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Spontaneous Hybridization between *Pinus sylvestris* L. and *P. mugo* Turra in Slovakia

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Abstract

Molecular evidence for spontaneous hybridization between *Pinus sylvestris* L. and *P. mugo* Turra in the putative hybrid swarm populations of the species in Slovakia was provided based on PCR-RFLP analysis of the cpDNA *trn*V-*trn*H region. Species-specific restriction profiles generated by *Hinf* I digests of the cpDNA products reliably identified *P. sylvestris* and *P. mugo* haplotypes of the embryos from open pollination. Simultaneous analysis of the respective cpDNA region in megagametophytes and embryos of individual seeds along with needles of a given maternal tree has enabled to score either the *P. sylvestris* or *P. mugo* haplotypes in the embryos illustrating hybridization patterns between the two species. Data obtained in this way indicate a

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relatively extensive hybridization which takes place between *P. sylvestris* and *P. mugo*. The extent of hybridization varied among populations as evidenced by the 41.1–58.7% proportion of hybrid embryos registered on the locality Habovka, and by the 8.3% and 2.7% proportions of hybrid embryos on the localities Tisovnica and Sucha Hora, respectively. The approach itself is recommended as a convenient method for monitoring the hybridization patterns in sympatric zones of the studied pine species.

Key words: Pinus sylvestris L., *P. mugo* Turra, hybridization, chloroplast DNA, restriction analysis.

Introduction

Hybrid zones of sympatric or allopatric species may represent intermediate stages in the divergence of populations up to the level of full species (HEWITT, 1988). They have considerable experimental potential for studies of the characters and processes involved in divergence and speciation. Several examples of such zones have been reported in the genus *Pinus*.

In North America, a spontaneous hybrid of jack pine (*P. banksiana* Lamb.) and lodgepole pine (*P. contorta* Dougl.) has originated from introgressive hybridization in contact zones of their natural areas in several places of Canada (MOSS, 1949; CRITCHFIELD and LITTLE, 1966; MIROV, 1967; SCOTTER, 1974; POLLACK and DANCIK, 1985; WAGNER et al., 1987, 1989). Introgression between *P. taeda* L. and *P. echinata* Mill. has been reported to take place near Mtn. Ida of the Montgomery County, Arkansas (EDWARDS-BURKE et al., 1997). Recently, EPPERSON et al. (2001) reported the introgression between *Pinus ponderosa* var. *scopulorum* and *P. arizonica* in southern Arizona and New Mexico. In Japan, the hybrid swarms were reported in the ecotonal zones of *P. pumila* (Pallas) and *P. parviflora* Siebold et Zucc. var. *pentaphylla* (Mayr) in central Honshu and on the island Hokkaido (SENJO et al., 1999; WATANO et al., 2004). Based on isozyme data, *P. densata* is supposed to had evolved as a new species via hybridization between *P. tabulaeformis* and *P. yunnanensis* in central China (WANG et al., 1990). The Mediterranean species *P. brutia* Ten. and *P. halepensis* Mill. were found to hybridize extensively in two small districts of the Rhodes island in Greece (PAPAJOANNON, 1936; BOSCHERINI et al., 1994; PANETSOS et al., 1997).

However, the oldest known example of introgression between pine species on European continent refers to the hybrid swarm populations of *P. mugo* and *P. sylvestris*. According to BUSINSKY (1998) the problem of hybrid zones in pines of Central Europe concerns exclusively *P. sylvestris* and *P. mugo*. The existence of spontaneous hybrids between these species has been postulated since the second half of the nineteenth, but discussions regarding their genetic status still persist (CHRIST, 1864; BRÜGGER, 1886; BERTSCH, 1906). Using allozyme polymorphism at 11 loci NEET-SARQUEDA (1994) has not been able to prove the introgressive hybrid zone hypothesis describing the intermediate form populations of these species in Switzerland as the mixed stands of *P. sylvestris* and *P. mugo*. Conversely, the artificial pollination data by WACHOWIAK et al. (2005) confirmed the compatible hybridological relationship between *P. sylvestris* and *P. mugo* illustrating the possibilities for their hybridization in overlapping zones. However, the recent studies of the authors based on cpDNA markers indicate a strong barrier against interspecific hybridization when *P. sylvestris* is used as a pollen donor (WACHOWIAK et al., 2006a; 2006b). This finding leaves the question of nature and relative proportions of these hybrids in nature opened.

The most recognized places where hybrid swarm populations of *P. mugo* x *P. sylvestris* have been reported are Rila Planina and Rodopy in Bulgaria (DOBRINOV, 1965; DOBRINOV and JAHZIDIS, 1971), the Dolina Nowotarska valley in Poland (STASZKIEWICZ and TYSZKIEWICZ, 1969; BOBOWICZ et al., 2000), Swiss Alps (NEET-SARQUEDA et al., 1988) and the Orava region in Slovakia (MUSIL, 1977; VIEWEGH, 1981). Some of these populations were characterized in detail using needle morphology and anatomy (STASZKIEWICZ, 1996; BOBOWICZ et al., 2000) as well as needle proteins and isoenzymes (PRUS-GLOWACKI and SZWEYKOWSKI, 1980; PRUS-GLOWACKI et al., 1981). Based on needle traits and phenology of reproductive organs, BORATYŃSKA et al. (2003) and BORATYŃSKI et al. (2003) recently reported of gene flow from *P. sylvestris* to *P. uliginosa* and to some extent reciprocally.

Paternal inheritance of chloroplast DNA (cpDNA) in conifers described originally by WAGNER et al. (1987) and confirmed subsequently by NEALE and SEDEROFF (1989) and MOGENSEN (1996) offers a unique opportunity for analysis of the process of hybridization of pines in molecular terms. The finding of species-specific cpDNA markers for *P. mugo* and *P. sylvestris* was a major step in making this opportunity realistic (WACHOWIAK et al., 2000). We have used this approach in analysing the spontaneous hybridization of these species in all the three putative hybrid swarm populations *P. mugo* x *P. sylvestris* occurring in Slovakia. Based on needle cross-sections, VIEWEGH (1981) has introduced the name *Pinus* x *celakovskyorum* ASCHERSON et GRAEBNER for the individuals of the hybrid swarm population in Habovka. Statistical treatment of the data on needle size and needle anatomy characteristics in individual trees by three different methods of cluster analysis indicates introgressive hybridization which takes place between the postulated hybrid individuals and the pure species individuals of *P. sylvestris* and *P. mugo* (VIEWEGH and ČAMBALOVA, 1993). In order to check quantitatively the extent of suspected hybridization, the individual trees of the putative hybrid swarm populations in Slovakia were subjected to the cpDNA analysis using cpDNA of their leaves and seeds from open pollination.

Materials and Methods

Three putative hybrid swarm populations of *P. mugo* x *P. sylvestris* growing on peatbogs in Habovka, Tisovnica and Sucha Hora of the Orava region, northern Slovakia, were subjected to molecular analysis. The neighbouring populations of the pure species *P. mugo* Turra in Roháče of the western part of the High Tatras Mts. and *P. sylvestris* in Hruštin served as a control. All cone-bearing trees were sampled. Because of temporal varia-

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tion in cone production, different trees were sampled in different years in Habovka. At the tree level, each individual was processed separately using needle cpDNA. At the seed level, each seed of a given tree was processed separately using its megagametophyte and embryo cpDNAs. The number of seeds scored for individual trees ranged between 6 and 11 except for the hybrid swarm in Habovka where only 1 to 3 seeds were analyzed in 2004, mainly because of the poor yield of cones and low quality of seeds. The number of trees representing individual populations together with the number of seeds scored are given in *Table 1*.

Table 1. – Populations and sample sizes used in the experiment.

Population	Species	Altitude	Year	Number of trees	Number of seeds
Habovka	putative hybrids	815 m	2004	35	70
Habovka	putative hybrids	815 m	2005	13	112
Tisovnica	putative hybrids	815 m	2005	20	252
Sucha Hora	putative hybrids	765 m	2005	34	325
Roháče	*P. mugo*	1450 m	2005	15	120
Hruštin	*P.sylvestris*	700 m	2005	15	135

Total DNA was extracted from young needles and seed tissues using the CTAB method of MURRAY and THOMPSON (1980). The *trn*V-*trn*H region of cpDNA was PCR amplified using the primer pair which consisted of 5'-GCTCAGCAAGGTAGAGCACC-3' and 5'-CTTGGTCCACTTGGCTACGT-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 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 1x TBE. The DNA fragments were visualized by UV fluorescence. The obtained PCR products were digested with the restriction enzyme *Hinf* I, which has been found to discriminate the cpDNA of *P. sylvestris* from that of *P. mugo* (WACHOWIAK et al., 2000; KORMUTAK et al., 2002). The generated fragments were fractioned electrophoretically in 8% polyacrylamide gels and 1x TBE buffer. Electrophoresis was run at 2.5 V.cm^{-1} for 3 hours. The gels were stained in 1x TBE with EtBr (0.5 mg.l^{-1}).

In the population Habovka, the proportions of progenies from conspecific and interspecific crosses (as assessed by the comparison of maternal and pollen haplotypes) were tested against the random-mating expectations using the χ^2-test. The heterogeneity of the proportions of hybrid progenies among maternal trees was tested using the χ^2-test. Because of small sample sizes, the distribution of the χ^2-statistics was derived from 10000 randomizations.

Results

The species-specific nature of *trn*V-*trn*H/*Hinf* I restriction profiles of *P. sylvestris* and *P. mugo* cpDNAs is based on differential occurrence of the 320 bp and 300 bp fragments, the former being typical for *P. sylvestris*, the latter for *P. mugo*. The remaining fragments of 750, 650, 280 and 200 bp size are uniformely shared by both species (*Fig. 1*, lanes 1–2).

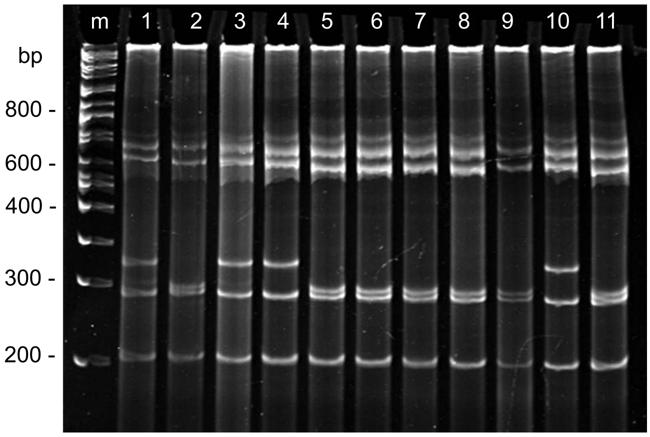

Figure 1. – *trn*V-*trn*H/*Hinf* I restriction profiles illustrating gene flow between *P. sylvestris* and *P. mugo*; m – size marker, lane 1 – *P. sylvestris* needles, lane 2 – *P. mugo* needles, lanes 3 – 11 – embryos originating from the same tree and exhibiting either *P. mugo* (lanes 5 – 9, 11) or *P. sylvestris* haplotype (lanes 3, 4, 10).

No variation between individual trees of putative pure species *P. mugo* and *P. sylvestris* was observed. Identical were also the restriction profiles of needles and megagametophytes of a given tree. Under these conditions, a simultaneous analysis of the respective cpDNA region using needles of a given tree along with the megagametophytes and embryos of individual seeds has enabled to score either *P. mugo* or *P. sylvestris* cpDNA haplotypes in the embryos (*Fig. 1*). This resulted in partitioning of individual seeds of a given tree according to the cpDNA haplotype of their embryos. The occurrence of embryos of *P. mugo* haplotype on a tree of *P. sylvestris* haplotype or vice versa was taken as an unumbiguous evidence of hybridization event between *P. sylvestris* and *P. mugo*.

The data obtained in this way are summarized in *Table 2*. It follows from the table that on the locality Habovka, the proportions of trees with the *P. sylvestris* and *P. mugo* haplotypes were 50.0% each. It is this population where the most extensive hybridization takes place as evidenced by 41.1% of hybrid embryos *P. mugo* x *P. sylvestris* recorded in 2004 along with 55.6% of hybrid embryos of reciprocal combination. Comparable situation was found also in 2005 except that introgression of *P. sylvestris* into *P. mugo* prevailed this year over the hybridization of *Pinus sylvestris* x *P. mugo*. Such proportions of hybrid progenies do not deviate significantly from the proportions expected under random mating between species (χ^2 = 1.03, P > 0.05; 2.47, P > 0.05 and 3.09, P > 0.05 with 3 df for 2004, 2005 and pooled samples from both years, respectively). Maternal trees with both maternal haplotypes differed significantly in the proportions of hybrid progenies in 2004 and in 2005 (*Table 3*). The observed proportions of hybrids ranged from 0 to 100% in both maternal species. The hybrid swarm populations in Tisovnica and Sucha Hora differed in this respect considerably. We have not

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Table 2. – Gene flow between *P. mugo* and *P. sylvestris* as revealed in their hybrid swarm populations and in populations of pure parental species.

Locality	Year	Trees		Total number of embryos scored	Embryo haplotype		Percentage of hybrid embryos
		Needle/ megagamet. haplotype	Number		*P. mugo*	*P. sylvestris*	
Habovka	2004	*P. mugo*	18	34	20	14	41.1
		P. sylvestris	17	36	20	16	55.6
Habovka	2005	*P. mugo*	6	46	19	27	58.7
		P. sylvestris	7	66	34	32	51.5
Tisovnica	2005	*P. mugo*	24	252	231	21	8.3
		P. sylvestris	0				
Sucha Hora	2005	*P. mugo*	34	325	316	9	2.7
		P. sylvestris	0				
Roháče	2005	*P. mugo*	15	120	120	0	0
Hruštin	2005	*P. sylvestris*	15	135	0	135	0

Note: Percentage of hybrid embryos expressed as the number of embryos of different haplotype per total number of embryos of all mother trees of a given haplotype.

detected *P. sylvestris* cpDNA haplotype in any of the sampled trees on these localities. As a consequence, only *P. mugo* x *P. sylvestris* hybrids were registered here reaching the average extent of 8.3% in Tisovnica and 2.7% in Sucha Hora. Nevertheless, like in Habovka, individual maternal trees significantly differed in the proportions of hybrid progenies (*Table 3*).

As expected, the individuals of pure species *P. mugo* from Roháče exhibited exclusively *P. mugo* cpDNA haplotype and produced only seeds of intraspecific crosses. The same is true of the pure species population *P. sylvestris* from Hruštin, where individuals were of *P. sylvestris* cpDNA haplotype at both the tree and seed levels (*Table 2*).

Table 3. – χ^2-test of heterogeneity of proportions of hybri progenies among trees in individual populations.

Population	Year	Maternal haplotype	χ^2	*P*
Habovka	2004	*P. mugo*	25.19	0.9825
		P. sylvestris	23.85	0.9804
	2005	*P. mugo*	21.03	0.9921
		P. sylvestris	28.26	0.9951
Tisovnica	2005	*P. mugo*	91.79	0.9999
Sucha Hora	2005	*P. mugo*	68.40	0.9960

Discussion

Scots pine and mountain dwarf pine are taxonomically related species of the subgenus *Diploxylon* (Hard Pines) occupying common position within the group *Lariciones* according to SHAW (1914) and/or within the section *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 artificial hybridization experiments, DUFFIELD (1952) included both these species in Group X, indicating some hybridological affinity between them. It was maintained since the beginning of the last century that the spontaneous hybridization between *P. sylvestris* and *P. mugo* is rather rare (PETERSEN, 1903). In connection to this, CHRISTENSEN and DAR (1997) have mentioned five types of obstacles preventing mutual hybridization between these species in nature. The differences in anthesis, in altitudinal and ecological ranges along with reduced number and viability of hybrid seeds and failure of the hybrids to become established are supposed to be responsible for a partial reproductive isolation of *P. sylvestris* and *P. mugo*. Nevertheless, rare as they appear, the existence of spontaneous hybrids of these species is generally acknowledged. The experimental evidence is primarily based on morphometric analysis of the hybrid swarm populations using phenotypic appearance of the trees (DOBRINOV and JAHZIDIS, 1971), bark, cone and needle characteristics, including needle cross-sections (MARCET, 1967; CHRISTENSEN and DAR, 1997; STASZKIEWICZ, 1996; BOBOWICZ et al., 2000; BORATYŃSKA et al., 2003) and phenology of reproductive organs (BORATYŃSKI et al., 2003). Biochemically such populations were characterized in terms of needle proteins and isoenzymes (PRUS-GLOWACKI and STEPHAN, 1998; PRUS-GLOWACKI and SZWEYKOWSKI, 1980; PRUS-GLOWACKI et al., 1981). Based on these data, the distance dendrograms and hybridity indexes have been derived supporting hybrid nature of *P. sylvestris* and *P. mugo* swarm populations. Paternal inheritance of cpDNA in conifers offers a more straightforward approach in screening introgressive processes at seed level. The difference in *trn*L-*trn*F intergenic spacer region of *P. elliottii* var. elliottii and *P. caribaea* var. *hondurensis* cpDNAs allowed to distinguish the hybrid progeny of these species from intraspecific and self progenies of the maternal species (SHEPHERD and HENRY, 2002). The same cpDNA marker was used by CHEN et al. (2002) in verifying putative hybrids *P. echinata* x *P. taeda*. Using *Bcl* I digest of the entire cpDNA, FILPPULA et al. (1992) were able to discriminate between *P. sylvestris* and *P. mugo* but not to prove the hybrid nature of suspected hybrid population *P. mugo* x *P. sylvestris* of the French Alps origin.

The species-specific marker for these species described by WACHOWIAK et al. (2000) refers to the variation in non-coding region between the *trn*F and *trn*L genes of cpDNA. The *Dra* I and *Hinf* I digests of the respective

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PCR product generate restriction patterns which reliably discriminate between *P. sylvestris* and *P. mugo*. The paternal inheritance of this marker has subsequently been proved by the authors on the example of reciprocal crosses between the parental species (WACHOWIAK et al., 2005). In presented study we used the *trn*V-*trn*H region of cpDNA that was found to differ in *P. sylvestris* and *P. mugo* after digestion with *Hinf* I and which did not exhibit intraspecific variation (KORMUTAK et al., 2002). In combination with the *trn*F-*trn*L/*Dra* I and *Hinf* I restriction patterns it broadens the chances for evaluation of events connected with gene flow between these species. Using this marker it was possible to provide direct molecular evidence for spontaneous hybridization between *P. sylvestris* and *P. mugo* at the seed level only. The opinions of some authors differ concerning the frequency of hybridization between these species. WACHOWIAK et al. (2005) have for example reported of incompatible nature of *P. mugo* x *P. sylvestris* combination. According to the authors, the reciprocal cross has also exhibited low compatibility between the parental species as evidenced by the yield of 36 empty seeds and 4 filled seeds obtained within the two mother trees of *P. sylvestris*. The outlined hybridological status of *P. mugo* x *P. sylvestris* crossing has repeatedly been confirmed by WACHOWIAK et al. (2006b) using 12 different clones of the paternal species. However, the involvement into artificial pollination of two additional clones of *P. sylvestris* (Hasl E 203, Schl 77/1) resulted in 35.6% and 56.8% yields of 1-year-old seedlings. It is worth of noting that our attempt with artificial hybridization of *P. sylvestris* and *P. mugo* resulted in relatively high yields of filled seeds ranging between 70.0% and 96.3% in *P. mugo* x *P. sylvestris* and between 13.5% and 83.4% in the reciprocal combination (KORMUTAK et al., 2005). This indicates fairly high hybridological affinity between the parental species. Presented restriction analysis data confirmed a relatively extensive gene flow between them also in zones of their sympatry. The detected frequency of *P. mugo* x *P. sylvestris* embryos (51.2%) and reciprocal ones (52.9%) in Habovka favours the isozyme based conclusion by PRUS-GLOWACKI and SZWEYKOWSKI (1983) about frequent occurrence of these hybrids in nature. We may only speculate whether reduced viability of hybrid seeds and/or low survival rate of hybrid individuals were the reasons of low frequency hybridization between *P. sylvetris* and *P. mugo* observed by CHRISTENSEN (1987) on the morphological basis. This figure differs considerably on individual localities studied, most probably due to the variable proportions of *P. sylvestris* and *P. mugo* individuals and their spatial distribution on these localities. Under conditions of the overlapping flowering of both parental species, the prevalence of *P. sylvestris* individuals has ensured a higher probability of pollination of *P. uliginosa* megastrobili by the pollen of prevailing species (BORATYŃSKI et al., 2003). We suppose that reduced occurrence of *P. sylvestris* trees accounts for the low frequency of hybridization of the species with *P. mugo* at the localities Tisovnica and Sucha Hora. The detrimental effect of climatic conditions cannot be excluded as well, as evidenced by the annual variation in frequency of introgression between the parental species at the locality Habovka in 2004 and 2005.

There was a significant variation in the proportion of hybrid progenies among individual trees in all hybrid swarm populations. One possible cause is the flowering phenology. Regarding the fact that *P. sylvestris* flowers earlier than *P. mugo* (BORATYŃSKI et al., 2003), only a small portion of trees of both maternal species is synchronized phenologically. The other reason might be genetic. Because of introgression, some trees with the *P. sylvestris* haplotype may contain a considerable portion of *P. mugo* genes and *vice versa*, so that they may be more prone to hybridization.

Irrespective of the variable extent of hybridization, the process was proved convincibly to take place between *P. sylvestris* and *P. mugo* in all the three putative hybrid populations in Slovakia. The method itself was shown to share a high diagnostic potential for scoring spontaneous hybridization between these species at seed level. However, it does not allow identification of the hybrid trees unless a species-specific marker for *P. sylvestris* and *P. mugo* mtDNAs is available.

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Primer Note: A New Set of Highly Polymorphic Nuclear Microsatellite Markers for *Nothofagus nervosa* and Related South American Species

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Abstract

Nothofagus is the main component of southern South American temperate forests. Overexploitation in the past has led to the loss of 40% of the original distribution range. Genetic diversity as well as biological processes shaping the distribution of the genetic variation (e.g. gene flow) constitutes basic knowledge for the implementation of conservation measures and for the definition of Evolutionary Significant Units. Nuclear microsatellites are the marker of choice for gene flow and fine-scale genetic structure studies. We enlarged a previous set of microsatellites (SSRs) for South American *Nothofagus* species, with special concern to *Nothofagus nervosa* (Phil.) Dim. et Mil. Five new SSRs are presented with allele numbers up to 12 in a single population. The primers transferred well to five related species (*N. obliqua* (Mirb.) Oerst, *N. glauca* (Phil.) Krasser, *N. dombeyi* (Mirb.) Oerst , *N. pumilio* (Poepp et Endl.) Krasser and *N. antarctica* (G. Forster) Oerst, with allele numbers up to 11. The high level of polymorphism promises a sufficient power for gene flow and parentage analyses.

Key words: Nothofagus, microsatellites, South America, gene flow, fine-scale genetic structure.

Introduction

Nothofagus is the dominant genus of southern South American temperate forests, with a total of ten endemic species. In the last century, over-exploitation, over

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