

Crossability of Putative Hybrids of *Pinus sylvestris* and *Pinus mugo* with Their Parents

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Abstract

Artificial hybridization experiment with the *Pinus sylvestris* and *Pinus mugo* putative hybrid individuals and their parents revealed a selective nature of their crossability conditioned by the identical chloroplast DNA (cpDNA) haplotypes of the crossed individuals. Efficiency of hybridization between hybrid individual H1 of *P. sylvestris* haplotype and *P. sylvestris* paternal tree was relatively high, as evidenced by 78.03% of filled seeds. On the contrary, the crossing of H1 individual with *P. mugo* was unsuccessful. Likewise, the crossability of the H2 individual of *P. mugo* haplotype with the paternal tree *P. mugo* resulted in 31.15% of filled seeds. A relatively high crossability was characteristic also for the H1 and H2 hybrid trees of different cpDNA haplotypes. A conclusion has been drawn postulating weakened reproduction barrier between hybrid swarm individuals and the paternal species *P. sylvestris* and *P. mugo*. Differentiation of the putatively hybrid individuals from the parental species is accompanied by the variation in cpDNA inheritance.

Keywords

Pinus sylvestris, *P. mugo*, Crossability, cpDNA

1. Introduction

The role of spontaneous hybridization between different species of plants during the evolution is widely discussed issue of the evolutionary biology [1] [2] [3]. The intermediate forms of plants generated in this way exhibit increased variability, varying adaptive potentials and different evolutionary prospects [4] [5].

Introgressive hybridization is in this context of special interest. It occurs between systematically related species of plants whose areas of natural distribution overlap to a lesser or larger extent offering the possibility for mutual hybridization in a contact zone. In case that F_1 hybrids are fertile, they have an opportunity to mate with each other or with the parental species [6]. Successive backcrossing of the hybrids with one or both parental species results in segregation of the backcrossed progenies which increases considerably their variability. Russian scientist [7] named this phenomenon “the hybrid mixing of species”. Subsequently, [8] has introduced the term “introgressive hybridization” for this type of hybridization substituting for the ecological and evolutionary significance of the process. In the genus *Pinus*, the introgressive hybrids were reported to occur in North America between the pairs of species *P. banksiana*—*P. contorta*, *P. ponderosa*—*P. arizonica* and *P. taeda*—*P. echinata* [9] [10] [11] [12] [13] as well as in Asia between the pairs of species *P. tabulaeformis*—*P. yunnanensis*, *P. pumila*—*P. parviflora* var. *pentaphylla* and *P. sibirica*—*P. pumila* [14] [15] [16] [17]. In Europe, an extensive study on the pattern of introgression between *P. brutia* and *P. halepensis* and its dynamics on the island Rhodes was followed by [18]. The other introgressive hybrid of pines on the continent is that between *P. sylvestris* and *P. mugo* occurring in several places in southern and central parts of Europe [19] [20] [21] [22]. In Slovakia, this hybrid may be found in the northern part of the country growing on the peat-bogs and on calcareous rocks [23] [24]. Different aspects of the hybrid populations have been studied so far, the main effort being focused on the evidence supporting hybrid nature of these populations [22] [25] [26] [27]. The authors [28] reported in this context also of a reciprocal gene flow between *P. sylvestris* and *P. uliginosa*, the latter being one of the three members of the *P. mugo* complex. The authors revealed 1% - 2% of hybrid individuals in the sympatric population of the species at the Węgliniec reserve in Poland, shedding some light on the interaction between hybridizing taxa. According to [29], we are still poorly equipped to tackle such fundamental problems of the hybrid swarms as the estimation of the proportions of hybridization events that may lead to speciation. Nevertheless, the quality of genotypic resolution afforded by the molecular markers has made it possible to view the hybrid zones as natural experiments and to study mating patterns, dispersal and genetic architecture of these zones [30]. In the present paper, we have been subjected to analyze the hybrid swarm population of *P. mugo* and *P. sylvestris* in north Slovakia aiming to the description of the complicated hybridological relationships between hybrid individuals of the swarm and their putative parental species *P. sylvestris* and *P. mugo*.

2. Materials and Methods

The putative hybrid swarm population of Scots pine (*Pinus sylvestris* L.) and mountain dwarf pine (*P. mugo* Turra) is located in northern Slovakia, near the village Zuberec, at the altitude of 815 m above sea level (a.s.l.). The past history

of the swarm is not clear. We suppose that hybrid swarm has evolved as a result of advanced hybridization between the parental species which has occurred during the recent period. It is registered under the name “Medzi Bormi” with the legislation status of natural reserve. The hybrid swarm grows on a peat-bog of the total area 6.5 hectares. Prevailing majority of the trees are of intermediate habitus between Scots pine and mountain dwarf pine but small groups and/or isolated individuals of typically parental trees also occur. The two individuals of the intermediate phenotype (hybrid 1-H1, hybrid 2-H2) exhibiting opposite chloroplast DNA (cpDNA) haplotypes (*P. sylvestris* vs *P. mugo*) served as mother trees in the artificial hybridization with *P. sylvestris* and *P. mugo* (Figure 1).

2.1. Pollen Collection and Handling

Both fresh and stored pollen was used in artificial pollination experiment. The pollen of the putative hybrid individuals serving as mother trees was harvested 5 days before pollen shedding. The pollen was collected into the paper bags, separately from each mother tree, transported to the laboratory on the same day and layered over the sheets on a sunny side of the laboratory. After two days' drying, the pollen was sieved and left additional 4 hours on a sheet to dry more completely. The dried pollen was placed in the test tubes with cottonwool stoppers which were kept in a desiccator with silica gel at 4°C until pollination. In the same way as described above, was also collected and processed the pollen of the paternal trees *P. mugo* and *P. sylvestris* except that dried pollen of these species was subjected to the long-term storage at -20°C and relative humidity of 27% - 30%. One tree of each *P. mugo* and *P. sylvestris* were involved in crossing experiment. The pollen of *P. mugo* was harvested in 2016 from a tree of the pure species population in Vratna valley of north Slovakia at the altitude of 1381 m a.s.l.



Figure 1. General view of mother trees with isolated female strobili on tested locality.

The same applies also to the *P. sylvestris* pollen which was harvested in 2017 from the pure species population in Smolenice, south Slovakia, at the altitude 320 a.s.l. In order to evaluate the actual quality of pollen grains, an *in vitro* germination test of both freshly collected and stored pollen has been done a few days before pollination. The cultivation medium consisted of 1.5% agar and 10% sucrose [31]. Each sample was tested in a triplicate. Following a 48 hour germination at 25°C in the dark, the proportion of germinating pollen grains was scored microscopically in a population of 300 pollen grains per tree. The length of pollen tubes was measured in 90 pollen grains of each tree.

2.2. Artificial Pollination

Artificial pollination experiment was done in 2019. The isolation of female strobili in mother trees was made 5 days before reaching maximal receptivity of female strobili when no indications on pollen release were observed in neither mother trees or in the neighbour trees. Female strobili were pollinated with a soft painting brush, each macrostrobilus being pollinated separately. During pollination, an isolating bag was removed for a moment from the shoot with receptive macrostrobili and put back immediately after their pollination. The isolation paper bags were definitely removed from the shoots upon closure of the ovuliferous bracts of pollinated female strobili and their development into young conelets (two weeks after pollination). At this stage of the experiment, the number of pollinated female strobili of individual crossing variants were recorded in each mother tree. During the pollination experiment, the preference was given to the crossing of putative hybrid individuals with *P. sylvestris* and *P. mugo*. Controlled outcrossing between the maternal trees H1 and H2 as well as their open pollination served as a control. Mature cones were collected at the beginning of November 2020 and dried at laboratory temperature. Hand-extracted and dewinged seeds were subjected to quality analysis according to both the attempted crossing variants of a given mother tree and in individual cones of a given variant. The filled and empty seeds served as the main criterion in quality assessment as revealed by the laboratory germination test.

The obtained experimental data on pollen and seed quality were statistically treated by the analysis of variance using ANOVA and Duncan grouping.

2.3. DNA Extraction and Processing

Total DNA was extracted from young leaves of the trees involved in experiment using the CTAB protocol [32]. Total DNA of embryos from both controlled and open pollination was extracted according to the modified CTAB protocol [33]. The extraction was preceded by *in vitro* germination of seeds in Petri dishes with wet cottonwool paper and subsequent excision of the embryos protruding from megagametophytes. Excised embryos were separately homogenized in a microtube using pestle and TRIS-HCl-CTAB extraction buffer, pH 8.0. The study on cpDNA transfer between the parental trees during controlled pollination and gene flow between the trees during open pollination was based on the

differences in the *trnV-trnH* region of cpDNA in *P. sylvestris* and *P. mugo*. The respective region was PCR amplified using the primer pair which consisted of 5'-GCTCAGCAAGGTAGAGCACC-3' and 5'-CTTGGTCCACTTGGCTACGT-3' [34]. The obtained PCR products were digested with the restriction enzyme *Hinf* I which was found to discriminate the cpDNA of *P. sylvestris* from that of *P. mugo* [33] [35]. The generated fragments were fractionated electrophoretically in 8% polyacrylamide gel and 1× TBE buffer. Electrophoresis was run at 2.5 V·cm⁻¹ for 3 h. The gels were stained in 1× TBE with EtBr (0.5 mg·l⁻¹). The hybrid nature of the progenies from controlled crossing and the modes of cpDNA transfer from the parents to their progenies were verified separately for each crossing variant. A set of samples involving the *trnV-trnH/Hinf* I digests of the corresponding parental trees and those of their hybrid embryos was run simultaneously by a polyacrylamide gel electrophoresis.

3. Results

3.1. Crossability Relationships

Stored pollen exhibited lower viability in one viability trait only in comparison with freshly collected pollen. In particular, it is true of the 2-years stored pollen of *P. sylvestris* with significantly reduced pollen tube length and with the 3-years stored pollen of *P. mugo* with markedly reduced pollen grain germination (Table 1).

The use of such pollen in artificial pollination has exerted a little effect on the outcome of the crossing experiment as evidenced by the proportion of filled seeds in the offspring of H1 × *P. sylvestris* (78.03%) and those in the control cross H1 × H2 in which the fresh pollen was applied (65.5%). It follows from Table 2 that similar situation has also been characteristic of the cross H2 × *P. mugo* with a comparable amount of filled seeds (31.15%) as in the offspring of the control H2 × H1 where fresh pollen was used (34.02%). The offspring from open pollination of mother trees H1 and H2 deviated conspicuously from the crosses mentioned above containing the lowest proportions of filled seeds.

Within context of the attempted crossing variants, the relationship between the cpDNA haplotype status of the parental trees and their crossability is of special interest. In spite of the opposite haplotypes of the mother trees H1 (*P.*

Table 1. Pollen viability of parental trees used in the experiment.

Species/hybrids	Germination [%]		Pollen tube length [µm]	
	Mean ± s.d.	Duncan grouping	Mean ± s.d.	Duncan grouping
<i>P. sylvestris</i>	85.6 ± 3.7	B	87.2 ± 33.8	B
<i>P. mugo</i>	70.6 ± 3.7	BC	114.2 ± 47.7	A
Hybrid 1—H1	82.3 ± 0.5	B	115.2 ± 39.5	A
Hybrid 2—H2	91.6 ± 1.5	A	109.5 ± 45.8	A

Note: Different letters of Duncan test refer to statistical significance of the differences between trees.

Table 2. Summary results of attempted crossing variants.

Crossing variants	Pollinated female strobili	Collected mature cones	Conelets dropping [%]	Total number of seeds	Filled seeds	
					Mean	[%]
H1 × H2	44	31	29.5	458	300	65.5
H1 × <i>P. sylvestris</i>	34	24	29.4	355	277	78.0
H1 × <i>P. mugo</i>	72	18	75.0	90	0	0
H1—open pollin.	-	24	-	463	174	37.5
H2 × H1	17	6	64.7	97	33	34.0
H2 × <i>P. sylvestris</i>	29	1	100	0	0	0
H2 × <i>P. mugo</i>	16	4	75.0	61	19	32.1
H2—open pollin..	-	15	-	257	37	14.4

sylvestris haplotype) and H2 (*P. mugo* haplotype), these trees intercross readily. The same is true of the crosses H1 × *P. sylvestris* and H2 × *P. mugo* with the parental trees sharing the same haplotypes, e.g. *P. sylvestris* and *P. mugo*, respectively. On the contrary, the crosses between the parental trees with opposite cpDNA haplotypes, i.e. those of H1 × *P. mugo* and H2 × *P. sylvestris* failed completely. The former yielded 90 seeds, all of which were empty, the latter had not produced seeds at all. Both these crossings were also remarkable by an enlarged extent of pollinated macrostrobili which desiccated during the period following pollination and dropped at the end of first growing season. In the cross H1 × *P. mugo* the number of pollinated female strobili was reduced of 75.0%, whereas in the H2 × *P. sylvestris* nearly 100% of conelets dropped during the scored period what is much higher figure than that in the rest of crossings attempted within the respective mother trees (Table 2). Statistical analysis confirmed significance of the differences between the four crossing variants attempted within H1 mother tree which are based on a sample of 1366 seeds ($P < 0.0001$). The same is true of the differences between the three crossing variants of the H2 mother tree with a total number of 415 sampled seeds ($P < 0.0001$).

At the level of individual cones, the conspicuous differences were registered in the content of their filled seeds. In the H1 × H2 control and in the H1 × *P. sylvestris* cross, it has ranged between 1 - 28 seeds per cone (2.5% - 10.0%) and in the cones obtained from open pollination of the H1 mother tree between 0 - 18 seeds (0% - 66%). In mother tree H2, this figure was reduced considerably, as evidenced by the 1 - 10 filled seeds per cone in the control cross H2 × H1 (5% - 47%), followed by the proportion of 0 - 7 filled seeds in the cross H2 × *P. mugo* (0% - 50%) and 0 - 6 filled seeds in the cones from open pollination (0% - 33%).

3.2. Variation in cpDNA Inheritance

Crossing variants attempted so far, along with the progenies from open pollination of mother trees were subjected to cpDNA analysis aiming at revealing the mode of cpDNA transmission from the parents to their progenies. The analysis

was based on the species-specific cpDNA *trnV-trnH/HinfI* restriction profiles of *P. mugo* and *P. sylvestris*. The former is distinguished by its 276 bp DNA fragment, the latter by the 282 bp DNA restriction fragment (**Figure 2**).

The two putatively hybrid individuals which served in the crossing experiment as mother trees were characterized by the opposite restriction profiles. The mother tree H1 exhibited *P. sylvestris* profile (S haplotype), the mother tree H2 was characterized by the *P. mugo* profile (M haplotype). Reciprocal crosses of these trees were found to inherit cpDNA in different ways. In the cross H2 × H1, the paternal inheritance of cpDNA was observed. All the 13 hybrid embryos extracted from 4 cones were of S haplotype (**Figure 3**).

On the contrary, in the cross H1 × H2 the biparental cpDNA transmission was revealed. Twenty hybrid embryos excised from 4 cones were of S haplotype constitution and 19 embryos of the same cross which originated from 19 cones exhibited M haplotype (**Figure 4**). Partial support for this mode of cpDNA inheritance was also provided by the progenies from open pollination of the H2 mother tree where 10 embryos of M haplotype and 7 embryos of S haplotype

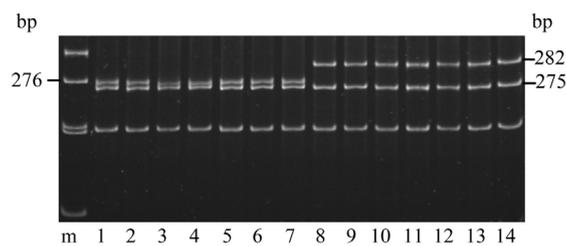


Figure 2. cpDNA restriction profiles in *P. mugo* (lanes 1 - 7) and *P. sylvestris* individuals (lanes 8 - 14); m-size marker.

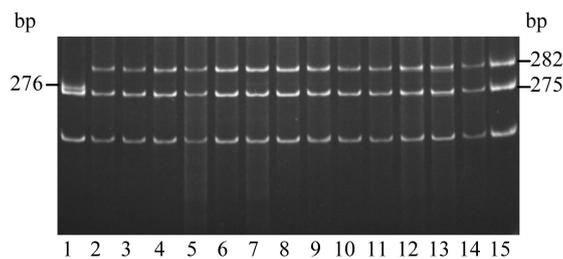


Figure 3. cpDNA restriction profiles of H2 × H1 hybrid embryos (lane 1-H2 mother tree, lanes 2 - 14-hybrid embryos, lane 15-H1 paternal tree).

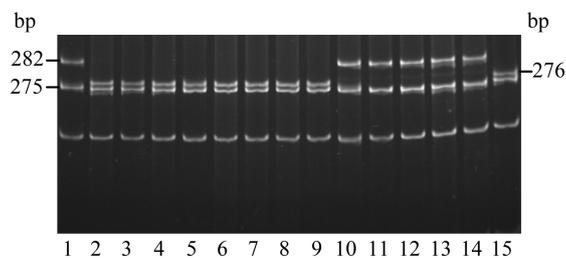


Figure 4. cpDNA restriction profiles of H1 × H2 hybrid seeds (lane 1-H1 mother tree, lanes 2 - 14-hybrid embryos, lane 15-H2 paternal tree).

were found. The four cones of the offspring contained the embryos of both M and S haplotypes with varying proportions in a given cone. The five cones contained the embryos of either M or S haplotype only. The offspring from open pollination of the H1 mother tree represented by 3 mature cones and 18 embryos shared uniformly S haplotype.

4. Discussion

Mutual hybridization of *P. sylvestris* and *P. mugo* in the overlapping parts of their distribution is considered to be one of the most significant evolutionary process leading to formation of the new taxa within *P. mugo* complex [36]. Hybrid swarms generated in this way include both the pure parental species and hybrids of varying degrees [6]. As the peat-bogs are untypical environments for *P. sylvestris* and *P. mugo*, it seems that parental species and hybrids undergo their strong natural selection. Controlled crosses may shed more light on reproductive isolation and strength of the genetic barriers between the parental species and hybrids [17]. Also, they could help answer the question if hybrids are less viable than parental species and therefore less competitive in stronger selective pressure of such environment [37]. The use of artificial hybridization approach in assessing the degree of genetic differentiation of the putative hybrid individuals of different cpDNA haplotypes from the species *P. sylvestris* and *P. mugo* resulted in the finding of a close relationship between the haplotype constitution of mother trees and haplotype of the paternal species. Within mother tree H1 of *P. sylvestris* haplotype, the highest crossing efficiency was characteristic for the crosses with the paternal species *P. sylvestris* of the same haplotype. Conversely, the crossing with *P. mugo* of *P. mugo* haplotype was unsuccessful. Likewise, in mother tree H2 of *P. mugo* haplotype, a relatively high crossability was registered in the crossing variant with *P. mugo* as compared with a total failure of the crossing with *P. sylvestris*. This pattern of crossability reflects the hybridological affinity between the pure species individuals of *P. sylvestris* and *P. mugo* as observed in the nature [28] [38] and confirmed experimentally by the artificial pollination experiments [39]. The only difference is that the yields of filled seeds of the crosses between the pure species individuals of *P. sylvestris* and *P. mugo* are lowered considerably in comparison with the amount of filled seeds in the crosses attempted within H1 and H2 mother trees [33] [35]. This may be taken as an indirect evidence supporting hybrid nature of the mother trees. An extensive dropping of the conelets in the crosses of the parental trees of different haplotypes ($H1 \times P. mugo$, $H2 \times P. sylvestris$) as compared with the control is another feature which reflects early development of pollinated macrostrobili in the reciprocal crosses of *P. sylvestris* and *P. mugo*. In the hybrids of the five-needle species *P. sibirica* and *P. pumila* of the subgenus *Strobus* (soft pines), the maternal trees of the hybrids were reported to cross better with their maternal parents used in the experiment as a pollen donor. The authors [17] have ascribed this superiority in crossability to the maternal effect what in terms of the cpDNA haplotype probably means the identity of the crossed individuals

in their cpDNA haplotypes. Being the members of the subgenus *Pinus* (hard pines), the species *P. sylvestris* and *P. mugo* differ from *P. sibirica* and *P. pumila* in the nature of their reproductive barriers. As shown by [40], the reproductive isolation between hard pine species relies on gametophytic incompatibility, whereas between soft pine species on abortive embryogenesis which is supposed to be less effective compared to the prezygotic reproductive barriers. This is probably the main reason why in the crosses between hybrid individuals *P. sibirica* × *P. pumila* and/or reciprocal with their parents the conelets dropping ranged between 40% - 50% only but in the crosses H1 × *P. mugo* and H2 × *P. sylvestris*, it reached the level of 78% and 100%, respectively. Accordingly, the amount of filled seeds in the crossing experiments with *P. sibirica* and *P. pumila* averaged at 8.2% in the cross of hybrid individuals with the paternal species *P. sibirica* and at 24.3% of filled seeds in the cross of the maternal trees of hybrids with *P. pumila* indicating extensive abortion of developing seeds. In our experiment with *P. sylvestris* and *P. mugo*, the corresponding values were found to be a 78.03% of filled seeds in the H1 × *P. sylvestris* cross and 31.15% of filled seeds in the cross H2 × *P. mugo*. This is much higher figure than in the crosses between the pure species individuals crosses *P. sylvestris* × *P. mugo* and *P. mugo* × *P. sylvestris* where the yields of sound seeds averaged at 10.8% and 15.0%, respectively [33]. It is obvious that genetic differentiation between the putative hybrids and the parental species *P. sylvestris* and *P. mugo* is conspicuously lowered in comparison with the differentiation between the pure species *P. sylvestris* and *P. mugo*.

Crossbreeding of the putative hybrid individuals H1 and H2 resulted in the relatively high proportions of filled seeds in the reciprocal crosses H1 × H2 (65.5%) and H2 × H1 (34.07%) suggesting a high degree of homology of their genomes. Except for the incompatible crossing variant H1 × *P. mugo*, the progenies from open pollination of mother trees contained the lowest amounts of filled seeds. A direct involvement of the ecological factors in pollen formation and pollen dispersal are assumed to be responsible for this phenomenon. The putative hybrid individuals H1 and H2 deviate from the species *P. sylvestris* and *P. mugo* not only in their reproductive behaviour but also in the mode of cpDNA inheritance. Within context of the experimentally proved paternal inheritance of cpDNA in the cross *P. sylvestris* × *P. mugo* [41], and exceptional, maternal inheritance of cpDNA in the reciprocal crosses *P. mugo* × *P. sylvestris* [33] [35] [42], the finding of a biparental cpDNA transmission from the parents H1 and H2 to the hybrid embryos modifies the commonly acknowledged patterns of paternal inheritance of cpDNA in the pines [10] [43]. Especially, it is true of the interbreeding of the maternal tree H1 with the paternal tree H2 which yielded the embryos of *P. sylvestris* haplotype, shared also by the maternal tree, along with the embryos of *P. mugo* haplotype characteristic for the paternal tree. In order to explain this phenomenon reasonably, we have to admit a lowered control on the presence of only one type of the chloroplasts in a zygote and/or zygotes of the respective mother tree.

5. Conclusion

Presented data on artificial hybridization have provided a conclusive evidence of genetic differentiation of the putative hybrid swarm in Zuberec from the parental species *P. sylvestris* and *P. mugo*. Crossability of the putative hybrid individuals with the parental species is much higher than reported crossability between individuals of the pure species individuals of *P. sylvestris* and *P. mugo* indicating weakening of the reproductive barrier in the hybrids. The process of hybridization is highly selective and is conditioned by the identity of the crossed individuals in their cpDNA haplotypes. Together with a prompt interbreeding between putative hybrid individuals, it provides a basis for further expansion of the swarm. The particular genetic status of the hybrid swarms accounts for a modified mode of gene flow between concerned taxa that is not so strict as between the species *P. sylvestris* and *P. mugo* and which poses some problems when dealing with the genetic structure of the swarm based on cpDNA markers.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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