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Phylogeography of Eurasian *Larix* species inferred from nucleotide variation in two nuclear genes

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Larch (*Larix* Mill.) is one of the most widely distributed tree genera in Eurasia. To determine population structure and to verify classification of five species and three varieties of the Eurasian *Larix* species, we investigated levels and patterns of nucleotide variation of two nuclear gene regions: the 4-coumarate coenzyme A ligase (4CL) and the coumarate 3-hydroxylase (C3H). In the 4CL region nucleotide diversity at silent sites (π_{sil}) varied between 0.0020 in *L. gmelinii* to 0.0116 in *L. gmelinii* var. japonica and in the C3H region between 0.0019 in *L. kaempferi* to 0.0066 in *L. gmelinii* var. japonica. In both gene regions statistically significant population differentiation (F_{ST}) was detected among adjacent refugial populations of some species suggesting limited gene flow and/or long time isolation of some refugial populations. On the other hand, populations of *L. sukaczewii* from northwestern Russia, which was glaciated 20,000 years ago showed no differentiation. This result is consistent with recent postglacial origin of these populations. Haplotype composition of some of the investigated Eurasian *Larix* species suggested that they are considerably diverged. Some haplotypes were unique to individual species. Our results indicate that more intensive sampling especially from known refugial regions is necessary for inferring correct classification of Eurasian *Larix* species and inferring their postglacial migration.

Key words: Eurasian *Larix*, nucleotide variation, nuclear gene, DNA sequence, population differentiation

INTRODUCTION

Larch species (*Larix* sp. Mill) are prominent components of the boreal forest. They are widely distributed across Eurasia and constitute 40% of its forest (Farjon, 1990). Relationships among Eurasian *Larix* species and their classification are still controversial. For instance, in western Urals and western Siberia some authors recognize only one species: *L. sibirica* (Bobrov, 1972; Bobrov, 1978; Farjon, 1990; Milyutin and Vishnevetskaia, 1995). On the other hand, two species (*L. sibirica* and *L. sukaczewii*) have been recognized in this region by some other authors (Dylis, 1947; Abaimov et al., 1998, 2002; Bashalkhanov et al., 2003). Similarly, there are different classifications of *Larix* species occurring in central and eastern Siberia. For instance, Milyutin and Vishnevetskaia (1995) recognized there only one species: *L. gmelinii*. On the other hand, *L. gmelinii* and two varieties (*L. gmelinii* var. olgensis and *L. gmelinii* var. japonica) have been recognized there by Farjon (1990). Yet another classification of the *Larix* species occurring in central and eastern Siberia was proposed by Abaimov et al. (2002) who recognized there: *L. cajanderi, L. gmelinii* and its three varieties: *L. gmelinii* var. olgensis, *L. gmelinii* var. japonica and *L. gmelinii* var. kamchatica. Several other classifications were also proposed for the *Larix* species from this region (Bobrov, 1978).

The advance and retreat of glaciers have significantly influenced the distribution and diversity of plant species. Climatic changes associated with glaciations led to large scale migration and reduction in population size and number followed by colonization and population expansion as the glaciers retreated (Pielou, 1991). During the last glacial maximum (LGM) approximately 18,000 ~ 20,000 years ago, a great part of the current taiga zone of the northern Eurasia, where the extant *Larix* species occur, was covered by ice (Svendsen et al., 1999; Tarasov et al., 2000), while most of Beringia (eastern Eurasia) remained ice-free during LGM (Hamilton and Thorson,

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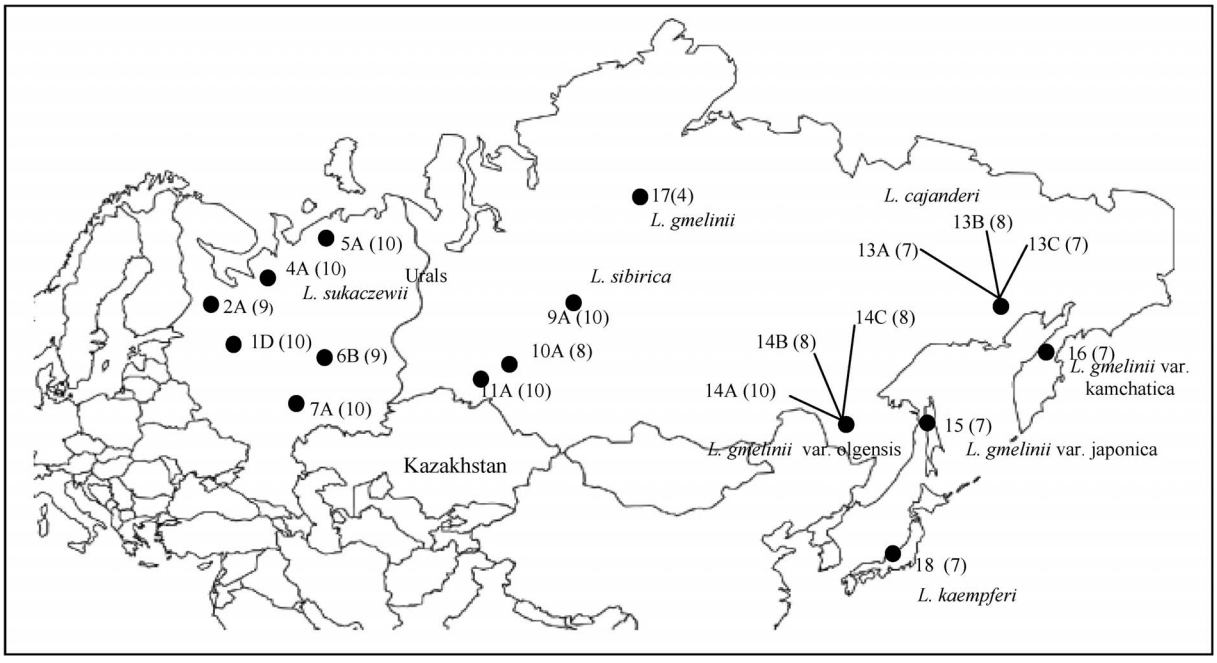

Fig. 1. Locations of the investigated *Larix* species from Eurasia, numbers of individuals are shown in parentheses near each population.

1983; Porter et al., 1983; Ananyeyev et al., 1993; Bennett, 1997). Both pollen and macrofossil evidence clearly indicate that *Larix* species survived during LGM in multiple and often isolated refugia located south of the Urals, southern Siberia and in western Beringia (Kremenetski, 1994; Tarasov et al., 2000; Andreev et al., 2002). Additional refugia were present in northern Kazakhstan, near the Sea of Azov and in the Yana-Indigirka lowland in the Russian Far East (Tarasov et al., 2000; Anderson et al., 2002). These refugia were suggested to serve as a source of *Larix* re-colonization of various regions of Eurasia during late Pleistocene (Semerikov et al., 1999).

Previous studies suggested that Eurasian *Larix* species are weakly diverged and have low levels of population differentiation (Lewandowski et al., 1991; Timerjanov, 1997; Semerikov et al., 1999, 2003; Semerikov and Lascoux, 2003; Wei and Wang, 2003, 2004a,b; Larionova et al., 2004). These findings are surprising taking into account complex and heterogeneous climatic history of Eurasia and certain reproductive features of *Larix* species such as limited seed and pollen dispersal (Duncan, 1954; Knowles et al., 1992), which serve to restrict gene flow among populations and therefore are expected to promote population differentiation.

Most of previous studies on DNA variation of Eurasian *Larix* species focused on non-coding and anonymous regions of nuclear genome and included only a single or very few species (Semerikov et al., 2003; Kozyrenko et al., 2004). The objectives of this study are to clarify population differentiation of Eurasian *Larix* species and to verify their classification. For this purpose we investigated levels and patterns of nucleotide variation of two partial nuclear gene regions: 4−coumarate coenzyme A ligase (4CL) and coumarate 3- hydroxylase (C3H). The 4CL and C3H gene regions play a key role in general phenylpropanoid metabolism in lignin biosynthesis. We examined 19 populations representing five species and three varieties from the genus *Larix* (Fig. 1). Four of the investigated populations of *L. sukaczewii* (1D ~ 5A) came from previously glaciated area in northwestern Russia. The other two populations (6B and 7A) came from putative refugia in southern Urals. Populations 10A and 11A of *L. sibirica* included in our study occur in or near putative refugia in southern Siberia. Populations of *L. cajanderi* (13A, 13B and 13C) are located near putative refugia in western Beringia. The remaining populations came from ice free areas in central and southeastern Siberia, Sakhalin Island, Kamchatka Peninsula and central Japan.

MATERIALS AND METHODS

Plant materials Seed samples were collected in 19 natural populations of the following five species and three varieties of Eurasian *Larix* species: *L. sibirica*, *L. sukaczewii, L. cajanderi, L. gmelinii, L. gmelinii* var. japonica, *L. gmelinii* var. kamchatica, and *L. gmelinii* var. olgensis (Abaimov et al., 2002). Seeds of *L. kaempferi* were collected in Japan. In this study we adopted classification proposed by Abaimov et al. (2002). Details about the locations and number of individuals of the investigated populations are presented in Fig. 1.

DNA isolation, PCR amplification and sequencing Megagametophytes, which represent haploid maternal tissue were isolated from germinating seed. Genomic DNA was isolated from megagametophytes using SDS method (Ish-Horowicz, 1989). Two partial gene regions (758 bp and 873 bp in alignment length) were amplified for the 4CL and C3H genes respectively. The PCR primers for the 4CL gene region were the same as those reported by Wang et al. (2000). The forward primer 5'-CCAATCCTTTYTACAAGCCG - 3' was located in exon 1 and the reverse primer 5' - CGGGGAARGGCTYCTTTGC-3' was located in exon 2. Primers for the C3H gene region were designed based on DNA sequences of *Pinus taeda* from GeneBank using primer3 program (http://fokker.wi.mit.edu/primer3/input-030.htm) (Rozen and Skaletsky, 2000). The forward primer 5'- CGAGCATTC-CCTATCTCC-3' was located in exon 1 and the reverse primer 5' - AACAAGCCCTGGATTCTCTG- 3' was located in exon 2. The PCR mixture was prepared to the total volume of 50 µl containing 50–100 ng DNA template, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 2.5 pM of each primers and 200 µM of each dNTP and 1 unit of Taq polymerase. Amplification was carried out as follows: 95°C for 3 min. followed by 35 cycles of 30 sec. at 95°C, 30 sec. at 55°C for annealing, 90 sec. at 72°C, and finally with 7 min. at 72°C for further extension. PCR products were purified using Wizard^{R} SV Gel and PCR Clean-Up System (Promega, USA). The purified products were directly sequenced using the BigDye^{TM} Terminator (v 3.1) and ABI Prism 3100 automatic sequencer

Table 1. Summary of polymorphic sites in the 4CL gene region including 26 haplotypes with 21 segregating sites, nucleotide positions relative to the beginning of the sequence are indicated by digits on the top, – indicates indels, * indicates nonsynonymous substitution

Nucleotide Positions	5 4	1 2 6	1 5 9	2 0 1	2 4 0	2 4 4	2 7 0	2 8 8	3 1 5	3 1 8	3 2 9	3 4 2	3 5 7	4 0 5	4 5 4	4 8 5	5 4 7	6 4 8	6 6 4	7 2 6	7 2 9
Haplotype						*	*								*	*	*			–	–
H1	T	G	G	G	G	C	C	C	T	C	G	C	C	G	A	T	C	C	C	T	–
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	T
H3	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	T	T
H4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
H5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	T	T
H6	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
H7	.	.	.	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	T	T
H8	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
H9	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	–
H10	A	A	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	T	T
H11	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
H12	A	A	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	T	T
H13	A	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	–
H14	A	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	T
H15	A	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	T	T
H16	A	A	.	.	.	.	.	.	.	.	.	.	T	.	G	.	.	.	.	T	T
H17	A	A	.	.	.	.	.	T	C	.	.	.	T	.	.	.	.	.	.	T	T
H18	A	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	T	T
H19	A	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	T	T
H20	A	A	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	T	T
H21	A	A	.	.	.	.	.	.	.	.	C	.	.	.	G	.	.	.	.	T	T
H22	A	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
H23	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T	T
H24	A	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T	T
H25	A	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	T	T
H26	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T

(Applied Biosystems, Foster City, CA, USA). Sequences obtained in this study have been deposited in the GenBank database under accession numbers EU280809 through EU280840 and EU280841 through EU280886 for the C3H and 4CL gene regions respectively.

Three copies of the 4CL gene (4CL-A1, 4CL-A2 and 4CL-B) exist in some *Larix* species (Wei and Wang, 2004a). However, we have several reasons to believe that the 4CL primers used in our study amplified only one particular copy (4CL-B) of the 4CL gene region. We compared the 4CL sequences obtained in our study with those reported by Wei and Wang (2004a). We found that the 4CL-A1 and 4CL-A2 copies are similar to each other and differ by only 14 nucleotides. On the other hand, they are highly diverged from the 4CL-B copy. The alignments between 4CL-A1 *vs*. 4CL-B and 4CL-A2 *vs*. 4CL-B copies differed by more than 50 nucleotides. Some of our sequences (28 out of 159) were identical with the 4CL-B region reported by Wei and Wang (2004a), while others differed by no more than 18 nucleotides. On the other hand, they differed from the other two copies by more than 50 nucleotides. In another study by Wang et al. (2000) where the same primers were used only one copy of the 4CL gene was detected in *L. gmelinii*. We compared the 4CL sequences obtained in that study with those reported by Wei and Wang (2004a) as well as with those obtained in our study. We found that similar to our results, the sequences obtained by Wang et al. (2000) represented the 4CL-B copy. Furthermore, if multiple and diverged copies were present in our material, we would expect to observe multiple peaks during sequencing using ABI 3100 sequencer, such as those reported by *e.g*., Gernandt and Liston (1999). Yet we did not observe such peaks. We therefore believe that direct sequencing method used in our study detected only one copy (4CL-B) of the 4CL gene region. Based on our data alone we cannot determine the reason why only this copy was amplified in our study and in that by Wang et al. (2000). Nevertheless, such selective amplification has been often reported in other studies and its possible causes have been reviewed by *e.g*., Wagner et al (1994).

Data analysis DNA sequences obtained for each gene region were checked and assembled using the ATGC program ver. 4 (GENETYX CORPORATION). Sequence alignments were done with CLUSTAL X program (Thomson et al., 1997) and adjusted manually using BioEdit program (Hall, 1999). DnaSP program ver. 4.0 (Rozas et al., 2003) was used to estimate the level of nucleotide diversity (π) (Nei, 1987) and nucleotide polymorphism (θ) (Watterson, 1975). Fixation index values (F_{ST}) (Hudson et al., 1992) were calculated without gaps

Table 2. Summary of polymorphic sites in the C3H gene region including 17 haplotypes with 17 segregating sites, nucleotide positions relative to the beginning of the sequence are indicated by digits on the top, * indicates nonsynonymous substitution

Nucleotide Positions	1 3	1 7 0 *	1 8 2	1 8 8	2 1 1	2 1 4	2 6 6	3 5 9	3 7 0	4 0 5	4 3 2	4 5 5	4 7 0	5 0 5	5 3 2	5 5 9	6 8 1
Haplotype																	
H1	A	G	A	C	G	T	G	G	T	C	T	T	G	G	C	G	G
H2	.	.	.	G	A	C	.	.	.	.	C	.	A	C	.	.	.
H3	.	.	.	.	.	.	.	.	.	A	.	C	.	C	.	.	.
H4	.	.	.	G	A	.	.	.	.	.	C	.	A	C	.	.	.
H5	.	.	.	.	.	.	.	.	.	.	C	.	.	C	A	.	.
H6	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
H7	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.
H8	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.
H9	.	.	.	.	.	.	.	A	.	.	C	.	.	C	.	.	.
H10	.	C	.	.	.	.	.	.	.	.	C	.	.	C	.	T	.
H11	.	.	.	G	A	C	.	.	.	.	C	.	A	.	.	.	.
H12	.	.	.	.	.	.	T	.	.	.	C	.	.	.	.	.	.
H13	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	T	A
H14	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
H15	.	.	T	G	A	.	.	.	.	.	C	.	A	C	.	.	.
H16	.	.	.	G	A	.	.	.	.	.	C	.	.	C	.	.	.
H17	G	.	T	G	A	.	.	.	.	.	C	.	A	C	.	.	.

using the Proseq program ver. 2.9 (Filatov, 2002) with 10,000 permutations to determine significance level. To infer relationships among haplotypes neighbor-joining (NJ) trees (Saitou and Nei, 1987) were constructed including gaps using CLUSTAL X program (Thomson et al., 1997), bootstrap values were calculated from 1000 replicates.

RESULTS

Nucleotide variation The sequences of haplotypes for 159 individuals were obtained for the two partial gene regions: 758 bp of the 4CL region and 873 bp of the C3H region (following alignment). In the 4CL region, there were 21 segregating sites and 26 haplotypes (Table 1). The first nucleotide position was numbered as +1 in both gene regions. In exon 1 (1 ~ 654 bp), there were 18 segregating sites including 13 synonymous substitutions and five non-synonymous substitutions (positions 244, 270, 454, 485 and 547). There were only one segregating site and two indels (insertion or deletion) in the intron (655 ~ 736 bp). On the other hand, there were no segregating sites in exon 2 (737 ~ 758 bp). In the C3H region, there were 17 segregating sites and 17 haplotypes (Table 2). In exon 1 (1 ~ 187 bp) there were five segregating sites including four synonymous substitutions and one non-synonymous substitution (position 70). Eleven segregating sites were found in the intron (188 ~ 588 bp). There was only one synonymous substitution in exon 2 (589 ~ 873 bp). No indels were detected in the C3H gene region.

Measures of nucleotide variation, nucleotide diversity (π) and nucleotide polymorphism (θ) were similar, so only π values for both gene regions are presented (Table 3). Total nucleotide diversity at all sites (π_T) in the 4CL region varied between 0.0007 in *L. gmelinii* and *L.*

Table 3. Summary of nucleotide diversity (π) in the 4CL and C3H gene regions

Populations	π for 4CL				π for C3H			
	π syn synonymous	π nonsyn nonsynonymous	π_{sil}	π_T	π syn synonymou	π nonsyn nonsynonymous	π_{sil}	π_T
L. sukaczewii	0.0079	0.0001	0.0057	0.0020	0.0036	0.0002	0.0026	0.0016
1D	0.0071	0.0000	0.0047	0.0016	0.0019	0.0000	0.0023	0.0013
2A	0.0073	0.0000	0.0049	0.0016	0.0021	0.0000	0.0021	0.0012
4A	0.0084	0.0040	0.0056	0.0021	0.0000	0.0000	0.0012	0.0007
5A	0.0100	0.0000	0.0086	0.0028	0.0033	0.0005	0.0025	0.0017
6B	0.0077	0.0004	0.0052	0.0020	0.0000	0.0000	0.0008	0.0005
7A	0.0060	0.0000	0.0040	0.0013	0.0100	0.0005	0.0050	0.0031
L. sibirica	0.0140	0.0000	0.0102	0.0033	0.0028	0.0005	0.0030	0.0020
9A	0.0135	0.0000	0.0099	0.0032	0.0033	0.0000	0.0025	0.0014
10A	0.0114	0.0000	0.0076	0.0025	0.0023	0.0015	0.0027	0.0022
11A	0.0140	0.0000	0.0108	0.0036	0.0036	0.0000	0.0040	0.0024
L. cajanderi	0.0083	0.0015	0.0072	0.0034	0.0000	0.0000	0.0035	0.0020
13A	0.0034	0.0011	0.0034	0.0019	0.0000	0.0000	0.0039	0.0023
13B	0.0094	0.0019	0.0081	0.0039	0.0000	0.0000	0.0025	0.0015
13C	0.0092	0.0015	0.0084	0.0038	0.0000	0.0000	0.0033	0.0020
L. gmelinii var. olgensis	0.0045	0.0008	0.0051	0.0022	0.0027	0.0000	0.0044	0.0026
14A	0.0045	0.0007	0.0053	0.0022	0.0000	0.0000	0.0034	0.0019
14B	0.0015	0.0011	0.0031	0.0017	0.0000	0.0000	0.0018	0.0011
14C	0.0073	0.0008	0.0070	0.0029	0.0080	0.0000	0.0072	0.0042
L. gmelinii var. japonica								
15	0.0138	0.0006	0.0116	0.0042	0.0106	0.0000	0.0066	0.0038
L.gmelinii var. kamchatica								
16	0.0057	0.0015	0.0061	0.0031	0.0000	0.0000	0.0039	0.0023
L. gmelinii								
17	0.0030	0.0000	0.0020	0.0007	0.0190	0.0000	0.0059	0.0034
L. kaempferi								
18	0.0034	0.0000	0.0023	0.0007	0.0044	0.0000	0.0019	0.0011

Table 4. Fixation index (F_{ST}) values for the two nuclear gene regions, 4CL (below diagonal) and C3H (above diagonal) for all pairwise population comparisons

Species	Pop.	1D	2A	4A	5A	6B	7A	9A	10A	11A	13A	13B	13C	14A	14B	14C	15	16	17	18
L. sukaczewii	**1D**		–0.106	–0.070	–0.033	0.007	0.203*	–0.081	0.131	–0.014	0.150*	0.521**	0.312*	0.257*	0.008	0.273*	0.137*	0.150*	0.567*	0.721**
	2A	–0.069		–0.049	–0.050	0.071	0.207	–0.077	0.119	0.004	0.141	0.526**	0.313*	0.258*	0.008	0.271**	0.130*	0.148*	0.571**	0.727**
	4A	–0.006	0.008		0.084	–0.035	0.235*	0.007	0.256*	0.057	0.222*	0.607**	0.391**	0.333*	0.014	0.327**	0.195*	0.209*	0.613*	0.789**
	5A	–0.009	0.007	–0.004		0.163	0.206*	–0.076	–0.020	–0.005	0.180*	0.486**	0.295*	0.249*	0.090	0.256**	0.125*	0.158	0.547*	0.685**
	6B	0.106	–0.060	–0.026	–0.009		0.264*	0.057	0.327*	0.075	0.334**	0.661**	0.457**	0.403*	0.088	0.374**	0.258**	0.276**	0.641*	0.823**
	7A	0.238**	0.133	0.087	0.123*	0.227**		0.206*	0.254*	0.208	0.295*	0.498**	0.365**	0.365*	0.229	0.213*	0.180	0.270**	0.356*	0.465**
L. sibirica	**9A**	–0.029	–0.006	0.028	–0.060	–0.032	0.158*		0.087	–0.038	0.200*	0.518**	0.318**	0.266*	0.046	0.278**	0.148*	0.165*	0.565*	0.713**
	10A	0.310*	0.237*	0.328*	0.306**	0.277*	0.407*	0.232		0.112	0.214*	0.464**	0.311*	0.281*	0.238*	0.257**	0.143*	0.214*	0.530*	0.646**
	11A	0.014	0.006	0.099	–0.005	0.003	0.215**	–0.061	0.093		0.204*	0.479**	0.303**	0.260*	0.091	0.272**	0.157*	0.175*	0.538**	0.667**
L. cajanderi	**13A**	0.114	0.112	0.131*	0.056	0.113	0.235**	0.096	0.443**	0.157*		0.220*	0.062	0.049	0.128	0.102	0.018	0.010	0.521*	0.631**
	13B	0.090	0.087	0.163*	0.098	0.071	0.306**	0.075	0.117	–0.001	0.163*		–0.077	0.016	0.446*	0.061	0.114	0.061	0.619**	0.741**
	13C	0.052	0.108	0.082	–0.004	0.034	0.293**	0.034	0.273*	0.025	0.079	–0.038		–0.127	0.196	0.013	0.003	–0.110	0.560*	0.678**
L. gmelinii	**14A**	0.259**	0.340**	0.207*	0.104	0.230**	0.454**	0.184**	0.535**	0.244*	0.232*	0.247*	–0.009		0.195	0.035	0.009	–0.116	0.563*	0.683**
var. olgensis	**14B**	0.390**	0.458**	0.328**	0.206*	0.353**	0.558**	0.282**	0.598**	0.326**	0.306*	0.300*	0.017	–0.067		0.222*	0.109	0.024	0.592*	0.753**
	14C	0.139*	0.210**	0.112	0.009	0.123	0.326**	0.066	0.457**	0.154*	0.090	0.177	–0.029	–0.039	0.000		–0.017	0.037	0.367*	0.415**
L. gmelinii var. japonica	**15**	0.105	0.151	0.126	0.007	0.086	0.314**	0.051	0.291*	0.042	0.152	0.045	–0.097	0.016	0.046	–0.014		–0.054	0.318	0.420**
L. gmelinii var. kamchatica	**16**	0.238*	0.301**	0.200*	0.068	0.216*	0.390**	0.138	0.494**	0.205*	0.216*	0.230	0.016	–0.025	0.000	–0.068	–0.008		0.527**	0.644**
L. gmelinii	**17**	0.570**	0.551**	0.533**	0.451**	0.533**	0.627**	0.423**	0.646**	0.450**	0.229	0.398*	0.373*	0.575*	0.617**	0.405*	0.427**	0.506**		0.272
L. kaempferi	**18**	0.590**	0.572**	0.553**	0.486**	0.553**	0.641**	0.467**	0.653**	0.479**	0.563**	0.503**	0.500**	0.642**	0.714**	0.550**	0.500**	0.583**	0.800**	

* P < 0.05
** P < 0.01

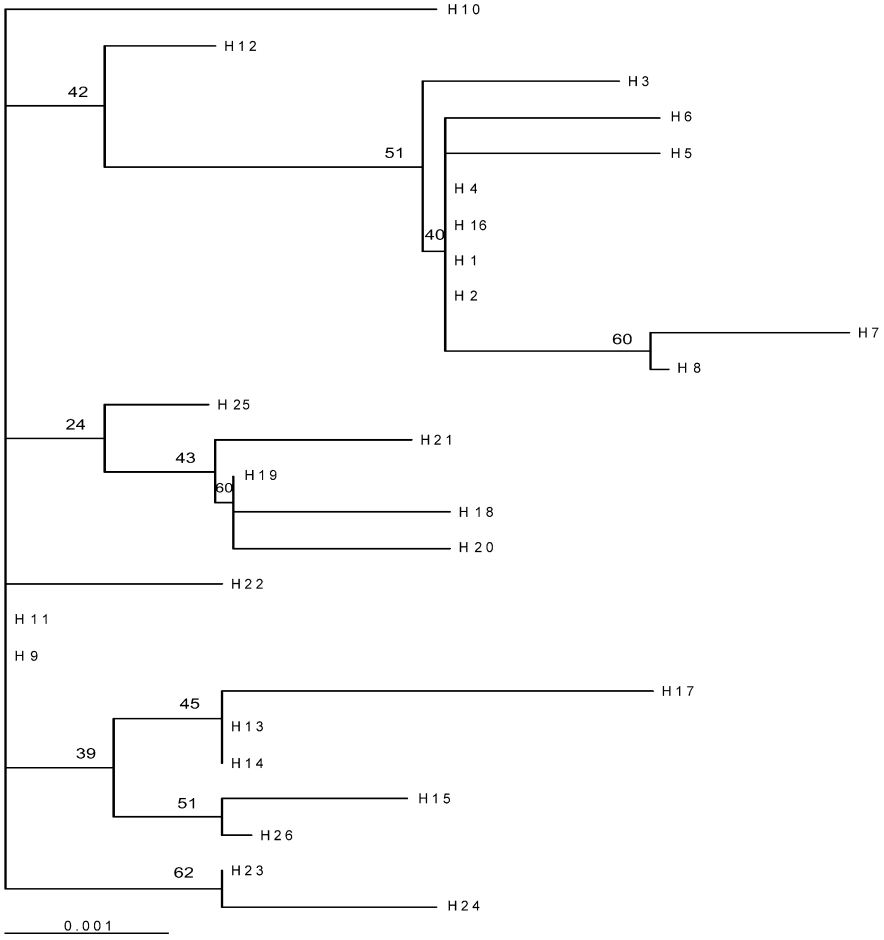

Fig. 2. Neighbor joining tree for the 4CL gene region, the numbers shown on the branches are bootstrap values based on 1000 replicates.

kaempferi to 0.0042 in *L. gmelinii* var. japonica. Nucleotide diversity at silent sites (π_{sil}) varied between 0.0020 in *L. gmelinii* to 0.0116 in *L. gmelinii* var. japonica. In the C3H region π_T varied between 0.0011 in *L. kaempferi* to 0.0038 in *L. gmelinii* var. japonica and π_{sil} varied between 0.0019 in *L. kaempferi* to 0.0066 in *L. gmelinii* var. japonica.

Population differentiation The values of F_{ST} for all pairs of populations were estimated by the method of Hudson et al. (1992) (Table 4). In the 4CL region, the F_{ST} values varied among pairs of populations and ranged between –0.069 (populations 1D-2A) to 0.800 (populations 17-18). In the C3H gene region, F_{ST} values ranged between –0.145 (populations 13C-14A) to 0.823 (populations 6B-18). In both regions, high and statistically significant F_{ST} values (> 0.15) were found for some pairs of populations of the same species *e.g.*, *L. sukaczewii* (populations 1D-7A) and *L. cajanderi* (populations 13A-13B). There were also significant F_{ST} values between some species *e.g.*, between population 7A of *L. sibirica* from putative *Larix* refugium in southern Siberia and most populations of other species included in our study. Significant F_{ST} values were also often observed in comparisons of *L. gmelinii* and *L. kaempferi* with other species. On the other hand, no population differentiation was observed among most *L. sukaczewii* populations except for comparisons involving population 7A. Similarly, weak population differentiation was observed among populations of *L. gmelinii* var. olgensis and in comparisons of this variety with populations of *L. gmelinii* var. japonica and *L. gmelinii* var. kamchatica.

Haplotype relationships and composition Relationships of the haplotypes for the 4CL and C3H regions were inferred using the neighbor-joining trees (Fig. 2 and Fig. 3 respectively). In both gene regions the haplotypes differed by only small number of mutational steps and except for *L. kaempferi* did not form groups corresponding to the taxonomic classification of the investigated species. In the 4CL region there were 26 haplotypes (designated as 4CL-H1 ~ H26) (Table 5), while in the C3H region

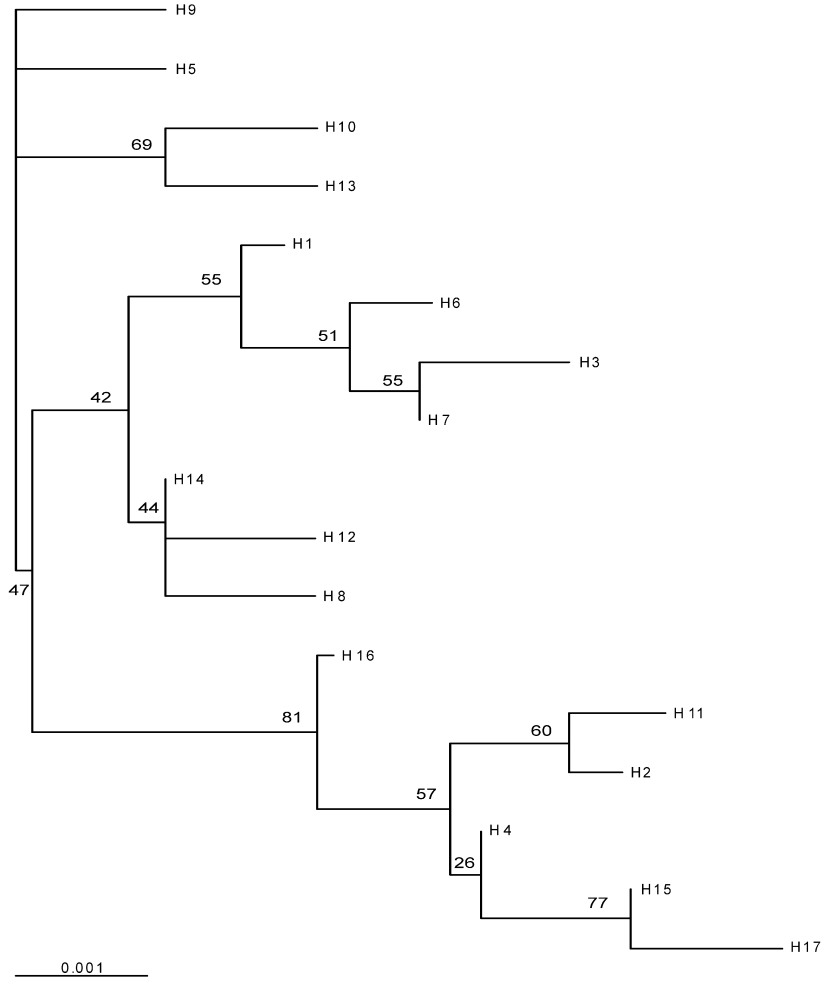

Fig. 3. Neighbor joining tree for the C3H gene region, the numbers shown on the branches are bootstrap values based on 1000 replicates.

there were 17 haplotypes (designated as C3H-H1 ~ H17) (Table 6). Some haplotypes *e.g*., 4CL-H15 and C3H-H14 were found in most populations. Haplotypes 4CL-H10, H12 and H13 and C3H-H10, H12 and H13 were found in some populations of *L. sibirica* and *L. sukaczewii* but were absent in the remaining species, while haplotypes 4CL-

Table 5. Haplotypes found in *Larix* populations in the 4CL gene region

Species	Hap. Pop.	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	Total
L. sukaczewii	1D	1			1					1		4			3													10
	2A	1			1							5	1	1														9
	4A						1					2	3	1	3													10
	5A	1									1	3	1			3				1								10
	6B		1				1					4			3													9
	7A									4	1		5															10
L. sibirica	9A				2						2	1		2		1				2								10
	10A			2	4						1		1															8
	11A				4						2	1				2				1								10
L. cajanderi	13A											2				1							1			3		7
	13B				1	3										2										2		8
	13C				1	1										3	1									1		7
L. gmelinii	14A														2	5		1								2		10
var. olgensis	14B															4	3										1	8
	14C														1	3	1		1	1						1		8
L. gmelinii var japonica	15							1	1							4				1								7
L. gmelinii var kamchatica	16															4	1				2							7
L. gmelinii	17																					1				3		4
L. kaempferi	18																							4	3			7
Total		3	1	2	14	4	2	1	1	5	7	22	11	4	12	32	6	1	1	6	2	1	1	4	3	12	1	159

Table 6. Haplotypes found in *Larix* populations in the C3H gene region

Species	Hap. Pop.	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	Total
L. sukaczewii	1D									1			1	1	7				10
	2A									1				1	7				9
	4A									1			1		8				10
	5A										1			2	7				10
	6B												2		7				9
	7A										1	4			5				10
L. sibirica	9A												1	2	7				10
	10A										3			1	4				8
	11A								3				2	2	3				10
L. cajanderi	13A	2						1		3					1				7
	13B							6							2				8
	13C							4							3				7
L. gmelinii	14A							5							5				10
var. olgensis	14B	1						1							6				8
	14C		1	3	1			1							2				8
L. gmelinii var. japonica	15						2	2							2			1	7
L. gmelinii var. kamchatica	16						1	2							3				7
L. gmelinii	17														1			3	4
L. kaempferi	18															2	5		7
		3	1	3	1	3	1	22	3	6	5	4	7	9	80	2	5	4	159

H7, H8, H16 ~ H18 and 4CL-H20 ~ H26, C3H-H1 ~ H7 and C3H-H15 ~ H17 were absent in *L. sibirica* and *L. sukaczewii* but present in the remaining species. On the other hand, some other haplotypes were species specific. For instance, haplotypes 4CL-H1, H2, H6, 4CL-H9 and C3H-H11 were specific to *L. sukaczewii*. Two of them (4CL-H2 and 4CL-H6) were rare, while haplotypes 4CL-H9 and C3H-H11 had high frequency in population 7A. Haplotypes 4CL-H3 and C3H-H8 were specific to populations 10A and 11A of *L. sibirica* respectively. Haplotypes 4CL-H5 and 4CL-H22 were specific to *L. cajanderi*, while haplotypes 4CL-H23, 4CL-H24 and C3H-H15 and C3H-H16 were specific to *L. kaempferi*. Haplotypes 4CL-H21 and C3H-H17 were specific to *L. gmelinii*, while haplotype C3H-H17 was shared only with *L. gmelinii* var. japonica.

DISCUSSION

Nucleotide diversity Most of the previous studies suggested that *Larix* has lower nucleotide diversity compared to other genera of the family Pinaceae (LePage and Basinger, 1995; Wang et al., 2000). However, the level of nucleotide diversity revealed in our study was in the same order or slightly higher than those reported for other conifers *e.g.*, *Cryptomeria japonica* (π_{sil} = 0.0039) over seven loci (Kado et al., 2003), *Cathaya argyrophylla* (π_{sil} = 0.0024) (Wang and Song, 2006), *Pinus tabuliformis*, *P. yunnanensis* and *P. densata* (π_{sil} = 0.0087 ~ 0.0128 over 7 loci, (Ma et al., 2006). It thus appears that contrary to previous suggestions *Larix* species have levels of nucleotide diversity comparable to other conifers.

Population differentiation Certain reproductive characteristics such as pollen lacking air sacks and low seed viability suggest that *Larix* species are likely to have high levels of population differentiation (Duncan, 1954; Knowles et al., 1992). Yet, most of the previous studies suggested weak population differentiation in several *Larix* species (Fins and Seeb, 1986; Lewandowski et al., 1991; Timerjanov, 1997; Jaquish and El-Kassaby, 1998; Semerikov et al., 1999, 2003; Semerikov and Lascoux, 2003; Wei and Wang, 2003, 2004a,b; Larionova et al., 2004). Taking into account huge size and heterogeneous climatic history of Eurasia and the aforementioned reproductive features of *Larix* species these results are surprising. Contrary to the previous studies, we often found high levels of population differentiation. Significant F_{ST} values were found for pairs of populations of the same species *e.g.*, populations 13A-13B of *L. cajanderi* and populations 6B-7A of *L. sukaczewii*. Significant F_{ST} values were also found between populations of different species *e.g.*, between *L. sukaczewii* and *L. sibirica*, *L. cajanderi* and *L. gmelinii* var. olgensis and between *L. gmelinii* and *L. kaempferi*. These results indicate that in contrast to previous suggestions some Eurasian *Larix* species are diverged and populations of individual species are often highly differentiated even if they are separated by short distances *e.g.*, populations 13A-13B of *L. cajanderi*, which are separated by less than two kilometers.

During the last glacial maximum (LMG) Eurasian *Larix* species survived in many distant and isolated refugia (Kremenetski, 1994; Tarasov et al., 2000; Andreev et al., 2002). Some of these refugia were located in southern Siberia (where populations 10A and 11A are located), south of Urals (where populations 6B and 7A are located) and in the Russian Far East (where populations 13A, 13B and 13C are located). Refugial populations are expected to be more differentiated, because they are likely to have evolved for a long time in isolation from each other. High population differentiation revealed in our study suggests that the present distribution of *Larix* species is a result of independent expansion and population mixing events involving multiple and genetically differentiated refugia.

Northwestern Russia, where populations 1D ~ 5A of *L. sukaczewii* occur, was heavily glaciated during LGM. Therefore, extant populations of *Larix* occurring in this region must be of recent, postglacial origin. This is consistent with our results, which showed that populations 1D ~ 5A are not differentiated because they share some haplotypes and the frequencies of these haplotypes are similar. Moreover, in both 4CL and C3H regions populations 1D ~ 5A shared some haplotypes with population 6B. They also had low F_{ST} values in comparisons involving this population. On the other hand, they showed significant F_{ST} values in comparisons with population 7A. In fact, population 7A also showed significant values in most comparisons with populations of other species included in our study. Populations 6B and 7A are located in southern Urals, which have often been suggested as *Larix* refugia during LGM (Kremenetski, 1994; Tarasov et al., 2000). Similarities between population 6B and populations 1D ~ 5A suggest that the part of the southern Urals where population 6B is located could be one of the sources of postglacial expansion of *L. sukaczewii* into the northwestern Russia. At the same time however, distinct character of population 7A from the same region indicates that populations in southern Urals are highly differentiated and that some of them did not contribute to post-glacial expansion of *L. sukaczewii* into northwestern Russia. It is also possible that the extant populations in northwestern Russia have originated from other refugial areas such as those near the Sea of Azov, which are now dominated by steppe vegetation or desert (Tarasov et al., 2000).

Currently *L. gmelinii* occurs in central Siberia. We found that it harbored a unique haplotype 4CL-H21, which was absent in all other species included in our study. It also harbored another haplotype C3H-H17, which was absent in other species except *L. gmelinii* var.

japonica. Furthermore, it showed significant F_{ST} values in comparisons with other species included in our study. These results suggest that the extant populations of *L. gmelinii* may have different origin from other Eurasian *Larix* species. The sources of postglacial expansion of *L. gmelinii* could be refugia located in the Russian Far East, which at the time of glaciations was only locally glaciated (Porter et al., 1983; Ananyeyev et al., 1993; Bennett, 1997). Fossil data also indicate that *Larix* species were present in this region during LGM (Kremenetski, 1994; Tarasov et al., 2000; Andreev et al., 2002). Postglacial expansion of *L. gmelinii* from the Russian Far East has been suggested by Semerikov et al. (1999). However, population of *L. gmelinii* included in our study was very different from populations of *L. cajanderi* located in the Russian Far East. It is therefore possible that the extant populations of *L. gmelinii* in central Siberia originated from other parts of Eurasia. Unfortunately, we have only a single population of *L. gmelinii* from central Siberia. Therefore, based on our data alone we can not infer more detailed picture of migration history of this species.

Classification of Eurasian *Larix* species There is no consensus regarding the number of *Larix* species in Eurasia and taxonomic position of some species is still an open and controversial issue. Many authors rejected status of *L. sukaczewii* as a species, declaring that *L. sukaczewii* and *L. sibirica* can not be distinguished (Bobrov, 1978; Milyutin and Vishnevetskaia, 1995). However, there is evidence showing that *L. sukaczewii* differs from *L. sibirica* in some morphological and biochemical features (Dylis, 1947, 1981; Milyutin et al., 1993; Abaimov et al., 1998; Bashalkhanov et al., 2003). We found that some haplotypes were unique to either *L. sukaczewii* or *L. sibirica*. For instance, haplotypes 4CL-H1, H2, H9 and 4CL-H14 were found in some populations of *L. sukaczewii* but were absent in all populations of *L. sibirica*. On the other hand, haplotype 4CL-H3 was found only in *L. sibirica* (population 10A) and absent in populations of *L. sukaczewii*. Similarly, haplotype C3H-H8 was present in population 11A of *L. sibirica*, while it was absent in populations of *L. sukaczewii*. On the other hand, haplotypes C3H-H9 and H11 were observed in some populations of *L. sukaczewii* but were absent in populations of *L. sibirica*. These differences in haplotype composition are in favor of the classification of *L. sukaczewii* and *L. sibirica* as different species. However, we also found low F_{ST} values in comparisons between populations of these two species *e.g.*, population pairs 1D-9A and 2A-9A. Furthermore, some populations of *L. sukaczewii* and *L. sibirica* (7A and 10A respectively) were significantly different from other populations of the same species. These results indicate that although some populations currently occurring in western and central Siberia may indeed belong to two or more different species, the current classification recognizing difference only between western and central Siberia (*e.g.*, Semerikov and Lascoux, 2003) is too simplistic and that further studies including additional populations especially from southern Urals and south-central Siberia are necessary to elucidate taxonomic status of *L. sukaczewii* and *L. sibirica*.

In central and eastern Siberia some authors recognized only a single species: *L. gmelinii* (Milyutin and Vishnevetskaia, 1995). Farjon (1990) had also recognized *L. gmelinii* in this region but with some varieties. On the other hand, other authors recognized there two different species: *L. cajanderi* and *L. gmelinii* (Bobrov, 1978; Abaimov et al., 1998, 2002). Our results show that there were differences in haplotype composition between *L. cajanderi* and *L. gmelinii*. *Larix cajanderi* harbored seven 4CL haplotypes and six of them (4CL-H4, H5, H11, H15, H16 and 4CL-H22) were absent in *L. gmelinii*. In the C3H region it harbored four C3H haplotypes and two of them (C3H-H1 and C3H-H9) were absent in *L. gmelinii*. Furthermore, the F_{ST} values between populations of *L. cajanderi* and *L. gmelinii* were higher than those within species. In the 4CL and C3H regions F_{ST} values between *L. cajanderi* (population 13B) and *L. gmelinii* were 0.398 and 0.619 respectively. F_{ST} values within *L. cajanderi* (between populations 13A-13B) were 0.163 and 0.220 in the 4CL and C3H regions respectively. These results support treatment of *L. cajanderi* and *L. gmelinii* as two different species. Similar to *L. sukaczewii* and *L. sibirica*, in *L. cajanderi* we also found high levels of population differentiation within very limited geographic area, which corresponds to LGM *Larix* refugium in the Russian Far East (Porter et al., 1983; Ananyeyev et al., 1993; Bennett, 1997). This further strengthens our suggestion that more intensive sampling especially from known refugial regions is necessary for correct classification of Eurasian *Larix* species and for inferring their postglacial migration.

Some authors regarded *L. gmelinii* var. olgensis as a separate species (Bobrov, 1978; Liu et al., 2006). However, other authors have argued that *L. gmelinii* var. olgensis is just a variety of *L. gmelinii* (Shi et al., 1998; Abaimov et al., 2002). Our results show that *L. gmelinii* var. olgensis has different haplotype composition than *L. gmelinii*. For instance, haplotypes 4CL-H14 ~ H16 and C3H-H3 and C3H-H7 were found in *L. gmelinii* var. olgensis and had high frequency but were absent in *L. gmelinii*. On the other hand, haplotypes 4CL-H21 and C3H-H17 were found in *L. gmelinii* but were absent in *L. gmelinii* var. olgensis. In addition, we found significant F_{ST} values in comparisons of population of *L. gmelinii* with populations of *L. gmelinii* var. olgensis as well as with populations of two other varieties of this species: *L. gmelinii* var. japonica and *L. gmelinii* var. kamchatica. These results indicate that *L. gmelinii* from central

Siberia is very different from its varieties occurring in the Russian Far East and Kamchatka Peninsula. Unfortunately, our sampling of *L. gmelinii* was very limited. Therefore, further studies including additional populations are necessary to determine relationships of this species and its varieties.

Phylogenetic studies suggested that Japanese larch *L. kaempferi* is closely related to *L. olgensis* (regarded as *L. gmelinii* var. olgensis in our present study) (Semerikov et al., 2003; Wei and Wang, 2003, 2004a). However, results of the present study do not support this suggestion. We found that in both gene regions haplotype composition of *L. kaempferi* differed from all other species included in our study. This was reflected in statistically significant F_{ST} values in comparisons with all other species indicating that *L. kaempferi* is among the most diverged Eurasian *Larix* species.

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