ISSN 1022-7954, Russian Journal of Genetics, 2014, Vol. 50, No. 3, pp. 253–260. © Pleiades Publishing, Inc., 2014.
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PLANT GENETICS

Mitochondrial DNA Variation in Olga Bay Larch (*Larix olgensis* A. Henry) from Primorsky Krai of Russia

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Received August 8, 2013; in final form, October 10, 2013

Abstract—Two mitochondrial DNA fragments, *nad*4(3c-4r) and *nad*5(1-2r), were sequenced in 58 larch accessions from the range of *Larix olgensis* A. Henry. Combinations of the *nad*4 polymorphic sites formed four haplotypes, two of which (H3 and H4) were unique and two (H1, H2) were common. Haplotype H1 was found only in pure *L. olgensis* from the vicinity of Olga Bay and in a number of accessions from the southern part of the range. Haplotype H2 was detected in the other samples from the range of Olga Bay larch, as well as in hybrid forms. Similarly to the *nad*4(3c-4r) fragment, the mtDNA fragment *UBC*460 was able to differentiate larch populations from the range of *L. olgensis* examined.

DOI: 10.1134/S1022795414030107

INTRODUCTION

Larix olgensis A. Henry is the only larch species in Primorsky krai of non-hybrid origin. The species has limited range and is listed in the Red Data Book of the Russian Federation [1]. Olga Bay larch was described in 1915 by British dendrologist A. Henry from the Olga Bay region of Primorsky krai. The small range of this species is located to the south of 45° N in the southwestern part of Primorsky krai, Russia. The range stretches southwards to Valentine Bay. It is limited in the east by the Sea of Japan and by the eastern slopes of Sikhote Alin in the west [2, 3]. There are literature data on the distribution of the Olga Bay larch in the north of the Korean Peninsula and in Northeast China (Jilin Province) [4]. Within the Russian part of the range, Olga Bay larch grows irregularly [2, 3]. The main stands of Olga Bay larch are found (from south to north) in the middle and upper flow of the Miloradovka River, in the middle part of Margaritovka River drainage, in the valley at the middle flow of one of the Ussuri River tributaries, and at the upper flow of the Arzamazovka and Tumanovka Rivers. Further, with some interruptions, the larch stretches northwards, encompassing the upper flow of Zerkal'naya River. In the rest of its range, the Olga Bay larch is rare, growing in separate groups of trees, which are rather distant from each other.

The larches from the south of the Far East are characterized by high hybridization intensity [5]. Hybridization results in variability of morphological characters and causes conflicting opinions on the taxonomy of larches. Some researchers recognize up to six larch species (*L. lubarskii*, *L. komarovii*, *L. amurensis*, *L. ochotensis*, *L. maritima*, *L. olgensis*) [2, 6, 7], while the others consider only the Olga Bay larch to be a pure species; all other forms observed are assigned to the complex formed as a result of introgressive hybridization between *L. gmelinii*, *L. cajanderi*, *L. kamchatica*, and *L. olgensis* [5, 8]. Hybridization absorbs the initial forms involved and can lead to the loss of the pure species *L. olgensis*. With respect to morphological characters, *L. olgensis* is slightly different from *L. komarovii*, which results in the commercial usage of this species, as with the other larch species [9].

Recent morphological studies revealed the presence of *L. komarovii* and transitional forms with a prevalence of the characters of one or another species in the range of *L. olgensis* [3]. Our population genetic studies performed with the use of RAPD markers (random amplified polymorphic DNAs) [10] showed that some populations from the range of *L. olgensis* were genetically different from samples taken from the site of species description (*L. olgensis locus classicus*). At the same time, the populations mentioned were genetically close to the hybrid species growing in neighboring territories, suggesting their hybrid origin. In some samples from the range of *L. olgensis*, an almost twofold reduction of the number of diploid cells, along with a higher level of mixoploidy compared to *L. olgensis locus classicus*, *L. sibirica*, and *L. gmelinii*, was observed, pointing to a possible hybrid status of these samples [11]. To confirm the conclusions based on the analysis of variation in nuclear markers, it seems reasonable to examine the variation

of the mitochondrial genome of larches from the range of *L. olgensis*. Unlike nuclear genes, which are propagated through seeds and pollen, mitochondrial DNA (mtDNA) in conifers is inherited along the maternal lineage and is transferred with seeds [12]. Mitochondrial markers provide a more pronounced population structure and longer preserves the traces of hybridization [13]. Because of this, analysis of the mtDNA variation can provide additional information on the taxonomic status of larches from the range of *L. olgensis*.

The objective of the present study was to explore the genetic variation and phylogenetic relationships of larch populations from the range of *L. olgensis* with the other Far Eastern larches, based on the mtDNA markers.

MATERIALS AND METHODS

Larch samples, consisting of one to 18 trees from 15 natural populations (Table 1, Fig. 1) were examined. Individual total DNA was isolated from fresh needles using the method described in [14] with modifications.

Ten samples of *L. olgensis* (OLG, MAR, ZK, ZER, VYS, PAV, LIS, GOR, CHER, VAL3) and one sample each of *L. sibirica* and *L. gmelinii* were tested using the *UBC*460 marker. Primers, PCR conditions, and the temperature regime for amplification of the *UBC*460 fragment are described in [15]. Variation of the fragment size was examined with the help of electrophoresis in 1% agarose gel.

The *nad*5 and *nad*4 sequences were examined in nine samples of Olga Bay larch (OLG, ARZ1, ARZ2, ZK, PAV, VAL1, VAL2, VAL3, MIN). Intron 1 of the NADH-dehydrogenase subunit 5 gene (*nad*5/1-2r) and intron 3 of the NADH-dehydrogenase subunit 4 gene (*nad*4/3c-4r) were amplified using primers described in [16]. PCR of the *nad*4 and *nad*5 fragments was carried out in the reaction mixture containing 65 mM Tris–HCl, pH 8.9; 16 mM $(NH_4)_2SO_4$; 2.1 mM $MgCl_2$, 0.05% Tween 20; 10 mM 2-mercaptoethanol; 0.2 mM each dNTP; 0.5 μM each primer; 30 activity units/mL of *Taq* DNA polymerase (Institute of Chemical Biology and Fundamental Medicine, Novosibirsk, Russia); and 50 ng of larch DNA. The reaction was run using the T3 Termocycler (Biometra, Germany). The reaction conditions included denaturation at 94°C for 2 min, followed by 40 cycles of amplification (94°C for 10 s; 68°C for 10 s; 72°C for 2 min) and a final extension at 72°C for 5 min. The fragments obtained were purified form primer excess and analyzed by means of electrophoresis in agarose gel. DNA was extracted from the gel using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Sweden).

Sequencing of the fragments was performed using the same primers as for the amplification and oligonucleotides 767F (5'-ATGGCAGCAAAGGAAGATA-3'), for the *nad*4 gene, 786F (5'-AGGTAATATCAAGTTGGTGAGC-3') and 1294F (5'-ACCATTTCTGCTCGTGCTA-3') for the *nad*5 gene. Direct sequencing of the PCR products was performed according to Sanger and the BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, United States). The reaction was carried out in the final volume of 20 μL, containing 0.3 to 0.5 pmol DNA fragments; 5 pmol sequencing primer; 1 μL BigDye Terminator Ready Reaction Mix; 4 μL BigDye Terminator 5× Sequencing buffer; and water to the final volume. The products of Sanger reaction were purified from unincorporated fluorescent labeled deoxynucleoside triphosphates on the Centri-Sep column (Princeton Separations, United States), dried in a vacuum concentrator (Eppendorf Concentrator 5301, Germany), and analyzed on the ABI PRISM 3130x1 automated sequencer (Applied Biosystems, United States) at the Genomics Core Facility, Siberian Branch of the Russian Academy of Sciences, Novosibirsk.

The sequences obtained were arranged in contiqs using the SeqMan software program from the DNASTAR package. The *nad*4 sequences, representing the Olga Bay larch haplotypes H1 through H4, were deposited in the GenBank database under the accession numbers KF453633 to KF453636.

The parameters of haplotype diversity (*Hd*), nucleotide diversity (*Pi*), and nucleotide polymorphism (θ) were obtained using the DnaSP v. 5.10.1 software program [17].

Analysis of the genetic relationships of larches from the range of *L. olgensis* was carried out based on *nad*4 haplotypes and using the neighbor-joining (NJ) method as implemented in the MEGA v. 5.2 software program [18]. The robustness of the branching order was evaluated using bootstrap analysis with 1500 pseudorandom replications. To identify possible genealogical relationships of the mtDNA haplotypes of the Primorye larches from the range of *L. olgensis*, the *nad*4(3c-4r) sequences of other larch species from the GenBank database (FJ572133 to FJ572141) were used. The sequence of *Pinus tabulaeformis* (EU276634) was used as outgroup. Mitotypes were analyzed using the method of statistical parsimony and visualized with the help of the TCS v. 1.21 software program [19].

RESULTS AND DISCUSSION

Polymorphism of the mtDNA *UBC*460 fragment in larches was described by Semerikov et al. [15] as the presence of the 2788-bp fragment in *L. sibirica* and *L. olgensis* and the 2300-bp fragment in other larch species (*L. decidua*, *L. gmelinii*, *L. kaempferi*, *L. laricina*). In our study, we demonstrated the presence of the long fragment in *L. sibirica*, *L. olgensis*, *L. gmelinii*, and in most of the trees from the samples VAL3 and

Table 1. Examined larch samples and variation of the mtDNA *UBC*460 fragment

Species	Population symbol	Location (coordinates)	Number of trees	*UBC*460, bp	
L. sibirica		Vicinity of the settlement of Lopatka, Krasnoyarsk krai (55°18′ N, 90°10′ E)	13	2900	–
L. gmelinii		Vicinity of the settlement of Tura, Evenkia (64°17′ N, 100°11′ E)	9	2900	–
L. olgensis (*locus classicus*)	OLG	Northwestern slope of Dundas Mountain, Olga Bay, Primorsky krai (43°41′44″ N, 135°13′45″ E)	18	2900	–
Samples from range of *L. olgensis*	ARZ1	Middle flow of Arzamasovka River (43°59′81″ N, 135°10′56″ E)	3	n/a	n/a
	ARZ2	Source of Arzamasovka River (44°02′97″ N, 135°11′65″ E)	6	n/a	n/a
	MAR	Middle flow of Margaritovka River (43°35′90″ N, 134°35′99″ E)	10	–	2600
	ZK	Zmeinyi Klyuch, tributary of Bol'shaya Ussurka River (44°42′16″ N, 135°39′08″ E)	11	–	2600
	ZER	Source of Zerkal'naya Ruver (44°16′93″ N, 134°53′59″ E)	10	–	2600
	VYS	Source of Vysokogorskaya River (44°29′13″ N, 135°23′51″ E)	11	–	2600
	PAV	Upper flow of Pavlovka River (44°17′34″ N, 134°52′05″ E)	10	–	2600
	LIS	Middle flow of Listvennaya River, tributary of Margaritovka River (43°26′45″ N, 134°39′45″ E)	12	2900	2600
	GOR	Gorbusha River, tributary of Rudnaya River (44°39′51″ N, 135°39′51″ E)	9	–	2600
	CHER	Source of Cheremukhovaya River, tributary of Dzhigitovka River (44°41′69″ N, 135°45′13″ E)	9	–	2600
	MIN	Source of Mineral'naya River, tributary of Avvakumovka River (43°39′17″ N, 134°41′25″ E)	1	n/a	n/a
	VAL1	2 km from the settlement of Valentine, coast of Bol'shaya Tikhaya Bay	11	n/a	n/a
	VAL2	2 km from the settlement of Valentine, deep into the coast of Bol'shaya Tikhaya Bay	8	n/a	n/a
	VAL3	2 km from the settlement of Valentine, 300 m from the coast of Bol'shaya Tikhaya Bay (43°07′34″ N, 134°19′22″ E)	12	2900	2600

n/a, sample was not analyzed; –, fragment was absent.

LIS. The short fragment was identified in the samples GOR, CHER, ZK, ZER, VYS, MAR, as well in three accessions from LIS and three accessions from VAL3 (Table 1). The presence of the short fragment in *L. gmelinii* from our study can probably be explained by the fact that accessions were collected in the transition zone between *L. sibirica* and *L. gmelinii* and were represented by hybrid forms with the morphological characters of *L. gmelinii*, while their mtDNA was inherited from *L. sibirica* [20]. At the same time, most of the samples from the range of *L. olgensis* examined were genetically different from the samples from *locus*

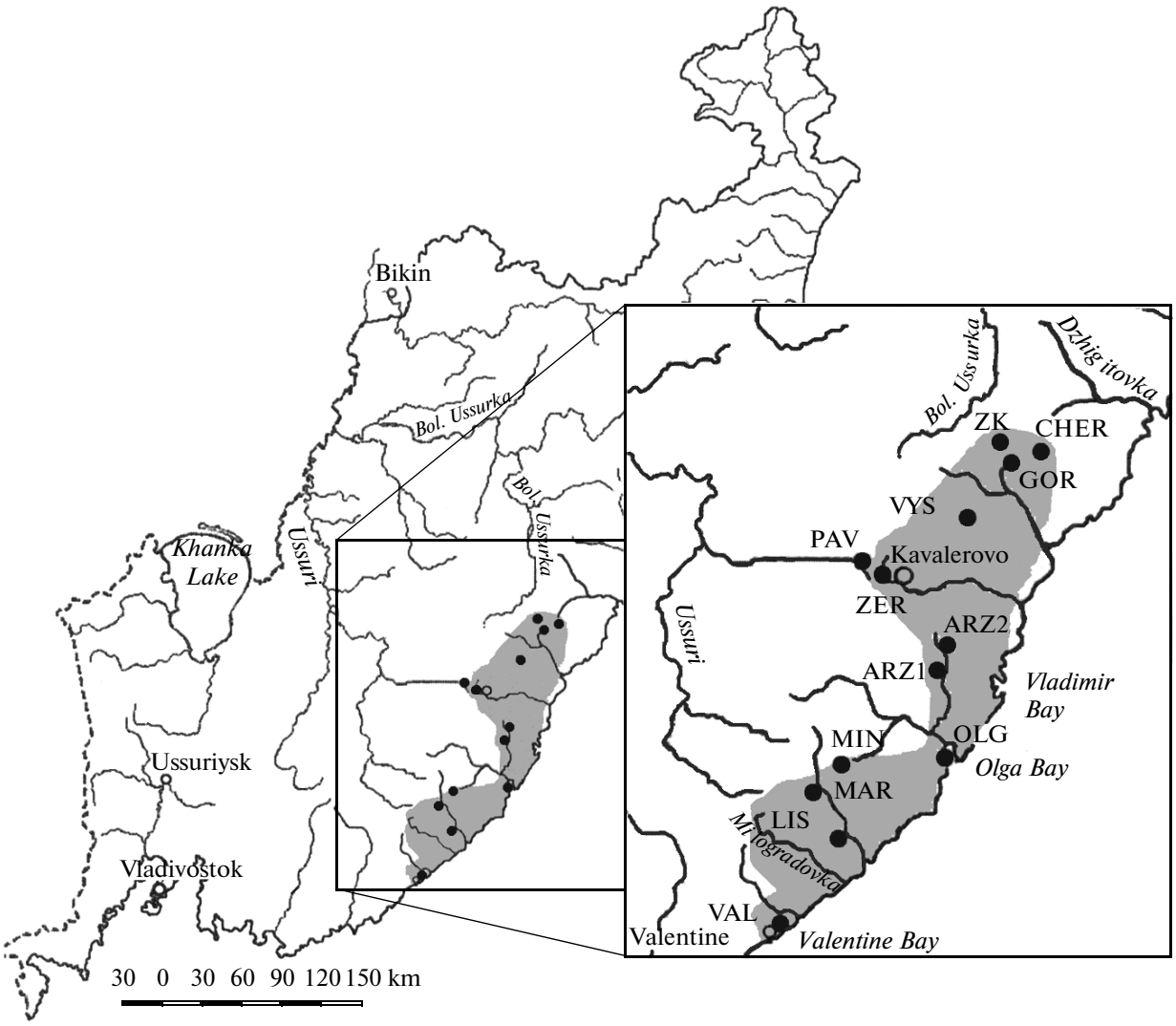

Fig. 1. Schematic map of Primorsky krai. Geographic distribution of examined larch populations. The range of Olga Bay larch is designated in gray (according to [2]); for population code see in Table 1.

classicus. These findings were congruent with the results of our earlier studies performed with the use of RAPD [10] and cytogenetic [11] analyses.

Nucleotide sequences of two mitochondrial genome fragments, *nad*4(3c-4r) and *nad*5(1-2r), were determined in 58 larch accessions from the range of *L. olgensis* (OLG, VAL1–3, ARZ1–2, PAV, ZK, MIN). Introns of the *nad*5 gene were found to be identical in all accessions. The size of the aligned sequence constituted 2143 bp. With respect to the third intron of the *nad*4 gene, the size of which constituted 1762 bp, the differences between the accessions were identified. These differences were determined by point transversions at four sites. Three of these transversions, at positions of 1433, 1601, and 1608 bp were informative in terms of maximum parsimony approach, and one transversion (1565 bp) was noninformative (Table 2).

The values of nucleotide diversity (*Pi*) and nucleotide polymorphism (θ) constituted 0.0008 and 0.00049, respectively. Combinations of the polymorphic sites identified in the Primorye larches formed four haplotypes. Two of these haplotypes (H3 and H4) were unique, while the two others (H1, H2) were common (Table 2). Haplotype diversity (*Hd*) constituted 0.482 ± 0.05; haplotype H1 was detected in 38 accessions. The latter were represented by all OLG accessions from *locus classicus*, all VAL1 accessions, three accessions from VAL2, and nine accessions from VAL3. Haplotype H2 was identified in five VAL2 accessions, two VAL3 accessions, and in all ARZ1, ARZ2, ZK, and PAV representatives. Haplotype H3 was detected in one VAL3 accession, and haplotype H4, in one MIN accession. In the NJ tree, the accessions formed two clusters (Fig. 2). One of the clusters was formed by the whole OLG sample from *locus clas-*

Table 2. Variable sites in the mtDNA *nad*4(3c-4r) fragment in larch populations

Haplotype	Symbol/population name	Number of samples	*nad*4							
			457	471	666	878	1433	1565	1601	1608
H1	OLG	15	G	T	G	C	A	T	G	C
	VAL1	11	G	T	G	C	A	T	G	C
	VAL2	3	G	T	G	C	A	T	G	C
	VAL3	9	G	T	G	C	A	T	G	C
H2	VAL2	5	G	T	G	C	C	T	T	A
	VAL3	2	G	T	G	C	C	T	T	A
	ARZ1	3	G	T	G	C	C	T	T	A
	ARZ2	6	G	T	G	C	C	T	T	A
	PAV	1	G	T	G	C	C	T	T	A
	ZK	1	G	T	G	C	C	T	T	A
	L. cajanderi	1	G	T	G	C	C	T	T	A
	L. × *czekanowskii*	1	G	T	G	C	C	T	T	A
	Kavalerovo	1	G	T	G	C	C	T	T	A
	Vanino	1	G	T	G	C	C	T	T	A
H3	VAL3	1	G	T	G	C	C	G	T	A
H4	MIN	1	G	T	G	C	A	T	T	A
	L. kurilensis ssp. *glabra*	1	G	T	G	C	A	T	T	A
H5	*L. kamtschatica* south	1	T	A	T	C	A	T	T	A
H6	*L. kamtschatica* north	1	G	T	G	C	C	T	G	A
H7	*L. sibirica*	1	G	T	G	C	A	T	T	C
H8	*L. kaempferi*	1	G	T	G	T	A	T	T	A

sicus, the whole VAL1 sample collected at the seashore, and several VAL2 and VAL3 accessions collected at a distance from the seaside. The second cluster included the remaining VAL2 and VAL3 accessions, as well as other larches carrying the short mtDNA *UBC*460 fragment. It should be noted that in the VAL3 sample a short *UBC*460 fragment was detected in the individuals with haplotype H2. Samples VAL2 and VAL3, containing two (H1, H2) and three (H1, H2, H3) haplotypes, respectively, were the most variable. Since the whole sample VAL1 contained the same haplotype as Olga Bay larch *locus clssicus*, it could be assigned to the pure species *L. olgensis*. Regarding this character, nine out of 12 VAL3 accessions and only three out of eight VAL2 accessions can be attributed to the pure species *L. olgensis*. The remaining VAL2 and VAL3 accessions, as well as other samples from the range of *L. olgensis* (ARZ1, ARZ2, ZK, PAV) carried haplotype H2, which was absent from the pure species *L. olgensis*. Thus, the mitochondrial *nad*4(3c-4r) marker, similarly to *UBC*460, differentiated accessions of *L. olgensis locus classicus*, as well as some of the accessions from the vicinity of the settlement of Valentive from the other Primorye larches, taken from the range of Olga Bay larch.

Comparative sequence analysis of the *nad*4(3c-4r) fragments from the larches growing in the range of *L. olgensis* and those from other larch species (*L. cajanderi*, *L.* × *czekanovskii*, *L. kurilensis* ssp. *glabra*, *L. sibirica*, *L. kaempferi*, *L. kamtschatica*) from Siberia and the Far East revealed eight haplotypes (Table 2).

In addition to the Primorye larch accessions mentioned, haplotype H2 was identified in the accession of *L. cajanderi*. In addition to the Primorye MIN accession mentioned, haplotype H4 was characteristic of the *L. kurilensis* ssp. *glabra* larch from Kamchatka peninsula. Haplotype H5 represented the *L. kamtschatica* accession from the south of Sakhalin Island, while haplotype H6 was found in the *L. kamtschatica* accession from the

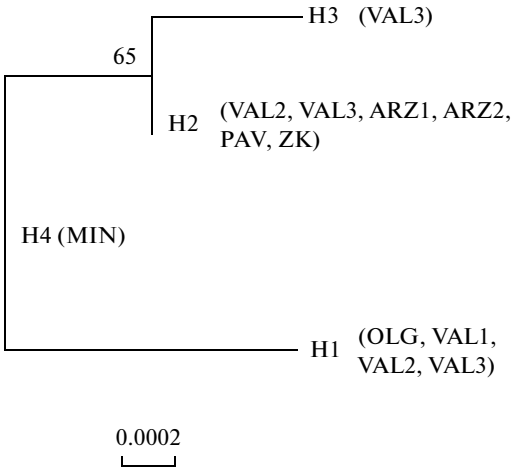

Fig. 2. NJ tree reflecting phylogenetic relationships of haplotypes (H1–H4) identified in populations from the range of *L. olgensis* (for population symbols see Table 1). Figures are the bootstrap support values (%).

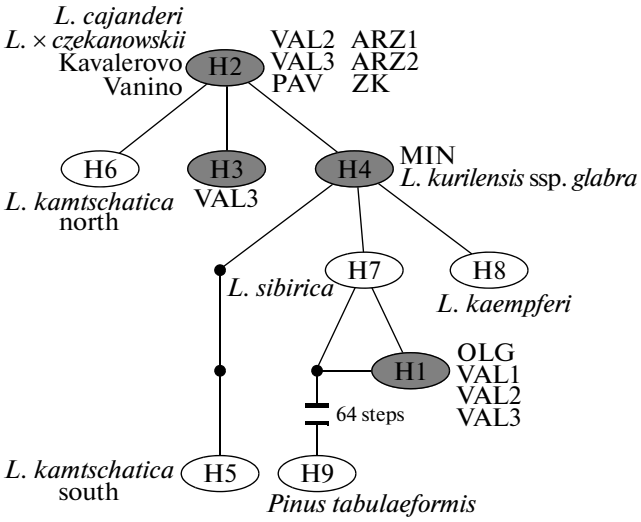

Fig. 3. Phylogenetic network of haplotypes, constructed based on the sequence comparison data for the mtDNA *nad*4(3c-4r) fragment in larches. Haplotypes identified in the range of Olga Bay larch are in gray; black circles, hypothetical haplotypes; lines between the haplotypes represent one mutational event.

northern part of Sakhalin Island. Haplotype H7 represented *L. sibirica*, while haplotype H8 represented *L. kaempferi*. The Primorye larch accessions were genetically close to the accessions from Kavalerovo and Vanino, the representatives of the species *L. cajanderi*, *L.* × *czekanovskii*, since all of them contain haplotype H2 (Table 2). The larches growing in the vicinity of the settlement of Kavalerovo are transitional forms with the characters of *L. olgensis* and *L. komarovii* [3]. *L. komarovii* itself is considered to be a hybrid species, the parental forms of which, according to Gukov [21], could be *L. olgensis* and *L. cajanderi*. The settlement of Vanino is the habitat of *L. amurensis*, which has a hybrid origin from *L. cajanderi* and *L. gmelinii* [21, 22]. The *L.* × *czekanovskii* larch represents a hybrid complex between *L. sibirica* and *l. gmelinii* [23]. Thus, mitotype H2 is the universal marker distinguishing hybrid forms between *L. sibirica*, *L. gmelinii*, *L. cajanderi*, and *L. olgensis*. From here it follows that all larches from the range of *L. olgensis* carrying mitotype H2 have a hybrid status and do not belong to the pure species *L. olgensis*.

The phylogenetic network of haplotypes, which shows mutational transitions between the samples of *L. olgensis* and other larches (Fig. 3), generally reflects the existing opinion on their genetic relationships. It can be seen that the accessions of the pure species *L. olgensis* (H1) are not only the neighbors of other Primorye haplotypes (H2–H4), but are located at the poles of haplotype network. Haplotype H1 is connected with the outgroup (H9) through 64 nucleotide substitutions. The *L. olgensis locus classicus* occupies basal position, which corresponds to the opinion that the *L. olgensis* typical mitotype is the most ancient among the mitotypes examined [12, 24]. This species stands three mutational steps apart from the geographically neighboring samples and other hybrid forms, which occupy upper position in the haplotype network (H2).

The closest neighbor of *L. olgensis* in the haplotype network is *L. sibirica* (H7). These species are separated by one nucleotide substitution (Fig. 3). In accordance with the literature data, *L. olgensis* and *L. sibirica* are considered as the genetically closest species [25]. Moreover, it is suggested that *L. sibirica* originated from *L. olgensis* [26]. *L. sibirica* is separated from *L. kurilensis* ssp. *glabra* from Kamchatka Peninsula (H4) by a single mutation. The relatedness of H7 and H4 can serve as argument in favor of the hypothesis that colonization of Kamchatka took place from the south of the Far East, through Sakhalin and Kuril Islands, rather than from the northern regions [12]. According to another hypothesis, Kamchatka larch maintained its presence in the peninsula and was involved into the processes of introgressive hybridization with *L. gmelinii* and *L. cajanderi* during the interglacial periods [24].

The neighborhood of haplotypes H4 and H8 fit into the above-mentioned pattern of the maternal gene flow movements. These findings agree with the proposal that in the past, the larches from Sakhalin Island formed a single species together with the larches of Japanese Islands [12]. It seems likely that the same gene flows determine the neighborhood of haplotypes from Kamchatka (H4) and southern Sakhalin (H5), which are separated, however, by three mutational steps. At the same time, larches from

northern Sakhalin (H6) are genetically closer to the transitional forms and hybrid species (H2) completing the chain of mutational changes, expressing more advanced evolutionary processes. The closeness of haplotypes H6 and H2 revealed is congruent with the data on the genetic similarity of larches from northern Sakhalin to the continental populations and the larches from southern Sakhalin to *L. kaempferi* [24].

Thus, the *UBC*460 and *nad*460 mitochondrial markers genetically distinguished larches from Olga Bay and the neighboring seashore populations from the most of the larches from the range of *L. olgensis* and confirmed the earlier data obtained with the help of RAPD markers [10] and karyological analysis [11]. The mitotype (H2) identified was typical to the samples from the range of *L. olgensis*, as well as to the transition forms between *L. olgensis*, *L. cajanderi*, *l. sibirica*, and *L. gmelinii*. These findings point to the hybrid status of most of the examined samples from the range of *L. olgensis*. The data on the mitochondrial genome variation indicate that the pure species *L. olgensis* occupies only the seashore area in the region of Valentine Bay and Olga Bay, while most of the range is occupied by transitional forms. Similar results were obtained in the recent study, based on analysis of the morphological characters of the generative organs of the representatives of Olga Bay larch [3]. The congruence of the data of morphological and population genetic analyses unambiguously indicate that in Primorsky krai, the species of *L. olgensis* is absorbed by other forms as a result of hybridization processes and is on the way to extinction.

ACKNOWLEDGMENTS

We thank E.V. Artyukhova and L.N. Spiridonova (Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok) for assistance in data processing, V.N. Gaponov (Forestry Department of Primorsky Krai), V.Yu. Minkhaidarov (Institute of Forestry and Forest-Park Management, Primorye State Agricultural Academy, Ussuriisk), and the employee of the Institute of Forest, Siberian Branch of the Russian Academy of Sciences (Krasnoyarsk) for assistance in sample collection.

This study was supported by the Program of the Presidium of the Russian Academy of Sciences (grant no. 12-I-P30-02), Integration Research Program of the Far Eastern Branch of the Russian Academy of Sciences and Siberian Branch of the Russian Academy of Sciences (grant no. 09-II-SO-06-003).

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Translated by N. Maleeva