

Application of amplified fragment length polymorphisms markers to study the hybridization between *Pinus sibirica* and *P. pumila*

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Abstract. The genetic diversity of *Pinus sibirica*, *P. pumila*, and their hybrids, in a mixed natural stand was studied using amplified fragment length polymorphisms (AFLP). The stand was situated in the northern slope of the Khamar-Daban Ridge. Hybrid trees were determined by their morphological traits in the field. Three AFLP primer combinations detected 167 bands in 35 putative hybrids, and in 25 *P. sibirica* and 26 *P. pumila* individuals. Genetic variation in the hybrids was the highest, and was the lowest in *P. sibirica*. Principal coordinate analysis clustered *P. sibirica*, *P. pumila* and the hybrid genotypes into three distinct groups. Nei's distance between the species was 0.193, whereas that between the hybrids and *P. sibirica* was 0.047, and between hybrids and *P. pumila* it was 0.074. Application of AFLP in the future studies promises to increase our knowledge of *P. sibirica* and *P. pumila* hybridization.

Keywords interspecies hybridization, five-needle pines, *Pinus sibirica*, *Pinus pumila*, amplified fragment length polymorphisms, AFLP, genetic variation.

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Introduction

Siberian stone pine (*Pinus sibirica* Du Tour) and Siberian dwarf pine (*P. pumila* (Pall.) Regel) are five-needle pines from the section *Quinquefoliae*, subgenus *Strobus*, genus *Pinus*. Much of the ranges of these species are located

in Russia. Natural hybrids between the species occur in the sympatric zone (Goroshkevich 1999, Goroshkevich et al. 2008). L.K. Pozdnyakov (1952) described the first putative hybrid in southern Yakutia. He noted the intermediate size and structure of the needle, and the intermediate size and mass of the seeds of the

hybrid tree. G.I. Galaziy (1954) found a putative hybrid in the upper reaches of the Langatuy River in the Khamar-Daban Ridge (1650 m above sea level). Later, V.N. Molozhnikov (1975) described tree-like forms of *P. pumila*, and assumed they were hybrids because they combined features of both *P. pumila* and *P. sibirica*. In these studies, the hybrids were described on the basis of their morphological traits. Hybridization between *P. pumila* and *P. sibirica* had remained controversial until the study of Politov et al. (1999). They showed that *P. sibirica* and *P. pumila* were differentiated by the allozyme locus *Skdh-2* and that putative hybrid individuals were heterozygous for the parent alleles at this locus (Politov et al. 1999). Since then, all putative hybrids have been identified using allozymes (Petrova et al. 2007, Petrova et al. 2008, Petrova et al. 2012).

Amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995) are an efficient method for investigating genetic variation and are an alternative to other methods commonly used in plant hybridization studies, such as allozymes, microsatellites and inter-simple se-

quence repeats. The AFLP method is cheap, provides unlimited numbers of genetic markers and does not require prior information of the target fragment sequence. Disadvantages of the AFLP method include difficulties in joining data arrays produced in separate experiments and the dominant nature of the markers, making it impossible to distinguish between dominant homozygous (1/1) and heterozygous (1/0) genotypes (Bensch & Åkesson 2005). The aim of this work was to study the genetic variation in a mixed population of *P. sibirica*, *P. pumila* and their hybrids, using AFLP markers.

Materials and methods

Branches from 25 *P. sibirica*, 26 *P. pumila* and 35 hybrid trees were collected in August 2011 from a mixed stand located on the northern slope of the Khamar-Daban Ridge (Fig. 1), which is approximately 24 km south-west of Lake Baikal near Chertovo Lake (51°29' N, 103°35' E, 1728 m above sea level).

Hybrids were identified by their morphologi-

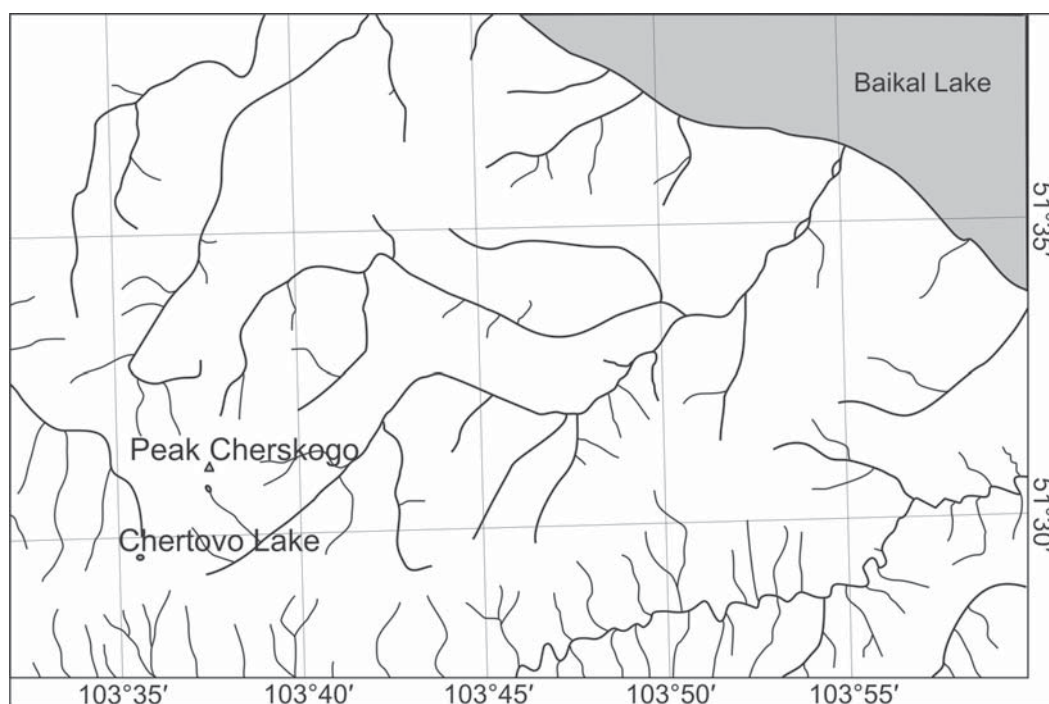


Figure 1 Location of studied mixed population

cal traits. The first of these traits was tree habit. Hybrids did not have a vertical trunk and the butt was often curved. Some hybrids did not have a pronounced trunk and their crown was formed by several trunk-branches. However, in contrast to *P. pumila*, not all the trunk-branches of the hybrid plant were prostrate. The second important trait was the color of the ripening cones, which were purple in hybrid cones, like those from *P. sibirica*. Additionally, the hybrid cones were larger than those of *P. pumila* but smaller than those of *P. sibirica*. In the depression near Chertovo Lake, the hybrids trees possessed the intermediate traits compared with parental species, whereas on the slopes, they had acquired a creeping form and could only be distinguished from *P. pumila* by their large, purple cones.

DNA was isolated from 0.1 g of fresh buds and needles according to the method of Devey et al. (1996) (cited in Ostrowska et al. 1998). The resulting DNA was then purified from RNA by incubation with RNase (100 µg/ml) at 65° C for 30 min. The DNA concentration was measured using spectrophotometer (Shimadzu). AFLP genotyping was based on a modified version of the method of Vos et al. (1995) and followed the protocol described by Samils et al. (2001), which uses fluorescently-labeled *EcoRI* primers. Three primer combinations that resulted in clear amplification profiles were selected for the analysis (*EcoRI* AGC NED™/*MseI* CCTG, *EcoRI* ACG JOI™/*MseI* CCTC, *EcoRI* ACT FAM™/*MseI* CCTC). Internal size markers (GeneScan™ – 500 ROX™ Size Standard) were used in each sample to size the fragments accurately. After the electrophoresis on a 3130 Genetic Analyzer instrument (Applied Biosystems, Applied Biosystems, Foster City, CA, USA), raw data were processed with the GeneMapper® software version 4.0 (Applied Biosystems). Only fragments ranging between 50 and 500 bp, and peaks with heights above 50 relative fluorescence units (rfu) were scored. All AFLP profiles were checked manually and the corresponding individuals were removed

from the analysis in case of bad amplification or presence of too many unique fragments. After visual inspection, only peaks with unambiguous interpretation were used for analysis.

Percentage of polymorphic loci (P), mean number of alleles per locus (N_a), expected heterozygosity (H_e) and an unbiased estimate of the expected heterozygosity (UH_e ; Nei 1978) were calculated based on presence/absence of AFLP fragments in the matrix using GENALEX 6.41 (Peakall & Smouse 2006) for the species and the hybrids. Nei's unbiased genetic distance among the studied species and hybrids was estimated (Nei 1978). Principal coordinates analysis (PCoA) was performed to visualize the genetic relationships among the studied individuals.

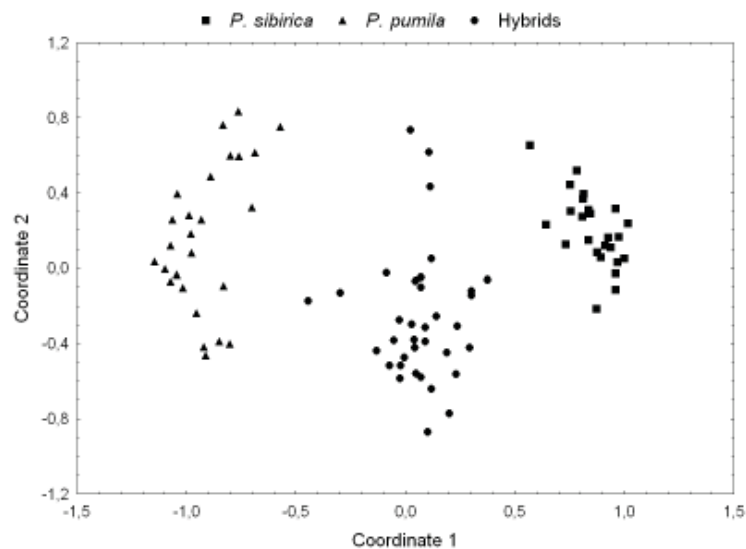
Results

In total, 167 AFLP fragments were obtained from 86 trees of the species and the hybrids. The numbers of bands produced by the primer combinations differed, with 48, 57 and 62 bands scored from *EcoAGC/MseCCTG*, *EcoACG/MseCCTC*, *EcoACT/MseCCTC*, respectively. The hybrids showed the largest genetic variation scores, whereas *P. sibirica* had the lowest (Table). The genetic variation scores for *P. pumila* were similar but smaller those for the hybrids.

AFLP analysis showed that all presumed hybrids were intermediate between parental species and clearly differed from them (Fig. 2). The PCoA coordinate 1 differentiated the three groups (species and hybrids) and coordinate 2 corresponded to variation within the group. The hybrid cluster was positioned between the parental species. Nei's distance between the species was 0.193, between *P. pumila* and hybrids was 0.074, and between *P. sibirica* and hybrids was 0.047, which indicated some bias of the hybrids toward the *P. sibirica* parents.

Table 1 Percentage of polymorphic loci (P), mean number of alleles per locus (N_a), expected heterozygosity (H_e) and an unbiased estimate of the expected heterozygosity (UH_e) of *P. sibirica*, *P. pumila* and their hybrids estimated by AFLP markers

Parameter	<i>P. sibirica</i>	Hybrids	<i>P. pumila</i>
P	58.080	83.830	80.840
N_a	1.395	1.808	1.707
H_e	0.178	0.265	0.261
UH_e	0.182	0.269	0.266

**Figure 1** Principal coordinate scatter plot showing the distinct distribution of *P. sibirica*, *P. pumila* and their hybrids into different clusters in the two dimensions

Discussion

Combining two significantly different genomes usually results in hybrids that are more genetically variable than their parental species (Anderson 1953, Stebbins 1959). Natural hybrids of *P. sibirica* and *P. pumila* are no exception. An allozyme survey of genetic diversity in *P. sibirica*, *P. pumila* and their hybrids (Petrova et al. 2007, 2012) showed that hybrids had the highest variation in all three mixed populations investigated, which corresponded with our results. Moreover, as in our study, the allozyme (Politov and Krutovsky 2004) and inter-simple sequence repeat markers (Liu et al. 2005) data also confirmed that *P. pumila* was more variable than *P. sibirica*.

How to explain an asymmetry in genetic distances between the hybrids and the parent species, which showed a slight bias of the hybrids toward *P. sibirica*? Perhaps this is normal for hybrids. For example, AFLP analysis of hybrids between *Populus ciliata* and *P. maximowiczii* showed more asymmetry and considerable shift of hybrids to one of the parental species on PCoA scatter plot (Chauhan et al. 2004). The observed asymmetry is probably associated with differentiated survival of different genotypes. Alternatively, the asymmetry could have occurred because of higher homozygosity of *P. sibirica* and consequently higher probability of offspring containing the band transmitted from *P. sibirica* parent.

Our earlier research suggested that the hy-

brids display sufficient seed production to form next hybrid generation, and the pollen of the hybrids pollinate the parental species. Consequently, they have a significant effect on the introgression of the species (Goroshkevich et al. 2008, Petrova et al. 2008, Vasilyeva 2011). In the near future, it would be interesting to study other groups of *P. sibirica* and *P. pumila* hybrids found in different locations of the sympatric zone. Taking into account the possibility of distinguishing F_1 and F_2 by means of AFLP markers (Miller 2000), we could further investigate the *P. sibirica*–*P. pumila* hybridization intensity and evolutionary consequences.

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