**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/HinfI 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 J. Angdžić, 1971), the Dolina Nowotarska valley in Poland (Staszkiewicz and Tyszkiel, 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, Boratynska 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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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 (Smelko 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’-GCTCAGCAAGGTAGACCC-3’ and 5’-CTTGGTCACCTTTGCTACG-3’ (Parducci and Szmidt, 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

<table>
<thead>
<tr>
<th>Combinations attempted</th>
<th>Number of seeds obtained</th>
<th>% of filled seeds</th>
<th>Degree of cross-ability</th>
<th>Germinability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pollinated macro-strobili</td>
<td>Mature cones</td>
<td>No. of filled seeds</td>
<td>No. of filled seeds tested</td>
<td>Germination %</td>
</tr>
<tr>
<td>P. sylvestris no. 1 selfing</td>
<td>42</td>
<td>23</td>
<td>81</td>
<td>18.5**</td>
</tr>
<tr>
<td>P. sylvestris no. 1 × P. sylvestris no. 4</td>
<td>41</td>
<td>25</td>
<td>79</td>
<td>100.0</td>
</tr>
<tr>
<td>P. sylvestris no. 1 open pollination</td>
<td>19</td>
<td>23</td>
<td>123</td>
<td>84.5**</td>
</tr>
<tr>
<td>P. sylvestris no. 1 × P. mugo no. 3</td>
<td>55</td>
<td>14</td>
<td>40</td>
<td>70.0**</td>
</tr>
<tr>
<td>P. sylvestris no. 2 selfing</td>
<td>53</td>
<td>20</td>
<td>106</td>
<td>19.0**</td>
</tr>
<tr>
<td>P. sylvestris no. 2 × P. sylvestris no. 4</td>
<td>59</td>
<td>31</td>
<td>165</td>
<td>96.3</td>
</tr>
<tr>
<td>P. sylvestris no. 2 open pollination</td>
<td>47</td>
<td>15</td>
<td>157</td>
<td>52.8**</td>
</tr>
<tr>
<td>P. sylvestris no. 2 × P. mugo no. 3</td>
<td>65</td>
<td>14</td>
<td>50</td>
<td>26.0**</td>
</tr>
<tr>
<td>P. sylvestris no. 3 selfing</td>
<td>27</td>
<td>11</td>
<td>100</td>
<td>26.0**</td>
</tr>
<tr>
<td>P. sylvestris no. 3 × P. sylvestris no. 4</td>
<td>48</td>
<td>21</td>
<td>114</td>
<td>70.1</td>
</tr>
<tr>
<td>P. sylvestris no. 3 open pollination</td>
<td>24</td>
<td>71</td>
<td>35.2**</td>
<td>25</td>
</tr>
<tr>
<td>P. sylvestris no. 3 × P. mugo no. 3</td>
<td>69</td>
<td>14</td>
<td>15</td>
<td>53.3</td>
</tr>
<tr>
<td>P. mugo no. 1 selfing</td>
<td>27</td>
<td>14</td>
<td>343</td>
<td>72.5**</td>
</tr>
<tr>
<td>P. mugo no. 1 × P. sylvestris no. 3</td>
<td>18</td>
<td>18</td>
<td>450</td>
<td>87.1</td>
</tr>
<tr>
<td>P. mugo no. 1 × P. mugo no. 3</td>
<td>18</td>
<td>18</td>
<td>118</td>
<td>68.6**</td>
</tr>
<tr>
<td>P. mugo no. 1 × P. sylvestris no. 4</td>
<td>33</td>
<td>15</td>
<td>133</td>
<td>13.5**</td>
</tr>
<tr>
<td>P. mugo no. 2 selfing</td>
<td>12</td>
<td>6</td>
<td>97</td>
<td>68.0**</td>
</tr>
<tr>
<td>P. mugo no. 2 × P. mugo no. 3</td>
<td>13</td>
<td>10</td>
<td>285</td>
<td>84.9</td>
</tr>
<tr>
<td>P. mugo no. 2 open pollination</td>
<td>34</td>
<td>608</td>
<td>91.6**</td>
<td>85</td>
</tr>
<tr>
<td>P. mugo no. 2 × P. sylvestris no. 4</td>
<td>10</td>
<td>8</td>
<td>315</td>
<td>83.4</td>
</tr>
</tbody>
</table>

1Statistically significant deviations from control at p > 0.05 (*) and p > 0.01 (**) 
2Crossability data referring to controlled outcrossing of the corresponding mother tree.
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* × *P. mugo* produced higher proportions of filled seeds than selfing in all three mother trees. The same was true of *P. mugo* no. 2 × *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* × *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* × *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 × *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 × *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 interspecific 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-Głowacki and Stephan (1998) used immunochemical and isoenzymatic methods to confirm the hybrid nature of both direct and reciprocal crossings of P. sylvestris and P. mugo. In accord with these findings, Glowacki and Stephan (1998) used immunochemical production of spontaneous hybrids at sites of their findings of differences in the pines. Together with reported differences in the liable in verifying the interspecific crosses of these prospects for more straightforward screening of natural hybrids in sympatry populations of P. sylvestris and P. mugo across Europe.

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REFERENCES


