Asymmetric introgression between *Pinus sibirica* and *Pinus pumila* in the Aldan plateau (Eastern Siberia)

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Abstract

Pinus sibirica and *P. pumila* are Asian five-needle pines with vast geographic distributions that are partially overlapping. Natural hybrids with intermediate morphology have been found previously, but there is a lack of evidence of ongoing introgression. The goal of our study was to elucidate the genetic structure of *P. sibirica* and *P. pumila* populations growing in the north-east of their sympatry zone (Aldan plateau) using cytoplasmic DNA markers. All studied *P. sibirica* and *P. pumila* trees had usual species-specific growth habits. Using *nad1* intron2 of mitochondrial DNA (mtDNA) and *trn*V of chloroplast DNA (cpDNA) we found that trees morphologically identified as *P. sibirica* had *pumila*-specific mtDNA. Moreover, some of them also had *pumila*-specific cpDNA. *P. pumila* trees were typical and had *pumila*-type cytoplasmic DNA markers. These results suggest that interspecific hybridization took place long ago and lead to introgression and cryptic hybrids with *P. sibirica* appearance and *P. pumila* mtDNA.

Keywords: *: gene flow, hybridization, introgression, gymnosperms, chloroplast DNA, mitochondrial DNA*

Introduction

At the present time, there are no doubts about the great importance of hybridization in plant evolution. Interspecific hybridization is widespread among trees and facilitated by anemophily and lack of strong selection pressure in high fecundity (Stebbins, 1959; Grant, 1981; Koropachinskiy and Milyutin, 2006). In tree species, including conifers, the main isolation mechanisms are geographic, ecological and phenological isolation which connected with incomplete reproductive isolation and, therefore, they are ineffective in preventing interspecific gene exchange.

Natural hybridization can lead to introgression. The term "introgression" was proposed by Anderson as back-crosses of hybrids with one or both parental species that resulted in gene transfer from one species to another (Anderson, 1953). Independently of Anderson, the phenomenon was also described by the Russian botanist E.G. Bobrov who called it "hybrid blending of the species" or in Russian "гибридное смешение видов" (Bobrov, 1944; 1961). First of all, introgression is caused by incomplete reproductive isolation that enables gene flow between parental species and F1 hybrids. Cross-pollination can result in two categories of reciprocal hybrids (Chen et al., 2004; Thórsson et al., 2001), or back-crosses can occur with one parental species and lead to unidirectional introgression (Petit et al., 2004; Burgess et al., 2005; Godbout et al., 2012).

Hybridization of Siberian stone pine (*Pinus sibirica* Du Tour) and Siberian dwarf pine (*P. pumila* (Pall.) Regel) is one of the many

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examples of interspecific hybridization in pines. However, the peculiarity of this pair of the species is the contrasting life forms; *P. sibirica* is an upright tree and *P. pumila* is a prostrate tree. Hybrids of the two species have been found in many parts of the huge area where the species grow together (Goroshkevich, 2004; Goroshkevich et al., 2008). The first genetic evidence of natural hybridization between *P. sibirica* and *P. pumila* was obtained by allozyme analysis (Politov et al., 1999). The hybrids have intermediate morphology and presumably they are F1 (Goroshkevich, 1999; Goroshkevich et al., 2008). They are fertile and have a significantly reduced but sufficient seed efficiency (Goroshkevich et al., 2008; Vasilyeva, 2014) and crossability with parental species (Vasilyeva and Goroshkevich, 2013). Previously we have identified back-crosses in hybrid populations in the northern Baikal region only at the embryo stage. We studied embryos from seeds and found that embryos from the hybrid trees partly resulted from pollination by *P. sibirica* and *P. pumila* pollen i.e. they were back-crosses (Petrova et al., 2007; 2008). Therefore, the search for hybrids morphologically similar to one of the parental species remains an important task in order to prove the ongoing introgression.

The chloroplast and the mitochondrial DNA in pines is paternally and maternally inherited, respectively, (Neale and Sederoff, 1989; Mogensen, 1996; Petit and Vendramin, 2007) and makes it possible to determine the direction of the gene flow. The goal of our study was to elucidate the genetic structure of *P. sibirica* and *P. pumila* populations growing in the north-eastern area of their sympatry zone (Aldan plateau) using cytoplasmic DNA markers and to clarify the introgression issue.

Materials and Methods

Sample collection

The Aldan plateau is characterized by an extreme continental climate. The temperature in the warmest month is 13–18 °C. The sum of the temperatures above 10 °C is 700–1400 °C. The frost-free season usually lasts 60–90 days. The climate is temperately humid with annual precipitation of 350–500 mm. The main forest tree is larch, which occupies about 76 % of the forest covered area. Approximately 12 % of the area is occupied by Siberian dwarf pine (Pozdnyakov, 1969). Siberian stone pine rarely forms pure stands in the area and is mainly found as admixture in the larch forest.

Adult trees of *P. sibirica* and *P. pumila* from two locations in the north-eastern part of their sympatry zone were the objects of the study (Fig. 1). The first location (Aldan, 58°20′35″ N, 125°16′57″ E, 750 m a.s.l.) is situated 5 km from Aldan town, near Leninskiy settlement. *P. sibirica* was found here in the mixed forest where the main species were *Larix sp.*, *Pinus sylvestris*, and *Betula sp.* and *Picea sp*. as admixtures. *P. pumila* occurred in the understory. *Betula divaricata, Duschekia fruticosa, Prunus padus, Sorbus sp., Juniperus sp., Salix sp.* and *Rosa sp.* also occurred in the understory. The second location (Tommot, 58°26′39″ N, 126°14′12″ E, 627 m a.s.l.) is 15 km from Tommot town, near the neglected Bezymyaniy settlement. *Pinus sibirica* was also found there in the mixed forest, but in this stand there was no *P. sylvestris*. *Pinus pumila, Duschekia fruticosa, Prunus padus, Sorbus sp., Juniperus sp.* and *Betula sp.* were in the understory.

Needles were collected from randomly chosen 59 *P. sibirica* individuals (15 trees in Aldan and 44 trees in Tommot) and 32 *P. pumila* individuals (15 trees in Aldan and 17 trees in Tommot). In both locations, average tree height of *P. sibirica* was about 16 m, trunk diameter was about 20 cm, and trees were about 120–150 years old. *P. pumila* had a common cup-like growth form and reached a height of about 4 m. We found no typical hybrids with intermediate habit in the populations studied. All trees had typical species-specific morphology.

Cytoplasmic DNA analysis

Nad1 intron2 locus was used as a mitochondrial (mtDNA) marker, as proposed previously by Gugerli et al. (2001). The fragment overlapping *rbcL-trnV* region (~3600 bp) was chosen as a suitable chloroplast DNA (cpDNA) marker because studied species differ in the restriction site for *Tru9I*; it is present in *P. pumila* and absent in *P. sibirica* (personal communication with Dr. Vladimir L. Semerikov). Analysis of *trnV* sequences of the studied species from GeneBank (AB455836.1, AB019870.1) showed that *P. sibirica* and *P. pumila* are indeed distinguished by the nucleotide substitution. To verify the cpDNA marker, several *P. sibirica* and *P. pumila* individuals from various geographical regions out of the sympatry zone were used (Table 1, Fig. 1). All the trees were grown in the clone archive and geographical cultures at the Kedr field station, managed by the Institute of Monitoring of Climatic and Ecological Systems SB RAS (56°13′ N, 84°51′ E, 78 m a.s.l., Tomskaya oblast, Russia).

Table 1
Sample list of reference *P. sibirica* and *P. pumila* trees used to test the cpDNA marker

Species	*Origin*	*Geographic coordinates*
P. sibirica	Republic of Khakassia	52°30' N, 90°05' E, 350 m a.s.l.
	Sverdlovskaya Oblast	57°15' N, 60°1' E, 300 m a.s.l.
	Yamalo-Nenets Autonomous Okrug	65°50' N, 78°10' E, 30 m a.s.l.
	Tomskaya Oblast	56°31' N, 84°39' E, 99 m a.s.l.
P. pumila	Kunashir Island	44°05' N, 145°49' E, 150 m a.s.l.
	Magadanskaya Oblast	59°34' N, 150°48' E, 116 m a.s.l.
	Primorsky Kray	43°41' N, 134°11' E, 1854 m a.s.l.
	Sakhalin Island	46°51' N, 143°09' E, 50 m a.s.l.

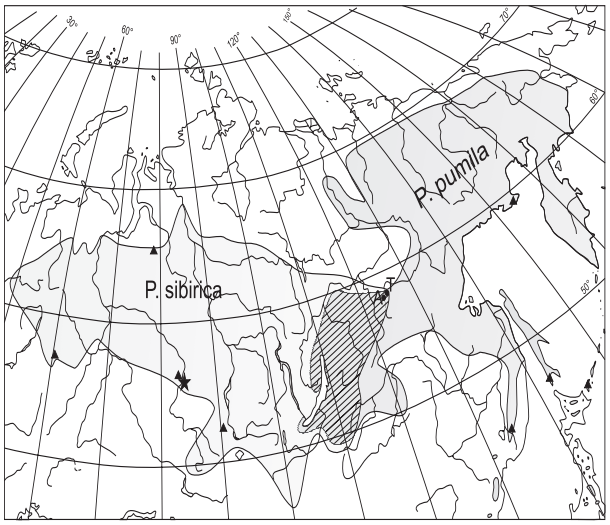

Figure 1
Geographic distribution of the species and their sympatry zone (shaded region). Studied populations: A – Aldan, T – Tommot. Asterisk – Kedr field station (Tomskaya oblast); triangle – the origin of *P. sibirica* and *P. pumila* trees used for *TrnV* verification.

Total DNA was extracted from the needles using the CTAB method (Doyle and Doyle, 1987). The DNA fragment of the *nad1* intron2 was amplified by PCR using primers previously described in the study of B. Demesure et al. (1995), namely forward: 5′-GCA TTA CGA TCT GCA GCT CA-3′ and reverse: 5′-GGA GCT CGA TTA GTT TCT GC-3′. *Nad1* intron2 locus was amplified with a typical mix of 40 µl PCR containing 1× *Taq* buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.2 µM of forward and reverse primers (Gugerli et al., 2001), approximately 100 ng of plant genomic DNA and 30 u/ml of Taq DNA Polymerase (Biosan, Russia). Cycling conditions for PCR consisted of an initial 1 min 30 s hot start at 95 °C, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 62 °C for 15 s and extension at 72 °C during 2 min 30 s, with a final incubation at 72 °C for 10 min and then remaining at 4 °C. Amplified products were analyzed by capillary electrophoresis using a Shimadzu MultiNA MCE-202 instrument (Shimadzu, Japan) with a DNA-12000 reagent kit, or by electrophoresis in 1.5 % agarose gel with 1× Tris-acetate-EDTA buffer.

Fragment *rbcL-trnV* was obtained by PCR amplification. The PCR mixture (40 µl volume) contained 1× *Taq* buffer, 0.2 mM dNTPs, 1.7 mM $MgCl_2$, 0.2 µM of selected forward (5′-TCG ATT CGT CCG ATC CACG-3′) and reverse (5′-TCG CAT TGG GCT CTT TCAT-3′) primers, approximately 100 ng of plant genomic DNA and 30 u/ml of Taq DNA Polymerase (Biosan, Russia). The cycling profile was as follows: hot start at 95 °C for 1 min 30 s, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 59 °C for 15 s and extension at 72 °C during 1 min and final hold at 4 °C. Post-PCR cleanup was conducted by absorption on AMPure XP magnetic particles (Agencourt, USA), and then fragments were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo, USA) and analyzed in an ABI 3130xl genetic analyzer (Applied Biosystems, USA). Sequencing of the fragments was carried out in the SB RAS Genomics Core Facility (ICBFM SB RAS, Novosibirsk, Russia). Indeed, a polymorphic position in the *rbcL-trnV* which discriminates studied species was located at *trnV* side (Table 2). A short fragment in *TrnV* intron determined interspecific differentiation: TGAA in *P. sibirica* and TTAA in *P. pumila*. Therefore, amplicons were sequenced using reverse primer and sequenced fragments had a discriminating position.

Table 2
Species-specific differences in cytoplasmic DNA in *P. sibirica* and *P. pumila*

Locus	*Species*	*Species-specific difference*	*Reference*
nad1 intron2	*P. sibirica*	Length (2530 bp)	Gugerli et al., 2001
mtDNA	*P. pumila*	Length (2181 bp)	
trnV cpDNA	*P. sibirica*	acaaagaTGAAgttcgatc*	GenBank: AB455836.1
			Given study (GenBank: MH443094, MH443095, MH443096, MH443097)
	P. pumila	acaaagaTTAAgttcgatc	GenBank: AB019870.1
			Given study (GenBank: MH443090, MH443091, MH443092, MH443093)

* Capital letters show restriction site for *Tru9I* that is present in *P. pumila* and absent in *P. sibirica*

Results

Using cytoplasmic DNA markers, we analyzed 59 *P. sibirica* trees and 32 *P. pumila* trees grown in the north-east of the sympatry zone. All *P. pumila* trees had species-specific cytoplasmic DNA loci (Table 3). In contrast, no *P. sibirica* trees had *sibirica*-specific mtDNA locus but *pumila*-like. CpDNA of the studied *P. sibirica* trees was frequently typical for the species, but some trees (17 %) had a *pumila*-specific *trnV* locus. Hence, all trees with *P. sibirica* appearance were hybrids.

Analyzed *trnV* intron was the same in all *P. pumila* trees, but some polymorphism was found in four individuals, three *P. pumila* trees and one *sibirica*-like hybrid. This polymorphism consisted of an insertion of a GAAA-motif (GeneBank: MH443098, MH443099). All obtained sequences with TGAA fragment in *TrnV* intron (*P. sibirica* variant) did not have this insertion. The reported hybrid had *pumila*-specific (TTAA) variant of *trnV* intron, and so this insertion was inherited only in *P. pumila*.

Table 3
Types of cytoplasmic genome found in *P. sibirica* and *P. pumila* trees identified morphologically

Cytoplasmic DNA loci	*Aldan*		*Tommot*	
	P. sibirica	*P. pumila*	*P. sibirica*	*P. pumila*
	N = 15	N = 15	N = 44	N = 17
nad1 intron2-*pumila* type	15	15	44	17
nad1 intron2-*sibirica* type	0	0	0	0
trnV-pumila type	1	15	9	17
trnV-sibirica type	14	0	35	0

Discussion

Only *P. sibirica* and *P. pumila* hybrids with intermediate traits have been described previously (Goroshkevich, 1999; 2004). Based on intermediate traits such hybrids were regarded as F1 (Goroshkevich et al., 2008). We suggest that traits of hybrids after multiple back-crosses with one of the parental species should be accompanied by their gradual shift in the direction of the species morphology.

Siberian stone pines investigated in the current study and originating from the north-east of their geographic distribution have a typical *P. sibirica* appearance. However, analysis of cytoplasmic DNA markers revealed traces of genetic exchange. We suggest these trees are nothing other than an echo of ancient introgression. Previously it was shown by means of allozyme analysis that species had different allele frequencies and that they were well differentiated from each other and from hybrids in the mixed hybrid populations from the Baikal region (Petrova et al., 2008; Petrova, Bender, 2010). Moreover, AFLP analysis also revealed clear division into two species and hybrid clusters in the mixed hybrid population in the south of the Baikal region (Vasilyeva and Semerikov, 2014). Therefore, we suppose that the hybridization pattern of the species is different in the different parts of the sympatry zone. On the one hand, the current process of gene flow between the species results in the intermediate F1 hybrid trees and occurs in the Baikal region. On the other hand, ancient introgression shown here occurs in the north-eastern part of the sympatry zone. The results obtained in this study suggest a repeated contact of the species ranges apparently due to global climate changes each of which could lead to hybridization. It is also possible that favorable conditions for genetic exchange were first developed in the north-eastern part of the current sympatry zone, and in the south-western part (i.e. the Baikal region) were sufficiently later.

It is interesting to note that the introgression of mtDNA we found was unidirectional. All trees morphologically identified as *P. sibirica* have *pumila*-specific mtDNA and mostly *sibirica*-specific cpDNA. However, there were no trees morphologically identified as *P. pumila* that have *sibirica*-specific cytoplasmic DNA markers. Flowering phenology and reproductive compatibility are of great importance for determination of the gene flow direction. It was shown that female cone development and pollen release in *P. pumila* occur earlier than in *P. sibirica* (Vasilyeva et al., 2010). In general, flowering phenology in these species is close enough to make individual variation and weather fluctuations provide gene flow in both directions. On the other hand, no evidence was found for unidirectional incompatibility between *P. sibirica* and *P. pumila* whose hybrids could be crosses with both parental species under controlled pollination conditions (Vasilyeva and Goroshkevich, 2013). Thus, unidirectional introgression of mtDNA could be caused by other currently undetermined factors.

It is difficult to discuss the timing of the introgression revealed because there is little available information concerning species origin and their geographic distribution path. Every recent study devoted to *Pinus* phylogeny has drawn attention to the possible effect of hybridization on establishment of a particular species or group of species (Syring et al., 2007; Tsutsui et al., 2009; Wang and Wang, 2014; Hao et al., 2015). It was shown in the five-needle pines that phylogenetic trees based on mtDNA, cpDNA and nuclear DNA did not coincide with each other (Tsutsui et al., 2009). Introgression was considered as one of the main factors leading to a phylogenetic incongruence. According to the cpDNA dataset, subsection *Strobus* (classification by Gernandt et al., 2005) splits into two groups, a Eurasian clade with North American *P. albicaulis* including all Eurasian species but not *P. peuce*, and the second group with the remaining five-needle pine species. In contrast, the mtDNA-based dataset demonstrated that group_2 includes all North American species and three Eurasian species, *P. pumila, P. koraiensis* and *P. peuce*, while the other species belong to a group_1. Eurasian *P. pumila, P. koraiensis* and North American *P. albicaulis* are characterized by cpDNA of the Eurasian clade and mtDNA of the group_2, mainly North American (Tsutsui et al., 2009). Hence, there is a reticulate component in the *P. pumila* origin possibly determining its predisposition to the interspecific genetic exchange. *P. pumila* hybridizes not only with *P. sibirica* but also with *P. parviflora* (Watano et al., 1995; 1996) which is very interesting for resembling of *P. pumila* and *P. sibirica* interspecific relation. *P. pumila* flowering also occurs earlier for several days compared with that of *P. parviflora* within the same locations (Ito et al., 2008). Pollen morphology is very similar in the *P. pumila / P. sibirica* pair (Kupriyanova and Litvintseva, 1974) and in the *P. pumila / P. parviflora* pair (Morita et al., 1999), making one-sided incompatibility unlikely. Hybridization of *P. pumila* and *P. parviflora* results in unidirectional introgression of mtDNA from the former species to the later one (Senjo et al., 1999). Thus, introgression takes place in the sympatry zone of *P. pumila* and closely related species (*P. sibirica* and *P. parviflora*). In both cases, hybrid trees morphologically

similar to upright species and having mtDNA of the prostrate species have been found. Why there are no hybrid trees with a *P. pumila* growth form and mtDNA of the upright species (*P. sibirica* or *P. parviflora*) remains unclear.

The novel information about the ancient introgression of *P. sibirica* and *P. pumila* resulting in formation of the cryptic hybrid populations of *P. sibirica* with a common habit but alien mtDNA could have an impact on understanding of the evolutionary relationships between the species and their differentiation in the sympatry zone. The most important questions to be further elucidated are geographic distribution of the ancient introgression and possible adaptive advantages of the cryptic hybrids over pure species.

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