

**ISOZYME DIFFERENTIATION OF *PINUS MUGO* TURRA AND *PINUS*
 × *PSEUDOPUMILIO* (WILLK.) BECK IN THE GIANT MOUNTAINS
 AND IN THE BOHEMIAN FOREST, CZECH REPUBLIC ¹**

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ABSTRACT

The genetic structure of two genera from the *Pinus mugo* complex was studied based on starch gel electrophoresis of dormant buds. In total 7 populations have been scored. Three subpopulations of *Pinus mugo* Turra originating from the Giant Mountains (Krkonoše Mts. – Northern part of the Czech Republic) and four subpopulations of *Pinus* × *pseudopumilio* (Willk.) Beck (a stabilized hybrid between *P. mugo* Turra and *P. rotundata* Link) originating from the Bohemian Forest peat bogs (Šumava Mts. – Southwestern part of the Czech Republic), in particular. The genotypic frequencies at 16 polymorphic enzyme loci of MDH, PGM, SKDH, LAP, FEST, PEROX, DIA, GOT, PEP, and GDH were scored. After our results *P. mugo* and *P. × pseudopumilio* are clustered into two clearly divorced groups, corresponding with the taxonomic status of these species and/or subspecies and geographic site. The *Got-A₁* allele was observed with low frequency in *P. mugo*. The *Lap-A1* occurred with relative high frequency in hybrid *P. × pseudopumilio*. Occurrence of some *Pgm*, *Skdh-A*, *Mdh-C*, *Fest-A* and *Got-B* alleles might be considered more like site-specific than species-specific.

Key words: Czech Republic, *Pinus mugo*, *Pinus* × *pseudopumilio*, isozymes, differentiation, genetic diversity

INTRODUCTION

The genus *Pinus* is one of the most widely distributed genera of conifers not only across Europe but also all over the Northern Hemisphere (PRAVDIN 1964, CRITCHFIELD & LITTLE 1966). Within distribution of Scots pine, it can grow in wide range of different ecological conditions, including extreme ones such as tundra, bogs, steppes, and mountains. Such a wide range of ecological conditions could favour the formation of variety ecotypes (GONCHARENKO *et al.* 1994). Pines covered only 3.4 % of the Czech Republic – in natural stage of tree species composition. Total amount of *Pinus* in the Czech Republic had increased during 18th century, especially due to artificial reforestation (SKALICKÝ 1988). Thus actual stage is even 17.5 % of forestland resources (COLLECTIVE 2000). It must be noted that the mentioned value did not represent only from the economic point of view the most important Scots pine (*Pinus sylvestris*) but also another species, including *P. mugo* and closely related species, subspecies and their hybrids. Eco-

logical resilience of pine species enables them to occupy many kinds of habitats. Following its behaviour a huge morphological variability is not surprising. Different morphological varieties of *Pinus* (*sensu lato*) are projecting into formation of specific ecotypes and so into many cases of taxonomical problems. In this paper, the classification of BUSINSKÝ (1998) is followed.

Introgressive hybridisation of pines has been described by many authors not only from the Czech Republic (BUSINSKÝ 1998, BUSINSKÝ & WEGER 1995, HOLUBIČKOVÁ 1965) but also from many other countries such as the countries of former Soviet Union (POLITOV *et al.* 1999, GONCHARENKO *et al.* 1995), Poland (SIEDLEWSKA & PRUS-GŁOWACKI 1995, BACZKIEWICZ & PRUS-GŁOWACKI 1997), Slovakia (KORMUŤÁK 1990, STASZKIEWICZ 1993), Switzerland (NEET-SARQUEDA 1994), Germany (SCHMID 2000), and Denmark (CHRISTENSEN 1987). On the other hand some authors do not agree with the previously described existence of introgressive hybrids and rejected the primary hypothesis on the ground of molecular markers (FILPPULA *et al.*

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1992, NEET-SARQUEDA 1994).

Occurrence of *Pinus sylvestris* L., *P. rotundata* Link, *P. mugo* Turra, and their hybrids *P.* × *pseudopumilio* (Willk.) Beck (*P. mugo* s.str. × *P. rotundata*), *Pinus celakovskiorum* A. et Gr. (*P. mugo* s.str. × *P. sylvestris*) – this hybrid is also known as *P.* × *rhaetica* Brøger (see CHRISTENSEN 1987 or STASZKIEWICZ 1993), *Pinus* × *digenea* Beck (*P. rotundata* × *P. sylvestris*), and also three-hybrid combination of *P. mugo* × *P. rotundata* × *P. sylvestris* was verified in the Bohemian Forest (BUSINSKÝ 1998). *Pinus uncinata* Ramond is not known not only from the Bohemian Forest but also from the whole Czech Republic and all cases when *P. uncinata* was mentioned were mistakes (BUSINSKÝ 1998).

All above-mentioned examples of pure pine species and its interspecific hybrids in the Bohemian Forest were described only by the morphological surveys. So the main aim of our research is to contribute to taxonomy of this species by using molecular gene markers – isozymes. First step of our project is the evaluation of genetic differentiation between most common pine on Bohemian Forest peat bogs *Pinus* × *pseudopumilio* (for detail description of this stabilized hybrid see JENÍK & SOUKUPOVÁ 1999) and *Pinus mugo* Turra.

Our research started in second half of the year 2001 in small electrophoretic laboratory by ěumava national park and should it be underlined that results presented in this paper must be considered as very first and preliminary only.

MATERIAL AND METHODS

Sampled populations

Dwarf mountain pine *P. mugo* Turra in the Bohemian Forest grows only on small and isolated rock islands (summits, scree, and cirques of the glacial lakes, particularly). Within the conditions of Czech Republic, the mountain range of the Giant Moun-

tains (Krkonoše Mts.) with the highest peak of the Czech Republic (Snežka Mt., 1602 m) is the typical example of area with indigenous *P. mugo* Turra. The area is also known for its tundra ecosystem above upper timber line (SOUKUPOVÁ *et al.* 1995) and cover of dwarf mountain pine *P. mugo* reaching over 2,000 hectares. That is why we sampled this material as *P. mugo* example rather than the Bohemian Forest ones. Populations of *Pinus* × *pseudopumilio* were collected in Bohemian Forest peat lands.

In total 269 individual trees from 7 populations were scored. Minimum distance between each plant was approximately 70 meters. All samples were taken during the dormant period, November 2001 and March 2002, particularly. Sampled trees were marked in the field and most of them also localized by the Global Positioning System (GPS). Branches with dormant buds were stored at refrigerator no longer than 1 month under –18 °C till analysis. For details and better orientation of populations assayed see Table 1 and Figure 1.

Isozyme analysis

Extracts for electrophoretic separation were prepared by homogenizing of diploid tissue of dormant buds. Methods of extraction followed CONKLE *et al.* (1982) and CHELIAK & PITEL (1984). Electrophoresis was carried out in horizontal chambers on 12–13 % starch gel made in Sigma. Histochemical enzyme staining of 11 enzyme systems was performed according to standard methods (CONKLE *et al.* 1982, CHELIAK & PITEL 1984, and MANCHENKO 1994) with some modifications. Zymogram explication followed RUDIN (1975, 1977), GULLBERG *et al.* (1985), PUGLISI & ATTOLICO (2000), and KONNERT (pers. comm.). The enzymes assayed, their abbreviations, the buffer systems upon which they run, and the numbers of scorable loci are listed in Table 2. For electrophoresis were used these buffers: A – continuous Tris-citrate pH 7.4 (KONNERT & MAURER 1995), B – discontinuous lithium borate/

Table 1. Geographic origin of subpopulations investigated.

Locations	Number of scored trees	Latitude (N)	Longitude (E)	Altitude (m a. s. l.)	Region
GM – 1	40	50°45'35"	15°32'39"	1320	The Giant Mountains
GM – 2	40	50°45'53"	15°32'40"	1270	The Giant Mountains
GM – 3	40	50°46'43"	15°32'53"	1390	The Giant Mountains
BF – 1	44	48°59'58"	13°39'30"	920	The Bohemian Forest
BF – 2	38	49°01'15"	13°31'40"	1120	The Bohemian Forest
BF – 3	25	49°04'10"	13°33'45"	1100	The Bohemian Forest
BF – 4	42	49°03'10"	13°36'30"	1060	The Bohemian Forest

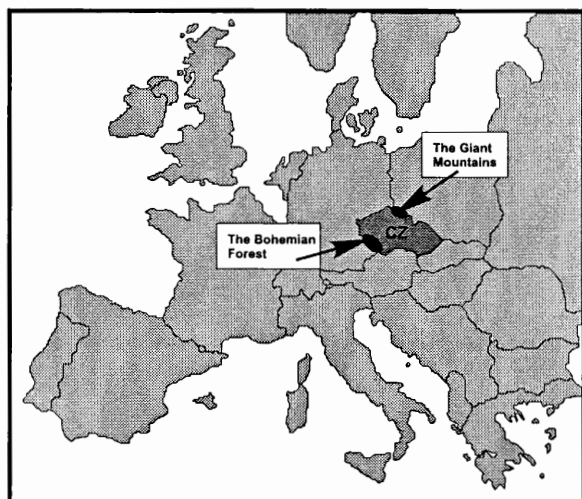


Figure 1. Location of region surveyed in the European context.

Tris-citrate pH 8.1/8.1 and C – discontinuous sodium borate/Tris-citrate pH 8.0/8.7 (POULIK 1957).

Enzymes of *P. mugo* and its hybrid *P. × pseudopumilio* have the same migrates rate. As for numbering of alleles scored they were indexed and so on. Within each enzyme systems the most anodally migrating loci was designated A, and slower loci according to the relative electrophoretic mobility, when the fastest allele was signed by 1, slower by 2 B, C etc.

Statistical analysis

Designation of genetic diversity, and differentiation values resulting from allele frequencies were performed with BIOSYS-1 software (SWOFFORD & SELANDER 1989). The average number of alleles per locus, percentage of polymorphic loci, expected

heterozygozity, observed heterozygozity as parameters of genetic structure were computed. Wright's fixation index (WRIGHT 1922) was computed in order to compare observed heterozygosities with panmictic model. Wright's F-statistic and Nei's G-statistic were computed using the statistic programme FSTAT (GOUDET 2001). Values of Nei's genetic distance were applied for dendrogram construction using the UPGMA method (SNEATH & SOKAL 1973).

RESULTS AND DISCUSSION

Genetic variation of 7 populations from two regions of the Czech Republic was studied. Some enzyme systems were difficult to score because of very faint banding patterns that could not be successfully interpreted (G-6-PDH, 6-P-GDH), similarly some loci of otherwise scored enzyme systems (*Got-C*, *Px-B*, *C*, *Fest-B*, *Dia-A*). Such enzymes and/or loci were excluded from the analysis. Also monomorphic IDH with only one observed allele was not taken into account. Nevertheless, polymorphism of 11 enzyme systems with 16 scorable loci was surveyed. In total, 52 allele variants were observed. Allelic frequencies are given in Table 5. The most variable loci in both genera, *P. mugo* and *P. × pseudopumilio*, were *Skdh-A* and *Per-A*. Intermediate level of variability appears at *Mdh-B* and *Mdh-C* and the remaining loci were slightly polymorphic. As can be seen from dendrogram of Nei's genetic distance (Fig. 2), two clearly separate clusters are perceptible.

Above all remarkable differences based on the allelic frequencies it can be noted allelic frequency of *Got-A₁* allele, which has been observed with relatively high frequency in all *P. × pseudopumilio* subpopulations, while in *P. mugo* subpopulations was found only in one case with very low frequency.

Table 2. Enzyme systems assayed, their abbreviations, Enzymes Commission codes, number of loci scored, and buffer systems used for electrophoresis.

Enzyme systeme	Abbreviation	E. C. code	Number of loci scored	Buffer
Fluorescent esterase	FEST	3.1.1.56	1	B
Diaphorase	DIA	1.6.4.3	1	B
Glutamate dehydrogenase	GDH	1.4.1.2	1	C
Glutamate oxaloacetate transaminase	GOT	2.6.1.1	2	C
Isocitrate dehydrogenase	IDH	1.1.1.42	1	A
Leucine aminopeptidase	LAP	3.4.11.1	2	B
Malate dehydrogenase	MDH	1.1.1.37	3	A
Peroxidase	PX	1.11.1.7	1	B, C
Phosphoenolpyruvate carboxylase	PEP	4.1.1.31	1	C
Phosphoglucomutase	PGM	2.7.5.1	1	A
Shikimate dehydrogenase	SKDh	1.1.1.25	2	B

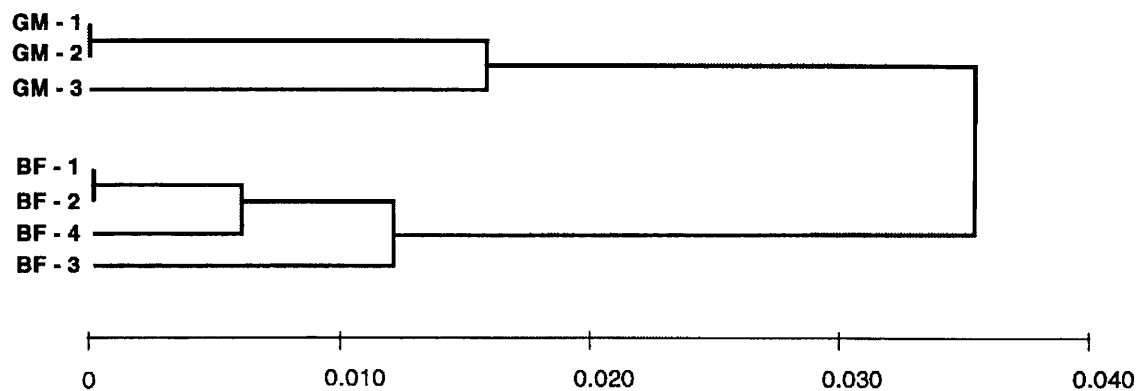


Figure 2. Dendrogram constructed on the basis of Nei's (1978) genetic distances using UPGMA method.

The low frequency of allele *Got-A₁* was observed in Polish populations (LEWANDOVSKI *et al.* 2002) and Switzerland population (NEET-SARQUEDA 1994) of *Pinus mugo*, too. Compared to other taxon of genus *Pinus*, high frequency of allele *Got-A₁* was observed in Polish populations of *Pinus sylvestris* (PRUS-GŁOWACKI & BERNARD 1994). According to fission of both species conformable results as in *Got-A₁* were observed in *Lap-A₁* allele, which was found only in *P. × pseudopumilio*. Absence or low frequency of allele *Lap-A₁* was observed in Polish and Switzerland populations of *Pinus mugo* (LEWANDOVSKI *et al.* 2002, NEET-SARQUEDA 1994). Low frequency of allele *Lap-A₁* was observed in other Eurasian taxon of genus *Pinus*, e.g. *Pinus pumila*, *Pinus sibirica* and *Pinus sylvestris* (GONCHARENKO *et al.* 1993a, GONCHARENKO *et al.* 1993b, GONCHARENKO *et al.* 1994). Differences

consist in occurrence of species-specific alleles proportions in *Skdh-A₁*, *Mdh-C₂*, *Fest-A₃* and *Got-B₂* were found in our study. Also PGM is showing the differentiation between both subspecies due to its monomorphism in all *P. mugo* subpopulations. In the study of Polish populations (LEWANDOVSKI *et al.* 2002) enzyme system PGM was observed by two loci but in our study we observed only one locus. Only one locus of system PGM was observed in population of *Pinus sibirica*, but it was not monomorphic (GONCHARENKO *et al.* 1993b). Genetic variation parameters observed in our study are summarized in Table 3.

Values of genetic variation of *P. mugo* subpopulations seem to be more balanced than those of *P. × pseudopumilio*. Especially in the Bohemian Forest BF – 2 subpopulation can be seen with some decrease of polymorphism. It may be due to

Table 3. Genetic variation in population of *P. mugo* originated in the Giant Mountains (GM) and *P. × pseudopumilio* originated in the Bohemian Forest (BF). *mSS* = mean sample size per locus; *mnA* = mean no. of alleles per locus; *pPL* = percentage of polymorphic loci; *H_o* = mean observed heterozygosity, *H_e* = mean expected heterozygosity, *F* = Wright's fixation index.

Population	<i>mSS</i>	* <i>mnA</i>	<i>pPL</i>	<i>H_o</i>	<i>H_e</i>	<i>F</i>
<i>Pinus mugo</i>						
GM – 1	39.6	2.6	81.3	0.158	0.223	0.291
GM – 2	39.7	2.6	87.5	0.211	0.230	0.083
GM – 3	39.6	2.4	75.0	0.196	0.251	0.219
Mean		2.53	81.27	0.188	0.235	0.198
<i>Pinus sylvestris</i>						
BF – 1	44.0	2.8	93.8	0.138	0.209	0.340
BF – 2	35.0	2.1	50.0	0.089	0.141	0.369
BF – 3	25.0	2.3	75.0	0.135	0.183	0.262
BF – 4	42.0	2.5	81.3	0.126	0.176	0.284
Mean		2.43	75.03	0.122	0.177	0.314

low number of trees scored, which is probably not sufficient to be representative for all gene pool. Concerning the mean numbers of alleles per locus and number of polymorphic loci no large differentiations have been found between both subspecies, but *P. mugo* subpopulations are more variable by means. Although, we analyzed only 80 trees of genus *P. mugo* it is evident that more than 81% of the loci in this species are polymorphic and the mean number of alleles per loci is greater than 2,5. The observed heterozygosity (H_o) is 0,188. Compare with Polish populations of *P. mugo* (LEWANDOVSKI *et al.* 2002) bigger variability of this taxon were observed in our study. For taxon *P. × pseudopumilio* we observed more than 75 % polymorphic loci and the mean number of alleles per loci is greater than 2.4. The observed heterozygosity is 0.122. Higher level of genetic variation in populations of *Pinus mugo* can be because of the isolation of populations of *P. × pseudopumilio*. Smaller level of genetic variability was observed in isolation populations of *Pinus sylvestris* (GONCHARENKO *et al.* 1994). As for fixation index – which are throughout positive – *P. × pseudopumilio* subpopulations are characterized by twice-higher values than those of *P. mugo*. High values of fixation index show deficiency of heterozygotes relative to expected in panmictic population. If Scots pine is partly self-pollinating species (LONGAUER *et al.* 1992, KÄRKKÄINEN *et al.* 1996) so dwarf pines must be the same.

All subpopulations of *P. × pseudopumilio* in the Bohemian Forest are characterized as more or less isolated islands in the middle of Norway spruce forests. The areas of those islands are mostly not wider than 2 hectares. Thus the loss of heterozygosity confirmed by high values of Wright's fixation indexes ($F = 0.314$) might be considered as a result of inbred depression.

The results of Wright's F-statistic and Nei's G-statistic showed a big influence in the populations, *Pinus mugo* and *Pinus × pseudopumilio* (Table 4). Even though the localities in Bohemian Forest are isolated they affected each other. F_{ST} and G_{ST} values were not high, 0.025 and 0.021, respectively. Thus

about 98 % of genetic variation resides within population and about 2 % among populations. F_{ST} enables us to estimate the level of gene flow (Nem) using formula $F_{ST} = 1/(1 + Nem)$. After our results gene flow was observed more than 9 migrant per population in the *P. × pseudopumilio*. This means that the possibilities for differentiation among these populations are small. Similar results were observed for populations of *Pinus mugo*. F_{ST} and G_{ST} values were 0.024 and 0.016, respectively. Thus almost 98 % of genetic variation consists within population and only 2 % among population. The gene flow was more than 10 migrants per population. If we compare both taxon we get values for F_{ST} and G_{ST} 0.052 and 0.046, respectively. They are higher values than in each taxon separately. However 95% of genetic variability is within populations and only 5% are due to interpopulation variation. It seems that the separation of both populations (Figure 2) is more because of geographic distance not because of the differences in genetic structure of the taxon.

The gene flow in other Eurasian species of genus *Pinus* varied from 5.56 (for *Pinus pumila*) to 18.98 (for *Pinus nigra*) and G_{ST} from 0.024 (for *Pinus nigra*) to 0.043 (for *Pinus pumila*) (GONCHARENKO *et al.* 1993a, GONCHARENKO *et al.* 1993b, GONCHARENKO *et al.* 1994, SILIN & GONCHARENKO 1996).

For values of Nei's genetic distance coefficient between subpopulations see Table 5. A cluster analysis based on NEI's (1978) unbiased genetic distances projecting into dendrogram by using UPGMA algorithm reveals a low level of genetic separation between subpopulations, but relatively huge differentiation between populations of the both, *P. mugo* and *P. × pseudopumilio*, respectively (Figure 2). The values of coefficient among the populations of *Pinus mugo* ranged from 0.003 to 0.013, averaging 0,008; of *Pinus × pseudopumilio* from 0.003 to 0.01, averaging 0.007. Coefficient compare both taxon ranged from 0.014 to 0.035. The average value of Nei's genetic distance coefficient in other species of genus *Pinus* varied from 0.012 (for *Pinus nigra*) to 0.022 (for *Pinus sibirica*)

Table 4. Nei's genetic distances between subpopulations surveyed.

Population	GM - 1	GM - 2	GM - 3	BF - 1	BF - 2	BF - 3	BF - 4
GM - 1	–						
GM - 2	.003	–					
GM - 3	.009	.013	–				
BF - 1	.016	.019	.033	–			
BF - 2	.014	.015	.029	.003	–		
BF - 3	.015	.014	.035	.010	.009	–	
BF - 4	.014	.016	.033	.007	.005	.008	–

Table 5. Allele frequencies at 16 analyzed enzyme loci.

Locus	Allele	GM – 1	GM – 2	GM – 3	BF – 1	BF – 2	BF – 3	BF – 4
<i>Mdh-A</i>	<i>N</i>	40	40	40	44	22	25	42
	1	.000	.013	.000	.011	.000	.000	.024
	2	1.000	.988	1.000	.989	1.000	1.000	.976
<i>Mdh-B</i>	<i>N</i>	38	39	39	44	22	25	42
	1	.197	.295	.192	.091	.114	.200	.179
	2	.724	.705	.769	.875	.886	.720	.702
<i>Mdh-C</i>	<i>N</i>	39	39	39	44	22	25	42
	1	.154	.051	.000	.068	.000	.180	.060
	2	.256	.346	.295	.045	.000	.080	.036
<i>Pgm</i>	<i>N</i>	40	40	40	44	38	25	42
	1	.000	.000	.000	.045	.000	.040	.024
	2	1.000	1.000	1.000	.909	1.000	.960	.845
<i>Skdh-A</i>	<i>N</i>	40	38	39	44	38	25	42
	1	.000	.000	.000	.125	.079	.040	.024
	2	.625	.632	.603	.818	.776	.940	.893
<i>Skdh-B</i>	<i>N</i>	40	39	40	44	38	25	42
	1	.038	.000	.050	.045	.000	.000	.048
	2	.900	.923	.813	.898	1.000	1.000	.929
<i>Lap-A</i>	<i>N</i>	40	40	40	44	38	25	42
	1	.000	.000	.000	.068	.013	.040	.000
	2	.988	.962	.975	.898	.934	.820	.952
<i>Lap-B</i>	<i>N</i>	40	40	39	44	38	25	42
	1	.013	.000	.026	.023	.000	.000	.000
	2	.962	.925	.885	.864	1.000	.980	1.000
<i>Fest-A</i>	<i>N</i>	40	40	40	44	38	25	42
	1	.162	.038	.040	.034	.000	.040	.000
	2	.713	.812	.760	.909	.908	.940	.917
<i>PxA</i>	<i>N</i>	39	40	40	44	38	25	42
	1	.026	.038	.013	.000	.039	.000	.083
	2	.051	.050	.175	.011	.026	.020	.095
	3	.077	.225	.125	.239	.211	.300	.155
	4	.603	.488	.400	.636	.513	.400	.560
	5	.179	.200	.200	.068	.066	.280	.024
	6	.064	.000	.038	.023	.092	.000	.036
<i>Dia-B</i>	<i>N</i>	40	40	40	44	38	25	42
	1	.000	.063	.050	.045	.000	.000	.060
	2	.988	.900	.950	.955	1.000	1.000	.905
	3	.012	.038	.000	.000	.000	.000	.036

Table 5. (continued).

Locus	Allele	GM - 1	GM - 2	GM - 3	BF - 1	BF - 2	BF - 3	BF - 4
<i>Got-A</i>	<i>N</i>	40	40	39	44	38	25	42
	1	.000	.039	.000	.136	.075	.120	.095
	2	0.988	.947	.987	.864	.913	.880	.905
		.013	.014	.013	.000	.012	.000	.000
<i>Got-B</i>	<i>N</i>	40	40	38	44	38	25	42
	1	.000	.025	.105	.057	.000	.080	.000
	2	.162	.162	.158	.023	.118	.100	.095
	3	.762	.788	.711	.750	.763	.780	.905
		.025	.013	.000	.000	.026	.000	.000
	.050	.013	.026	.170	.092	.040	.000	
<i>Pep</i>	<i>N</i>	40	40	40	44	38	25	42
	1	1.000	.975	1.000	.977	1.000	1.000	1.000
	2	.000	.025	.000	.023	.000	.000	.000
<i>Gdh</i>	<i>N</i>	37	40	40	44	38	25	42
	1	.095	.050	.087	.170	.105	.000	.048
	2	.905	.950	.913	.830	.895	.980	.952
	3	.000	.000	.000	.000	.000	.020	.000

(GONCHARENKO *et al.* 1993a, GONCHARENKO *et al.* 1993b, GONCHARENKO *et al.* 1994, SILIN & GONCHARENKO 1996).

CONCLUSION

Although *P. mugo* and *P. × pseudopumilio* are very closely related, the results presented in this paper shall be interpreted as significant confirmation of 2 separate groups. The separation seems to be because of geographic distance more than genetic variability. But *P. × pseudopumilio*, hybrid between *P. mugo* and *P. rotundata* a typical phenomenon of Bohemian Forest peat bogs might be considered as stable one. Nevertheless, research is on its beginning and will continue especially by investigation of *P. rotundata*, *P. sylvestris* and *P. mugo* in the Bohemian Forest region.

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