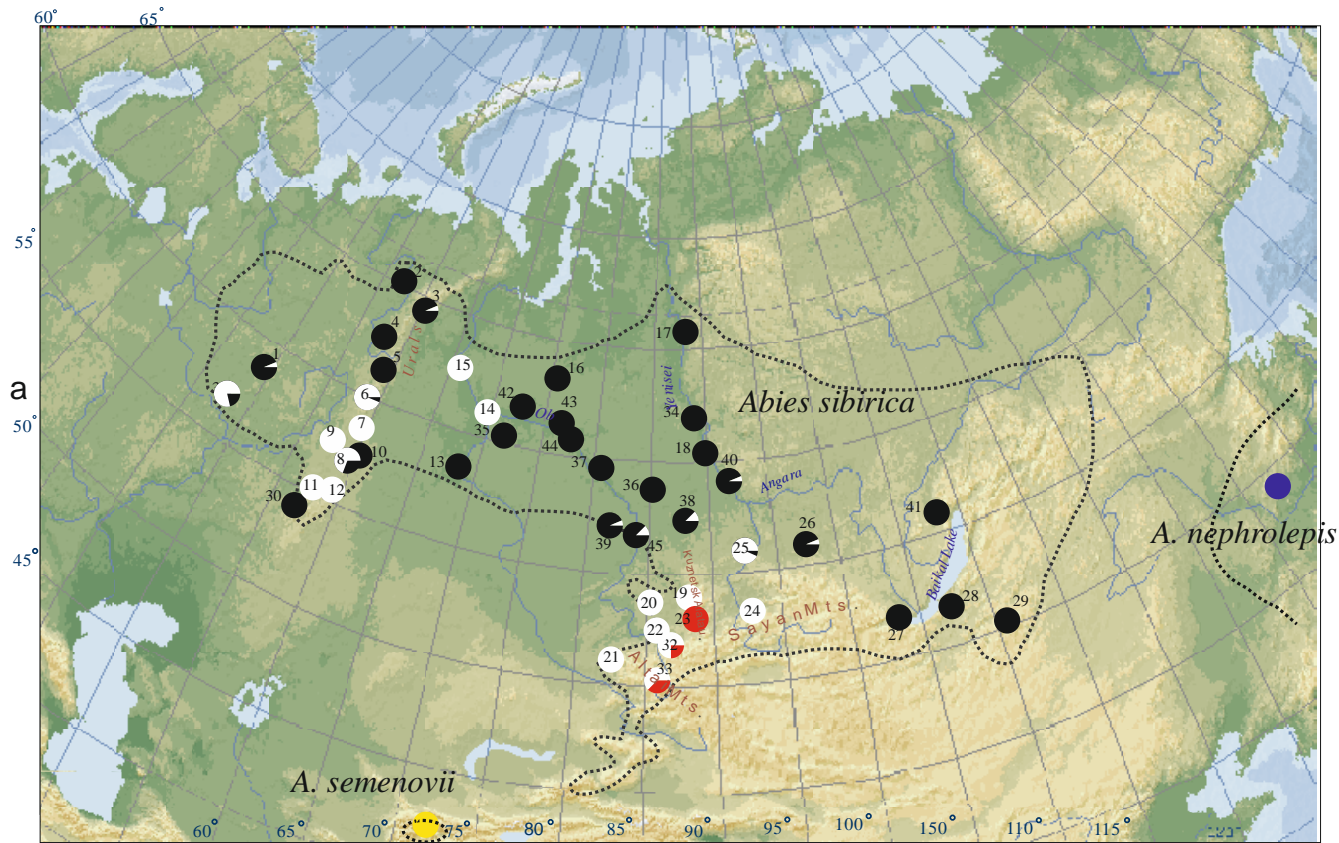
585

## 586 **Figure captions**

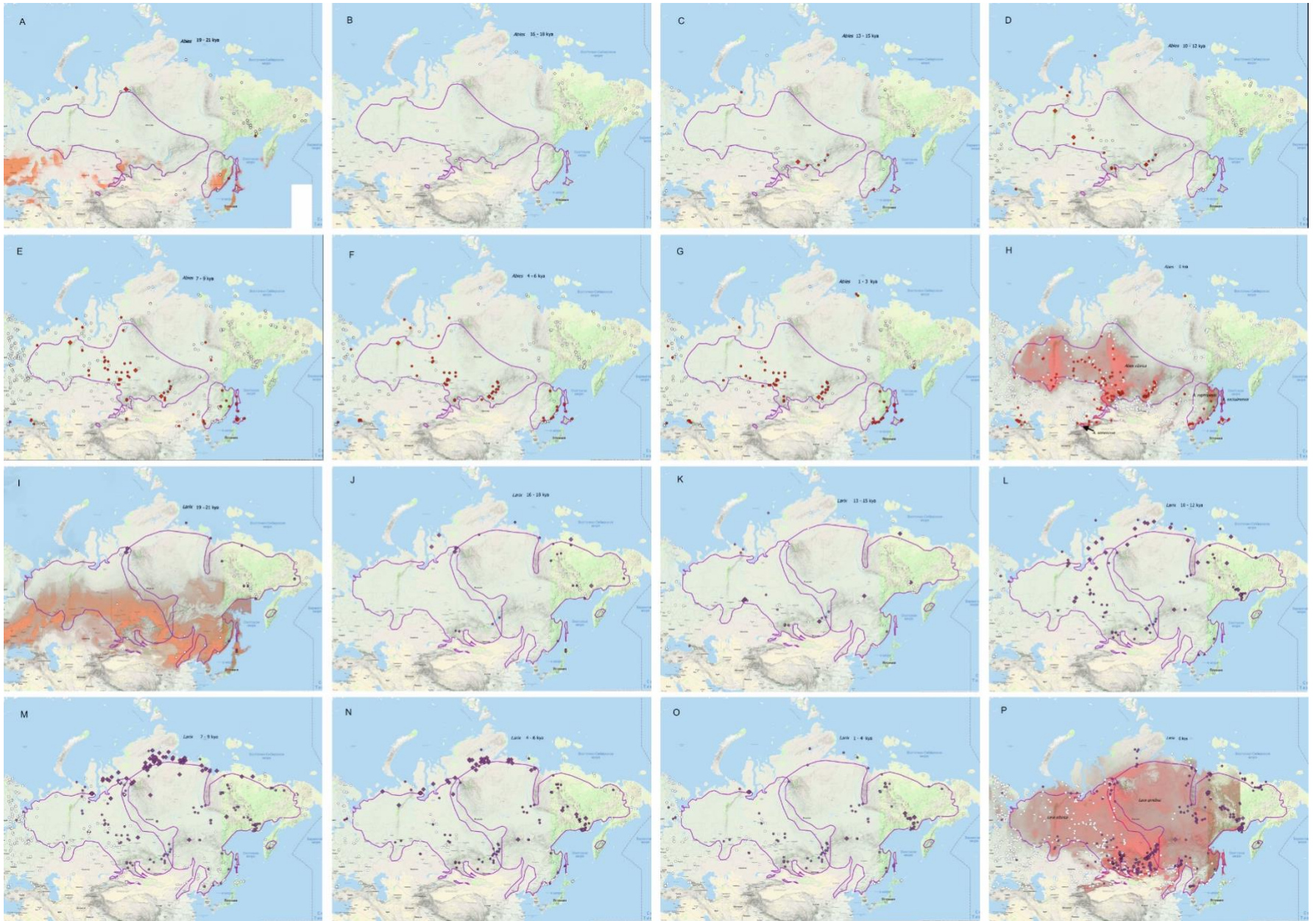
587

588 **Fig. 1. (a)** Geographic distribution of the studied fir populations. The mitotype frequencies in each  
 589 population are represented as a pie diagram. See population names in Table S1 (Supplementary  
 590 material). **(b)** A network of three mitotypes (M1-M3) found in *Abies sibirica* in Eastern Europe and  
 591 Northern Asia and two mitotypes found in *A. semenovii* (M4) and *A. nephrolepis* (M5).

592 **Fig. 2.** Mapped pollen and macrofossil paleorecords of *Abies* sp. and *Larix* sp. Modern ranges are  
593 outlined by the thick purple line. Existed pollen records for particular time interval are depicted as  
594 white circles, pollen records of *Abies* – as red circles, *Abies* macrofossils – as red diamonds, pollen  
595 record of *Larix* – as plume circles, *Larix* macrofossils – as plume diamond. Distributions of species  
596 predicted by MAXENT for LGM (A and I) and present day (H and P) are highlighted by the tone of  
597 red color. More saturated red color reflects conditions more appropriate for the species, less  
598 saturated - less suitable conditions. Time span (kya) for *Abies*: A – 18.5–21.5, B – 15.5-18.5, C –  
599 12.5–15.5, D – 9.5–12.5, E – 6.5–9.5, F – 3.5–6.5, G – 0.5–3.5, H - 0–0.5, and *Larix*: I – 18.5–21.5,  
600 J – 15.5-18.5, K – 12.5–15.5, L – 9.5–12.5, M – 6.5–9.5, N – 3.5–6.5, O – 0.5–3.5, P - 0–0.5.



601



Supplementary material

603

**A range-wide study of the mitochondrial DNA diversity of the Siberian fir  
indicates multiple post-glacial colonization centers**

604

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Vladimir L. Semerikov, Svetlana A. Semerikova, Yuliya A. Putintseva, Natalia V. Oreshkova,

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Konstantin V. Krutovsky

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609 **Table S1.** Studied fir populations and their mitotypes.

No.	Population	Location (Northern latitude / Eastern longitude)	Sample size	Mitotype frequencies	Diversity, $H_e$
<i>A. sibirica</i>					
1	Viyatka	58°40' / 49°30'	16	M1:1,M2:15	0.094
2	Pechera	65°00' / 57°30'	12	M2:12	0
3	Manya	64°30' / 60°50'	16	M1:1,M2:15	0.094
4	Pechero-Ilychsky	61°50' / 57°00'	24	M2:24	0
5	Denegkin	60°09' / 59°57'	16	M2:16	0
6	Kongakovskii	59°40' / 59°10'	16	M1:15,M2:1	0.094
7	Kushva	58°18' / 59°41'	16	M1:16	0
8	Homutovka	56°48' / 59°57'	13	M1:9,M2:4	0.346
9	Chusovoy	57°17' / 57°49'	16	M1:16	0
10	Tavatuy	56°50' / 60°20'	16	M2:16	0
11	Sim	54°59' / 57°41'	12	M1:12	0
12	Taganay	55°10' / 59°40'	16	M1:16	0
13	Tobolsk	58°12' / 68°16'	16	M2:16	0
14	Khanty-Mansiysk	61°00' / 69°10'	16	M1:16	0
15	Oktyabrskoye	62°35' / 66°05'	16	M1:16	0
16	Noyabrsk	63°12' / 75°29'	16	M2:16	0
17	Turuhansk	65°48' / 87°59'	16	M2:16	0
18	Yartsevo	60°14' / 90°15'	16	M2:16	0
19	Kemerovo	55°20' / 86°05'	24	M1:24	0
20	Salair	53°35' / 85°70'	24	M1:24	0
21	Kolyvan	51°10' / 82°50'	18	M1:18	0
22	Karasuk	51°58' / 85°57'	16	M1:16	0
23	Tashtagol	52°40' / 88°00'	16	M3:16	0
24	Tanzybey	52°50' / 93°00'	16	M1:16	0
25	Divnogorsk	55°55' / 92°30'	15	M1:14,M2:1	0.100
26	Taishet	55°57' / 98°00'	16	M1:1,M2:15	0.094

No.	Population	Location (Northern latitude / Eastern longitude)	Sample size	Mitotype frequencies	Diversity, $H_e$
27	Sludyanka	51°38'/103°42'	24	M2:24	0
28	Ulan-Ude	51°50'/106°42'	16	M2:16	0
29	Sohondinskii	49°30'/111°00'	16	M2:16	0
30	Inzer	54°18'/57°23'	16	M2:16	0
31	Yoshkar-Ola	56°42'/47°55'	11	M1:8,M2:3	0.327
32	Artybash <sup>1</sup>	51°48'/87°15'	16	M1:9,M3:3	0.409
33	Multa	50° 00' /85° 49'	8	M1:5,M3:3	0.534
34	Bor	61°30'/90°10'	16	M2:16	0
35	Salym	60°03'/71°27'	16	M2:16	0
36	BelYar	58°26'/85°06'	16	M2:16	0
37	Kargasok	59°00'/80°51'	16	M2:16	0
38	Teguldet	57°18'/88°14'	16	M1:2,M2:14	0.175
39	Bakchar	57°02'/82°03'	16	M1:1,M2:15	0.094
40	Eniseysk	58°25'/92°09'	16	M1:1,M2:15	0.094
41	Severobaikalsk	55°42'/109°03'	16	M2:16	0
42	Pochekuika	61°22'/73°46'	16	M2:16	0
43	Visokii	61°06'/76°00'	16	M2:16	0
44	Aleksandrov	60°25'/77°50'	8	M2:8	0
45	Tomsk	56°34'/84°00'	8	M1:1,M2:7	0.188
	Total		697		
		<i>A. nephrolepis</i>			
	Obluchye	49°01'/131°05'	1	M5:1	-
		<i>A. semenovii</i>			
	Sary-Chelek	41°54'/71°56'	1	M4:1	-

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611

612 **Fig.S1.** Genotyping of Siberian fir mitochondrial DNA markers using the SSCP method (Fujita et al.,  
613 1994) in a non-denaturing polyacrylamide gel. Eight lines (from left to right) correspond to eight  
614 individuals with mitotypes M3, M1, M1, M3, M2, M2, M2, M2. Four variable electrophoretic zones of  
615 single-stranded fragments correspond to the markers A167, A65, A126 and A37 (from top to bottom),  
616 each of which has two alleles differing in one nucleotide and different mobility, due to conformational  
617 polymorphism. Marker A167 in these eight trees has a nucleotide: G, G, G, G, T, T, T, T. Marker A65 -  
618 A, A, A, A, C, C, C, C, respectively. Marker A126 - C, C, C, C. A, A, A, A. A37 - A, C, C, A, C, C, C,  
619 C.  
620

