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Hybridisation processes in sympatric populations of pines *Pinus sylvestris* L., *P. mugo* Turra and *P. uliginosa* Neumann

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Abstract. Natural hybridisation was postulated between the closely related pine species *Pinus sylvestris* and the *P. mugo* complex, however no clear evidence on propagation of mature hybrids in nature has been documented so far. To test the hybridisation hypothesis we applied chloroplast DNA (*cp*DNA) markers and isozymes in the analyses of 300 individuals representing the variety of morphological forms in the sympatric populations of *P. sylvestris*, *P. mugo* and *P. uliginosa* at the peat bog complex in the Sudety Mts., Poland. Additionally, the haplotypes of paternally inherited *cp*DNA of 149 open pollinated progeny derived from seeds were compared to the haplotypes of parental trees to access the intensity and direction of contemporary hybridisation. The morphologically highly variable polycormic (multi-stemmed) hybrids between *P. mugo* and *P. uliginosa* were identified. The second group of hybrids was found among the monocormic (single-stemmed) *P. sylvestris*-like individuals carrying the *cp*DNA from *P. mugo* complex. Hybrids of *P. sylvestris* as a pollen donor and *P. mugo* or *P. uliginosa* as a mother were not found, either in the group of examined trees, or among the open pollinated progeny. The results indicate that numerous hybrids can exist in the sympatric population of the species studied and that gene flow can successfully proceed from *P. mugo* complex to *P. sylvestris*. Hybridisation and ecological selection seems to play a significant role in diversification and evolution of the investigated species.

Keywords: *P. sylvestris*; *P. mugo*; *P. uliginosa*; hybridisation; molecular markers; reproductive barrier; sympatric population; speciation

Natural hybridisation is recognised as an important process leading to diversification and adaptive evolution in plants and animal species (Lewontin and Birch 1966, Arnold 1997). There are many examples, which show that hybrid genotypes may have equivalent or even higher fitness as compared to parental species and can be favoured in a given environment. Even in case of initially reduced fertility or viability of hybrids from early generations, the gene flow can proceed in the populations leading to propagation of hybrids and speciation (Arnold et al. 1999).

The genus *Pinus* is the largest in conifers and is divided into two monophyletic subgenera

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including *Haploxylon* (subgenus *Strobus*) and *Diploxylon* (subgenus *Pinus*). It contains about one hundred species widely distributed in the northern hemisphere and some tropical and subtropical areas (Critchfield and Little 1971). Hybridisation in pines was detected in several species including *P. halepensis* and *P. brutia* in Turkey (Bucci et al. 1998), *P. contorta* and *P. banksiana* in Canada (Wagner et al. 1987), *Pinus pumila* and *P. pentaphylla* in Japan (Watano et al. 1996) or *P. taeda* and *P. echinata* in the USA (Chen et al. 2004).

Natural hybridisation was postulated also between closely related Scots pine (*P. sylvestris*) and the taxa from *P. mugo* complex including dwarf mountain pine (*P. mugo* Turra) and peatbog pine (*P. uliginosa* Neumann) (Siedlewska 1994, Boratyński et al. 2003). *P. sylvestris* is the most widespread forest tree species in Europe and Asia whereas *P. mugo* is an endemic species typical to the mountain regions of Europe (Critchfield and Little 1971). *P. uliginosa* was described in the Central Sudetes (Neumann 1837) where it grows mainly on peat bogs. The present distribution of Scots pine is a result of postglacial migration from several glacial refugia (Willis and Andel 2004, Cheddadi et al. 2006). It is supposed that recolonisation created the zones of secondary contacts between isolated local populations from ice free regions which survived the last glacial maximum with populations from southern refugia. As the ranges of *P. mugo* and *P. sylvestris* overlapped in some part of their distribution, hybridisation between the species was supposed to contribute to high diversification within *P. mugo* complex (Christensen 1987a). It was also suggested that *P. uliginosa* could result from ancient cross-pollination between *P. sylvestris* and the taxa from *P. mugo* complex (Prus-Głowacki et al. 1998, Lewandowski et al. 2000).

At present *P. sylvestris* and *P. mugo* have mostly allopatric distribution. Some sympatric populations of the species were reported on low lying peatbogs from post glacial period occupied by relict populations of *P. mugo* and surrounded by extensive forest stand of other conifers including *Picea abies*. The natural hybridisation between the species was studied in several populations, however the biometric studies were limited by the lack of diagnostic characters suitable for identification of hybrids. The estimates of hybridisation intensity based on anatomical and morphological techniques varies from rare formation of hybrids (Christensen and Dar 1997) to the formation of putative hybrid swarms (Staszkiewicz 1993). The hybridisation hypothesis was tested with the use of serological techniques and isozymes. Mixed traits of antigenic proteins as compared to putative parental species were found in the populations suggesting the possibility of hybridisation (Prus-Głowacki et al. 1981). No fixed differences in isozymes were found between the *P. sylvestris* and *P. mugo* complex. In the majority of the peat bog populations the allele frequencies in polycormic individuals were similar to those observed in dwarf mountain pine from continuous range, which suggested rather the low extent of hybridisation (Filppula et al. 1992, Neet-Sarqueda 1994, Odrzykoski 2002). No evidence of ongoing hybridisation was found in the studies applying RFLP markers (Filppula et al. 1992, Odrzykoski 2002). In the controlled crossing experiments, the hybridisation barriers between *P. sylvestris* and *P. mugo* were observed by Wachowiak et al. (2005a, 2006a), whereas Kormutak et al. (2005) successfully crossed the species in both directions.

Recently, DNA markers of paternally inherited chloroplast DNA were described for *P. sylvestris* and *P. mugo* complex (Wachowiak et al. 2000, Wachowiak et al. 2006a). The markers were applied in hybridisation studies in two putatively hybridising populations of the species. The ongoing but very rare hybridisation was detected in *P. sylvestris* and *P. uliginosa* population but no evidence of the existence of hybrid trees was found (Boratyńska et al. 2003, Wachowiak et al. 2005b). The ongoing hybridisation was also detected in the sympatric population of *P. sylvestris* and *P. mugo* but only one hybrid tree was identified (Wachowiak et al. 2006b). The study questioned the existence of a hybrid swarm between the species in the investigated population.

In the study, we applied DNA markers and isozymes to identify hybrids in the sympatric

population of *P. sylvestris*, *P. mugo* and *P. uliginosa* from the "Torfowisko pod Zieleńcem" reserve in Poland. The occurence of the three pine species in a very diverse habitat of the peat-bog complex gives unique opportunity for studying adaptive evolutionary processes involving natural hybridisation. Specifically, we asked the question if natural hybridisation takes place in this population and leads to propagation of hybrids trees? Then, if the observed patterns of hybridisation are consistent with our previous investigations in two populations of different species composition? And finally, what can be the evolutionary consequences of hybridisation in the studied group of taxa? We demonstrate here that hybridisation can proceed in natural population of *P. sylvestris*, *P. mugo* and *P. uliginosa* and may produce many fertile hybrids competing with parental species. High intensity of hybridisation accompanied by ecological selection seems to be meaningful for the evolution of the sympatric populations of the analysed taxa.

Materials and methods

Study area and sampling. Plant material was collected at the "Torfowisko pod Zieleńcem" reserve (called hereafter Zieleniec reserve) which is the largest peat bog complex in the Sudety Mountains, the southwest part of Poland. The formation of peat started about 9.000 to 7.500 years ago and at present the reserve covers the area of about 156 ha. *P. sylvestris* are found mostly on dryer part of the peat bog growing in close vicinity of the taxa from *P. mugo* complex including *P. mugo* Turra and *P. uliginosa* Neumann. 300 individuals representing the phenotypic forms observed at Zieleniec reserve were collected from the area of the entire peat bog. These included individuals classified as *P. sylvestris* (85 in total), *P. mugo* (37), *P. uliginosa* (66) and 112 oligo- and polycormic (multistemmed) individuals of atypical morphology, which could not be classified to either of the above taxa. Selected phenotypic traits, i.e. growth form, bark colour of the upper part of trunk and main branches, colour and shape of needles and setting angle of conelet from the previous year were used for preliminary taxonomic classification. Samples of one-year old twins including winter buds were collected from selected trees. Additionally, 149 open pollinated seeds from seven trees were analysed. Mixed pool of seeds from a few cones was analysed separately for each individual. The seeds were germinated for two weeks and the seedlings were used for further analyses. The seeds were derived from one *P. sylvestris*, one *P. uliginosa*, two *P. mugo* and three individuals identified in the course of the analysis as hybrids.

DNA extraction and cpDNA markers application. The needles of mature trees (ca. 100 mg of fresh material) and the whole two-weeks old seedlings were used for DNA extraction following the CTAB (cetyltrimethylammonium bromide) protocol (Wachowiak et al. 2006a). Species diagnostic to *P. sylvestris* and *P. mugo* c*p*DNA haplotypes were defined with the use of two DNA markers. One of them represents single nucleotide restriction site polymorphism in the *trn*L-*trn*F region (Wachowiak et al. 2000). It can be detected with the use of PCR-RFLP method and *Dra*I restriction enzyme which leads to undigested PCR product for *P. sylvestris* (haplotype S) and digested (two bands) for *P. mugo* (haplotype M). The sequence analysis of this region in *P. uliginosa* indicated its identity to *P. mugo* (Wachowiak et al. 2005b). PCR-amplification was carried out in a total volume of 15 µl containing about 10 ng of template DNA, 2.5 mM $MgCl_2$, 100 µM of each of dNTP, 0.2 µM each of primer and 0.25 U *Taq* polymerase (Fermentas, Lithuania) with the respective 1x PCR buffer following the cycle profile and primers as previously reported (Wachowiak et al. 2000). The PCR products (10 µl) were subjected to the over night restriction analyses at 37°C. After digestion, the samples were separated in 2% agarose gel, stained with ethidium bromide and analysed under UV light.

The second species-diagnostic DNA marker originated from the chloroplast microsatellite region Pt41093 (Vendramin et al. 1996). Teufel (unpublished) found that the length differences in this region varied from 86 to 92 bp (> 86) for *P. mugo* and from 78 to 82 bp (< 82) for *P. sylvestris* and thus clearly distinguishes between the two species. This result was further confirmed in the analyses of individuals from controlled crosses (Wachowiak et al. 2006a). *P. uliginosa* length variation of Pt41093 microsatellite region is within the range for *P. mugo*. PCR-amplification was carried out in a total volume of 25 µl containing about 20 ng of template DNA, 2.5 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each primer and 0.25 U of *Taq* polymerase with the respective 1x PCR buffer (Fermentas, Lithuania). PCR was run in a

Personal Cycler (MJ Research, USA). The PCR products were separated in a 8% polyacrylamide gel (39:1 acrylamide:bisacrylamide, Sigma), stained with ethidium bromide and analysed under UV light.

The haplotype analyses. Both PCR-RFLP and microsatellite markers were applied to determine the haplotypes of mature trees. The data were compared to the phenotype of each individual. PCR-RFLP marker was applied in the analyses of open pollinated progeny derived from individuals classified on the basis of morphological traits as *P. sylvestris* (26 seedlings from one tree), *P. mugo* (28 seedlings from two trees), *P. uliginosa* (30 seedlings from one tree) and from three individuals tentatively classified as *P. sylvestris* but discovered to carry the *cp*DNA haplotype of *P. mugo* complex (65 seedlings). The species-diagnostic *cp*DNA haplotypes of progeny were compared to the haplotypes of mother trees. The results of haplotype analyses were compared to the outcomes of the previous studies of trees and an open pollinated progeny from the sympatric population of *P. sylvestris* and *P. uliginosa* (Wachowiak et al. 2005b) and *P. sylvestris* and *P. mugo* (Wachowiak et al. 2006b).

Isozyme analyses. Out of 300 individuals genotyped at two marker loci five groups of trees were selected for isozyme studies (Table 1). These included 34 *P. sylvestris* individuals (PUZ), 28 *P. mugo* (PMZ), 32 *P. uliginosa* (PUZ), a group of 29 *P. sylvestris* with *cp*DNA of *P. mugo* complex (*P. sylvestris*-like H1) and a group of 30 morphologically variable, oligo- and polycormic individuals, which could not be phenotypically classified as a pure species (Polycormic H2). Electrophoresis in starch gel was used for isozymes studies following the separation, staining procedures and genetic interpretation of the results as described by Odrzykoski (2002). All samples were genotyped at 10 enzymatic loci including: 6-phosphogluconate dehydrogenase (6PGD – E.C. 1.1.1. 44), malate dehydrogenase (MDH – 2 loci – E.C. 1.1.1.37), glutamate dehydrogenase (GDH – E.C. 1.4.1.3), shikimate dehydrogenase (SHDH – 2 loci – E.C. 1.1.1.25), diaphorase (DIA – E.C. 1.6.99), glutamate-oxalacetic transaminase (GOT – 3 loci – E.C. 2.6.1.1). Allelic variants *6Pgd*B2 and *Mdh*C2 were previously found to be more frequent in *P. mugo* in comparison to *P. sylvestris*, and called semi-diagnostic by Odrzykoski (2002). The allele frequency was compared between the five groups and with the allele frequency of pines studied by Wachowiak et al. (2006b). They included three samples of polycormic (BP 5, BP 7, BP 9) and three

Table 1. Location of populations and the sample size of present and the reference isozyme studies

No.	Species /sample	N	Location	Reference
1	*P. sylvestris* PSZ	34	Torfowisko pod Zieleńcem, Sudety Mts	Present study
2	*P. mugo* PMZ	28	Torfowisko pod Zieleńcem, Sudety Mts	Present study
3	*P. uliginosa* PUZ	32	Torfowisko pod Zieleńcem, Sudety Mts	Present study
4	*P. sylvestris*-like H1[a]	29	Torfowisko pod Zieleńcem, Sudety Mts	Present study
5	Polycormic H2[b]	30	Torfowisko pod Zieleńcem, Sudety Mts	Present study
6	*P. mugo* DB	51	Dubrawiska, Tatra Mts.	Odrzykoski 2002
7	*P. mugo* ZT	40	Żółta Turnia, Tatra Mts.	Odrzykoski 2002
8	*P. mugo* ST	56	Stawy Toporowe, Tatra Mts	Odrzykoski 2002
9	*P. mugo* BP5[c]	64	Bór na Czerwonem, Nowy Targ	Odrzykoski 2002
10	*P. mugo* BP7[c]	87	Bór na Czerwonem, Nowy Targ	Odrzykoski 2002
11	*P. mugo* BP9[c]	42	Bór na Czerwonem, Nowy Targ	Wachowiak et al. 2006b
12	*P. sylvestris* BM1[d]	49	Bór na Czerwonem, Nowy Targ	Odrzykoski 2002
13	*P. sylvestris* BM2[d]	50	Bór na Czerwonem, Nowy Targ	Odrzykoski 2002
14	*P. sylvestris* BM3[d]	72	Bór na Czerwonem, Nowy Targ	Wachowiak et al. 2006b
15	*P. sylvestris* ZF	101	Puszcza Zielonka, Poznań	Myczko 2001
16	*P. sylvestris* PN20	53	PN-20 seed orchard, Olsztyn	Odrzykoski 2002

[a] *P. sylvestris*-like hybrids with *cp*DNA from *P. mugo* complex; [b]oligo- and polycormic multistemmed individuals of atypical morphology, which on the base of *cp*DNA haplotypes and isozymes studied were concluded to represent hybrids between *P. mugo* and *P. uliginosa*; [c]polycormic pines assumed to represent mostly pure *P. mugo*; [d]monocormic pines assumed to represent pure *P. sylvestris*

monocormic (BM 1, BM 2, BM 3) pines from different regions from sympatric population of *P. sylvestris* and *P. mugo* from Bór na Czerwonem peat bog and the reference pure *P. mugo* and *P. sylvestris* (Odrzykoski 2002). *P. mugo* from Tatra Mts. (Poland) originated from Dubrawiska (DB), Żółta Turnia (ZT) and Wyżni Staw Toporowy peat bog (ST). Samples of *P. sylvestris* come from Puszcza Zielonka in Poland (Myczko 2001) and from the seed orchard PN-20 that contains trees from northern Poland (Odrzykoski 2002). The list of reference population samples is presented in Table 1. GenAlex software was used to calculate allelic frequencies and Nei's (1978) genetic distances between all groups. The genetic distances were used to conduct cluster analysis in MEGA 3 using the Unweighted Pair Group Method with Arithmetic Mean.

Results

Identification of hybrids. The results of *cp*DNA haplotypes analysis of 300 trees are summarised in Table 2. No other haplotypes than previously described for *P. sylvestris* and *P. mugo* complex (*P. mugo* and *P. uliginosa*) were observed in the examined group of individuals. The whole group of 37 *P. mugo* individuals, 66 *P. uliginosa* individuals and 112 oligo- or polycormic individuals of differentiated morphology had *cp*DNA haplotypes diagnostic for *P. mugo* complex. Among the 85 individuals tentatively classified as *P. sylvestris* only 50 displayed *cp*DNA haplotypes typical for the species. The remaining 35 *P. sylvestris*-like individuals carried *cp*DNA haplotypes diagnostic for *P. mugo* complex (*P. mugo* and *P. uliginosa*).

The *cp*DNA haplotypes of an open pollinated progeny are presented in Table 3. The hybrid seedlings with species diagnostic *cp*DNA haplotypes discordant with the haplotype of a parental tree were detected for the *P. sylvestris* progeny as well as for the *P. sylvestris*-like individuals but carrying the *P. mugo cp*DNA. The results of previous *cp*DNA haplotype studies of trees and open-pollinated progeny in sympatric populations of *P. sylvestris*/*P. uliginosa* and *P. sylvestris*/*P. mugo* are also summarised in Table 2 and Table 3.

Isozyme analysis. The frequencies of the most common alleles at 10 studied loci among the groups of individuals are presented in Table 4. Two semidiagnostic alleles for *P. mugo* (*6Pgd*B2 and *Mdh*C2) were the most frequent among *P. mugo* and *P. uliginosa* from Zieleniec reserve, three *P. mugo* populations from the reference group and among the

Table 2. The *cp*DNA haplotypes and number of hybrid trees identified among the selected taxa from Zieleniec reserve and the results from the reference studies in a sympatric populations of *P. sylvestris* and *P. mugo* complex (*P. mugo* and *P. uliginosa*). Hybrids were found among the *P. sylvestris*-like individuals and oligo- and polycormic individuals as revealed from *cp*DNA and isozyme studies. M – haplotypes species diagnostic for *P. mugo* complex, S – for *P. sylvestris*

No	Taxa/pines	Location	Number of trees	*cp*DNA haplotypes		Number of hybrids	Reference
				M	S		
1	*P. sylvestris*	Torfowisko pod Zieleńcem	85	35	50	35	Present study
2	*P. mugo*	Torfowisko pod Zieleńcem	37	37	0	0	Present study
3	*P. uliginosa*	Torfowisko pod Zieleńcem	66	66	0	0	Present study
4	Polycormic H2	Torfowisko pod Zieleńcem	112	112	0	112	Present study
5	*P. uliginosa*	The Stołowe Mts.	32	32	0	0	Wachowiak et al. 2005b
6	*P. uliginosa*	Low Silesian Pinewood	28	28	0	0	Wachowiak et al. 2005b
7	*P. sylvestris*	Low Silesian Pinewood	8	0	8	0	Wachowiak et al. 2005b
8	Polycormic	Bór na Czerwonem	42	42	0	0	Wachowiak et al. 2006b
9	Monocormic	Bór na Czerwonem	72	1	71	1	Wachowiak et al. 2006b

Table 3. The *cp*DNA haplotypes and number of hybrids identified among the open pollinated progeny derived from selected individuals (taxa) from Zieleniec reserve and the results from the reference studies. Hybrid seedlings of F1 generation were found among the progeny from *P. sylvestris* and *P. uliginosa*. Hybrids of further than F1 generations were produced by hybrid individuals of *P. sylvestris*-like phenotype. M – haplotypes species diagnostic for *P. mugo* complex, S – for *P. sylvestris*

Taxa/pines	Location	Number of progeny	*cp*DNA haplotypes		Number of hybrids	Reference
			M	S		
P. sylvestris	Zieleniec reserve	26	15	11	15	Present study
P. mugo	Zieleniec reserve	28	28	0	0	Present study
P. uliginosa	Zieleniec reserve	30	30	0	0	Present study
P. sylvestris-like H1	Zieleniec reserve	65	59	6	65	Present study
P. uliginosa	Low Silesian Pinewood	487	480	7	7	Wachowiak et al. 2005b
P. sylvestris	Low Silesian Pinewood	329	7	322	7	Wachowiak et al. 2005b
Polycormic	Bór na Czerwonem	43	43	0	0	Wachowiak et al. 2006b
Monocormic	Bór na Czerwonem	22	17	5	17	Wachowiak et al. 2006b

polycormic pines from Bór na Czerwonem reserve, which in the study by Wachowiak et al. (2006b) were considered to represent mostly pure *P. mugo*. Both alleles were also the most frequent among the group of polycormic individuals from Zieleniec reserve, whereas the group of *P. sylvestris*-like individuals with *cp*DNA of *P. mugo* complex had the higher frequency of only *Mdh*C2 allele. Contrary, *P. sylvestris* from Zieleniec reserve similarly to monocormic pines from Bór na Czerwonem reserve (considered by Wachowiak et al. (2006b) as a pure *P. sylvestris*) and the two reference *P. sylvestris* populations had the most frequent allele *6Pgd*B1 and *Mdh*C1. In the remaining loci the most frequent alleles were shared between the analysed groups. The exception was allele *Gdh*1, which was more frequent in *P. uliginosa* from Zieleniec reserve and *P. mugo* from Żółta Turnia and the allele *Sdh*2 more frequent in *P. mugo* from Zieleniec reserve and the group of polycomic pines from this area.

Genetic distances between the groups are presented in Table 5 and the relationships between populations are further demonstrated on a dendrogram (Fig. 1). *P. mugo* and *P. uliginosa* from Zieleniec reserve cluster together ($D_N = 0.002$) and they show also very close genetic distance to polycormic pines from this area carrying the *cp*DNA of *P. mugo* complex ($D_N = 0.003$, $D_N = 0.005$). The polycormic pines show close genetic distance to the reference allopatric populations of *P. mugo* (populations 6–8; $D_N = 0.025$, $D_N = 0.033$ and $D_N = 0.064$, respectively) and to the *P. mugo* from Bór na Czerwonem reserve (population 9–11; $D_N = 0.039$, $D_N = 0.031$ and $D_N = 0.045$, respectively). Contrary, this group of polycormic pines show very high genetic distance to *P. sylvestris* from Zieleniec reserve ($D_N = 0.153$), to the reference *P. sylvestris* from allopatric populations (average $D_N = 0.137$) and to the group of *P. sylvestris* from Bór na Czerwonem reserve (average $D_N = 0.129$). A genetic distance among the subspecies is higher or equal to 0.05 (Nei 1987).

P. sylvestris-like individuals from Zieleniec reserve, which had the *cp*DNA of *P. mugo* complex, form a separate cluster with monocormic pines including *P. sylvestris* from Zieleniec reserve and the reference *P. sylvestris* populations. The genetic distance between the *P. sylvestris*-like individuals and *P. sylvestris* from Zieleniec reserve is 0.030 which is similar to the average genetic distance between *P. sylvestris*-like individuals and the remaining populations (12–16) of *P. sylvestris* (average $D_N = 0.031$). This is lower as for *P. sylvestris*-like individuals and the reference *P. mugo* (pop-

Table 4. Frequency of the most common alleles at ten loci in 16 populations from Table 1. In bold – frequency of semidiagnostic alleles for *P. mugo*

Locus		Taxa from Zieleniec reserve					*P. mugo*					*P. sylvestris*					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
6PGD-B	1	.750			.574								.688	.630	.710	.624	.642
	2		**.646**	**.625**		**.700**	**.931**	**.900**	**.825**	**.662**	**.625**	**.700**					
MDH-A	1	.956	1	1	.982	1	.990	.950	1	.992	.994	.952	.984	.970	.979	.955	.943
MDH-C	1	.735											.750	.640	.715	.725	.784
	2		**.786**	**.823**	**.536**	**.800**	**.882**	**.900**	**.658**	**.700**	**.830**	**.714**					
GDH	1		.500	.516		.500		.613									
	2	.588	.500		.554	.500	.647		.798	.600	.568	.619	.828	.710	.694	.650	.613
SDH-A	1	.853		.500	.603		.500	.675	.746	.719	.614	.714	.844	.820	.764	.827	.868
	2		.554	.500		.633	.500										
SDH-B	1	.956	.889	.919	1	.967	.922	.950	1	.938	.932	.964	.922	.920	.913	.950	.962
DIA-C	1	.779	.944	.952	.804	.950	.922	.938	1	.873	.898	.869	.875	.900	.910	.787	.830
GOT-A	1	1	.960	.940	1	1	.951	1	1	.968	.983	.927	1	1	.993	1	.972
GOT-B	1	.516	.944	.893	.654	.931	.725	.888	.737	.797	.756	.702	.625	.704	.664	.598	.585
GOT-C	1	.720	.794	.773	.775	.762	.755	.838	.566	.800	.813	.846	.672	.640	.549	.655	.642

ulations 6–8, average $D_N = 0.062$), but similar to *P. mugo* and *P. uliginosa* from Zieleniec reserve ($D_N = 0.043$ and $D_N = 0.039$, respectively). The average genetic distance between the reference *P. mugo* (population 6, 8) and *P. sylvestris* (population 15–16) is about $D_N = 0.12$.

Discussion

The presented study documents for the first time, that natural hybridisation in the sympatric population of *P. sylvestris*, *P. mugo* and *P. uliginosa* can lead to propagation of numerous hybrid trees. We identified two groups of hybrids. First one is formed by morphologically highly variable oligo- and polycormic individuals which show close genetic identity to both *P. mugo* and *P. uliginosa*. Their genetic distance to *P. sylvestris* from Zieleniec reserve and to *P. sylvestris* from the two reference populations are even higher that between pure *P. sylvestris* and *P. mugo*. This observation and the fact that all individuals in this group display the haplotypes of plastid DNA diagnostic for *P. mugo* complex indicate that they are hybrids between *P. mugo* and *P. uliginosa* with no evidence on contribution of *P. sylvestris*. No evidence on ongoing hybridisation with *P. sylvestris* as a pollen donor was also found in the analyses of seeds from *P. mugo* and *P. uliginosa*. The close genetic distance of oligo- and polycormic hybrids to both *P. mugo* and *P. uliginosa* suggests that some of them may represent backcrosses. It is likely that such hybrids are presented in our selected groups of putatively pure *P. mugo* and *P. uliginosa* as both groups show much lover genetic distance as found between allopatric populations of the species in other studies (Prus-Głowacki et al. 1998, Lewandowski et al. 2000).

The second group of hybrids was identified within monocormic individuals, tentatively classified on the basis of morphological traits as *P. sylvestris*. In the group of 85 *P. sylvestris*-like individuals, 35 had *cp*DNA from *P. mugo* complex. As chloroplast genome is inherited in paternal line in these species (Wachowiak 2005a), the result indicates that they are hybrids between *P. sylvestris* as a mother and *P. mugo* or

Table 5. Nei's (1978) genetic distances between studied populations (Table 1) based on allelic frequencies of ten isozyme loci

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*P. sylvestris* PSZ	***															
2	*P. mugo* PMZ	.134	***														
3	*P. uliginosa* PUZ	.125	.002	***													
4	*P. sylvestris*-like H1	.030	.043	.039	***												
5	Polycormic H2	.153	.003	.005	.051	***											
6	*P. mugo* DB	.169	.026	.025	.068	.025	***										
7	*P. mugo* ZT	.154	.030	.022	.068	.033	.020	***									
8	*P. mugo* ST	.097	.058	.055	.049	.064	.031	.050	***								
9	*P. mugo* BP5	.072	.029	.025	.024	.039	.028	.022	.021	***							
10	*P. mugo* BP7	.089	.023	.018	.028	.031	.023	.022	.026	.005	***						
11	*P. mugo* BP9	.073	.036	.031	.025	.045	.026	.025	.020	.004	.006	***					
12	*P. sylvestris* BM1	.014	.128	.125	.038	.147	.151	.158	.071	.065	.084	.070	***				
13	*P. sylvestris* BM2	.014	.093	.088	.025	.110	.118	.113	.054	.041	.057	.048	.005	***			
14	*P. sylvestris* BM3	.012	.113	.108	.033	.129	.152	.148	.076	.066	.083	.074	.008	.005	***		
15	*P. sylvestris* ZF	.004	.111	.105	.023	.126	.136	.129	.069	.055	.074	.057	.009	.007	.007	***	
16	*P. sylvestris* PN20	.007	.129	.122	.036	.147	.157	.145	.082	.065	.087	.069	.008	.008	.011	.006	***

P. uliginosa as a pollen donor. The hybrids reflect the pattern of contemporary hybridisation at this area as revealed by the analyses of an open pollinated progeny. More than 50% of *P. sylvestris* progeny was of hybrid origin carried the *cp*DNA from the *P. mugo* complex. As there is no difference in *cp*DNA discovered so far between *P. mugo* and *P. uliginosa*, the applied markers do not allow to indicate which of the two taxa from *P. mugo* complex participated in fertilisation. However, our previous studies showed that *P. sylvestris* can be fertilized in nature by *P. uliginosa* (Wachowiak et al. 2005b) and *P. mugo* (Wachowiak et al. 2006b). Therefore, it seems that both *P. mugo* and *P. uliginosa* can be a pollen donor to produce monocormic *P. sylvestris*-like hybrids.

The hybrids resembling one of the parental types were found in other pine species including *P. taeda* and *P. echinata* (Chen et al. 2004). These studies suggested that they were not F1 individuals but most likely the early generation backcrosses. As shown in our study, the haplotypes diagnostic to both *P. sylvestris* and *P. mugo* were found among the seeds derived from *P. sylvestris*-like hybrids, which indicates their fertility and potential for backcrosses. Further than F1 generation of hybrids were also detected in controlled crosses between *P. sylvestris* and *P. montana* (*P. mugo* complex) (Wachowiak et al. 2006a). In our study, the *P. sylvestris*-like hybrids showed closer genetic distance to pure *P. sylvestris* that *P. mugo*. Therefore it seems that some of the monocormic hybrids can represent backcrosses with *P. sylvestris*. However, it is unknown if their closer similarity to *P. sylvestris* can result from selection among the hybrids.

In the previous studies, the hybrid seeds and one hybrid tree of *P. sylvestris* as a mother and *P. mugo* as a pollen donor were detected in the sympatric population of both species from Bór na Czerwonem reserve in Poland (Wachowiak et al. 2006b). No evidence on reciprocal hybridisation in this population was found either in the group of open pollinated progeny or among the trees. The evidence on reciprocal but very rare ongoing hybridisation was found in the analyses of seeds in the sympatric population of *P. sylvestris* and

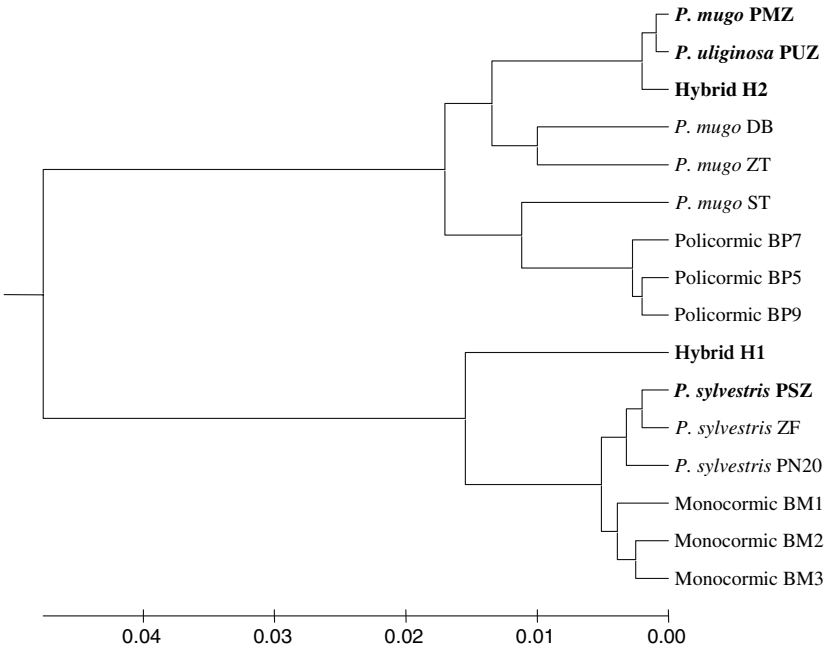

Fig. 1. Dendrogram constructed on the basis of genetic distances between populations presented in Table 5. In bold - populations from Zieleniec reserve including group of *P. sylvestris*-like (H1) and Polycormic (H2) pines, considered on the base on isozymes and *cp*DNA as a hybrids

P. uliginosa from Węgliniec reserve in Poland. However, no hybrid trees were detected there (Wachowiak et al. 2005b). As shown in the presented study, if the three taxa occur sympatrically, two groups of mature hybrids are produced. The results from the three populations of different species composition suggest that *P. sylvestris* participate in hybridisation as a mother tree. The results show also free natural hybridisation within *P. mugo* complex (between *P. mugo* and *P. uliginosa*).

Hybridisation in pines was reported to lead to formation of hybrid zones of different genetic structure (Watano et al. 2004), formation of hybrids resulted from bi-directional introgression and backcrosses (Chen et al. 2004) or to rare hybridisation events limited to individuals of F1 generation (Bucci et al. 1998). The so far estimates of hybridisation intensity and the species composition in the sympatric populations of *P. sylvestris* and *P. mugo* varied from rare formation of F1 hybrids to the formation of putative hybrid swarms (Staszkiewicz and Tyszkiewicz 1972, Christensen 1987b, Bobowicz 1990, Neet-Sarqueda 1994). It seems that similarly to the Zieleniec reserve propagation of F1 and next generations of hybrids can freely proceed in the sympatric populations of the taxa from the *P. mugo* complex. Consequently, the past and contemporary hybridisation within the *P. mugo* complex could account for the variety of morphological forms reported in other populations of *P. mugo* (Staszkiewicz and Tyszkiewicz 1969, Filppula et al. 1992, Neet-Sarqueda 1994). However, the so far data do not support the hypothesis that hybridisation with only *P. sylvestris* could transform the population of *P. mugo* or *P. uliginosa* into a hybrid swarm.

The existence of viable hybrids, well adapted to the specific microhabitats of complex environments may lead to their dissemination followed by mutual competition with the parental types (Harrison 1990). The hybrids within *P. mugo* complex show such signs of expansion at the Zieleniec reserve. Their amount in the entire population can be estimated for more than thirty

percent and they constitute the majority of individuals in the central, less humid parts of the peat bog. They gradually replace *P. mugo* and *P. uliginosa* in this region. The remaining area is inhabited by the mixture of the pure species and hybrids, in some parts of the peat bog occasionally represented by a single individual. The dryer and more solid external parts of the peat bog are mostly occupied by *P. uliginosa*, *P. sylvestris* and *P. sylvestris*-like hybrids. The number of *P. sylvestris*-like hybrids seems to be similar to *P. sylvestris* and they are less numerous in this population than the remaining pine taxa. However, *P. sylvestris*-like hybrids produce seeds and the viability of analysed seedlings seems to be similar to the pure species. Therefore it is likely that these hybrids can strength the competition with parental species and in the future potentially dominate the external parts of the peat bog.

The results of presented study support the hypothesis that past hybridisation events in the contact zones between *P. sylvestris* and other closely related pine species could play a significant role in the evolution of the *P. mugo* complex. The example from the Zieleniec reserve demonstrates (1) the existence of viable and fertile hybrids, (2) ecological selection which influence their distribution in the complex microhabitats and (3) the limited gene flow among a certain groups of taxa including the parental species, which creates excellent conditions for further diversification of the taxa from this area. Both theoretical models and experimental studies show that ecological selection can promote diversification of hybrids and speciation (Gross and Rieseberg 2005). It seems likely that the ancient hybridisation processes in the contact zones similar to the Zieleniec reserve between *P. sylvestris* and *P. mugo* complex, including an isolated population which survived the glacial maxima, could play a role in speciation in *Pinus*. Homoploid hybrid speciation in pines, which could potentially involve recombination speciation (Lai et al. 2005), is well documented in *P. densata* from Tibetan Plateau, a hybrid between *P. tabulaeformis* and *P. yunnanensis* (Wang et al. 2001). Additionally, different *P. densata* populations were found to have unique evolutionary histories and most likely independent hybrid origins (Song et al. 2003). However, more studies are needed to evaluate how common could be speciation in pines through hybridisation.

Previous studies showed that *P. mugo* is more resistant than *P. sylvestris* to some pathogens including needle cast (*Lophodermium seditiosum*). These observations motivated the attempts of controlled crosses between the species to produce hybrids for breeding purposes (Prus-Głowacki and Stephan 1998). The hybrids identified in presented study, especially the *P. sylvestris*-like ones, need detailed biometric and biochemical investigations to access their breeding values and their potential use, as in case of other pine hybrids (Dungey 2001). The applied methods proved to be useful for the analyses of microevolutionary processes going on in the sympatric populations of *P. sylvestris* and *P. mugo* complex and could be implemented in similar studies in other putatively hybridising populations. Identification of hybrids gives unique opportunity for more complex studies including the genetics of adaptive variation in this group of taxa.

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