

Resource Article

1 **Siberian larch (*Larix sibirica* Ledeb.) chloroplast genome and**
2 **development of polymorphic chloroplast markers**

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15 **Abstract**

16 **Background:** The main objectives of this study were sequencing, assembling and annotation
17 of chloroplast genome of one of the main Siberian boreal forest tree conifer species Siberian
18 larch (*Larix sibirica* Ledeb.) and detection of polymorphic genetic markers – microsatellite loci
19 or simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs).

20 **Results:** We used data of the whole genome sequencing of three Siberian larch trees from
21 different regions - Urals, Krasnoyarsk, and Khakassia, respectively. Sequence reads were
22 obtained using the Illumina HiSeq2000 in the Laboratory of Forest Genomics at the Genome
23 Research and Education Center in the Siberian Federal University. The assembling was done
24 using the Bowtie2 mapping program and the SPAdes genomic assembler. The genome
25 annotation was performed using the RAST service. We used the SciRoKo program for the SSRs
26 search, and the Bowtie2 and UGENE programs for the SNPs detection. Length of the assembled
27 chloroplast genome was 122,561 bp, which is similar to 122,474 bp in the closely related
28 European larch (*Larix decidua* Mill.). As a result of annotation and comparison of the data with

29 existing data available only for three larch species - *L. decidua*, *L. potaninii var. chinensis*
30 (complete genome 122,492 bp) and *L. occidentalis* (partial genome of 119,680 bp), we
31 identified 110 genes, 34 of which represented tRNA, 4 rRNA and 72 protein-coding genes. In
32 total, 13 SNPs were detected; two of them were in the *tRNA-Arg* and *Cell division protein FtsH*
33 genes, respectively.

34 **Conclusions:** The complete chloroplast genome sequence was obtained for Siberian larch for
35 the first time. The reference complete chloroplast genomes, such as one described here would
36 greatly help in the chloroplast resequencing and search for additional genetic markers using
37 population samples. The results of this research will be useful for further phylogenetic and gene
38 flow studies in conifers.

39 **Keywords:** Chloroplast genome, *Larix sibirica*, Sequencing, Siberian larch, SNPs, SSRs

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44

45 **Background**

46 Chloroplast genome in conifers, including larch species [1] has a unique, strictly paternal
47 inheritance via pollen, unlike angiosperms, where it has a maternal inheritance via seeds [2]. It
48 allows tracing paternal gene flow and lineages separately from maternal (mitochondrial genes)
49 and bi-parental (nuclear genes) ones. Therefore, chloroplast DNA sequences are the most
50 important source of genetic markers to study distribution of paternal genes and paternally based
51 molecular phylogenetic relationships in conifers.

52 Larch species, as well as many other conifer species are the main boreal forest tree species,
53 which comprise ~30% of the world's forested lands [3]. Boreal forests play very important
54 ecological role, but are also affected by the global climate change. On one hand, they suffer

55 now from more frequent and drastic droughts, but on the other hand their area is expanding in
56 the northern regions, and their tree line is moving towards north creating an ecotone, a highly
57 dynamic transition area [4]. It is important to know how much of paternal associated gene flow
58 by pollen contributes into establishing this zone compared to the maternal and bi-parental
59 contributions by seeds. Such studies require chloroplast markers. Next generation sequencing
60 (NGS) technique allows whole chloroplast genome sequencing in multiple individuals and
61 makes a search for the molecular genetic markers more efficient. For instance, Parks *et al.* [5]
62 nearly completely sequenced chloroplast genomes in 37 pine species using NGS. They found
63 significant amount of variation (especially in two loci *ycf1* and *ycf2*) that provided them with
64 additional data for inferring intrageneric phylogeny of genus *Pinus*.

65 Whole chloroplast genome comparison across different species and genera allows also
66 studying organelle evolution and how it is associated with speciation and dispersal. Complete
67 chloroplast genome sequences are available in NCBI Genbank for multiple plant species,
68 including conifers. However, most of them represent the *Pinus* genus, and only three chloroplast
69 genomes are available for the *Larix* genus: complete for European (*Larix decidua* Mill.;
70 AB501189.1) and Chinese (*L. potaninii* var. *chinensis* Beissn.; KX808508) larch and partial for
71 Western larch (*L. occidentalis* Nutt.; FJ899578.1).

72 Variation in the chloroplast genome is effectively used in phylogenetics at different levels.
73 It allowed discriminating different subgenera and genera. For instance, Cronn *et al.* [6]
74 compared chloroplast genome sequences of seven pine and one spruce species and found three
75 regions that have deletions corresponded to the subgenera specific deletions in three genes:
76 *ycf12* (78 bp at the nucleotide starting position 51051), *psaM* (93 bp at position 51442) and
77 *ndhI* (371 bp at position 101988), respectively. These are common deletions in the chloroplast
78 genome in the pine species of the subgenus *Strobus* (i.e., *P. gerardiana*, *P. krempfii*, *P.*

79 *lambertiana*, *P. longaeva*, *P. monophylla*, *P. nelsonii*, *P. koraiensis*); the corresponding genes
80 were present in the subgenus *Pinus* (*P. contorta*, *P. ponderosa*, *P. thunbergii*) and in spruce
81 *Picea sitchensis* [6].

82 Variation in the chloroplast genome can be also effectively used in discriminating different
83 populations of the same species. For instance, Whittall *et al.* [7] demonstrated a strong
84 differentiation between mainland and island populations of Torrey pine (*Pinus torreyana*) based
85 on 5 SNPs found in the entire chloroplast genome of 120 Kbp.

86 **Methods**

87 We used data of the whole genome sequencing of three Siberian larch trees generated by
88 Illumina HiSeq2000 [8]. DNA samples were isolated from needles and haploid callus of three
89 Siberian larch trees, representing different regions in Russia – Ural Mountains, Krasnoyarsk
90 Region and Khakassia Republic, respectively. *Larix decidua* Mill. [9] and *L. occidentalis* Nutt.
91 [5] chloroplast genomes were used as reference (NCBI Genbank accession numbers
92 AB501189.1 and FJ899578.1, respectively). We did not use the chloroplast genome of *L.*
93 *potaninii* [10] as a reference, because it was assembled by using the chloroplast genome of *L.*
94 *decidua* (NC_016058; [9]) as a reference, but we used it in the comparative analysis. The paired-
95 end (PE) and mate-pair (MP) libraries with fragment sizes of 400-500 bp (Ural and Krasnoyarsk
96 trees) and 300-400 bp (Khakassia tree), respectively, were used for sequencing via 2×100
97 cycles by Illumina HiSeq2000.

98 The sequence reads were mapped to the reference chloroplast genomes using the Bowtie2
99 software [11], which is good for mapping short sequence reads to medium-sized and large
100 genomes. This software implements an algorithm to derive FM-index based on Burrows-
101 Wheeler Transform. The SPAdes genome assembler has been used to assemble the larch

102 genome, which implements the De Bruijn graph approach [12]. The Rapid Annotation service
103 with Subsystem Technology (RAST) has been used for annotation [13].

104 The first step in our assembly procedure consisted of mapping short reads to the available
105 chloroplast genome references of *L. decidua* and *L. occidentalis* using the Bowtie2 software.
106 Then, the aligned reads were assembled by SPAdes. Obtained contigs were aligned again on
107 the reference of *L. decidua* using BLAST. At the third step, the selected contigs were verified
108 to get the “trusted” status. Then, the assembly was carried out using SPAdes. The final step of
109 the assembly was the scaffolding, which was done using the generated contigs and MP reads
110 using the SSPACE program [14].

111 Considering a well-known fact that chloroplast organelle originated from cyanobacteria, and
112 that, therefore, chloroplast genes are still very similar to the bacterial ones, the RAST service,
113 which was designed for annotation of bacterial and archaeal genomes, was used for the larch
114 genome annotation. The annotation obtained by the RAST contained both confirmed known
115 genes and predicted genes, potentially coding hypothetical proteins. In order to clarify the roles
116 of these hypothetical coding regions our annotation was compared with annotations of two
117 closely related species *L. decidua* and *L. occidentalis*, respectively. In addition, some fragments
118 of the genome have been also selectively aligned with BLAST. Sites of hypothetical proteins
119 confirmed by BLAST were identified and recorded.

120 SNPs were search using the Bowtie2 and UGENE [15] software (option *Call Variants with*
121 *SAMtools*). The search was done across the three above mentioned trees. First, reads of Urals
122 and Khakassian trees were mapped to the finally assembled genome of the Krasnoyarsk tree.
123 The resulting *sam*-file together with the assembled genome was used by the UGENE program
124 to search for SNPs.

125 **Results**

126 The total length of the final Siberian larch chloroplast genome assembly was 122,560 bp, which
127 is very close to 122,474 bp in closely related European larch (*Larix decidua*). The annotation
128 through the comparison with available data for *L. decidua* and *L. occidentalis* identified 110
129 genes, from which 34 represented tRNA genes, 4 rRNA and 72 protein-coding genes. In three
130 trees 13 SNPs were detected. Two of them were found in the coding regions of the *tRNA-Arg*
131 and *Cell division protein ycf2* genes.

132 We used available software, such as Bowtie2, BLAST and SPAdes to assemble chloroplast
133 genome using reads generated in the whole genome sequencing of Siberian larch project. We
134 used SSPACE for scaffolding and the RAST service for annotation of obtained chloroplast
135 genome. We developed a procedure that allowed us to successfully extract chloroplast genome
136 specific reads and then assemble and annotate the resulting sequences. We identified and
137 verified 110 coding regions representing 38 RNA and 72 protein genes, which is equal to the
138 number of genes in chloroplast sequences of *L. decidua* and *L. potaninii* and close to 105 genes
139 in a partial chloroplast genome sequence of *L. occidentalis*. A gene map of the genome was
140 generated using OGDRAW [16] and presented in Fig. 1. Search for SNPs using UGENE
141 revealed a relatively small number of SNPs (Fig. 2; Additional file 1), but it is only preliminary
142 data based on a limited sample size.

143 **Discussion**

144 The chloroplast genome variation in most plants is often limited due to a relatively low
145 frequency of mutations in this organelle. For example, the mutation rate of the chloroplast
146 genome in pines is approximately $0.2-0.4 \times 10^{-9}$ synonymous substitutions per nucleotide per
147 year [17, 18]. However, with an average length of 120-160 Kbp and 130 genes chloroplast

148 genomes are sufficiently large and complex and include structural and point mutations that
149 reflect population differentiation and evolutionary divergence [6].

150 Unlike angiosperms, conifer chloroplast DNA (cpDNA) lacks large inverted repeats (IR),
151 but contains dispersed repetitive DNA that is associated with structural rearrangements. In
152 addition to large dispersed repeated sequences, conifer cpDNA also possess a number of small
153 repeats. It contains variable numbers of tandem repeats of 124 to 150 bp in size, which are
154 associated with polymorphic rearranged region near *trnK-psbA*, where the *psbA* gene has been
155 duplicated [19].

156 Most variation in the chloroplast genome is associated with microsatellite loci [20, 21].
157 However, these markers have a too high mutation rate that can lead to the incorrect phylogenetic
158 inferences [22-24]. SNPs could be better markers for phylogenetic inferences, and comparative
159 complete chloroplast genome studies are needed to discover these markers. The reference
160 complete chloroplast genomes, such as one described here would greatly help in the chloroplast
161 resequencing and search for SNPs using population samples.

162 **Conclusions**

163 The complete chloroplast genome sequence was obtained for Siberian larch for the first time.
164 Annotation and comparison of the obtained data with data available only for two other larch
165 species helped us identify and verify 110 coding regions representing 38 RNA and 72 protein
166 genes. Total 13 SNPs were detected; two of them were in the coding regions of the genome.
167 The results of this research will be useful for further phylogenetic and gene flow studies in
168 conifers.

169 **Additional files**

170 **Additional file 1:** excel file representing the Siberian larch chloroplast genetic variant data.

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181 **Availability of data and materials**

182 The annotated chloroplast genome of *L. sibirica* has been deposited in the NCBI GenBank with
183 the accession number MF795085.

184 **Authors' contributions**

185 EIB and YAP assembled and annotated the chloroplast genome, analysed the data and wrote the
186 draft paper, NVO prepared and sequenced DNA libraries, KVK designed and coordinated
187 research and wrote the paper.

188 **Ethics approval and consent to participate**

189 Not applicable.

190 **Consent for publication**

191 Not applicable.

192 **Competing interests**

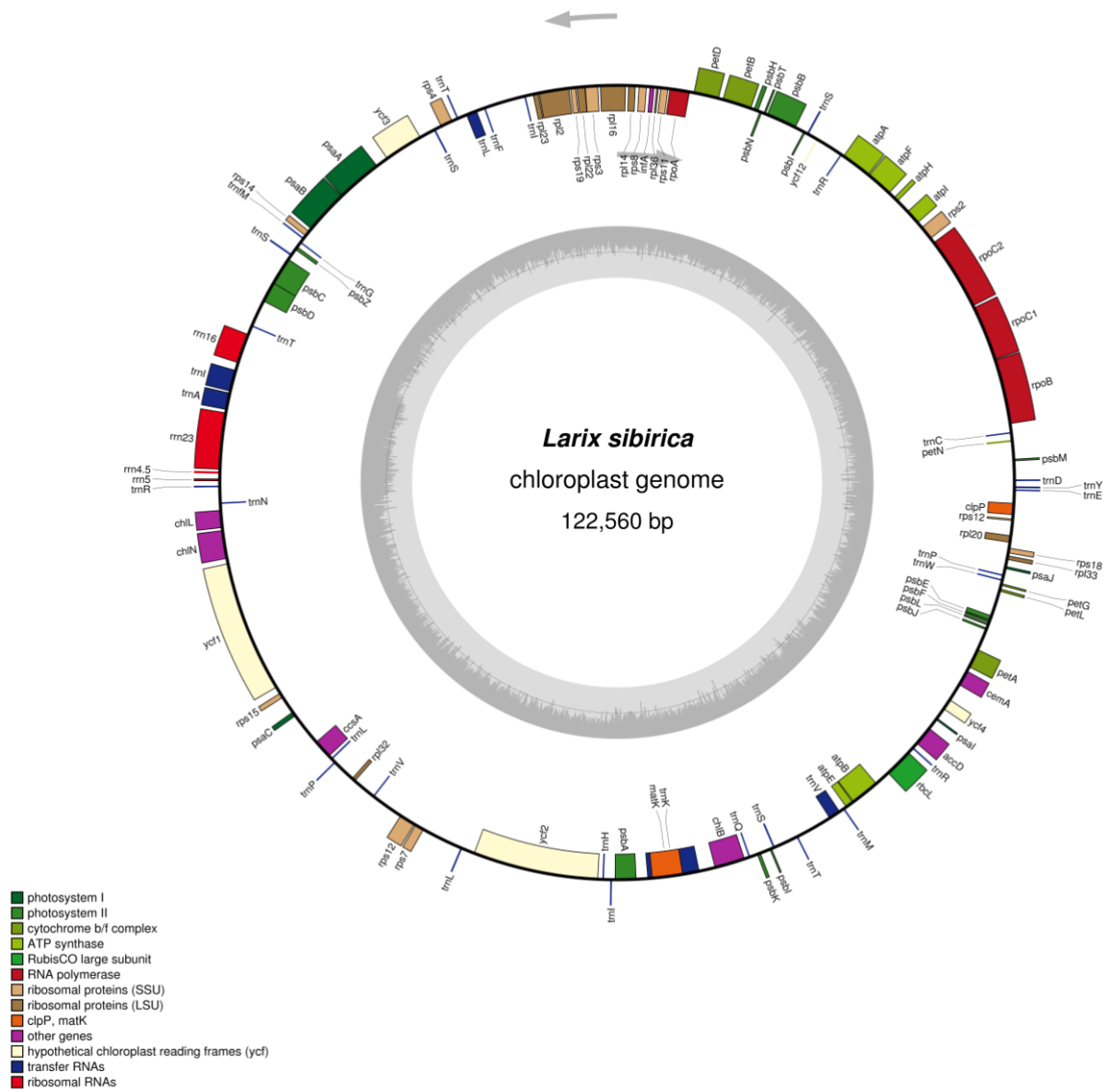
193 The authors declare that they have no competing interests.

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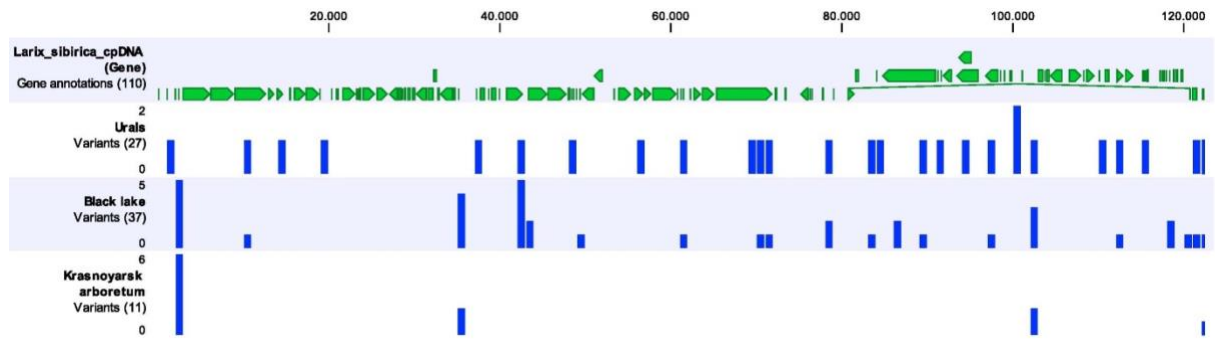
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- 267



269

270 **Fig. 1** Gene map of the *Larix sibirica* chloroplast genome. Genes belonging to different
 271 functional groups are color-coded. The dark and light grey in the inner circle represents the
 272 GC and AT content, respectively

273



274

275 **Fig. 2** Variation detected in the *Larix sibirica* chloroplast genome (see also Additional file 1)