



ARTIFICIAL HYBRIDIZATION OF *PINUS SYLVESTRIS* L. AND *PINUS MUGO* TURRA

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Artificial hybridization experiments revealed a relatively high degree of crossability between *Pinus sylvestris* and *P. mugo*, ranging between 0.15 and 0.98. Differences between *P. sylvestris* × *P. mugo* and reciprocal hybrids of the species were observed. Hybrid seeds of *P. sylvestris* × *P. mugo* shared higher germination potential than seeds from selfing, controlled intraspecific crossing, and open pollination. The corresponding value in reciprocal combination was slightly lower. The hybrid nature of the seeds obtained was shown by restriction analysis of chloroplast DNA (*cpDNA*) using the *trnV-trnH/Hinf*I primer-enzyme combination.

Key words: *Pinus*, cross-pollination, seed viability, *cpDNA*.

INTRODUCTION

Scots pine (*Pinus sylvestris* L.) and mountain dwarf pine (*P. mugo* Turra) are systematically related species of the subgenus *Diploxylon* (Hard Pines) occupying a common position within the group *Lariciones* according to Shaw (1914) and/or within the sections *Eupitys* and *Pinus* according to Pilger (1926) and Price et al. (1998). Little and Critchfield (1969) placed these pines in the section *Pinus*, subsection *Sylvestres* Loud. Based on hybridization experiments, Duffield (1952) placed both these species in Group X, indicating some hybridological affinity between them. The existence of spontaneous hybrids between *P. sylvestris* and *P. mugo* has been postulated since the second half of the nineteenth century, but discussions of the genetic status of the putative hybrids persist (Christ, 1864; Brügger, 1886; Bertsch, 1906). The most recognized places in Europe where hybrid swarm populations of *P. mugo* × *P. sylvestris* have been reported are Rila Planina and Rodopy in Bulgaria (Dobrinov, 1965; Dobrinov and Jagdzidis, 1971), the Dolina Nowotarska valley in Poland (Staszkiwicz and Tyszkiewicz, 1969; Bobowicz et al., 2000), Swiss Alps (Net-Sarqueda et al., 1988) and the Orava region in Slovakia (Musil, 1977; Viewegh, 1981).

Several attempts at artificial hybridization of these species have produced viable hybrids (Dengler, 1932; Schmidt, 1951; Marcet 1967). The hybrid families were characterized in terms of needle morphology and anatomy (Christensen and Dar, 1997; Bobowicz et al., 2000) and to a lesser extent also needle proteins and isozymes (Prus-Glowacki and Stephan, 1998). However, there are no available data quantifying the degree of crossability between the parent species. Christensen and Dar (1997) mentioned in this connection the reduced number and viability of hybrid seeds, along with physiological and ecological differences preventing a higher frequency of hybridization and introgression among *P. mugo* and *P. sylvestris*. Based on needle traits and the phenology of reproductive organs, Boratyńska et al. (2003) and Boratyński et al. (2003) recently reported gene flow from *P. sylvestris* to *P. uliginosa* and to some extent reciprocally. To quantitatively characterize the hybridological affinity between these species, the present work attempted artificial hybridization of *P. sylvestris* and *P. mugo*, and made a cytological study of pollen-ovule interaction during the first period of growth.

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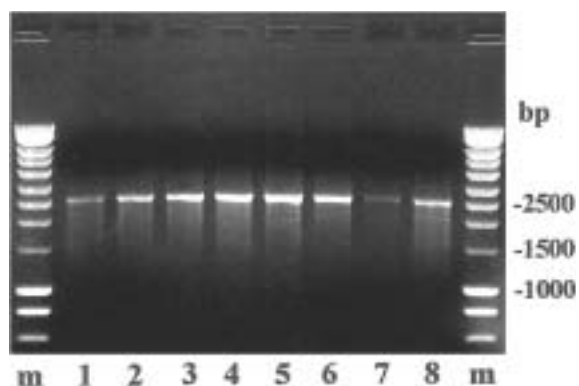


Fig. 1. PCR products of amplified *trnV-trnH* gene region. m – size marker; lane 1 – *P. mugo* needles; lanes 2–4 – megagametophytes of three hybrid seeds of *P. mugo* × *P. sylvestris*; lanes 5–7 – corresponding embryos of *P. mugo* × *P. sylvestris*; lane 8 – *P. sylvestris* needles.

MATERIALS AND METHODS

ARTIFICIAL POLLINATION AND SEED VIABILITY TEST

Three mother trees of Scots pine (*Pinus sylvestris* L.) and two mother trees of mountain dwarf pine (*P. mugo* Turra) were used in the artificial hybridization experiment. The *P. sylvestris* trees grow in a natural stand of the species in Velká Lehota, western Slovakia (575 m a.s.l.), and the *P. mugo* trees in Popradské Pleso in the High Tatras, eastern Slovakia (1500 m a.s.l.). In addition, one tree of each species was selected from the respective localities as a male parent (*P. sylvestris* no. 4, *P. mugo* no. 3). Artificial pollination was done in 2002 using freshly collected pollen by a standard procedure for conifers, with the use of paper bags and syringe pollinators. All five mother trees were used as pollen donors in self-pollination variants. Isolators were put on the macrostrobili before pollen shedding, and removed after complete closure of the bract scales of the conelets. Mutual crossability of Scots pine and mountain dwarf pine was tested within all five mother trees used. Besides the interspecific crossing mentioned, variants with selfing, open pollination and controlled intraspecific crossing of each of the mother trees were performed (Tab. 1). The variant with controlled intraspecific crossing served as a control. The degree of crossability between *P. sylvestris* and *P. mugo* was expressed as the ratio between the percentage of viable seeds of the interspecific cross and the percentage of viable seeds in the corresponding control from intraspecific outcrossing of a given tree.

Seed viability was estimated by *in vitro* germination tests, using only filled seeds after removal of the empty ones (Tab. 1). The seeds were allowed to germinate on wet filter paper in Petri dishes at 25°C for 15 days. The results of crossing experiments and germination tests were analyzed statistically with the z-test

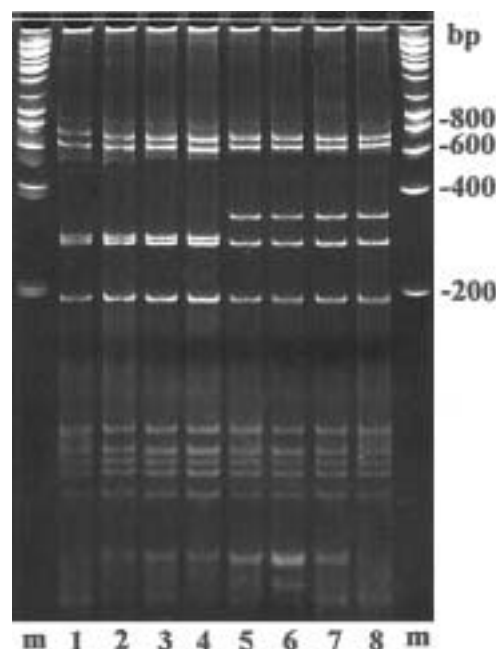


Fig. 2. Restriction profiles of *trnV-trnH* gene region generated by *Hinf*I. Lane designations as in Figure 1.

(Šmelko and Wolf, 1977). Besides seed quality, the relative proportions of pollinated macrostrobili and collected mature cones were considered in estimating crossability between *P. sylvestris* and *P. mugo*.

EMBRYOLOGICAL STUDY OF DEVELOPING OVULES

To reveal the nature of pollen-ovule interaction during the first growing season, self-pollinated ovules and those of *P. sylvestris* × *P. mugo* crossing at conelet stage were subjected to comparative embryological analysis. Excised ovules were fixed in Navashin fixative for 12–24 h, washed with tap water, dehydrated in an ethanol series with toluene in the middle step, and finally embedded into paraffin. Following removal of the paraffin, longitudinal sections of the ovules were stained with hematoxylin and embedded in Canada balsam (Pazourková, 1986).

CHLOROPLAST DNA (*cpDNA*) MARKER ANALYSIS

Total DNA was extracted from young needles and seeds of the same mother trees using the CTAB method of Murray and Thompson (1980). The *trnV-trnH* region of *cpDNA* was PCR amplified using the primer pair consisting of 5'-GCTCAGCAAGGTAGAGCACC-3' and 5'-CTTGGTCCACTTGGCTACGT-3' (Parducci and Szmidi, 1999). DNA amplification was performed at 94°C for 4 min followed by 35 cycles at 93°C for 1 min, 56°C for 1 min and 72°C for 2 min. The last strand

TABLE 1. Results of artificial hybridization between *Pinus sylvestris* and *P. mugo*

Combinations attempted	Number		No. of seeds obtained	% of filled seeds ¹	Degree of crossability ²	Germinability	
	Pollinated macrostrobili	Mature cones				No. of filled seeds tested	Germination % ¹
<i>P. sylvestris</i> no.1 selfing	42	23	81	18.5**		15	80.0
<i>P. sylvestris</i> no.1 × <i>P. sylvestris</i> no.4	41	25	79	100.0		79	86.0
<i>P. sylvestris</i> no.1 open pollination		19	123	84.5**		104	77.8
<i>P. sylvestris</i> no.1 × <i>P. mugo</i> no.3	55	14	40	70.0**	0.70	28	92.8
<i>P. sylvestris</i> no.2 selfing	53	20	106	19.0**		20	70.0
<i>P. sylvestris</i> no.2 × <i>P. sylvestris</i> no.4	59	31	165	96.3		159	55.3
<i>P. sylvestris</i> no.2 open pollination		47	157	52.8**		83	68.6*
<i>P. sylvestris</i> no.2 × <i>P. mugo</i> no.3	65	14	50	26.0**	0.26	13	76.9
<i>P. sylvestris</i> no.3 selfing	27	11	100	26.0**		26	42.3
<i>P. sylvestris</i> no.3 × <i>P. sylvestris</i> no.4	48	21	114	70.1		88	53.4
<i>P. sylvestris</i> no.3 open pollination		24	71	35.2**		25	52.0
<i>P. sylvestris</i> no.3 × <i>P. mugo</i> no.3	69	14	15	53.3	0.76	8	62.5
<i>P. mugo</i> no.1 selfing	27	14	343	72.5**		67	26.8
<i>P. mugo</i> no.1 × <i>P. mugo</i> no.3	18	18	450	87.1		49	38.7
<i>P. mugo</i> no.1 open pollination		35	118	68.6**		81	69.1**
<i>P. mugo</i> no.1 × <i>P. sylvestris</i> no.4	33	15	133	13.5**	0.15	56	43.2
<i>P. mugo</i> no.2 selfing	12	6	97	68.0**		65	43.0**
<i>P. mugo</i> no.2 × <i>P. mugo</i> no.3	13	10	285	84.9		95	93.6
<i>P. mugo</i> no.2 open pollination		34	608	91.6**		85	81.1
<i>P. mugo</i> no.2 × <i>P. sylvestris</i> no.4	10	8	315	83.4	0.98	45	86.6

¹Statistically significant deviations from control at $p > 0.05$ (*) and $p > 0.01$ (**)

²Crossability data referring to controlled outcrossing of the corresponding mother tree.

elongation at 72°C was allowed an additional 10 min. To confirm successful amplification of the *cpDNA* region, 2 µl of the PCR products were separated by electrophoresis in 0.8% agarose gel containing ethidium bromide (0.5 mg l⁻¹) in 1 × TBE. The DNA fragments were visualized by UV fluorescence. The obtained PCR products were digested with the restriction enzyme *HinfI*, which has been found to discriminate the *cpDNA* of *P. sylvestris* from that of *P. mugo* (Kormuťák et al., 2002). The generated fragments were fractionated electrophoretically in 8% polyacrylamide gels and 1 × TBE buffer. Electrophoresis was run at 2.5 Vcm⁻¹ for 3 h. The gels were stained in 1 × TBE with EtBr (0.5 mg l⁻¹).

RESULTS

The hybrid nature of the seeds from artificial hybridization of *P. sylvestris* and *P. mugo* was confirmed by restriction analysis of the *trnV-trnH* region of *cpDNA* in hybrid seeds and in the corresponding parental trees. No variation in the size of the PCR products of both species was observed; it ranged around 2530 bp (Fig. 1). Following their digestion with *HinfI*, the two species-specific haplotypes of *cpDNA* were recognized. Figure 2 shows that needles of *P. mugo* no. 2 and female

gametophytes of hybrid seeds of *P. mugo* no. 2 × *P. sylvestris* shared the *P. mugo* haplotype, which consisted of 700, 600, 320, 300 and 190 bp fragments. Essentially the same restriction profile was characteristic for *P. sylvestris* also, except for the 350 bp fragment distinguishing the species from *P. mugo* which contained a 320 bp fragment instead. Because *cpDNA* in conifers is inherited paternally, the presence of the 350 bp fragment in hybrid embryos unequivocally demonstrates their hybrid origin.

The degree of crossability between *P. sylvestris* and *P. mugo* varied considerably depending on the parent trees crossed, but compatibility characterized the interspecific crossings in all five mother trees involved (Tab. 1). In combination with the same paternal tree, *P. mugo* no. 3, *P. sylvestris* mother trees no. 1, 2 and 3 exhibited 0.70, 0.26 and 0.76 degrees of crossability, respectively. Reciprocal crossings of mother trees *P. mugo* no. 1 and no. 2 with *P. sylvestris* tree no. 4 resulted in 0.15 and 0.98 degrees of crossability. These values indicate relatively high hybridological affinity between *P. sylvestris* and *P. mugo*. In terms of filled seed percentage, the controlled intraspecific crossings were the most effective among the crossing variants attempted so far. The only exception was *P. mugo* mother tree no. 2, which produced a greater number of viable seeds from open pollination than from its cross-

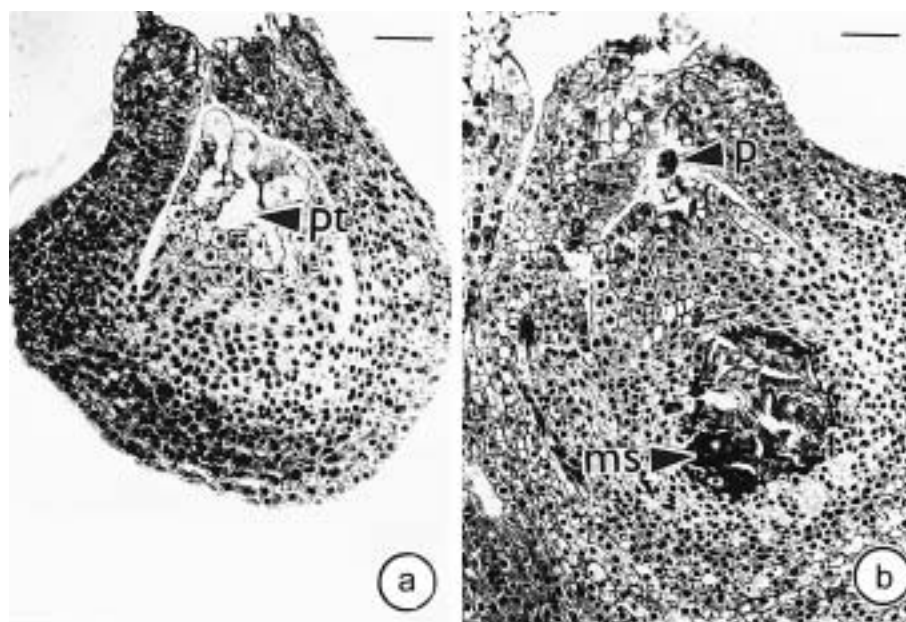


Fig. 3. Longitudinal sections of ovules in *P. sylvestris* no.1 \times *P. mugo* no. 3 crossing, showing progressively developing ovules with pollen tubes penetrating nucellar tissue (a), and abortive ovules with dormant pollen at top of nucellus and degenerating megaspore (b). p – pollen; pt – pollen tube; ms – megaspore. Bar = 600 μ m.

ing with *P. mugo* no. 3. In contrast to the intraspecific crossings, the variants with selfing produced the lowest proportions of filled seeds in the majority of mother trees. Especially conspicuous was the inbreeding effect of selfing in *P. sylvestris*, with the percentages of filled seeds ranging from 18.5% to 26% only. The corresponding values in *P. mugo* were much higher, reaching 72.5% and 68% in the two mother trees. The interspecific crossing *P. sylvestris* \times *P. mugo* produced higher proportions of filled seeds than selfing in all three mother trees. The same was true of *P. mugo* no. 2 \times *P. sylvestris* no. 4 crossings.

The hybrid seeds were of high quality, as evidenced by the data presented in Table 1. The seeds of *P. sylvestris* \times *P. mugo* reached the highest percentage of in vitro germination, surpassing the rest of the variants in the three mother trees of *P. sylvestris*. Likewise, hybrid seeds of *P. mugo* \times *P. sylvestris* exhibited high viability, reaching 43.2% and 86.6% germination; in this respect they lagged only behind *P. mugo* no. 1 open pollination and *P. mugo* no. 2 \times *P. mugo* no. 3. The results on the germination potential of selfed progeny of *P. sylvestris* and *P. mugo* were in sharp contrast to their filled seed percentages. In spite of having a lower proportion of filled seeds, the percentage of germinated *P. sylvestris* seeds from selfing was higher than in *P. mugo*, which produced more filled seeds from selfing.

Embryological analysis of the ovules during the first growing season confirmed an affinity between

P. mugo pollen and nucellar tissue of *P. sylvestris* ovules. Pollen germinated at the top of the nucellus in a small fraction of the ovules at this stage of their development (Fig. 3a). Penetration of pollen tubes into the nucellar tissue enables survival of the ovules during winter, and also provides a necessary stimulus for development of the conelets into mature cones during the second growing period. However, along with normally proceeding fertilization, deviation from this pattern was noted in ovules of *P. sylvestris* no. 1 \times *P. mugo*. Pollen grains remained dormant at the top of the nucellus, causing abortion of the megaspore in the central part of the ovule (Fig. 3b).

DISCUSSION

The present results of artificial hybridization show relatively high hybridological affinity between *P. sylvestris* and *P. mugo*. Dengler (1932) and Schmidt (1951) maintained that the two species are crossable. Our data indicate a broad range of crossability between these species. In some exceptional combinations of parental trees, the efficiency of filled seed production seemed comparable with intraspecific crossings, particularly when *P. mugo* was used as the maternal species. However, when the numbers of pollinated macrostrobili and collected mature cones are taken into account, the genetic differentiation of *P. sylvestris* and *P. mugo* becomes much more apparent. In interspecific

crossings with *P. sylvestris* used as a maternal species, the survival rate of conelets during the first growing season was half that observed in the controlled intraspecific crossings. The corresponding figure in variants with self-pollination of the mother tree was higher than in *P. sylvestris* × *P. mugo* combinations. Since conelet survival is determined by the ability of pollen grains to germinate at the top of the ovular nucellus (Sarvas, 1962), the massive dropping of *P. sylvestris* × *P. mugo* conelets indicates that only a small fraction of *P. mugo* pollen have this ability. The corresponding ability of *P. sylvestris* pollen in *P. mugo* × *P. sylvestris* crossings is probably higher, indicating differences in the efficiency of both direct and reciprocal crossings of *P. sylvestris* and *P. mugo*. In light of these findings, we conclude that crossability between *P. sylvestris* and *P. mugo* is relatively high, high enough to ensure the production of spontaneous hybrids at sites of their sympatric occurrence. Validating these hybrids, Prus-Glowacki and Stephan (1998) used immunochemical and isoenzyme methods to confirm the hybrid nature of controlled crosses between *P. montana* var. *rostrata* and *P. sylvestris*. Our approach, based on previous findings of differences in the *trnV-trnH* spacer of *cpDNA* in *P. sylvestris* and *P. mugo*, also proved reliable in verifying the interspecific crosses of these pines. Together with reported differences in the *trnF-trnL* gene region of *cpDNA* in the respective species (Wachowiak et al., 2000), this work offers additional prospects for more straightforward screening of natural hybrids in sympatric populations of *P. sylvestris* and *P. mugo* across Europe.

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