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CLONAL STRUCTURE OF SMALL ISOLATED POPULATIONS OF *PINUS MUGO* TURRA FROM PEATBOGS IN THE TATRA MTS

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This study examined genetic variability at 11 allozyme loci and the clonal structure of peatbog populations of dwarf mountain pine (*Pinus mugo* Turra) from three small isolated stands in the Tatra Mts, Poland. The percentage of clones in the peatbog populations was high, 48.45% on average. Genetic variability was lower in these populations than in populations from the continuous range of the species. The mean number of alleles per locus (A) was 2.2. Estimates of total (H_T = 0.231) and within-population (H_S = 0.224) genetic diversity were very similar. Differentiation between populations was low (D_{ST} = 0.7%; G_{ST} = 2.1%). Gene flow (Nm = 8.11) was also relatively low because of their geographic isolation. Wright's fixation index (F) for all examined enzymatic loci demonstrated that the populations were in Hardy-Weinberg equilibrium.

Key words: *Pinus mugo* Turra, genetic variability, isozymes, clones, isolated populations, gene flow.

INTRODUCTION

Small isolated populations generally differ in genetic structure from populations occurring within the continuous range of the species. Very often they have a unique gene pool which differs from the gene pool of central populations (Jimenez et al., 1999; De Matthaeis et al., 2000; Prus-Głowacki et al., 2003). In such populations, the founder effect and genetic drift are significant factors. In addition, small population size and being outside the continuous range prevent free gene exchange; inbreeding results in increasing homozygosity and decreasing polymorphism. This kind of situation favors clonal propagation of some plant species as a life strategy (Wright, 1969; Kimura, 1983; Hamrick and Godt, 1996; Mitka, 1997; Shea and Furnier, 2002). In conifers, the unique genetic structure of isolated populations is exemplified by Yeh and Layton's (1979) description of genetic variability in marginal and central populations of *Pinus contorta*, and Hamrick et al.'s (1989) study of *Pinus ponderosa* from marginal and continuous range populations. Prus-Głowacki et al. (2003) demonstrated lower genetic variability in marginal populations of *Pinus sylvestris*.

Dwarf mountain pine (*Pinus mugo* Turra) grows in the mountains of Central and Southern Europe (Carpathians, Sudeten, Alps, Apennines, Balkan mountains, Pyrenees), in the subalpine zone above the treeline (Bugała, 1991). The species is highly resistant to extreme climatic and soil conditions, also inhabiting very poor biotopes of oligotrophic high peatbogs (Mirek and Piękoś-Mirkowa, 1996). In such places, *P. mugo* forms small island populations (Obidowicz, 1973, 1996a). The dwarf mountain pine does not spread outside the peatbogs since it is a photophilic species (Christensen, 1987), and outside the peatbogs it cannot compete with the spruce trees that surround the *P. mugo* stands in the Tatras.

The genetic structure of *P. mugo* peatbog populations in the Tatra Mts can be affected by several factors: (1) their small size and patchiness; (2) their spatial and phenological isolation, which restricts gene flow via pollen and seeds from other peatbog populations and from the continuous range of dwarf mountain pine; and (3) their ability to form clones through offshoots which take root (Seneta and Dolatowski, 1997)

The genetic differentiation and spatial distribution of genotypes in *P. mugo* populations are poorly known. Most investigations have been accessory to studies on *P. mugo* × *P. sylvestris* hybrids (Prus-Głowacki and Szweykowski, 1977, 1983; Prus-Głowacki et al., 1998; Filppula et al., 1992; Siedlecka and Prus-Głowacki, 1994; Lewandowski et al., 2000; Odrzykoski, 2002).

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The aim of the present study was to analyze the genetic structure of small isolated populations of dwarf mountain pine in peatbogs of the Tatra Mts, in order to determine (1) the level of variability in the studied populations, (2) the participation of clones in the studied populations, (3) whether the populations differ significantly from each other, and (4) how the populations differ from the continuous range of the species.

MATERIALS AND METHODS

Material was collected from three peatbog populations of *P. mugo* in the Tatra Mts in southern Poland. The peatbogs are located below the subalpine zone, at 1100–1380 m a.s.l. *P. mugo* individuals form very dense thickets there, making it difficult to distinguish particular individuals. In each population, winter buds were collected from rooted shoots at 3 m intervals along a transect. Hence the number of collected samples reflected the size of the population. Three hundred samples were collected from the largest population in the peatbog at Dolina Kościeliska (DK), and 86 and 64 from two smaller peatbog populations at Wielka Pańszczycka Młaka (WPM) and Waksmundzka Młaka (WM), respectively (Tab. 1, Fig. 1).

Isoenzyme analysis was used to examine the genetic and genotypic structure of the populations. The methods used for horizontal starch-gel electrophoresis, the staining procedure for isozymes, and genetic interpretation have been described by Rudin and Ekberg (1978), Szmidt and Yazdani (1984), Yazdani et al. (1985) and Gullberg et al. (1985). The following enzyme systems were assayed in all populations: two loci of glutamate oxaloacetate transaminase (GOT, E.C. 2.6.1.1), alcohol dehydrogenase (ADH, E.C. 1.1.1.1), diaphorase (DIA, E.C. 1.8.1.4), fluorescent esterase (FEST, E.C. 3.1.1.23), glutamate dehydrogenase (GDH, E.C. 1.4.1.2), glucose–6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.44), two loci of malate dehydrogenase (MDH, E.C.1.1.1.37), and two loci of shikimate dehydrogenase (ShDH, E.C.1.1.1.25).

Individual samples (ramets) in a population with the same multilocus isozyme pattern were tentatively assigned to a single clone. The formula of Aspinwall and Christian (1992) was used to calculate the prob-

TABLE 1. Number of studied samples (N), number of genotypes (G) and percentage of clones and geographic coordinates of the studied populations of *P. mugo* in the Tatra Mts

Population	Samples (N)	Number of genotypes (G)	Clones (%)
Wielka Pańszczycka Młaka peatbog (WPM) 49°16' 08"N 20°02'50"E 1260 m a.s.l.	86	63	26.74
Waksmundzka Młaka peatbog (WM) 49°15' 30"N 20°03'50" E 1380 m a.s.l.	64	25	60.94
Dolina Kościeliska peatbog (DK) 49°13'40"N, 19°52'00"E 1100 m a.s.l.	300	127	57.66
Average			48.45

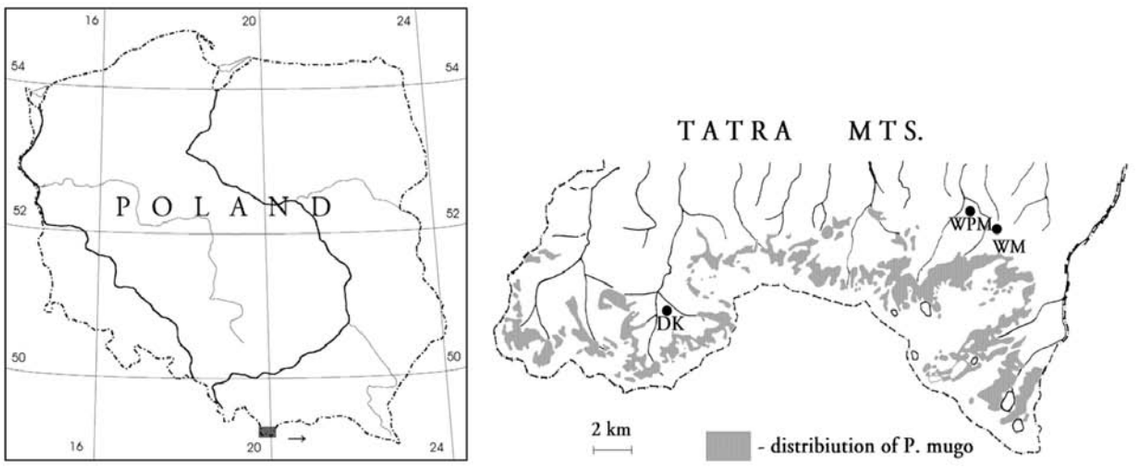

Fig. 1. Location of studied populations of *Pinus mugo*. Distribution of *P. mugo* in the subalpine belt of the Tatra Mts is according to Mirek and Piękoś-Mirkowa (1996).

TABLE 2. Allele frequencies at 11 polymorphic enzyme loci in the studied populations of *P. mugo*

Locus	Allele (n)	Population			Mean
		WPM	WM	DK	
Adh	1	0.944	0.950	0.937	0.944
	2	0.056	0.050	0.008	0.038
	3	-	-	0.056	0.019
Dia	1	1.000	1.000	0.925	0.975
	2	-	-	0.075	0.025
FEst	1	0.846	0.967	0.803	0.872
	2	0.038	0.033	0.173	0.081
	3	0.115	-	0.024	0.046
Gdh	1	0.278	0.350	0.622	0.417
	2	0.685	0.650	0.374	0.570
	3	0.037	-	0.004	0.013
Got-A	1	0.907	1.000	0.945	0.951
	3	0.093	-	0.051	0.048
	4	-	-	0.004	0.001
Got-B	1	0.760	0.750	0.764	0.758
	2	0.240	0.250	0.232	0.241
	3	-	-	0.004	0.001
Mdh-A	1	1.000	1.000	0.996	0.999
	2	-	-	0.004	0.001
Mdh-C	1	0.102	0.033	0.102	0.079
	2	0.898	0.967	0.898	0.921
Pgd	1	0.157	0.117	0.114	0.129
	2	0.824	0.833	0.858	0.838
	3	0.019	0.050	0.028	0.032
Shdh-A	1	0.577	0.750	0.528	0.618
	2	0.404	0.250	0.462	0.372
	3	-	-	0.012	0.004
	5	0.019	-	-	0.006
Shdh-B	1	0.962	1.000	0.905	0.956
	3	0.038	-	0.095	0.044

ability that ramets with the same multilocus pattern belonged to the same genet (clone). Genetic parameters were estimated using GEN software (Nowak-Bzowy and Bzowy 1998, unpublished), including total number of alleles, percentage of polymorphic loci, mean number of alleles per locus (A/L), number of genotypes per locus (G/L), observed heterozygosity (H_o), expected heterozygosity (H_e) and genotype polymorphic index (Pg). Deviations from Hardy-Weinberg expectations for each polymorphic locus in each population were calculated using Wright's fixation index $F = 1 - (Ho/He)$ (Wright, 1922). For all loci we calculated total allelic diversity (H_T), diversity within populations (H_S), gene diversity between populations (D_{ST}), the coefficient of gene differentiation ($G_{ST} = D_{ST}/H_T$) and number of migrants per generation (Nm) (Slatkin, 1985). Allelic frequencies were used to calculate genetic distances (D) and genetic similarities (I) for pairwise comparisons between populations (Nei, 1972, 1978). Clonal diversity (D_G) was assessed according to the following formula (Pielou, 1969; Ellstrand and Roose, 1987):

$$D_G = 1 - \Sigma\{[n_i\,(n_i - 1)] / N\,(N - 1)\}$$

where ni is the number of individuals in clone i, and N is the sample size. D_G values range from 0 to 1, and higher values correspond to greater clonal diversity. The genotypic evenness index (E) scales D_G to the level of polymorphism within the population (Fager, 1972; Ellstrand and Roose, 1987; Eckert and Barrett, 1993):

$$E = (D_{G\,observed} - D_{G\,min}) / (D_{G\,max} - D_{G\,min})$$

where

$$D_{G\,min} = (C - 1)\,(2N - C) / N\,(N - 1)$$

and

$$D_{G\,max} = N\,(C - 1) / C\,(N - 1)$$

RESULTS

Considering the three sites together, 30 alleles were detected in total, including 8 rare alleles (with frequencies >0.05) and 5 very rare alleles (with frequencies >0.01). All alleles except one (*Shdh-A 5*) were found in the DK population, while the WM and WPM populations contained 19 and 24 alleles, respectively. The mean number of alleles per locus (A/L) varied from 1.7 at WM to 2.7 at DK, with a mean of 2.2 for the three populations (Tabs. 2, 3).

Population-specific alleles were found at WPM and DK. Five such private alleles were found in the DK population with frequencies of 0.056 (*Adh 3*), 0.075 (*Dia 2*), 0.004 (*Got-A 4*), 0.004 (*Got-B 3*) and 0.004 (*Mdh-A 2*), and one in the WPM population (*Shdh-A 5*, 0.019). Four of the private alleles were rare alleles, and two occurred at higher than 5% frequency.

All examined loci were polymorphic in one population, DK (Tab. 3). The average fraction of polymorphic loci was 81.8%. The lowest genotype polymorphism was found in the WM population (Pg = 0.227); in the WPM and DK populations it was Pg = 0.323 and Pg = 0.362, respectively.

In isozymatic studies, significant fractions of the samples were found to have same multilocus genotype, so they were classified in the same clone. The calculated probability that ramets with the same multilocus pattern are the same genet (clone) was 95%. The fraction of clones was high in the studied populations, 48.45% on average. The lowest percentage of clones was found in the WPM population (26.74%), and the highest in the WM population (60.94%) (Tab. 1).

Clonal diversity (D_G) was similar in all three stands (0.950–0.987). The evenness index (E) was higher in the DK population than in the others (Tab. 4).

TABLE 3. Genetic parameters based on variation at 11 enzyme loci in three peatbog *P. mugo* populations, characterized by total number of allele and rare alleles. *P* – percentage of polymorphic loci; *A/L* – mean number of alleles per locus; *G/L* – genotypes per locus; *Ho* – observed heterozygosity *He* – expected heterozygosity; *Pg* – genotype polymorphism index; *F* – fixation index; Standard deviations given in parentheses

Population	Total no. alleles	Rare alleles	*P*	A/L	*G/L*	Ho	*He*	*Pg*	F
WPM	24	5	81.8	2.2 (0.2)	2.7	0.211 (0.058)	0.220 (0.068)	0.323	0.037
WM	19	3	63.6	1.7 (0.2)	2.0	0.148 (0.056)	0.156 (0.063)	0.227	0.049
DK	29	8	100.0	2.7 (0.2)	3.0	0.251 (0.064)	0.242 (0.061)	0.362	-0.037
Mean	24.0	5.3	81.8	2.2	2.3	0.203	0.206	0.304	0.016

Analysis of Wright's fixation index (*F*) demonstrated that the studied populations were close to the Hardy-Weinberg equilibrium. A slight excess of homozygotes was observed in the WPM (0.037) and WM (0.049) populations, while the DK population demonstrated a slight excess of heterozygotes (-0.037) (Tab. 3).

Mean observed (*Ho*) and expected (*He*) heterozygosity based on 11 loci were similar to each other in two populations (DK, WPM). Observed heterozygosity was low in the WM population (*Ho* = 0.148), much lower than in WPM (*Ho* = 0.211) and DK (*Ho* = 0.251) (Tab. 3).

Total allelic diversity based on mean allelic frequencies at all sites was H_T = 0.231 (SD ± 0.067), similar to genetic diversity within populations (H_S = 0.224) (Tab. 5). Inter-population gene diversity (D_{ST} = 0.7%) and the inter-population differentiation coefficient (G_{ST} = 2.1%, SD ± 0.015) were relatively low. This means that ~98% of total genetic variability in the studied populations was related to intra-population variability.

The mean number of migrants per generation (*Nm*) in the populations was 8.11, indicating limited gene flow (Tab. 5).

Genetic similarities (*I*) and genetic distances (*D*) (Nei, 1972) are presented in Table 6. The highest similarity (*I* = 0.994) was observed between the WM and WPM populations. Populations DK and WM were genetically the most distant from each other.

DISCUSSION

Vegetative propagation is a life strategy of many plant species. Artificial cloning is often used in forest tree breeding and gene conservation programs, in comparative studies of the resistance of particular genotypes to harmful biotic and abiotic factors, or for improvement of trees through biotechnology manipulations. However, cloning is not well studied in natural populations of coniferous species. In our study, allozyme analysis demonstrated significant fractions of clones in the studied populations of *P. mugo*. The highest percentage of clones was noted in the WM

TABLE 4. Genetic diversity in the studied populations: multilocus genetic diversity (D_G) observed, minimum and maximum and evenness index (*E*)

Population	$D_{G\,observed}$	$D_{G\,min}$	$D_{G\,max}$	*E*
WPM	0.982	0.856	0.993	0.919
WM	0.950	0.705	0.982	0.884
DK	0.987	0.664	0.995	0.976

TABLE 5. Total genetic diversity (H_T), genetic diversity within populations (H_S), genetic diversity between populations (D_{ST}), proportion of total genetic diversity among populations (G_{ST}) (Nei, 1972), and number of migrants per generation (*Nm*) for each locus in the studied populations of *P. mugo*

Locus	H_T	H_S	D_{ST}	G_{ST}	Nm
Adh	0.120	0.120	0.000	0.000	200.74
Dia	0.086	0.083	0.003	0.035	7.77
FEst	0.283	0.273	0.009	0.032	7.28
Gdh	0.512	0.465	0.046	0.090	2.50
Got-A	0.108	0.106	0.002	0.018	16.63
Got-B	0.365	0.365	0.000	0.000	1366.65
Mdh-A	0.005	0.005	0.000	0.000	158.88
Mdh-C	0.168	0.167	0.001	0.006	35.99
Pgd	0.268	0.267	0.001	0.004	98.62
Shdh-A	0.500	0.489	0.011	0.022	11.05
Shdh-B	0.125	0.122	0.003	0.027	11.71
Mean	0.231	0.224	0.007	0.021	8.11

TABLE 6. Nei's measures of genetic similarity (*I*) (above diagonal) and genetic distance (*D*) (below diagonal) for populations of *P. mugo*

Population	WPM	WM	DK
WPM	-	0.994	0.984
WM	0.006	-	0.982
DK	0.016	0.018	-

population: ~61% of the analyzed samples were clones. The result was similar in the DK population, where clones constituted 57.66% of the collected samples. The fraction was lower (26.74%) in the WPM population. This means that ~48% of the individuals formed clones in the studied populations. Dwarf mountain pine growing in its continuous range had lower percentages of clones: 9.7% on limestone substrate, and 34.3% on granite (Prus-Głowacki, unpublished data). The higher representation of clones on the peatbog stands may reflect more advantageous conditions for vegetative reproduction: high soil moisture and a thicker peat layer, both of which promote root development from offshoots. Moreover, these populations may be very old, representing a relict of the glacial epoch (Obidowicz, 1996a,b). Some individuals may be several hundred or even a thousand years old, sufficient time for vegetative propagation and the formation of large clones. An example of such a clonal structure of *P. mugo* in the Tatra Mts is the presence of only two genotypes (clones) in a peatbog population below Ostry Wierch Mt. (Bączkiewicz and Prus-Głowacki, 1997).

Peatbog populations of *P. mugo* exhibited lower allozyme variability in the number of alleles per locus (A/L = 2.2) than did populations from the continuous range of the species (A/L = 3.0) (Prus-Głowacki et al., 1998). This difference may be the result of the genetic isolation and small effective population size of the peatbog populations, leading to inbreeding and genetic drift. The largest population, DK, proved to be the most variable: all the studied loci in that population were polymorphic. Four monomorphic loci (*Dia*, *Got-A*, *Mdh-A*, *Shdh-B*) and no rare alleles were found in the WM population. In a study of 6 populations growing above the treeline (continuous range), Prus-Głowacki et al. (1998) found that 2 populations showed polymorphism at all examined loci, 2 populations contained one monomorphic locus, and 2 populations contained two monomorphic genes. The same loci that Prus-Glowacki et al. (1998) and Odrzykoski (2002) found to be monomorphic (*Mdh-A*, *Got-A*, *Dia*) were also monomorphic in the studied peatbog populations.

The DK population was the most diverse in terms of the total number of alleles and the number of genotypes per locus. The lowest number of genotypes and alleles was found in the sample from WM, possibly reflecting the small size of that population (Tab. 3). The mean number of alleles per locus (A/L) was 2.2 (1.7 in WM, 2.7 in DK). In populations growing in the continuous range of dwarf mountain pine the mean number of alleles per locus was higher, ranging between 2.7 and 3.2 (Prus-Głowacki et al., 1998). Apparently the peatbog populations manifest an impoverished gene pool. The differences in the frequency of alleles concern mainly the rare alleles, which are much less represented in the peatbog populations then in those from the continuous range. The exception was the rare allele *Got-A* 3, which was relatively frequent (0.093 and 0.051) at two sites located far from each other (WPM, DK). Adaptation of *P. mugo* to the conditions of the peatbog biotope may involve directional selection favoring specific genotypes, and can lead to genetic uniformity. The WPM and WM populations exhibited high genetic similarity. Nei's genetic distance between the two populations was D = 0 .006 (Tab. 6). On the other hand, the populations are close to each other and may have constituted a single unit in the past. After the last glacier receded, *P. mugo* was common in the lower parts of mountains (Obidowicz, 1996a,b). With the warming of the climate, the occurrence of the species at such elevations was restricted to peatbogs, representing relicts of the glacial epoch (Christensen, 1987). From this point of view, the private alleles may represent the remnants of the primary gene pool. It cannot be ruled out that the unique genetic structure of the populations resulted from the founder effect. The WPM peatbog, however, is relatively young, not more than 4500 years old (Obidowicz, 1973, 1996a), so the presence of dwarf mountain pine there may be due to migration of seeds originating from the high mountains.

The H_o value from WM (0.148), lower than from populations from the continuous range of dwarf mountain pine (0.250) (Prus-Głowacki et al. 1998), may reflect selection linked to specific environmental conditions, or the founder effect, or finally to small effective population size. For the studied peatbog populations the average observed heterozygosity was H_o = 0.203. The level of heterozygosity in *Pinus sylvestris*, a species related to *P. mugo*, ranges from a few percent to around 60% for particular loci, but on average does not exceed 30% (Filppula et al., 1992; Prus-Głowacki et al., 1998; Prus-Głowacki and Stephan, 1994; Lewandowski et al., 2000). The studied populations were close to Hardy-Weinberg equilibrium, with a slight excess of homozygotes in the WPM and WM populations (F = 0.037 and 0.049) and small excess of heterozygosity (F = –0.037 in DK) (Tab. 3). The level of inbreeding in dwarf mountain pine is thought to be low (Filppula et al., 1992). The small population size of the studied stands may have induced inbreeding, but such a pattern is not visible in these peatbog populations.

In coniferous trees, most isoenzymatic variation has been observed within populations, and only a small fraction of it represents inter-population variability. For example, in *P. sylvestris* populations the G_{ST} coefficient was found to vary from ~2.5% to 7.6%, and D_{ST} to average ~2% (Gullberg et al., 1985; Prus-Głowacki et al., 1993; Prus-Głowacki and Stephan, 1994; Prus-Głowacki and Bernard 1994; Prus-Głowacki et al., 1998; Goncharenko et al., 1994). In populations from the continuous range of *P. mugo*, G_{ST} was rather low at ~2.9% (Prus-Głowacki et al., 1998; Odrzykoski, 2002); it was also low at 2.1% for the studied sites. This

could be an effect of harsh environmental conditions exerting selection pressure and unifying the genetic composition of these peatbog populations. Isolated populations of other conifer species demonstrate higher inter-population variability: for example, for *P. muricata* G_{ST} = 14% (Millar, 1983), and for *P. sylvestris* 4.0% (Prus-Głowacki and Stephan, 1994) and 7.0% (Prus-Głowacki and Bernard, 1994). The studied populations exhibited a degree of genetic isolation. Gene exchange between these *P. mugo* populations and the main range is probably restricted. Gene flow is lower for the three *P. mugo* populations (Nm = 8.1) than the average for coniferous anemogamous species (Nm = 14.7) (Prus-Głowacki et al., 1998).

The study revealed a higher percentage of clones and lower genetic variability in the peatbog populations than in populations from the continuous range. This pattern of genetic structure is a consequence of specific environmental conditions, isolation, and small population size.

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