PAN

POLSKA AKADEMIA NAUK

PAN
POLSKA AKADEMIA NAUK
ODDZIAŁ W KRAKOWIE

Hybridization Processes in Putative Hybrid Swarms of Scots Pine and Mountain Dwarf Pine as Revealed by Chloroplast DNA

Andrej Kormutak[*], Martina Brana, Peter Manka, Martin Galgoci,
Jana Libantova, Vladimir Camek, Peter Bolecek, Dusan Gömöry

*Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences,
Akademická 2, P.O.Box 39A, SK-950 07 Nitra, Slovak Republic*

Gene flow among individual trees of *Pinus sylvestris* and *P. mugo* putative hybrid swarms in Slovakia was followed at four localities using the species-diagnostic cpDNA *trnV-trnH/Hinf* I restriction profile. Variable proportions of *P. sylvestris* and *P. mugo* haplotypes were revealed among the sampled localities. Low between-habitus consistency of the trees and their cpDNA haplotypes indicates the hybrid nature of the swarms. Molecular analysis based on mutual comparison of the haplotypes of a given tree and its embryos suggests direct and reciprocal hybridization between trees of the *P. sylvestris* and *P. mugo* haplotypes. Besides conspecific embryos resulting from hybridization of trees with the same haplotype (*P. sylvestris* × *P. sylvestris* and *P. mugo* × *P. mugo*), hybrid embryos of *P. sylvestris* × *P. mugo* (8.03%) and *P. mugo* × *P. sylvestris* (11.50%) were also detected in open-pollinated offspring. The results are discussed from the standpoint of primary and introgressive hybridization between the parental species.

Key words: cpDNA, embryos, gene exchange, hybrid swarms, *Pinus sylvestris*, *P. mugo*.

INTRODUCTION

Scots pine (*Pinus sylvestris* L.) and mountain dwarf pine (*P. mugo* Turra) are taxonomically distinct species representing subsection *Pinus* of the hard pines (Price et al., 1998). In Slovakia they are distributed allopatrically at 250–1650 m a.s.l. (Scots pine) and 680–2054 m a.s.l. (mountain dwarf pine) (Futák et al., 1966). In some places in Slovakia they have an extrazonal distribution at 765–1172 m a.s.l. Owing to a weak reproductive barrier, the species intercross spontaneously in contact zones, forming hybrid swarms. These swarms were reported to occur on peat bogs and on calcareous rock in northern Slovakia and in the adjoining Nowy Targ valley in Poland (Businsky, 1999; Staszkiewicz, 1996; Staszkiewicz and Tyszkiewicz, 1969). Based on needle anatomy, Viewegh (1981) postulated the hybrid nature of the swarm at the Zuberec peat bog locality in the western part of the High Tatras. Several intermediate forms of needle anatomy have accordingly been posited between *P. mugo* subsp. *mughus* and *P. sylvestris* along with pure-species individuals of *P. sylvestris*. Both primary hybridization between

P. mugo and *P. sylvestris* resulting in early-generation hybrids and introgressive hybridization between the hybrids and parental species are predicted to occur at this locality. It is not excluded that introgression resulting from multiple backcrosses may occur here. In fact there are other places in Europe with suspected *P. sylvestris* and *P. mugo* hybrid swarms. Their hybrid nature has not yet been confirmed convincingly by molecular methods.

Based on 11 allozyme loci, Neet-Sarqueda (1994) found no evidence for introgressive zones in three populations in Switzerland consisting of forms intermediate between *P. uncinata* and *P. sylvestris*. Using isoenzymes and chloroplast DNA (cpDNA), Filppula et al. (1992) were not able to prove the hybrid origin of three putative hybrid swarms in the Czech Republic and one putative hybrid swarm in Slovakia. No evidence for the hybrid swarm hypothesis was found at isozyme and cpDNA levels in sympatric populations of *P. mugo* and *P. sylvestris* in the northern foothills of the Polish Tatras (Wachowiak et al., 2006). On the other hand, analysis of morphologically variable forms in sympatric populations of *P. sylvestris*, *P. mugo* and *P. uligi-*

---

[*]e-mail: nrgrkorm@savba.sk

TABLE 1. Populations studied and sample sizes

Population	Species	Altitude	Number of analyzed trees	Number of analyzed seeds
Hruštín	*P. sylvestris*	700 m	10	97
Vrátna valley	*P. mugo*	1230 m	10	99
Zuberec	hybrid swarm	815 m	19	233
Obšívanka	hybrid swarm	1000 – 1172 m	16	192
Suchá Hora	hybrid swarm	765 m	21	259
Tisovnica	hybrid swarm	815 m	14	307

nosa at a peat bog complex in the Sudety Mts. indicates numerous hybrids, with gene flow from the *P. mugo* complex to *P. sylvestris* (Wachowiak and Prus-Glowacki, 2008). The availability of species-specific chloroplast haplotypes offers a straightforward means of estimating paternal gene flow within hybrid swarms (Wachowiak et al., 2000). Here we estimated the proportions of conspecific and heterospecific fertilization in selected trees of the four hybrid swarms in Slovakia. The frequency of hybrid seeds was recorded from individual trees using a species-diagnostic cpDNA marker. We also assessed the hybrid nature of the swarms based on comparisons of the phenotype and cpDNA haplotype of scored trees.

MATERIALS AND METHODS

SPECIES AND POPULATIONS

Three putative hybrid swarms of Scots pine (*Pinus sylvestris* L.) and mountain dwarf pine (*P. mugo* Turra) growing at peat bog localities in Zuberec, Sucha Hora and Tisovnica, northern Slovakia, were subjected to molecular analyses. Also analyzed was a putative hybrid swarm of the species growing on calcareous rock at the Obšívanka locality in the Mala Fatra region. The control groups were adjoining pure-species populations of *P. sylvestris* at Hruštín and *P. mugo* in the Vratná valley. Table 1 lists the tested populations, their elevation, the number of scored trees and the total number of analyzed seeds. The locations of the populations are shown on a map of Slovakia in Figure 1.

MATERIALS AND DNA ANALYSES

Only cone-bearing trees were used in the experiment. During cone collection in autumn 2011 the habitus of each putative hybrid swarm tree scored
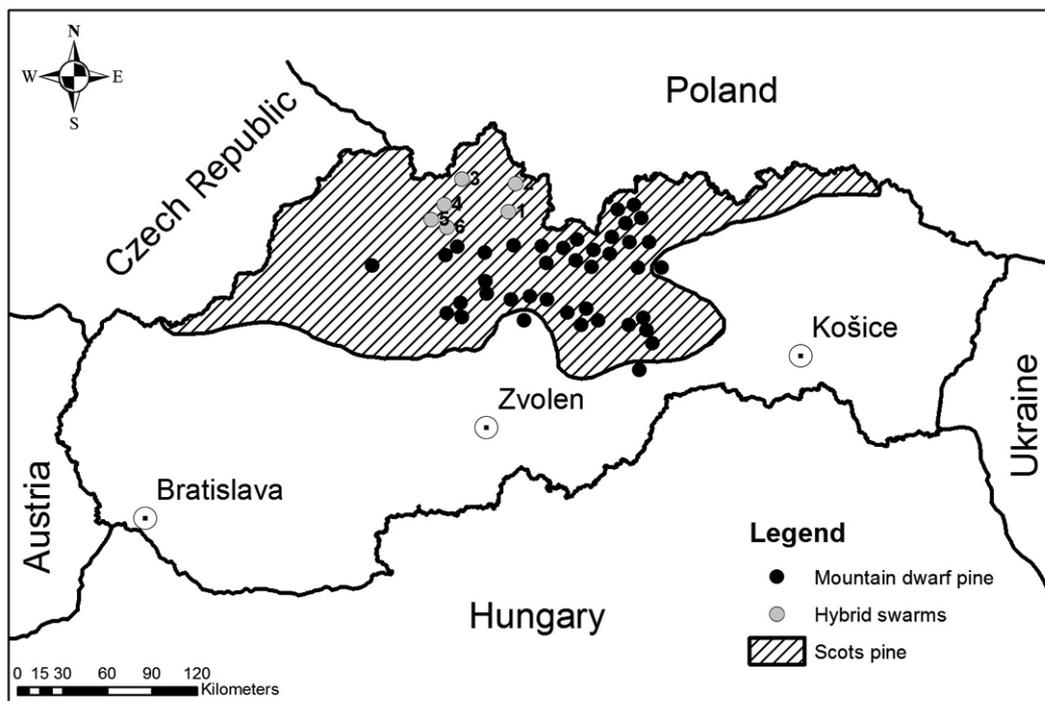
Fig. 1. Natural distribution of Scots pine and mountain dwarf pine in Slovakia and locations of their putative hybrid swarms. 1 – Zuberec; 2 – Suchá Hora; 3 – Tisovnica, 4 – Obšívanka.

TABLE 2. Number and frequency of conspecific and hybrid embryos in mother trees of different haplotypes and localities

Population	Tree haplotype	Number of trees	Number of seeds analyzed	Number of conspecific embryos	Number of hybrid embryos
Hruštín	*P. sylvestris*	10	97	97 (100%)	0
	P. mugo	0	0	0	0
Vrátna valley	*P. sylvestris*	0	0	0	0
	P. mugo	10	99	99 (100%)	0
Zuberec	*P. sylvestris*	6	77	64 (83,1%)	13 (16,9%)
	P. mugo	13	156	120 (76,5%)	36 (23,5%)
Obšívanka	*P. sylvestris*	12	132	109 (82,5%)	23 (17,5%)
	P. mugo	4	60	57 (95,0%)	3 (5,0%)
Suchá Hora	*P. sylvestris*	9	120	118 (98,3%)	2 (1,7%)
	P. mugo	12	139	126 (90,6%)	13 (9,4%)
Tisovnica	*P. sylvestris*	14	307	286 (93,1%)	21 (6,9%)
	P. mugo	0	0	0	0

was recorded. Phenotype was assigned to individual trees based on the combination of their monocormic or polycormic stem characteristics and their total height (*P. mugo*, *P. sylvestris*, intermediate).

Total DNA was extracted from needles of all mother trees (Tab. 2) by the CTAB method (Murray and Thompson, 1980). In vitro germination of mature seeds in Petri dishes with wet cottonwool paper preceded DNA extraction from embryos. Embryos protruding from the seed coat were excised and separately used for DNA extraction with a SiMax[TM] Genomic DNA extraction kit (SBS Genetech, Peking). Due to the reduced fecundity of the trees in the swarms, only 9–12 embryos per tree were analyzed, and the tested seeds originated from different cones of a given tree.

The *trn*V-*trn*H region of cpDNA was PCR-amplified with a primer pair as described by Parducci and Szmidt (1999). The obtained PCR products were digested with restriction enzyme Hinf I and the generated fragments were fractionated electrophoretically in 7.5% polyacrylamide gels and 1× TBE.

Differences in the frequencies of hybrid seeds between *P. sylvestris* and *P. mugo* at each locality were tested using the χ^2 test.

RESULTS

Amplification of cpDNA *trn*V-*trn*H gave a PCR product of 1860 bp in length in both *P. sylvestris* and *P. mugo* (Fig. 2). Digestion of the product with *Hinf* I generated species-specific restriction profiles which differed in the parental species by 680 bp and 700 bp fragments. Except for the 670 bp fragment commonly shared by the species, *P. sylvestris* also pos-

sessed a 700 bp fragment and *P. mugo* a 680 bp fragment. No variation of the chloroplast haplotype was found between the control *P. sylvestris* trees at
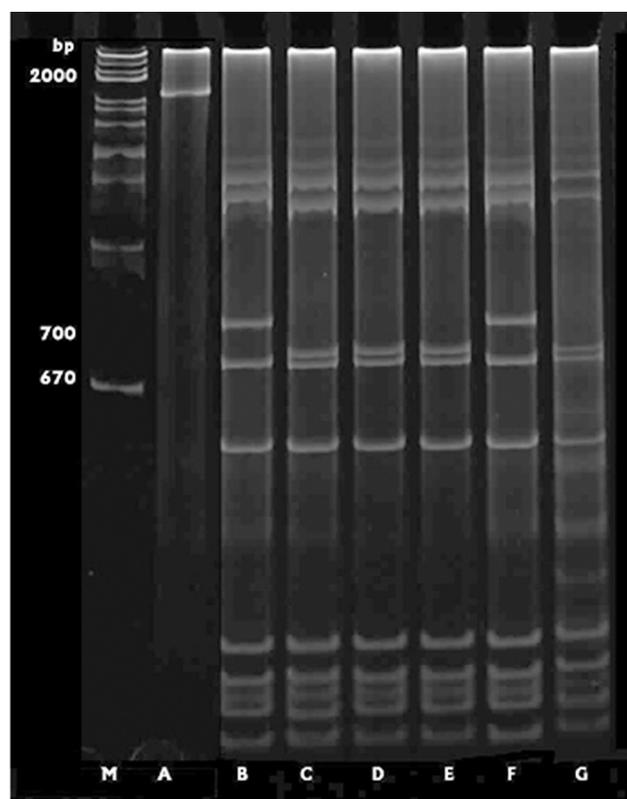

Fig. 2. Putative hybrid swarm embryo heterogeneity. cpDNA restriction profiles of mother tree with *P. sylvestris* haplotype (lane B) and its five embryos showing *P. mugo* haplotype (lanes C,D,E,G) and *P. sylvestris* haplotype (lane F). M – size marker; A – PCR product.

the Hruštín locality, nor between the *P. mugo* trees at the control locality in the Vratná valley.

Taking advantage of paternal inheritance and species-specific chloroplast haplotypes, we examined gene exchange between the two species, indicating intra- or interspecific gene flow (Wagner et al., 1987; Wachowiak et al., 2005). The chloroplast DNA haplotype revealed in the needle cpDNAs of each *P. sylvestris* or *P. mugo* tree was compared with the haplotypes of individual embryos of the same tree using cpDNA *trn*V-*trn*H/*Hinf* I restriction profiles. Finding the same haplotype in a given tree and its embryo suggests intraspecific gene flow, whereas a finding of different haplotypes indicates an interspecific hybridization event (Tab. 2).

The obtained data show that the tested trees of the putative hybrid swarms are either *P. sylvestris* or *P. mugo* haplotypes, differing in frequency of occurrence between localities. The only exception was the hybrid swarm in Tisovnica, uniformly exhibiting the *P. sylvestris* haplotype in all 14 trees scored there. This result is independent of their *P. sylvestris* or *P. mugo* habitus. According to morphological classification, 9 of the 16 trees tested at the Zuberec locality are *P. mugo*, 2 are *P. sylvestris* and 8 are of intermediate morphology. Of the 9 resembling *P. mugo* at that locality, 4 trees shared the *P. mugo* haplotype and 5 the *P. sylvestris* haplotype. The two trees with a *P. sylvestris* habitus shared the *P. mugo* haplotype. Seven of the 8 trees with intermediate habitus were of the *P. mugo* haplotype and one was of the *P. sylvestris* haplotype.

The situation was similar at the Obšívanka locality. The group of 16 tested trees contained 11 trees of the *P. mugo* habitus, 4 trees of the *P. sylvestris* habitus and one tree of intermediate morphology. Surprisingly, the great majority of the trees resembling *P. mugo*, 10 of the 11, showed the *P. sylvestris* haplotype. The 4 trees of the *P. sylvestris* habitus shared the *P. mugo* haplotype. The one tree of intermediate morphology shared the *P. sylvestris* haplotype.

The group of trees at the Suchá Hora locality consisted of 13 trees with the *P. mugo* habitus and 8 trees with the *P. sylvestris* habitus. Nine trees of the *P. mugo* phenotype shared the *P. sylvestris* haplotype and the other 4 the *P. mugo* haplotype. Only one of the 8 trees of the *P. sylvestris* habitus exhibited that haplotype; 7 were of the *P. mugo* haplotype.

The Tisovnica locality yielded an even greater discrepancy between morphology and cpDNA haplotype. Five trees had *P. mugo* morphology, 2 trees had *P. sylvestris* morphology, and 7 were intermediate. Irrespective of their morphology, all the trees shared the *P. sylvestris* haplotype.

The lack of correspondence between phenotype and cpDNA haplotype in these trees, together with the haplotype diversity of the putative hybrid swarms, may be taken as indirect evidence of their hybrid nature.

At seed level, the pure-species *P. sylvestris* and *P. mugo* populations contained only conspecific embryos originating from intraspecific *P. sylvestris* × *P. sylvestris* and *P. mugo* × *P. mugo* pollination, respectively. The prevalence of conspecific embryos also characterized the hybrid swarms, which in addition contained embryos from interspecific *P. sylvestris* × *P. mugo* and reciprocal pollination. Their frequency varied considerably depending on the haplotype of the maternal trees and the locality. Mother trees of the *P. sylvestris* haplotype contained between 1.67% and 17.42% hybrid *P. sylvestris* × *P. mugo* seeds, as compared with 5.00–23.08% hybrid *P. mugo* × *P. sylvestris* seeds in mother trees with the *P. mugo* haplotype (Tab. 2).

It is evident that mother trees of *P. sylvestris* haplotype readily hybridize with *P. mugo* at the Obšívanka locality. The *P. mugo*-haplotype mother trees at the Suchá Hora locality hybridize readily with *P. sylvestris*. At the Zuberec locality, on the other hand, mother trees of the *P. sylvestris* and *P. mugo* haplotypes are similar in their tendency to hybridize with trees of the other haplotype (χ^2=1.225; P=0.2683).

Of the 735 seeds with the *P. sylvestris* haplotype harvested from 41 trees of hybrid swarms, 676 seeds contained conspecific embryos (91.97%) and 59 seeds had hybrid *P. sylvestris* × *P. mugo* embryos (8.03%). Of the 452 seeds with the *P. mugo* haplotype originating from 29 trees of hybrid swarms, 400 seeds had conspecific embryos (88.50%) and 52 had hybrid *P. mugo* × *P. sylvestris* embryos (11.50%). These figures do not represent a significant difference in the frequency of occurrence of hybrid embryos in maternal trees of the *P. mugo* haplotype in comparison with the frequency of hybrid embryos in maternal trees of the *P. sylvestris* haplotype, indicating relatively wide gene exchange between individuals of the putative hybrid swarms (χ^2=3.592; P=0.0580).

DISCUSSION

The hybrid swarm populations in this study share numerous individuals of intermediate morphology, with growth habitus combining the multi-stemmed character of *P. mugo* and the height growth of *P. sylvestris*, along with the trees resembling pure *P. sylvestris* and pure *P. mugo*. Complete correspondence between phenotype and cpDNA haplotype was confirmed only in pure-species individuals. The hybrid swarms varied in this respect, exhibiting the two haplotypes. The haplotype heterogeneity of the putative hybrid swarms and the haplotype uniformity of the pure-species populations are the most

conspicuous features differentiating these groups of populations. This is preliminary but important corroboration of Viewegh's (1981) postulate, based on needle anatomy data, of the hybrid origin of the swarm in Zuberec, and it has application to the other three putative hybrid swarms we studied.

The presence of trees with intermediate habitus differing in cpDNA haplotype at the localities with hybrid swarms suggests the possibility of both primary and introgressive hybridization. As representatives of the subgenus *Diploxylon* (hard pines), *P. sylvestris* and *P. mugo* are supposed to be reproductively isolated by the gametophytic incompatibility which operates within this subgenus (Kriebel, 1975). Our earlier study showed that incompatibility between these species is not complete. Besides retardation of *P. mugo* pollen tube growth in the nucellar tissue of the *P. sylvestris* ovule, abortive embryogeny was found to act as an additional barrier lessening the crossability of the *P. sylvestris* × *P. mugo* interspecific cross (Kormuták, 1984). Low as it is, this crossability may generate F_1 hybrids in a contact zone, initiating subsequent crossings between the interspecific hybrids themselves, and backcrossings with the parental species. As a result, hybrid swarm populations may evolve at the respective localities, involving individuals of both parental species and hybrids of several kinds.

The relatively high rate of direct and reciprocal hybridization events between trees of the *P. sylvestris* and *P. mugo* haplotypes may be ascribed to their hybrid nature. Under natural conditions and in controlled crosses, viable offspring was obtained between the two species only when *P. mugo* was the pollen donor (Wachowiak et al., 2005, 2006; Wachowiak and Prus-Glowacki, 2008). In this connection we should mention that our experiments with controlled interspecific crossings of *P. mugo* × *P. sylvestris* conducted in two different years yielded 13.5% and 83.4% filled seeds of the total number of 133 and 315 seeds obtained from 3 maternal trees of *P. mugo* (Kormuták, 1988; Kormuták et al. 2005).

Apparently the hybrid individuals of the putative hybrid swarms are more susceptible to pollination with *P. sylvestris* pollen than with *P. mugo* pollen. Some trees with the *P. mugo* haplotype may contain a considerable portion of *P. sylvestris* genes, which may enhance their hybridological affinity for *P. sylvestris*. It is reasonable to suppose that the crossability of these hybrid trees with *P. mugo* would be modified as well. A satisfactory explanation of this aspect of the hybridological relationships between hybrid swarm individuals depends on confirming or disproving their hybrid nature. Morphoanatomical studies by Marcet (1967) and Christensen and Dar (2003) based on needle size and internal structure have contributed to a resolu-

tion of the question but have not settled it. Our study demonstrated that cpDNA markers are very good for scoring gene flow between the trees of the swarms but that alone they are limited in their ability to reveal the hybrid nature of these trees. Finding a species-specific mtDNA marker for the parental species *P. sylvestris* and *P. mugo* would greatly improve the protocol for diagnosing these organellar DNAs. This is a challenge for further research aimed at elucidating the genetic nature of the swarms and specifying the nature of hybridization processes in them.

AUTHORS' CONTRIBUTIONS

AK cone collection, PCR analysis; MB PCR analysis; PM, MG and PB cone collection, seed extraction; JL PCR analysis; VC cone collection; DG statistics. The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENT

REFERENCES

Businsky R. 1999. Taxonomic study on *Pinus mugo* complex and its hybrid populations. *Acta Pruhoniciana* 68: 123–143.

Christensen K, and Dar GH. 2003. A morphometric study of hybridization between *Pinus mugo* and *P. sylvestris* (Pinaceae). *Acta Horticulturae* 615: 211–221.

Filppula S, Szmidt AE, and Savolainen O. 1992. Genetic comparison between *Pinus sylvestris* and *P. mugo* using isozymes and chloroplast DNA. *Nordic Journal of Botany* 12: 381–386.

Futák J, Jasičová M, and Schidlay E. 1966. *Flora of Slovakia.* Part II, Slovak Academy of Sciences Publisher, Bratislava [In Slovak].

Kormuták A. 1984. Some cytological and biochemical aspects of interspecific incompatibility in pines (*Pinus* sp.). *Acta Dendrobiologica* 7/84. VEDA, Bratislava.

Kormuták A, and Lanáková M. 1988. Biochemistry of reproductive organs and hybridological relationships of selected pine species (*Pinus* sp.). *Acta Dendrobiologica*. VEDA, Bratislava.

Kormuták A, Ostrolucká M, Vooková B, Pretová A, and Fečková M. 2005. Artificial hybridization of *Pinus sylvestris* L. and *Pinus mugo* Turra. *Acta Biologica Cracoviensia Series Botanica* 47: 129–134.

Kriebel HB. 1975. Interspecific incompatibility and inviability problems in forest trees. *Proceedings of the 14th Meeting of the Canadian Tree Improvement Association*, Part 2, 28–30 August 1973, 67–84, Fredericton, New Brunswick.

MARCET E. 1967. Über den Nachweis spontaner Hybriden von *Pinus mugo* Turra und *Pinus sylvestris* L. aufgrund von Nadelmerkmalen. *Berichte der Schweizerischen Botanischen Gesellschaft* 77: 314–361.

MURRAY MG, and THOMPSON WF. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Research* 8: 4321–4325.

NEET-SARQUEDA C. 1994. Genetic differentiation of *Pinus sylvestris* L. and *P. mugo* aggr. populations in Switzerland. *Silvae Genetica* 43: 207–215.

PARDUCCI L, and SZMIDT A. 1999. PCR-RFLP analysis of cpDNA in the genus *Abies. Theoretical and Apllied Genetics* 98: 802–808.

PRICE RA, LISTON A, and STRAUSS SH. 1988: Phylogeny and systematics of *Pinus*. In: Richardson DM [ed.], *Ecology and Biogeography of* Pinus, 49–68. Cambridge University Press, Cambridge.

STASZKIEWICZ J. 1996. Natural hybrids of *Pinus mugo* × *P. sylvestris* (Pinaceae) in Tatra Mts. *Fragmenta Floristica et Geobotanica, Series Polonica* 3: 23–30.

STASZKIEWICZ J, and TYSZKIEWICZ M. 1969. Natural hybrids of *Pinus mugo* Turra × *Pinus sylvestris* L. in Nowy Targ valley. *Fragmenta Floristica et Geobotanica* 15: 187–212.

VIEWEGH J. 1981. Variability of *Pinus mugo* × *Pinus sylvestris* hybrid swarm on peat bogs near Zuberec in Orava. *Folia Dendrologica* 8: 41–62.

WACHOWIAK W, and PRUS-GŁOWACKI W. 2008. Hybridisation process in sympatric populations of pines *Pinus sylvestris* L., *P. mugo* Turra and *P. uliginosa* Neumann. *Plant Systematics and Evolution* 271: 29–40.

WACHOWIAK W, LEŚNIEWICZ K, ODRZYKOSKI I, AUGUSTYNIAK H, and PRUS-GŁOWACKI W. 2000. Species specific cpDNA markers useful for studies on the hybridisation between *Pinus mugo* and *P. sylvestris*. *Acta Societatis Botanicorum Poloniae* 69: 273–276.

WACHOWIAK W, LEWANDOWSKI A, and PRUS-GŁOWACKI W. 2005. Reciprocal controlled crosses between *Pinus sylvestris* and *P. mugo* verified by a species-specific cpDNA marker. *Journal of Applied Genetics* 46: 41–43.

WACHOWIAK W, STEPHAN BR, SCHULZE I, PRUS-GŁOWACKI W, and ZIEGENHAGEN B. 2006. A critical evaluation of reproductive barriers between closely related species using DNA markers – a case study in *Pinus*. *Plant Systematics and Evolution* 257: 1–8.

WACHOWIAK W, ODRZYKOSKI J, MYCZKO Ł, and PRUS-GŁOWACKI W. 2006. Lack of evidence on hybrid swarm in the sympatric population of *Pinus mugo* and *P. sylvestris*. *Flora* 201: 307–316.

WACHOWIAK W, and PRUS-GŁOWACKI W. 2008: Hybridisation processes in sympatric populations of pines *Pinus sylvestris* L., *P. mugo* Turra and *P. uliginosa* Neumann. *Plant Systematics and Evolution* 271: 29–40.

WAGNER DB, FURNIER GR, SAGHAI-MAROOF MA, WILLIAMS SM, DANCIK BP, and ALLARD RW. 1987. Chloroplast DNA polymorphism in lodgepole pines and jack pines and their hybrids. *Proceedings of the National Academy of Sciences U.S.A.* 84: 2097–2100.