

Genetic Differentiation of Larch Populations from the *Larix olgensis* Range and their Relationships with Larches from Siberia and Russian Far East

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Random amplification polymorphic DNA (RAPD) markers were used to assess genetic differentiation between twelve larch populations from various parts of the *Larix olgensis* A. Henry range and to reveal the genetic relationships between these populations and pure larch species (*L. sibirica*, *L. gmelinii*, *L. cajanderi*, *L. kamtschatica* and *L. kaempferi*) and hybrid larch species (*L. amurensis*, *L. komarovii* and *L. ochotensis*) from Siberia and Russian Far East. Analysis of Nei's genetic distance values and molecular variance revealed a significant differentiation between the *L. olgensis* range populations that was similar in magnitude to that among species. It was found that these populations are as genetically distant from population collected *L. olgensis locus classicus* as from other pure larch species. At the same time, the similarity of the *L. olgensis* range populations to hybrid species located in adjacent territories was shown. A possible scenario to explain these results is suggested.

Key words : *Larix olgensis*, RAPD, genetic differentiation, AMOVA

INTRODUCTION

According to different sources, several larch species exist across the Russian Far East (Kolesnikov, 1946; Dylis, 1961; Bobrov, 1972; Gukov, 1976; Kabanov, 1977). Some of them are morphologically distinct species or the so-called pure species (*Larix gmelinii* (Rupr.) Rupr., *L. cajanderi* Mayr, *L. kamtschatica* (Rupr.) Carr. and *L. olgensis* A. Henry). The others have complicated hybrid origins that were the cause of difficulty in the determination of their taxonomic status (*L. amurensis* Kolesn., *L. maritime* Sukacz., *L. ochotensis* Kolesn., *L. komarovii* Kolesn. and *L. lubarskii* Sukacz.). The greatest number of these hybrid species occurred in the Primorye and Khabarovsk territories, as a result of climate oscillations and the geological history of these regions. Absence of glaciations in the majority of these ter-

ritories was also an important factor to maintain such diversity.

In the territory of the Primorye located in the southern part of the Russian Far East, only *Larix olgensis* has no hybrid origin (Bobrov, 1972; Koropachinski, 1989). This species was first described in 1915 based on specimens collected from Olga Bay by A. Henry (Bobrov, 1972). This species also occurs on the north part of the Korean Peninsula and in the northeast part of China (Bobrov, 1972). In the Primorye, *L. olgensis* has a narrow fragmented range, located south of 45°N, restricted by the eastern macroslope of the southern Sikhotealin Mountains and the Sea of Japan coast (Gukov, 1976). *L. olgensis* is a vulnerable species and is included in the Red List of Endangered Species of Russian Federation (Red Book., 1988). Taxonomic status of some larch populations from this range is doubtful because they have high variability of morphological characteristics due to introgressive hybridization with nearby larch species. This issue could be resolved using molecular

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markers.

Recently, the population genetic investigations of Pinaceae representatives were carried out using molecular genetic markers, namely allozymes (Potenko and Razumov, 1996; Goncharenko and Silin, 1997; Semerikov *et al.*, 1999; Semerikov and Lascoux, 1999), randomly amplified polymorphic DNA (RAPDs) (Isabel *et al.*, 1995; Lee *et al.*, 2002; Nkongolo *et al.*, 2002; Kozyrenko *et al.*, 2004a, b), microsatellite (SSRs) (Karhu *et al.*, 2000; Khasa *et al.*, 2000; Isoda and Watanabe, 2006) and other means. However, allozymes do not represent a random sample of genomes and thus, may bias some population genetic inferences (Aagard *et al.*, 1998). DNA molecular markers are more informative to study plant species genetic variability and population structure because they allow analysis of coding and non-coding regions of the genome. Of these markers, RAPDs are very polymorphic markers (Williams *et al.*, 1991) that are considered to be a useful technique to determine genetic variability parameters and to compare closely related species and genera (Weising *et al.*, 1995).

The goals of this study were (i) to analyze genetic differentiation of the larch populations from the *L. olgensis* range in the Primorye outlined by Gukov (1976) using multilocus RAPD markers and (ii) to reveal the relationships of these populations with morphologically distinct and hybrid larch species from Siberia and Russian Far East.

MATERIAL AND METHODS

Twenty natural larch populations were sampled from their natural ranges in Siberia and the Russian Far East. The specimens of *L. kaempferi* were selected from the Botanical Gardens of Moscow (Figure 1; Table 1).

The genomic DNA was isolated from young needles using the technique of Isabel *et al.* (1995). The polymerase chain reaction (PCR) was conducted in the thermal cycler UNO II 48 (Biometra, Germany) with a set of decamer arbitrary primers (Operon Technologies Inc., USA), applying the reaction mixture and temperature regime described previously (Kozyrenko *et al.*, 2001). The amplification products were separated by electrophoresis in 1.4% agarose gels in the presence of ethidium bromide and examined under UV light. The size of each RAPD fragment was determined by comparison with the standard molecular weight marker, λ DNA double digested with *EcoRI* and *HindIII* (Fer-

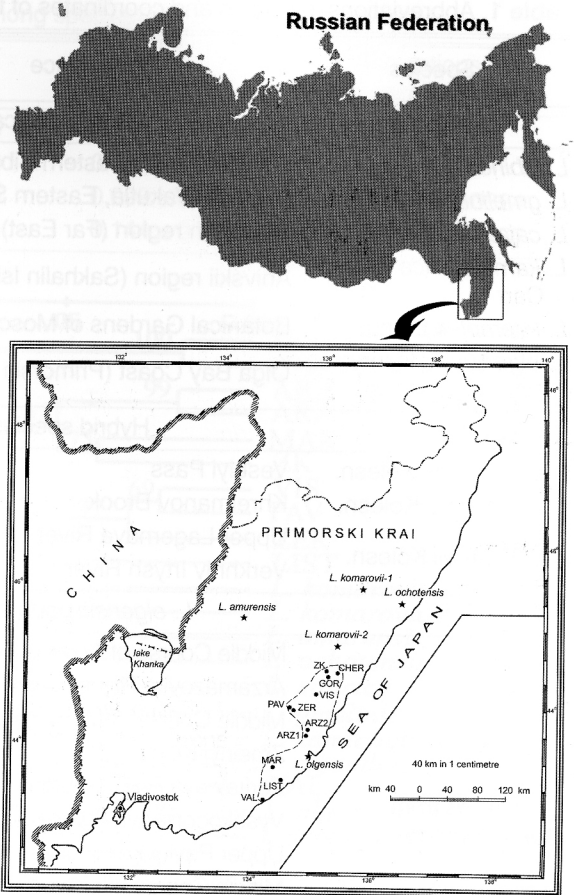


Figure 1. Geographic distribution of the larch populations studied. Sample locations of pure and hybrid species are shown by asterisks; sample locations of populations from the *L. olgensis* range are indicated with circles. The *L. olgensis* range in the Primorye outlined by Gukov (1976) is shown by dashed line. For codes of the populations, see Table 1.

mentas, Lithuania). Fragments within the range of 500-1700 bp were scored for the presence (1) or for absence (0) of a band. All RAPD runs were repeated at least twice and only the reproducible bands were scored. Nei's (1978) genetic distances (D_N) were estimated using the TFGPA package software (Miller, 1997). To visualize the genetic relationship between populations, a dendrogram was constructed based on D_N values matrix, applying the unweighted pair group method of cluster analysis using arithmetic averages (UPGMA) with the bootstrap analysis estimates of the branching order reliability (1000 replications). The molecular differentiation between population pairs was quantified with the pairwise F_{ST} and the apportionment of genetic variation within and among populations was determined by analysis of molecular variance (AMOVA), using ARLEQUIN v. 2.000 (Schneider *et al.*, 2000). For each analysis, 1000

Table 1. Abbreviations, locations and coordinates of the larch populations studied.

Species	Provenance	Code	Latitude / Longitude	Sample size
Morphologically distinct species				
<i>L. sibirica</i> Ledeb.	Tomsk region (Western Siberia)	Sib	56°30 N / 84°49 E	11
<i>L. gmelinii</i> (Rupr.) Rupr.	Evenkia (Yakutia, Eastern Siberia)	Gme	69°19 N / 87°59' E	13
<i>L. cajanderi</i> Mayr	Magadan region (Far East)	Caj	59°37 N / 150°50 E	11
<i>L. kamtschatica</i> (Rupr.) Carr.	Anivskii region (Sakhalin Island)	Kam	46°43 N / 142°36 E	7
<i>L. kaempferi</i> Lamb.	Botanical Gardens of Moscow	Kae		4
<i>L. olgensis</i> A. Henry (<i>locus classicus</i>)	Olga Bay Coast (Primorski Krai)	Olg	43°41'44 N / 135°13'45" E	18
Hybrid species from Primorski Krai				
<i>L. ochotensis</i> Kolesn.	Veselyi Pass	OCH	45°29 N / 137°00' E	6
<i>L. amurensis</i> Kolesn.	Khreimanov Brook	AMU	45°28 N / 134°08 E	9
	Upper Lagernaya River	KOM1	45°42 N / 136°21 E	14
<i>L. komarovii</i> Kolesn.	Verkhniy Irtys River	KOM2	45°03 N / 135°48 E	9
<i>L. olgensis</i> populations from Gukov's range				
	Middle Course of Arzamazovka River	ARZ1	43°59'11 N / 135°10'56" E	9
	Arzamazovka River Source	ARZ2	44°02'27 N / 135°11'65" E	11
	Middle Course of Margaritovka River	MAR	43°35'90 N / 134°35'99" E	10
	Zmeinyi Kluch	ZK	44°42'16 N / 135°39'08" E	11
	Zerkalnaya River Source	ZER	44°16'93 N / 134°53'59" E	10
<i>L. olgensis</i> from Gukov's range	Vysokogorskaya River Source	VIS	44°29'13 N / 135°23'51" E	11
	Upper Pavlovka River	PAV	44°17'34 N / 134°52'05" E	10
	Middle Course of Listvennaya River	LIST	43°26'45 N / 134°39'45" E	12
	Gorbusha River	GOR	44°39'51 N / 135°39'51" E	9
	Cheremukhovaya River Source	CHER	44°41'69 N / 135°45'13" E	9
	Valentin Bay Coast	VAL	43°07'34 N / 134°19'22" E	13

permutations were performed to obtain significance levels. To test for a correlation between pairwise F_{ST} and geographical distances (in km) among populations, a Mantel test was performed using TFGA (Miller, 1997). UPGMA and NJ (Neighbor-joining) methods using the NTSYS package (Rohlf, 1998) were also applied to obtain the dendrograms based on F_{ST} as interpopulation genetic distances.

RESULTS AND DISCUSSION

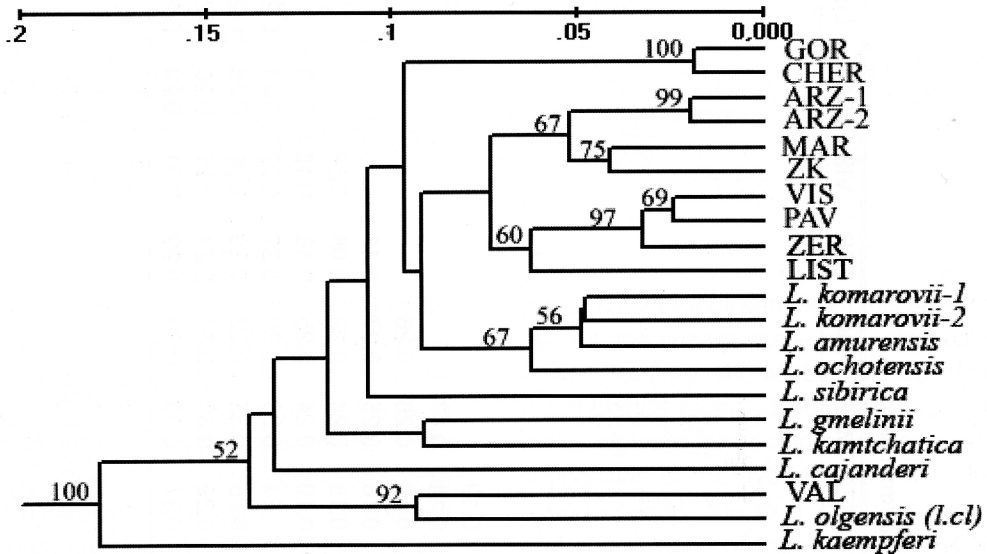
RAPD analysis was carried out using eleven primers previously chosen from a set of primers that were effective in PCR reactions with larch DNA templates (OPA-01, OPA-09, OPA-11, OPA-12, OPA-20, OPB-10, OPB-11 and OPC-02) (Sazonova *et al.*, 2001). A total of 189 RAPD loci were obtained and scored for 217 surveyed individuals (23.6 loci per primer, on average). Only six loci were monomorphic for all samples, while 183 loci (96.8%) were found to be polymorphic. Of these

polymorphic loci, 177 occurred in all populations with different frequencies, four loci were present only in the hybrid species and *L. olgensis* range populations and two polymorphic loci were found in the above mentioned hybrid species and *L. olgensis* populations, but were absent in the Olga Bay sample (*locus classicus*).

The Nei's genetic distances among the different larch species populations ranged from 0.092 to 0.225 (Gme-Kam and Kae-Caj, respectively), D_N distances among hybrid species populations varied from 0.049 to 0.068 (KOM1-AMU and KOM1-OCH, respectively) and genetic distances among the *L. olgensis* range populations were from 0.018 to 0.154 (GOR-CHER and MAR-VAL, respectively) (data not shown). D_N values between some of the populations studied corresponded to the interspecies level. It should be noted that the average Nei's genetic distance among *L. olgensis* range populations was higher than the average D_N among hybrid species populations (0.082 and 0.056, respectively) (Table 2). Nevertheless, these

Table 2. The average values of genetic distances (D_N) among species and populations.

Larch populations	Pure species	Hybrid species	Populations from <i>L. olgensis</i> range
Pure species	0.156 ± 0.0285		
Hybrid species	0.134 ± 0.0308	0.056 ± 0.0097	
Populations from <i>L. olgensis</i> range	0.130 ± 0.0328	0.099 ± 0.0184	0.082 ± 0.0324
<i>L. olgensis</i> (<i>locus classicus</i>)	0.161 ± 0.0225	0.144 ± 0.0064	0.121 ± 0.0165

**Figure 2.** UPGMA dendrogram of the populations and *Larix* species based on Nei's (1978) genetic distance. Bootstrap values (based on 1000 permutations) are indicated in each node. The codes for the populations are shown in Table 1.

populations have more resemblance to hybrid species ($D_N = 0.099$) than to samples from *locus classicus* ($D_N = 0.121$) (Table 2).

Thus, these results showed that *L. olgensis* populations in the Primorye are genetically heterogeneous and more distant from the *locus classicus* sample than from hybrid species populations. It seems that this could be explained by their long-term isolation in the conditions of a mountain landscape.

The UPGMA cluster analysis (Figure 2) based on Nei's genetic distances indicates that most of the *L. olgensis* range populations grouped together according to geographical distribution, except for GOR, CHER and VAL samples. Group GOR-CHER was jointed to cluster that was formed by two groups, including *L. olgensis* populations and hybrid species populations, respectively. Pure species were sequentially associated with this large cluster. It is notable that *L. olgensis* (*locus classicus*) was distant from both the hybrid species and populations from the *L. olgensis* range outlined by Gukov (1976). *L. kaempferi* was distant from all taxons studied. The resulting dendrogram (Figure

2) showed the genetic resemblance of the populations studied to hybrid species. It allows us to suppose hybrid nature of these populations. It was interesting that the Olg sample (*locus classicus*) grouped together with the VAL sample (the southernmost point of the collection) on the dendrogram. This grouping could be explained by similar ecological growing conditions of these populations (rocky coast of Sea of Japan), which are optimum for this species.

All pairwise differences (F_{ST}) between populations were significant ($P < 0.00001$) (Table 3). UPGMA and NJ dendrograms based on pairwise F_{ST} values (not shown) have similar topologies to the dendrogram based on Nei's genetic distance (Figure 2). According to the literature (Genetic Diversity Analysis... 2004), genetic differentiation is considered small when F_{ST} falls within 0 to 0.05, moderate when F_{ST} is 0.05 to 0.15, large for F_{ST} ranges from 0.15 to 0.2 and, at last, very large when the value of F_{ST} equals 0.25 and higher. Our results (Table 3) showed that pure species have very large differentiation, but hybrid species have moderate and large differentiation, whereas *L.*

Table 3. Pairwise F_{ST} among all populations. All distances were significant at $P < 0.05$ (1000 permutations).

Populations	Kae	Caj	Sib	Gme	Kam	KOM1	KOM2	AMU	OCH	ARZ1	ARZ2	MAR	ZK	ZER	VIS	PAV	LIS	GOR	CHER	VAL	
Caj	0.47																				
Sib	0.41	0.47																			
Gme	0.29	0.41	0.38																		
Kam	0.39	0.42	0.41	0.29																	
KOM-1	0.42	0.42	0.31	0.32	0.38																
KOM-2	0.37	0.37	0.32	0.29	0.36	0.13															
AMU	0.34	0.39	0.27	0.29	0.34	0.12	0.15														
OCH	0.38	0.34	0.32	0.31	0.36	0.18	0.16	0.14													
ARZ1	0.34	0.33	0.33	0.27	0.31	0.27	0.26	0.23	0.22												
ARZ2	0.29	0.34	0.29	0.24	0.29	0.24	0.21	0.17	0.24	0.07											
MAR	0.34	0.39	0.35	0.24	0.32	0.31	0.26	0.29	0.29	0.19	0.19										
ZK	0.33	0.39	0.36	0.25	0.31	0.33	0.28	0.27	0.28	0.17	0.14	0.17									
ZER	0.41	0.33	0.35	0.35	0.39	0.29	0.26	0.23	0.22	0.17	0.16	0.26	0.26								
VIS	0.37	0.34	0.36	0.32	0.36	0.31	0.27	0.26	0.29	0.23	0.18	0.25	0.27	0.11							
PAV	0.37	0.32	0.29	0.30	0.37	0.23	0.20	0.21	0.19	0.16	0.15	0.24	0.23	0.06	0.08						
LIS	0.36	0.29	0.29	0.29	0.31	0.28	0.26	0.23	0.24	0.19	0.19	0.24	0.25	0.18	0.19	0.19					
GOR	0.41	0.33	0.35	0.33	0.36	0.29	0.29	0.27	0.22	0.22	0.24	0.33	0.30	0.27	0.27	0.24	0.24				
CHE	0.39	0.38	0.36	0.34	0.39	0.31	0.29	0.28	0.21	0.25	0.26	0.35	0.32	0.29	0.31	0.25	0.28	0.03			
VAL	0.41	0.37	0.36	0.39	0.44	0.35	0.36	0.36	0.32	0.29	0.29	0.36	0.34	0.27	0.27	0.25	0.26	0.32	0.36		
Olg	0.38	0.37	0.37	0.37	0.38	0.35	0.35	0.34	0.31	0.31	0.31	0.33	0.33	0.29	0.31	0.29	0.23	0.34	0.35	0.26	

Table 4. Summary of analysis of molecular variance (AMOVA). The analysis was based on RAPD phenotypes, consisting of 189 band states.

Source of variation	df	Variance components	Percentage of variation	F_{ST}
Among pure species	5	12.967	38.65	0.38646*
Within pure species	58	20.587	61.35	
Among hybrid species	3	3.789	14.53	0.14528*
Within hybrid species	25	22.291	85.47	
Among population from <i>L. olgensis</i> range (excluding locus classicus sample)	10	6.931	23.80	0.23800*
Within population from <i>L. olgensis</i> range (excluding locus classicus sample)	104	22.191	76.20	
Among all population from <i>L. olgensis</i> range	11	7.681	25.64	0.25636*
Within all populations from <i>L. olgensis</i> range	121	22.279	74.36	

* $P < 0.00001$

olgensis populations have various levels of differentiation. It was interesting that large differentiation was found between the Olg sample collected from *L. olgensis locus classicus* and all populations from the range outlined for this species in the Primorye by Gukov. This differentiation corresponds to differentiation between these populations and pure species samples (Gme, Kae, Sib, Caj and Kam; Table 3). This result indicated that isolation between these populations existed for a long time and gene flows were restricted by geographic barriers. Mantel test also supports an isolation-by-distance model for the genetic structure of these populations ($r=0.4179$; $p=0.0028$), indicating that some populations located at short geographic distances show closer genetic affinities and, conversely, some populations spatially separated are not as genetically related.

To assess the overall distribution of diversity within and among populations studied, an AMOVA analysis was completed (Table 4). Most of the total genetic variation was found within the studied populations. These results are consistent with the general observation that woody species and especially conifers maintain most of their variation within populations. Genetic differentiation was 39% among pure species and 15% among hybrid species, but among populations from the *L. olgensis* range, including the sample from *locus classicus*, genetic differentiation was equal to 26%, which also is evidence of long-term isolation of these populations from each other.

In conclusion, our results show that all of the *L. olgensis* range populations studied, previously considered as *L. olgensis* based on morphological traits (Gukov, 1976), are genetically distant from

the *locus classicus* sample of this species. Consequently, the current range of *L. olgensis* in the Primorye Territory should be revised. The genetic resemblance revealed between populations from the *L. olgensis* range and hybrid species supports supposition of the hybridization with the adjacent hybrid species due to a string of fragmentations and connections of these populations as well as conditions of climate change and heterogeneity of the Sikhote-Alin Mountains landscape.

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