# GENETIC ANALYSIS OF THE PUTATIVE HYBRID POPULATION *PINUS SYLVESTRIS* × *PINUS MUGO* IN SLOVAKIA[1]

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## ABSTRACT

Morphometric and genetic analyses of the putative hybrid population *Pinus sylvestris* × *P. mugo* at the locality „Medzi Borami", Slovakia, were made using morphometric traits of cones and needles, artificial hybridization between the respective species and restriction analysis of chloroplast DNA (*cp*DNA). A low degree of crossability between *P. sylvestris* and *P. mugo* was confirmed experimentally supporting the idea of a spontaneous hybridization between the species. According to the cone size and number of stomatal rows on ventral side of needles the putative hybrid population „Medzi Borami" occupied intermediate position between the neighbour populations of *P. sylvestris* and *P. mugo*. Restriction analysis of *cp*DNA revealed the prevalence of *P. sylvestris* haplotypes in the putative hybrid population.

**Key words:** *Pinus sylvestris* L., *P. mugo* Turra, putative hybrid population, cones, needles, chloroplast DNA, restriction fragment length polymorphism

## INTRODUCTION

*Pinus mugo sensu lato* represents taxonomically critical group of pines which involves a complex of *P. mugo* varieties including its introgressive hybrid with *P. sylvestris* (VIEWEGH 1981). The trees of the complex occupy preferentially the upper limit of the timber line but occasionally they may be also found at the lower altitudes. In particular, it is true of the putative hybrid combination *P. mugo* × *P. sylvestris* growing on the peat bogs where it exhibits all the intermediary forms between a tree-like and bush-like habitus. The introgression between *P. mugo* and *P. sylvestris* was ascribed to the accidental contact of the species during glacial and postglacial migration and to human activity (BOBOWICZ 1990). In Europe, the Scots pine grows jointly in the neighbourhood of the taxa forming *P. mugo* complex southwards from the line of the last glacial period (HOLUBIČKOVÁ 1965, MUSIL 1973). As a result, the distinct intermediary hybrids between *P. mugo* and *P. sylvestris* were postulated to exist almost everywhere where these species overlap (KAŇÁK 1983). The hybrid swarms have accordingly been reported to occur in the Rila Planina and Rodopy Mountains in Bulgaria (DOBRINOV & JAGZIDIS 1961, DOBRINOV 1965), in the Nowy Targ Valley, Poland (STASZKIEWICZ & TYSZKIEWICZ 1969, BOBOWICZ *et al.* 2000), in the Swiss Alps (NETT-SARQUEDA *et al.* 1988) and in the Orava region, Slovakia (MUSIL 1977, VIEWEGH 1981). The evidence concerning hybridity of these swarms is based primarily on morphological traits of their needles and cones (MUSIL 1975, KRZAKOWA *et al.* 1984, BOBOWICZ 1990) and on needle anatomy (STASZKIEWICZ & TYSZKIEWICZ 1968, 1972). To a lesser degree, the antigenic properties of needles (PRUS-GŁOWACKI I *et al.* 1978, 1980, 1981), isoenzyme polymorphism and variation of polyphenol compounds (PRUS-GŁOWACKI & SZWEYKOWSKI 1983, KRZAKOWA *et al.* 1984, KRZACZEK & URBANIAK 1985, BOBOWICZ *et al.* 2000) were used in identifying the hybrids. Some controversies have however emerged between the results of morphological classification of the hybrid swarm populations and the data obtained by the molecular biology methods. Using isoenzyme and restriction fragment length polymorphism (RFLP) of chloroplast DNA (*cp*DNA) approaches FILPPULA *et al.* (1992) demonstrated that postulated hybrid complex *P. mugo* × *P. sylvestris* from former Czechoslovakia is in fact

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a mixed stand of the pure species *P. mugo* and *P. sylvestris*. Also, using isoenzyme markers ODRZYKOSKI & WACHOVIAK (2002) put under question the hybrid nature of the postulated hybrid population in a peat-bog preserve „Bór na Czerwonem" in Poland.

In order to partition the specimen of the putative hybrid complex at the locality „Medzi Borami", Slovakia, a comparative study of both parental species and the putative hybrid individuals was done using restriction analysis of *cp*DNA. The comparison has been paralleled by the crossing experiment aiming at assessment of the extent of mutual hybridization between *P. sylvestris* and *P. mugo*.

## MATERIALS AND METHODS

### Artificial hybridization experiments

The crossability relationship between *P. sylvestris* L. and *P. mugo* Turra was tested by means of artificial hybridization experiments using four mother trees of the former and two mother trees of the latter which grow in Arboretum Mlyňany. The conventional technique of controlled pollination of pines was applied in the experiments (VIDAKOVIČ & BORZAN 1973) during which only fresh pollen was used. Paper bags serving as isolators were removed from pollinated female strobili after complete closing of the ovuliferous scales. Except for the interspecific combinations *P. sylvestris* × *P. mugo* and reciprocal the variants with selfing, controlled cross-pollination and open pollination of the maternal trees were also used serving as a control (Table 1).

### Restriction analysis of chloroplast DNA

The young needles were collected from 41 individuals of the putative hybrid complex *P. sylvestris* × *P. mugo* at the locality „Medzi Borami" as well as from 19 individuals of the neighbour population *P. sylvestris* L. in Oravský Biely Potok and from 16 individuals of *P. mugo* stand at Popradské Pleso.

Total DNA was extracted from 0.5 g of fresh needles using protocol by MURRAY & THOMPSON (1980). Three regions of chloroplast DNA (*cp*DNA) genome were subjected to PCR-RFLP analysis. The *rbc*L gene region was amplified by a pair of primers designed by TSUMURA *et al.*(1996), the *psb*C gene region (psII 44 kd) by a pair of primers designed by DEMESURE *et al.* (1995) and the intergenic spacer region *trn*V-H by a pair of primers designed by PARDUCCI & SZMIDT (1999). The PCR reaction was carried out in a total volume of 25 µl. The obtained PCR products have been digested with seven restriction endonucleases. Total digestion volume was separated on 8% non-denaturating polyacrylamide gels in 1× TBE.

## RESULTS

The main conclusion drawn from the artificial hybridization experiments concerns the low degree of mutual crossability between *P. sylvestris* and *P. mugo*. It follows from the data pooled in Table 1 that both *P. sylvestris* × *P. mugo* combination and reciprocal yielded only negligible amount of filled seeds suggesting low compatibility between the parental species. The 0.2 and 0.67 yields of filled seeds per cone in *P. sylvestris* × *P. mugo* and *P. mugo* × *P. sylvestris* crossings, respectively, lagged in efficiency behind all the remaining variants tested so far including the selfed progeny of *P. sylvestris* with conspicuously reduced share of filled seeds. Low compatibility between the parental species was apparent at the conelet level already. The survival

**Table 1. Summary of results achieved during artificial hybridization of *P. sylvestris* and *P. mugo*.**

| Crossing | Number of pollinated female flowers | Number of collected mature cones | Total number of seeds obtained | Average number of seeds per cone | Average number of filled seeds per cone |
|---|---|---|---|---|---|
| *P. sylvestris* self-pollination | 380 | 124 | 2041 | 17.9 | 3.6 |
| *P. sylvestris* cross-pollination | 592 | 241 | 4042 | 17.6 | 13.4 |
| *P. sylvestris* open pollination | | 135 | 2484 | 18.7 | 14.4 |
| *P. sylvestris* × *P. mugo* | 235 | 23 | 198 | 8.6 | 0.2 |
| *P. mugo* – self-pollination | 475 | 108 | 4611 | 45.0 | 25.8 |
| *P. mugo* – cross-pollination | 171 | 42 | 2207 | 42.8 | 26.4 |
| *P. mugo* – open pollination | | 91 | 2559 | 30.7 | 18.0 |
| *P. mugo* × *P. sylvestris* | 541 | 76 | 1898 | 10.6 | 0.6 |

**Table 2. Differential occurrence of *Hinf* I, *Cla* I and *Taq* I restriction fragments in *trn* V-H intergenic spacer region of *P. sylvestris* and *P. mugo*.**

| Enzyme | Species | Fragment (bp) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 5090 | 4072 | 3000 | 3054 | 2050 | 2036 | 1635 | 1018 |
| *Hinf* I | *P. sylvestris* | | + | | + | | | + | + |
| | *P. mugo* | | | | | | | + | + |
| *Cla* I | *P. sylvestris* | | | + | | | + | | + |
| | *P. mugo* | + | | | | | + | | + |
| *Taq* I | *P. sylvestris* | + | | | | | + | + | + |
| | *P. mugo* | + | | | | + | | + | + |

rate of conelets in *P. sylvestris* × *P. mugo* combination averaged at 9.7 % only as compared with the corresponding values of 32. 6 % in *P. sylvestris* – selfing and of 40.7 % in *P. sylvestris* – cross-pollination variants. The same tendency has also been characteristic for the *P. mugo* crossing variants except that survival rate of *P. mugo* × *P. sylvestris* conelets was slightly increased reaching 14 % (Table 1). At the seed level, retarded development of conelets in both interspecific combinations resulted in a profound reduction of a total amount of seeds per cone. Of interest is in this connection also a high efficiency of selfing and outcrossing in *P. mugo* as compared with *P. sylvestris*.

The partitioning of the population „Medzi Borami" based on the *cp*DNA restriction analysis data revealed the prevalence of *P. sylvestris* haplotypes at the locality. Of the 21 fragment/enzyme combinations tested, only the combinations of *trn*V-H primer with *Hinf* I, *Cla* I and *Taq* I have produced the restriction patterns which have enabled to differentiate between the species *P. sylvestris* and *P. mugo*. In particular, it is true of the 4072 bp fragment of *cp*DNA generated by *Hinf* I that has been detected in *P. sylvestris* but which is lacking in *P. mugo*. Differential occurrence of the 3000 bp and 5090 bp fragments has on the other hand been characteristic for the restriction profiles generated by *Cla* I restriction nuclease, the former being present in *P. sylvestris* and the latter in *P. mugo* digests. The last difference detected in the intergenic spacer region *trn*V-H is that revealed by *Taq* I endonuclease. The restriction profile of *P. sylvestris* possessed in this case the 2036 bp fragment, whereas *P. mugo* 2050 bp fragment on the basis of which it was possible to discriminate between them (Table 2). The above mentioned differences concerned all the individuals of a given species. Consequently, the absence of intraspecific variation in restriction patterns of the respective species has enabled to analyze the genetic structure of the putative hybrid population. The results of this analysis summarized in Table 3 indicate the predominance of *P. sylvestris* haplotypes in the putative hybrid population „Medzi Borami".

## DISCUSSION

**Table 3. Number of individuals in the putative hybrid complex *P. sylvestris* × *P. mugo* sharing *P. sylvestris* and *P. mugo* haplotypes.**

| Enzyme | *P. sylvestris* haplotype | *P. mugo* haplotype | Total number of individuals |
|---|---|---|---|
| *Hinf* I | 26 | 12 | 38 |
| *Cla* I | 28 | 13 | 41 |
| *Taq* I | 23 | 10 | 33 |

The hybrid nature of the putative hybrid populations *P. mugo* × *P. sylvestris* at the locality „Medzi Borami" in Slovakia and in the neighbourhood Valley of Bialy Potok in the High Tatra Mts., Poland, has been quoted recently on the morphological and anatomical grounds (STASZKIEWICZ 1994, 1996). Also, the natural hybridization between *P. mugo* and *P. sylvestris* was admitted to occur at the lower altitudes of *P. mugo* range (SCHÜTT 1959, DOBRINOV & JAGZIDIS 1961, MIROV 1967). Our attempt to hybridize these species artificially resulted in a negligible yield of sound seeds in *P. sylvestris* × *P. mugo* crossing and in a slightly increased amount of filled seeds in the reciprocal crossing. This may be taken as an indication of the low crossability between the parental species. Based on 22 isozyme loci and RFLP analysis of *cp*DNA,

ODRZYKOSKI & WACHOWIAK (2002) were not able to confirm the hybrid swarm hypothesis concerning peat-bog preserve „Bór na Czerwonem" in Poland. Contrary to the results of biometric studies, the authors suggest that fraction of hybrids within this population is low. It is worth to mention in this connection that PRUS-GŁOWACKI & STEPHAN (1998) have reported about immunochemical and isoenzymatic characteristics of the hybrids from controlled crossings between *P. montana* var. *rostrata* and *P. sylvestris*. Unfortunately, no data concerning the degree of crossability have been provided. However, it seems that despite of their taxonomic relatedness, the species *P. mugo* and *P. sylvestris* intercross with difficulty. This supports the conclusion made by MIROV (1967) about irregularity of *Pinus* species in respect to their hybridization. It is reasonable to believe that even negligible hybridological affinity between these species may contribute to the hybrid swarms formation in zones of their sympatry.

Although the needle and cone traits were reported to reflect hybridity of these swarms in a varying degree, in general, they are considered to be suitable markers of the kind. According to BOBOWICZ *et al.* (2001) of the six morphological needle traits analyzed in the $F_1$ hybrids from controlled crossing *P. montana* var. *rostrata* and *P. sylvestris* , the stomatal rows were the main trait responsible for distinction between all the three specimen compared. The same hybrids subjected to the isozyme and immunological analyses were found to contain some „novel" proteins which were not present in the parents (PRUS-GŁOWACKI & STEPHAN 1998). Enriched antigenic patterns have also been characteristic for the needles of individuals from the hybrid swarm population in Klodzka Valley, Poland, differing from the putative parental species by the number of antigens (PRUS-GŁOWACKI *et al.* 1981). Together with asymetric inheritance of the isoenzymes by the hybrid swarm individuals of *P. mugo* × *P. sylvestris* at locality „Bór na Czerwonem" these data were reported to provide additional evidence supporting the introgressive character of the trees from the populations mentioned above (BOBOWICZ *et al.* 2000). No such evidence has however been obtained in case of the hybrid swarm population analyzed in present study, *e.g.* in population from the locality „Medzi Borami". Analysing the same population by means of three enzyme systems and 8 loci, LANÁKOVÁ (1992) was not able to differentiate between the parental species and the putative hybrid individuals. Also, FILPPULA *et al.* (1992) found little differentiation between the 4 suspected hybrid populations from former Czechoslovakia and/or from Germany on the one hand and the parental species *P. sylvestris* and *P. mugo* on the other hand. With special reference to the hybrid swarm population from locality „Medzi Borami", the authors clustered it together with *P. mugo* individuals, whereas the remaining 3 hybrid populations with *P. sylvestris*. At the level of *cp*DNA, the authors succeeded in differentiation between *P. sylvestris* and *P. mugo* which differed with regard to their 8.8 kb and 7.1 kb fragments generated by *Bcl* I. However, the *cp*DNA of the 3 suspected hybrid populations from Bohemia and Germany was identical with the *cp*DNA from the pure *P. sylvestris* population. As far as the hybrid population „Medzi Borami" is concerned, only preliminary conclusion can be made regarding its genetic nature. Our data refer to the restriction profiles of *cp*DNA isolated from needles of individual trees which allow to partition the hybrid swarm population according to the *P. sylvestris* and *P. mugo* haplotypes only. The prevalence of the former was found in the population „Medzi Borami" as compared with the prevalence of *P. mugo* genotypes in the hybrid swarm population „Bór na Czerwonem" in Poland based on isozyme gene markers (ODRZYKOSKI 2002). In order to confirm or deny the hybrid nature of the putative hybrid population „Medzi Borami", the involvement of seed material into restriction analysis is a necessary prerequisite. The *cp*DNA markers described above together with those proposed recently by WACHOWIAK *et al.* (2000) allow efficient discrimination between *P. sylvestris* and *P. mugo* and hence a detailed analysis of the genetic structure of their hybrid populations.

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