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Crossability of *Pinus sibirica* and *P. pumila* with their hybrids

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Abstract

Crossability of *Pinus sibirica* and *P. pumila* hybrids and their parental species was studied using the controlled pollination method. *Pinus sibirica* and its hybrids were represented by grafts at the “Kedr” field station southeast of Tomsk Oblast, Russia; the parental species was of local provenance, with its hybrids obtained from the Southern Baikal region. In the case of *P. pumila*, trees were pollinated in a wild stand located in the Upper Angara River delta. Parental species had the highest number of filled seeds under open pollination. When they were pollinated with hybrid pollen, the trees showed nearly two-fold reductions in the number of filled seeds. Hybrids tended to abort most ovules during the first year of female cone development, resulting in a high seed abortion rate and consequent low seed production. The number of filled seeds obtained from hybrids was low, with levels ranging from 8.2 to 24.3%. Because of weak reproductive isolation between hybrids and parental species, crosses are inevitable and lead to

species introgression. The hybrids probably contribute to interspecies genetic exchange both through hybrid seed production following pollination by parental species and by hybrid pollen distribution.

Key words: hybridization, *Pinus sibirica*, *P. pumila*, controlled pollination, reproductive isolation.

Introduction

Interspecies hybridization is common in plants, and quite frequently contributes to their evolution (RIESEBERG, 1997; RIESEBERG and CARNEY, 1998; ARNOLD et al., 1999). In woody plants, the widespread occurrence of natural hybridization is closely connected with anemophily, a non-specialized type of pollination (KOROPACHINSKIY and MILYUTIN, 1979; 2006). In conifers, natural hybridization usually leads to introgression, i.e., genetic exchange between species (GRANT, 1981; KOROPACHINSKIY, 1992; KOROPACHINSKIY and MILYUTIN, 2006).

Among five-needle pines, Siberian stone pine (*Pinus sibirica* Du Tour) and Siberian dwarf pine (*P. pumila*

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[Pall.] Regel) have the largest natural habitats, occupying about 5 and 6 million km², respectively (CRITCHFIELD and LITTLE, 1966). *Pinus sibirica* is widely distributed throughout Russia. Its northern border extends from the Izhma River to the middle course of the Pechora River and the Northern Urals, then east to the Yenisei River (up to 68°N), and then slightly south to the upper reaches of the Aldan River. Its eastern border is in Transbaikalia, stretching along the Yablonovy Range into *P. sibirica*'s southernmost point in northern Mongolia (about 46°30' N). The southern border encompasses the Altai Mountains and extends westward to the southern part of the West Siberian Plain and thence to its western border in the Ural Mountains (Flora of USSR, 1934).

Pinus pumila is distributed between 69° and 35°N, i.e., from the northern timberline in Yakutia to Honshu Island. From west to east, its range extends from Lake Baikal to the Kamchatka Peninsula, although the middle portion of its western border deviates strongly to the east. This deviation is due to low snowfall in winter and the centuries-old impact of forest fires (UTKIN et al., 2001).

Pinus sibirica and *P. pumila* have extensively overlapping ranges, with areas of sympatry encompassing nearly 1 million km² (Fig. 1). This overlapping territory includes the Aldan Uplands, the Stanovoye Uplands, the Vitim Plateau, the Khentey-Chikoiskoye Uplands, and the mountain ridges surrounding Lake Baikal, excluding its western shores.

Hybrids are frequently found in their parental species' communities. The effect of these hybrids on population processes is determined by their fertility and ability to backcross with parental species (GRANT, 1981; RIESEBERG, 2001). Controlled crossing experiments can be used to study reproductive isolation and estimate the

strength of genetic barriers between species and/or hybrids. These crossing techniques have been widely applied to investigate affinity and incompatibility sources in conifers, especially in larches (AVROV, 1982), silver firs (KORMUTAK et al., 2008), spruces (FOWLER, 1987; MAJOR et al., 2005), two-needle pines (McWILLIAM, 1959; CRITCHFIELD, 1966; WACHOWIAK et al., 2005; KORMUTAK et al., 2005; LEWANDOWSKI and WISNIEWSKA, 2006), and five-needle pines (KRIEBEL, 1972; CRITCHFIELD, 1975; TITOV, 1977; BLADA, 1994; FERNANDO et al., 2005).

The earliest studies of natural hybridization between *P. sibirica* and *P. pumila* focused solely on morphology of a few putative hybrids (POZDNYAKOV, 1952; GALAZIY, 1954; MOLOZHNIKOV, 1975). Hybridization between these species was confirmed by isoenzyme analysis (POLITOV et al., 1999). Natural hybrids between *P. sibirica* and *P. pumila* are now known to frequently occur in areas where their habitats overlap (GOROSHKEVICH, 1999; GOROSHKEVICH et al., 2008a,b). The hybrids are fertile, and are characterized by intermediate-sized cones and seeds and considerably reduced seed efficiency compared with the parental species (GOROSHKEVICH et al., 2008a). In previous studies, hybrid seed efficiency varied depending on terrain and year of collection, with 1.8–25.1% of ovules growing into filled seeds with well-developed embryos (GOROSHKEVICH, 2004; VASILYEVA et al., 2006; PETROVA et al., 2007). When studying hybrid reproductive performance, knowledge of the degree of their reproductive isolation from parental species is needed to determine the intensity, direction, and probable results of hybridization. The aim of our study was to determine crossability of *P. sibirica* and *P. pumila* hybrids and their parental species using the controlled pollination method.

Figure 1. – Geographic distribution of *P. sibirica* and *P. pumila*. 1 – “Kedr” field station, 2 – Upper Angara River delta where controlled pollination of *P. pumila* was conducted, 3 – northern macroslope of Khamar-Daban Ridge is point of origin of hybrids used in the experiment.

Materials and Methods

The study was carried out from 2005 to 2011 at two locations in Russia. The first location was the “Kedr” field station, managed by the Institute of Monitoring of Climatic and Ecological Systems and situated 30 km south of Tomsk (56°13'N 84°51'E, 78 m above sea level). The second location was a wild stand of *P. pumila* in the Upper Angara River delta near Nizhneangarsk (55°47'N 109°33'E, 487 m above sea level). Grafts of eight *P. sibirica* clones of local provenance were used as maternal plants. For hybrid pollination, we used grafts represented by 11 hybrid clones originating from the northern macroslope of the Khamar-Daban Ridge (Fig. 1). Graftings were performed in 1997, with local *P. sibirica* trees used as rootstocks. Hybrid individuals were identified in the field based on tree habit and color of ripening cones. Compared with parental species, hybrids are intermediate in habit (GOROSHKEVICH et al., 2008a) and have purple cones of similar color to those of *P. sibirica*. All hybrids used in the study were presumed to be F1. We pollinated five *P. pumila* trees in the Upper Angara River delta, which were separated from one another by a distance of at least 50 m to ensure they were genetically distinct. *Pinus sibirica* pollen used in the experiment was a blend of pollen from several trees of local origin, while that of *P. pumila* was a blend from different trees in the wild stand in the Upper Angara River delta. Hybrid pollen was collected during the pollination year from the same grafts that were pollinated; only six clones were used because the remainder lacked microstrobili. *Pinus sibirica* pollen was collected a year prior to pollination, because pollen release in this species occurs slightly later than in cloned hybrids (VASILYEVA et al., 2010). *Pinus pumila* pollen was similarly collected beforehand, as *P. pumila* trees in the Upper Angara River delta release their pollen after that of *P. sibirica* and hybrids in the clonal archive. Collected pollen was stored below freezing, with calcium chloride used as a moisture absorbent.

For controlled pollination, female cones were isolated by placing Kraft paper bags over them approximately two days before pollen release. After isolation, we carefully observed the development of the covered cones, and pollinated them twice during their receptive phase. Paper bags were removed upon complete closure of cone scales.

The number of pollinated and measured cones varied each year over the course of the experiment, depending on the type of pollination. Only undamaged cones collected from 2006 to 2009 were measured, with seeds from the remaining cones saved for further analysis. Length, maximal diameter, and number of scales and seeds were measured for each cone. The number of potential ovules developing into formed seeds (both full-grown and aborted) per cone, expressed as a percentage, was estimated using the formula

$$SFF = \frac{Nse}{0.6 \times 2Nsc} \times 100\%$$

where *SFF* is the seed formation frequency, *Nse* is the number of seeds, *Nsc* is the number of scales, and the

coefficient 0.6 represents the average fraction of fertile scales in *P. sibirica* cones (GOROSHKEVICH and KHUTORNOY, 1996). Seeds were categorized as either full-grown or aborted, with the quality of full-grown seeds checked using X-ray analysis (SHCHERBAKOVA, 1965).

Collected data were statistically analyzed using Statistica 6.0 software. Mean, standard deviation, and maximum and minimum values were calculated. Comparison of means of cones taken from different crosses was performed using ANOVA and Duncan's *post hoc* test. We used the χ^2 (chi-squared) test to compare proportions of filled seeds.

Results

During the year of pollination, conelet abortion under open pollination was minimal (around 10%), with rates in hybrids and parental species differing only slightly. Under controlled pollination with hybrid pollen, conelet drop in the parental species increased to 40–50%. Hybrids exhibited 40–50% conelet abortion under controlled pollination, regardless of pollen source.

In both hybrids and parental species, mature cones retained their distinctive morphological features, regardless of pollination method; this was particularly apparent with respect to the number of scales (Table 1). No significant differences in cone length or diameter were observed, most likely because of the different numbers of full-grown seeds in cones. In regard to cone seed content, the presented cross variants differed from one another to varying extents, with the smallest values observed in the hybrids. The number of seeds in cones of parental species depended on pollen source, with reduced numbers observed when hybrid pollen was used.

Based on the estimated frequency of seed formation, open pollination was successful (Fig. 2). When parental species were pollinated by hybrid pollen, seed formation was initiated in ovules at levels approximately 75% of those observed under open pollination. Compared with parental species, the frequency of seed formation was quite low in hybrids. The highest value (54%) was obtained upon pollination with *P. sibirica*, whereas the lowest recorded value (21%) resulted from crossbreeding between hybrids. Both full-grown (normal size) and aborted (n-fold smaller) seeds were found in the cones. Under both open and controlled pollination, crosses involving parental species as maternal plants had seed abortion rates below 10%. In contrast, the use of hybrids as maternal plants led to seed abortion rates of 25% or greater, with no significant differences observed with respect to cross type or pollen used (Fig. 2).

Analysis of full-grown seed quality revealed the presence of many empty seeds and seeds with undeveloped endosperm, regardless of cross type. The highest percentages of filled seeds were recorded from open-pollinated parental species and the lowest from hybrids, with intermediate values observed from parental species pollinated with hybrid pollen (Table 2). Seed quality varied from one year to the next, but relative proportions among the different crosses remained similar. Different

Table 1. – Traits of *Pinus sibirica* (sib), *P. pumila* (pum) and their hybrids (hyb) cones obtained as a result of open (OP) and controlled pollination.

Trait		sib OP	sib×hyb	hyb×sib	hyb×hyb	hyb OP	hyb×pum	pum×hyb	pum OP
Number of measured cones		28	23	20	20	158	9	13	31
Cone length (mm)	Mean	65 a	62 a	45 b	38 c	40 bc	43 bc	38 c	43 bc
	Std.Dev.	9	11	7	6	8	8	10	8
	Min–Max	46–86	40–89	31–55	26–50	17–55	35–56	29–59	28–57
Cone diameter (mm)	Mean	44 a	44 a	34 b	30 c	31 c	32 bc	27 d	30 c
	Std.Dev.	5	6	4	4	5	4	7	3
	Min–Max	35–52	36–55	27–39	22–37	18–40	27–39	16–40	25–35
Number of scales	Mean	85 a	84a	55 b	56 b	54 b	56 b	45 c	42 c
	Std.Dev.	8	11	6	6	8	8	9	6
	Min–Max	67–102	50–97	47–67	43–67	36–83	43–63	35–62	30–50
Number of seeds	Mean	76 a	52 b	36 c	14 e	20 de	27 cd	23 d	35 c
	Std.Dev.	17	20	15	11	11	12	21	16
	Min–Max	34–104	14–94	7–60	3–45	2–67	8–49	1–63	11–66
Number of full-grown seeds	Mean	74 a	49 b	24 cd	10 f	14 ef	17 def	20 de	31 c
	Std.Dev.	17	19	10	7	8	8	21	15
	Min–Max	33–101	13–91	6–46	2–29	1–44	8–31	1–63	10–65

Note: for a given variable, means not followed by the same letter are significantly different ($p \leq 0.05$).

Figure 2. – Seed formation frequency and seed abortion rate in different crosses. Designation of cross types is the same as in the tables.

pollination schemes gave rise to different percentages of filled seeds. Chi-squared tests found significant differences ($p < 0.001$) in filled seed percentages among all but two pairs of crosses. There were no significant differences between the backcrosses *P. sibirica* × hybrids and *P. pumila* × hybrids ($p = 0.161$), or between crosses where hybrids were maternal plants, i.e., hybrids × hybrids vs.

hybrids × *P. pumila* ($p = 0.302$). We also examined the quality of seeds from hybrid cones. Throughout the study period, hybrids, regardless of cross type, tended to have nearly identical small numbers of filled seeds. In 2011, however, the number of filled seeds obtained from open-pollinated hybrids was lower than the number derived from hybrids under controlled pollination.

Table 2. – Seed quality of *Pinus sibirica* (sib), hybrids (hyb) and *P. pumila* (pum) under open (OP) and controlled pollination in different years.

Cross type	sib OP	sib×hyb	hyb×sib	hyb×hyb	hyb OP	hyb×pum	pum×hyb	pum OP
2006								
Number of analyzed seeds	270	103	150	— ¹	495	—	—	—
Number of filled seeds	189	51	30	—	100	—	—	—
Percentage of filled seeds	70.0	49.5	20.0	—	20.2	—	—	—
2007								
Number of analyzed seeds	—	350	799	—	1533	116	—	—
Number of filled seeds	—	216	38	—	113	12	—	—
Percentage of filled seeds	—	61.7	4.8	—	7.4	10.3	—	—
2008								
Number of analyzed seeds	—	245	105	59	—	77	—	—
Number of filled seeds	—	94	18	11	—	22	—	—
Percentage of filled seeds	—	38.4	17.1	18.6	—	28.5	—	—
2009								
Number of analyzed seeds	612	582	—	164	712	81	264	852
Number of filled seeds	430	205	—	17	103	3	95	691
Percentage of filled seeds	70.3	35.2	—	10.4	14.5	3.7	36.0	81.1
2011								
Number of analyzed seeds	—	517	—	211	824	199	—	—
Number of filled seeds	—	162	—	65	159	78	—	—
Percentage of filled seeds	—	31.3	—	30.8	19.3	39.2	—	—
Total								
Number of analyzed seeds	882	1797	1054	434	3564	473	264	852
Number of filled seeds	619	728	86	93	475	115	95	691
Percentage of filled seeds	70.2 b²	40.5 c	8.2 f	21.4 d	13.3 e	24.3 d	36.0 c	81.1 a

Note: ¹ – given crosses are not conducted in this study year.² – values not followed by the same letter are significantly different ($p \leq 0.05$).

Discussion

With respect to cross pollination of hybrids and parental species, incompatibility obviously plays a major role in seed efficiency. It is commonly believed that incompatibility manifests itself at every stage of seed development, whether during pollen germination or embryo development. This is clearly observed in deceleration of all development processes along with their association failures (BANNIKOVA, 1986; JOHNSON, 2010). Five-needle pine cones develop over two growing seasons. During the first year of cone development, pollination takes place, with pollen subsequently germinating and pollen tubes growing into the nucellus. The following year, the female gametophyte begins growing intensively; the megaspore, archegonia, and ovules are formed, and fertilization and embryo development then occur (FERGUSON, 1901; NEKRASOVA, 1972; OWENS and MOLDER, 1977). In five-needle pines, incompatibility manifests itself during the second year of cone development, and is usually evidenced by fertilization failure or rapid deteri-

oration of the newly formed embryo (HAGMAN and MIKKOLA, 1963; KRIEBEL, 1972; FERNANDO et al., 2005).

Low seed formation frequency is typical in cross-pollinating species, including *P. sibirica* and *P. pumila* (WIENS, 1984). In our study, we discovered that a significant number of ovules, both in parental species and hybrids, died during the year of pollination. Although it is surprising that parental species lost a third to a half of ovules (depending on the pollination method), hybrid losses were much higher, i.e., three-fourths. This fact clearly indicates the inferiority of most hybrid ovules, namely, their inability to develop into well-formed embryo-containing seeds. As evidenced by incompatibility, maternal genotype, rather than interaction of male and female reproductive systems, is therefore the most important factor influencing hybrid seed efficiency. Some pine species, for example *P. sylvestris* (KÄRKKÄINEN et al., 1999) and *P. sibirica* (GOROSHKEVICH and KHUTORNOY, 1996) in their non-hybridizing populations, have individuals with genetically conditioned low seed

efficiency. Low seed efficiency is apparently the norm in *P. sibirica* and *P. pumila* hybrids rather than the exception.

CRITCHFIELD (1975) suggested that the magnitude of genetic barriers between two species can be expressed by crossability, i.e., the percentage ratio of number of germinable seeds from crosses between two species to the number of seeds from within-species crosses. In a similar fashion, crossability between parental species and hybrids can be represented as the percentage ratio of the number of filled seeds in a cone from species pollinated by hybrid pollen to the same number from open-pollinated species. The number of filled seeds in a cone can be obtained by multiplying the proportion of filled seeds (Table 2) by the number of full-grown seeds (Table 1). Based on these calculations, the crossability of maternal parents *P. sibirica* and *P. pumila* with hybrids were 38.2% and 28.6%, respectively. Considering that crossability values can vary from 0 to 100%, the calculated values are lower than average, but are typical for most compatible *Pinus* species (CRITCHFIELD, 1975). It is also worth mentioning that while five attempts were made to cross *P. sibirica* with hybrids, only one experiment was performed between *P. pumila* and hybrids. To confirm the values obtained in this study, additional crossing attempts must thus be made between *P. pumila* and its hybrids.

When hybrids were backcrossed with parental species (hybrids $\times$ *P. sibirica* and hybrids $\times$ *P. pumila*), the number of filled seeds was never high (averaging 8.2 and 24.3%, respectively). In addition, crosses between two hybrids were not useful as controls, as the resulting number of filled seeds was lower than those obtained from the crosses between hybrids and *P. pumila*. It was furthermore difficult to differentiate between influences due to incompatibility vs. those arising from poor hybrid ovule quality.

The results of our study demonstrate that reproductive isolation of hybrids from their parental species is weak and cannot prevent them from crosses, thus leading to introgression. Absence of total reproductive isolation between hybrids and their parental species is common among many other hybridizing *Pinus* species (LEDIG, 1998). Of many examples, introgression is typical for *P. sylvestris* and *P. uncinata* (JASINSKA et al., 2010), *P. taeda* and *P. echinata* (CHEN et al., 2004), *P. contorta* and *P. banksiana* (CRITCHFIELD, 1985), *P. montezumae* and *P. hartwegii* (MATOS and SCHAAL, 2000), and *P. pumila* and *P. parviflora* (SENJO et al., 1999).

Despite the great variability observed during the course of the study in the number of filled seeds obtained from crosses between hybrids and their parental species, we found that hybrids and *P. pumila* have greater crossability than hybrids and *P. sibirica*. This observation is corroborated by the results of a genetic study of natural hybrids and their parental species in the Baikal region. In that study, hybrids whose embryos were formed primarily by pollination with *P. sibirica* pollen had twice as many empty seeds, and four times more seeds with undeveloped endosperm,

than hybrids pollinated by *P. pumila* (PETROVA et al., 2007). In all probability, hybrids cross better with their maternal parental species, meaning that the observed superior crossability is nothing more than a maternal effect. To prove this hypothesis and define the genetic contribution of each individual hybrid genotype for each species, additional studies are required.

In conclusion, controlled pollination of *P. sibirica* and *P. pumila* hybrids and their parental species was carried out for the first time in our study. Our results demonstrate that pollination of parental species by hybrid pollen led to considerably decreased seed efficiency. Low seed efficiency in the hybrids is due to their genetic character, with some ovules unable to develop into sound seeds. As a consequence, weak reproductive isolation between hybrids and their parental species can lead to introgression. This introgression can be bidirectional because of reciprocal back crossing between hybrids and their parental species.

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