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**Short communication**

# Reciprocal controlled crosses between *Pinus sylvestris* and *P. mugo* verified by a species-specific *cp*DNA marker

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**Abstract**. A species-specific marker of *cp*DNA (paternally inherited in pines) was used to verify the hybrid origin of seedlings from controlled reciprocal crosses between *Pinus sylvestris* and *P. mugo*. A very low degree of compatibility between those two species has been revealed. In the three consecutive years of experiments, no filled seeds were obtained in the combination with *P. mugo* as the seed parent. From *P. sylvestris* as the seed parent and *P. mugo* as the pollen donor, we succeeded to obtain four filled seeds (about 1 %), but only in one year. The seedling obtained from the seeds had *cp*DNA haplotypes specific to *P. mugo*, which proves their hybrid origin. This method enables verification of the result of controlled crosses. The importance of the results has been discussed in the aspect of postulated natural hybridisation in sympatric populations of the two species.

**Key words**: controlled crosses, *cp*DNA marker, hybridisation, *Pinus mugo*, *Pinus sylvestris.*

The Scots pine (*Pinus sylvestris*) is a widespread tree species and the main forest-forming component in Europe and Asia. *P. sylvestris* is closely related to the dwarf mountain pine (*P. mugo*), which is endemic to European mountainous regions and also occurs on some peatbogs from the postglacial period (Critchfield and Little 1966). Individuals with intermediate or mixed phenotypic characters with regard to *P. sylvestris* and *P. mugo* were found in sympatric populations of the species (Prus-Głowacki and Szweykowski 1980; Yurukov and Tashew 1992). Studies on their hybridisation were conducted through artificial crossing. These experiments were also carried out to determine phylogenetic relationships among pine species and for breeding purposes (Kormutak and Lanakova 1988; Prus-Głowacki and Stephan 1998). The production of filled seeds has mostly been used as a criterion of successful crosses. However, due to the lack of species-specific markers the hybrid origin of the seeds from these experiments and the individuals from sympatric populations has not been proved so far. Nowadays, the application of DNA markers of the plastid genome, which is paternally inherited in pines (Wagner 1992), enables verification of the results of controlled crosses. The comparative analyses of species-specific *cp*DNA haplotypes of paternal tree and F1 progeny make it possible to confirm hybridization.

In the presented study a species-specific *cp*DNA marker was applied to verify the hybrid origin of the progeny from controlled crosses between *P. sylvestris* and *P. mugo*. The study aimed to check the crossability between the species in the aspect of their postulated natural hybridisation. Two individuals of *P. sylvestris*

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originating from the seed orchard in Zwierzyniec near Kórnik in Poland and two of *P. mugo* from the Arboretum of the Institute of Dendrology, Polish Academy of Science, were used in the reciprocal controlled crosses. One individual from each species was used as the pollen donor. Controlled crosses were carried out according to the procedure by Moulalis et al. (1976) in three consecutive years, from 1998 to 2000. In May, two weeks before the expected flowering, about 50 female strobili of each species were isolated in polyethylene bags when flower buds were large enough and the peduncle was visible. Two days before the expected pollination, male strobili were collected. Pollination was carried out during the fifth stage of the female strobili development, namely when cone scales were open and stood almost at a right angle to the axes of the strobili. The pollination was repeated 2–3 days later, depending on the prevailing weather conditions. The pollen was applied into the bags by a medicine dropper, with a rubber blower equipped with a valve. As a result, in the 3-year experiment on *P. mugo* as maternal individuals and *P. sylvestris* as the pollen donor, only 3 mature cones were obtained, all with undeveloped seeds. Conelet abortion took place mainly the following May, after pollination. In the crosses with *P. sylvestris* as maternal individuals, about 70% of conelets developed into mature cones but most of them contained empty or undeveloped seeds. Only in 2001, we succeeded to obtain 4 filled seeds. Two of them originated from one *P. sylvestris* tree, along with 254 empty seeds, whereas the other two, with 182 empty seeds, originated from the other individual.

The method developed by Dumolin et al. (1995) was used to extract DNA from fresh needle material (50–100 mg) of the parental trees and from whole 3-week-old seedlings grown from the seeds obtained in the controlled crosses. We applied the previously described species-specific DNA marker of the *trn*L-*trn*F region (Wachowiak et al. 2000). This PCR-RFLP marker represents a *Dra*I restriction site polymorphism in the above region, which leads to one band of PCR products for *P. sylvestris* and two bands for *P. mugo*. The digested PCR products are separated in a 2% (w/v) agarose gel and visualized under UV after staining with ethidium bromide. Figure 1 present the *cp*DNA haplotypes of the parental trees and their progeny. The four individuals obtained from the crosses with *P. sylvestris* as the seed tree and *P. mugo* as the pollen donor displayed a *cp*DNA marker specific to *P. mugo*, which unambiguously indicates that they are hybrids. This result also proves the usefulness of *cp*DNA markers for identification of hybrids.

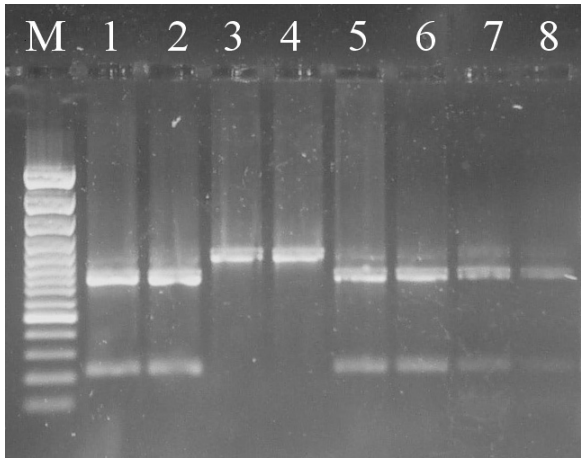


Figure 1. PCR products of *trn*L-*trn*F *cp*DNA region digestion with *Dra*I enzyme in *P. mugo* (lanes 1–2); *P. sylvestris* (lanes 3–4); and *P. sylvestris* (♀) × *P. mugo* (♂) (lanes 5–8). M = 100-bp marker (Ladder Plus, Fermentas)

The very low degree of crossability between these two species (about 1%) observed in this study is consistent with previous reports. Moulalis et al. (1976) obtained 31 full and germinating seeds by pollination of 309 female strobili from 7 *P. sylvestris* trees with *P. mugo* pollen. The results presented above, together with the results of other experiments (Kormutak 1990), prove that crosses between *P. sylvestris* as the seed parent and *P. mugo* as the pollen donor are possible in spite of the small efficiency of that process.

Controlled crosses should be repeated for several years in order to formulate general conclusions concerning crossability of the studied species and to exclude the influence of the used provenances, biotic factors, and environmental conditions during and after pollination (Moulalis et al. 1976). Therefore, the absence of filled seeds in the reciprocal crosses conducted in the presented study does not exclude the possibility of hybridisation with *P. mugo* as the seed parent. Successful crosses in this combination were obtained in previous studies (Kormutak and Lanakova 1988). However, due to the lack of diagnostic markers, it was not possible then to confirm species identity of parental trees as well as the hybrid origin of seeds and to exclude uncontrolled pollination in the course of the experiments.

The absence of diagnostic characters enabling identification of the hybrids has not allowed formulating so far any coherent concept of the postulated processes of *P. sylvestris* and *P. mugo* natural hybridisation. Therefore, the estimated frequency of hybridisation based on biometric and biochemical analyses ranges from rare formation of hybrids (Christensen and Dar 1997; Odrzykoski 2002) to formation of hybrid swarms (Staszkiewicz 1993). The presented results suggest a limited gene flow and existence of hybridisation barriers between these species. The application of described *cp*DNA markers in the analyses of parental individuals and their progeny from sympatric populations of the species will permit to determine if hybridisation barriers revealed in artificial experiments occur also in natural conditions.

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