Plant Syst. Evol. 221: 15–24 (2000)

Plant Systematics and Evolution

Allozyme investigations on the genetic differentiation between closely related pines – *Pinus sylvestris*, *P. mugo*, *P. uncinata*, and *P. uliginosa* (Pinaceae)

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Received August 23, 1998
Accepted September 8, 1999

Abstract. In eight natural European populations of four closely related taxa of pines (*Pinus sylvestris*, *P. mugo*, *P. uncinata*, and *P. uliginosa*) starch-gel electrophoreses revealed altogether 58 alleles at 15 loci from nine enzyme systems. With Nei's genetic distance (D) the largest mean genetic distance (D = 0.171) was found between *P. sylvestris* and *P. mugo*, a distance corresponding to that between other closely related pine species. Mean genetic distances between the other taxa were less than half that value and characteristic for subspecies or varieties rather than for species. On the basis of our results we suggest that both, *P. uliginosa* and *P. uncinata*, could be the result of the ancient hybridization between *P. sylvestris* and *P. mugo*.

Key words: Pinaceae, *Pinus sylvestris*, *P. mugo*, *P. uncinata*, *P. uliginosa*. Allozymes, genetic distance, hybridization.

Scots pine (*Pinus sylvestris* L.) is the most widespread coniferous species of Europe and Asia. It occurs from the Atlantic coast on the West to the Pacific on the East, covering a distance of about 14000 km (Boratyński 1991). The species grows under extremely different conditions which favours the formation of local ecotypes and varieties (e.g. Giertych 1991). The range of dwarf mountain pine (*Pinus mugo* Turra) is restricted to the montane regions of Central and South Europe. (Fig. 1) The species is a characteristic component of subalpine communities, where it forms thickets above the upper forest limit, up to an altitude of about 2700 m (Meusel et al. 1965). Occasionally, it also grows on peat-bogs in the montane forest zone (Jalas and Suominen 1973). The mountain pine (*P. uncinata* Ramond ex DC.) occurs mostly in the Pyrenees and Alps (Fig. 1). It forms forests in the upper mountain forest zone at altitudes of 1400–2700 m in the Pyrenees (Franco Amaral 1986), but also grows on peat bogs as *P. mugo*. Peat-bog pine (*Pinus uliginosa* Neumann) was described from the peat bogs of the Sudety Mountains. Contradictory views exist as to the taxonomic rank of *P. uliginosa*. Previously it was treated as a "small" species or sometimes included in *P. uncinata* (e.g. Gaussen et al. 1964). After biometrical studies *P. uliginosa* was considered either as a hybrid between *P. mugo* and *P. sylvestris* (Staszkiewicz and Tyszkiewicz 1969, Staszkiewicz and Tyszkiewicz 1972), or a hybrid between *P. mugo* and *P. uncinata* (Holubičkova 1965). These different views on the systematic position of this taxon stimulated numerous detailed investigations on its vari-

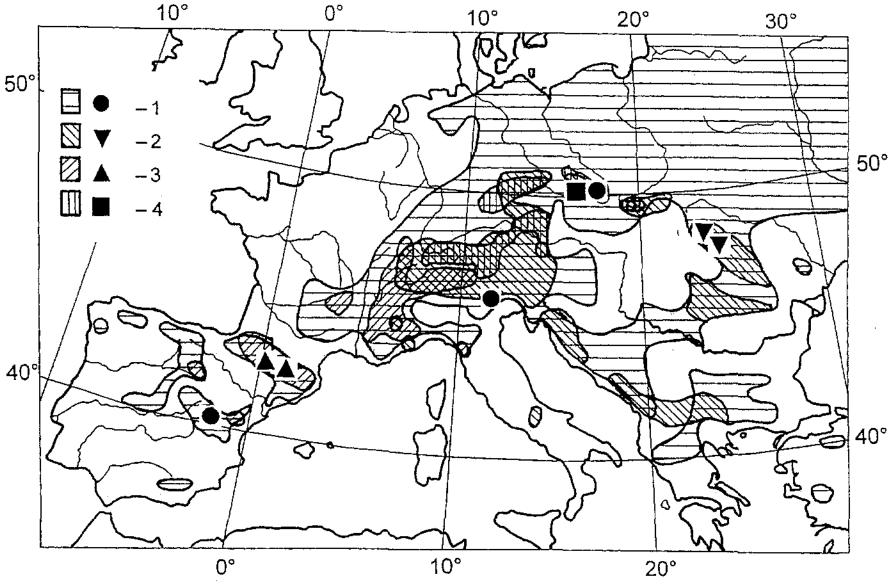

Fig. 1. Geographic location of the investigated populations of *Pinus sylvestris* (1), *P. mugo* (2), *P. uncinata* (3) and *P. uliginosa* (4) against the background of taxon ranges

ability (Holubičkova 1965, Marcet 1967, Staszkiewicz and Tyszkiewicz 1969, Szweykowski 1969, Krzakowa et al. 1984, Prus-Głowacki and Szweykowski 1983, Siedlewska and Prus-Głowacki 1995, Christensen and Dar 1997, Lauranson-Broyer et al. 1997).

In spite of all these studies on natural and anthropogenic populations of *P. uliginosa*, *P. uncinata*, *P. mugo* and *P. sylvestris* their systematic position is still not settled. Some authors are of the opinion that *P. mugo* and *P. uncinata* are two subspecies of *P. mugo s.l.*. According to Christensen (1987) *P. uncinata* is a western subspecies of *P. mugo* (= *P. mugo* subsp. *uncinata*), and typical *P. mugo* (= *P. mugo* subsp. *mugo*) is an eastern subspecies. Following this point of view *P. uliginosa* is a hybrid between the two above-mentioned subspecies of *P. mugo* (= *P. mugo* nothosubsp. *uliginosa*). However, hybrids between *P. sylvestris* and *P. mugo* subsp. *mugo* or *P. sylvestris* and *P. mugo* subsp. *uncinata*, known as *P. rhaetica* Brügger, are similar to *P. uliginosa* and sometimes indistinguishable (Staszkiewicz and Tyszkiewicz 1972). *P. uncinata* is also intermediate between *P. mugo* subsp. *mugo* and *P. sylvestris*, and the two last taxa sometimes are considered to be the ancient parents of *P. uncinata* (Gams 1928/29, Holubičkova 1965, Staszkiewicz and Tyszkiewicz 1972). Hybridization between *P. mugo* subsp. *mugo* and *P. sylvestris* was supposed to be quite frequent in the Alps and Carpathians (Marcet 1967, Staszkiewicz and Tyszkiewicz 1969, Szweykowski and Bobowicz 1983). However, according to others real hybrids are not frequent in natural mixed stands of these taxa (Christensen 1987, Filppula et al. 1992, Neet-Sarqueda 1994).

The aim of our present study was 1) to determine the level of genetic differentiation and clarify the phylogenetic relationships between the closely related *P. sylvestris*, *P. mugo* and *P. uncinata* on the basis of analysis of 15 allozyme loci, and 2) to contribute to the taxonomic position of the controversial *P. uliginosa*.

Material and methods

The present study is based on seeds from 72 *Pinus sylvestris*, 42 *P. uncinata* and 78 *P. mugo* individuals. Dormant winter buds from 47 trees of *P. uliginosa* were collected on the locus classicus in Batorów (Poland). The geographical origins of the investigated populations are described in Table 1 and shown in Fig. 1. In previous studies of the variability of these pines mostly Central European populations were usually considered.

Table 1. Origin and size of samples used for allozyme analyses

Taxa	Pop. No.	Abbr.	Total no. of trees	Origin
Pinus sylvestris				
	1	(Sie)	20	Sierra de Guadarrama, Spain
	2	(Mor)	28	Morter, Italy
	3	(Szcz)	24	Szczeliniec, Poland
Pinus uncinata				
	4	(Bar)	15	Barranco de Vallibierna, Spain
	5	(Tos)	27	Tossal de l'Orri de Rubio, Spain
Pinus uliginosa				
	6	(Bat)	47	Batorów, Poland
Pinus mugo				
	7	(CzA)	39	Czarnohora A, Ukraine
	8	(CzB)	39	Czarnohora B, Ukraine

We have collected material from the most distant populations of *P. mugo* s. str. and *P. uncinata* within our reach in order to exclude the influence of hybrid contacts. Populations of *P. sylvestris* were taken not only from Central Europe, but also from the Iberian Peninsula and the Italian Alps.

Individual trees were analysed using 8 to 10 macrogametophytes or, in case of *P. uliginosa*, extracts from two buds. The following 9 enzyme systems encoded by 15 loci were studied (Enzyme Commission number and locus abbreviations in parentheses): fluorescent esterase (EC 3.1.1.2, *Fle*), glutamate dehydrogenase (EC 1.4.1.2. *Gdh*), glutamate oxalo-acetate transaminase (EC 2.6.1.1, *Got 1*, *Got 2*, *Got 3*), isocitrate dehydrogenase (EC 1.1.1.42, *Idh*), leucine aminopeptidase (EC 3.4.11.1, *Lap 1*, *Lap 2*), malate dehydrogenase (EC 1.1.1.37, *Mdh 1*, *Mdh 3*), 6-phosphogluconate dehydrogenase (EC 1.1.1.44, *6Pgd 1*, *6Pgd 2*), phosphoglucomutase (EC 2.7.5.1, *Pgm 1*, *Pgm2*), superoxide dismutase (EC 1.15.1, *Sod 1*). The separation of isoenzymes on starch gels and the genetic interpretation of the results were performed as described by Rudin and Ekberg (1978), Szmidt and Yazdani (1984) and Goncharenko et al. (1994). Alleles at each locus were numbered according to the electrophoretic migration of allozymes. The most anodally migrating band was named 1, the next 2, and so on.

Genetic variability was described by the average number of alleles per locus (Na), the percentage of polymorphic loci P1 (0.95% criterion), P2 (0.99% criterion) and expected heterozygosity (He) (Nei 1975). Genetic differences between populations were measured by the genetic distance index (D) of Nei (1975). D values were clustered using the unweighted pair group method (UPGMA) (Sneath and Sokal 1973).

Results

Out of 15 loci analysed in 8 populations, 13 were polymorphic in at least one population, and 2 loci were completely monomorphic (*Idh* and *Sod 1*). Fifty eight alleles were observed. Allozyme frequencies at some loci varied markedly between populations (Table 2), but the most common alleles were the same in all eight populations analysed at more than half of the loci (*Got 1*, *Got 3*, *Idh*, *Lap 2*, *Mdh 1*, *6Pgd 1*, *Pgm 1*, *Pgm 2*, *Sod 1*), independent of the investigated taxa. Differences were especially well marked at *Gdh*, *Mdh 3* and *6Pgd 2*, and manifested between populations and taxa as different combinations or frequencies of shared alleles. The frequency of allele 3 at locus *Mdh 3* was high in *Pinus sylvestris*, moderate in *P. uliginosa*, low in *P. uncinata* and very low in *P. mugo*, whereas that of allele 5 was low in *P. sylvestris*, moderate in *P. uncinata* and *P. uliginosa* and very high in *P. mugo*. Similarly, the frequency of allele 1 at *6Pgd 2* was high in *P. sylvestris*, moderate in

Table 2. Frequencies of alleles in the investigated populations

	Taxa and populations							
	Pinus sylvestris			*P. uncinata*		*P. uliginosa*	*P. mugo*	
	Sie	Mor	Szcz	Bar	Tos	Bat	CzA	CzB
Fle								
1	0.03	0	0.04	0	0	0.02	0.01	0.03
2	0.40	0.86	0.75	0.93	0.91	0.79	0.90	0.55
3	0.05	0.07	0.13	0	0.04	0.10	0.09	0.32
4	0.52	0.07	0.08	0.07	0.05	0.09	0	0.10
Gdh								
1	0.10	0.50	0.23	0.61	0.52	0.05	0.12	0.27
2	0	0	0.04	0.03	0.09	0.43	0.65	0.38
3	0.90	0.50	0.73	0.33	0.32	0.27	0.23	0.35
4	0	0	0	0.03	0.07	0.25	0	0
Got 1								
1	0	0.02	0.02	0	0	0.01	0	0
2	1	0.98	0.98	1	0.98	0.84	1	1
3	0	0	0	0	0	0.14	0	0
4	0	0	0	0	0.02	0.01	0	0
Got 2								
1	0	0	0.04	0	0	0.01	0	0
2	0	0.05	0.06	0	0.02	0	0.04	0.08
3	0.28	0.32	0.35	0.54	0.54	0.34	0.21	0.10
4	0.57	0.63	0.55	0.43	0.44	0.65	0.75	0.82
5	0.05	0	0	0	0	0	0	0
6	0	0	0	0.03	0	0	0	0
7	0.10	0	0	0	0	0	0	0
Got 3								
1	0.03	0.02	0	0	0	0	0	0
2	0.32	0.21	0.35	0.33	0.30	0.12	0.06	0.14
3	0.03	0.13	0	0.03	0	0.01	0.05	0.04
4	0.62	0.64	0.65	0.64	0.70	0.87	0.89	0.82
Idh								
1	1	1	1	1	1	1	1	1
Lap 1								
1	0	0	0	0.03	0.06	0	0	0
2	0.83	0.91	0.85	0.73	0.74	0.96	0.19	0.78
3	0.05	0	0.15	0.23	0.18	0.04	0.46	0.18
4*	0.12	0.09	0	0	0.02	0	0.35	0.04
Lap 2								
1	0.07	0	0.08	0.03	0	0	0	0.03
2	0.88	0.89	0.88	0.67	0.85	0.89	0.73	0.95
3	0.05	0.11	0.04	0.30	0.15	0.11	0.27	0.01
4	0	0	0	0	0	0	0	0.01

Table 2 (continued)

Mdh 1								
1	0	0.02	0	0	0	0.09	0	0
2	1	0.98	1	1	1	0.89	1	1
3	0	0	0	0	0	0.02	0	0
Mdh 3								
1	0	0.05	0	0	0	0	0	0
2	0.10	0.02	0	0	0	0	0	0
3	0.70	0.70	0.67	0.13	0.15	0.39	0.03	0.02
4	0	0	0	0.10	0.05	0	0	0.04
5	0.15	0.21	0.29	0.77	0.80	0.61	0.97	0.94
6	0.03	0	0	0	0	0	0	0
7*	0.02	0.02	0.04	0	0	0	0	0
6Pgd 1								
1	0.05	0.07	0.02	0.03	0	0.01	0.01	0.04
2	0	0	0	0	0.02	0.01	0.23	0.18
3	0.58	0.70	0.67	0.57	0.70	0.87	0.75	0.77
4	0.37	0.21	0.31	0.40	0.28	0.11	0.01	0.01
5	0	0.02	0	0	0	0	0	0
6Pgd 2								
1	0.80	0.68	0.73	0.30	0.30	0.32	0	0
2	0	0	0	0	0.02	0	0.03	0.01
3	0.20	0.30	0.27	0.70	0.68	0.68	0.97	0.99
4	0	0.02	0	0	0	0	0	0
Pgm 1								
1	0.03	0.14	0.06	0	0.02	0.05	0	0
2	0.95	0.86	0.92	0.97	0.96	0.95	0.97	1
3	0.02	0	0.02	0.03	0.02	0	0.03	0
Pgm 2								
1	0	0	0	0.03	0	0.33	0	0.19
2	1	1	0.98	0.90	0.96	0.63	0.82	0.68
3	0	0	0.02	0.07	0.04	0.04	0.18	0.13
Sod 1								
1	1	1	1	1	1	1	1	1

* - null allele

P. uncinata and *P. uliginosa* and absent in *P. mugo*, while that of allele 3 was low in *P. sylvestris*, moderate in *P. uncinata* and *P. uliginosa* and very high in *P. mugo*. At locus *Gdh* the most frequent in *P. sylvestris* was allele 3, in *P. uncinata* allele 1, in *P. uliginosa* and *P. mugo* allele 2. We found 10 population-specific alleles in the investigated material, but they occurred in very low frequencies and only two of them (allele 3 at *Got 1* and allele 7 at *Got 2*) reached a frequency of 10%.

A summary of measures of the genetic variability at 15 loci for the analysed groups of populations is given in Table 3. Generally, a high and similar level of allozyme variation was observed. The mean number of alleles per locus (Na) ranged from 2.4 in *P. uncinata* and *P. mugo* to 2.7 in *P. uliginosa*. The proportion of the polymorphic loci P1 and P2 ranged from 57% and 70% in *P. mugo* to 80% and 87% in *P. uliginosa*. Expected heterozygosity (He) was also high and ranged from 0.22 in *P. mugo* to 0.28 in *P. uliginosa*.

Table 3. Genetic variability at 15 loci in the investigated populations (standard errors in parentheses)

Taxa/population	Na	P1	P2	He
Pinus sylvestris				
1. Sierra	2.5	67	67	0.25 (0.06)
2. Morter	2.5	67	80	0.26 (0.05)
3. Szczeliniec	2.4	67	80	0.26 (0.05)
Mean	2.5	67	76	0.26
P. uncinata				
4. Barranco	2.3	67	73	0.27 (0.06)
5. Tossal	2.5	60	80	0.25 (0.06)
Mean	2.4	64	77	0.26
P. uliginosa				
6. Batorów	2.7	80	87	0.28 (0.05)
P. mugo				
7. Czarnohora A	2.2	53	73	0.21 (0.05)
8. Czarnohora B	2.5	60	67	0.22 (0.06)
Mean	2.4	57	70	0.22

Although our data are based on limited material, genetic distances (Table 4) clearly demonstrate that *P. sylvestris* is distinct from *P. mugo* (mean value of D = 0.171). The mean genetic distances between all the other investigated species were clearly lower, but rather similar in all pairs. *Pinus uncinata* and *P. uliginosa* had the smallest genetic distance values of the five combinations (D=0.058). Genetic distances between *P. uncinata*, *P. uliginosa* and *P. mugo* were larger and reached D = 0.073 and D = 0.065, respectively. The highest genetic distance values were observed between *P. sylvestris* and *P. uncinata* (D =0.081), and between *P. sylvestris* and *P. uliginosa* (D = 0.086). Mean genetic distances within taxa were always smaller than distances between taxa. Relationships between the analysed populations are shown in the form of a dendrogram (Fig. 2).

Discussion

Levels of genetic variation at allozyme loci found in the populations of *Pinus* species analysed in this study were high and similar to those reported earlier for other conifers (Loveless and Hamrick 1984). The mean values of genetic variation of the selected species should be considered with caution because of the small number of populations analysed. However, in the case of *P. sylvestris* parameters of genetic variation obtained in the present study are very similar to those reported in earlier studies (Gulberg et al. 1985, Mejnartowicz and Bergmann 1985, Wang et al. 1991, Goncharenko et al. 1994).

Pinus sylvestris and *P. mugo* are closely related species, but well distinguishable morphologically and biochemically. Allozymic distinctness between *P. sylvestris* and *P. mugo* has been earlier demonstrated (Prus-Głowacki and Szweykowski 1983, Filppula et al. 1992, Neet-Sarqueda 1994, Goncharenko et al. 1995,

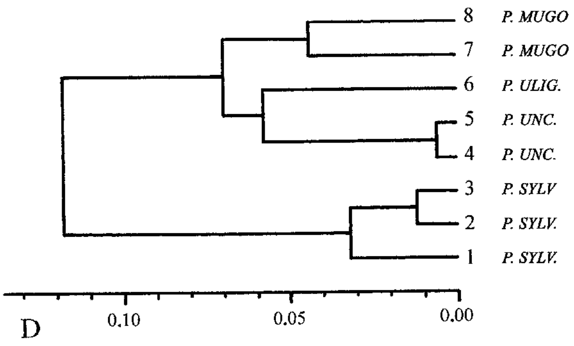

Fig. 2. UPGMA dendrogram based on Nei's genetic distances. Population numbers as in Table 1

Siedlewska and Prus-Głowacki 1995). Corresponding to our results, Nei's genetic distances between the analysed populations of these two species usually ranged rom 0.1 to 0.2, conforming to genetic distances between other closely related pine species (Miller et al. 1988, Wang et al. 1990, Goncharenko et al. 1995). The mean genetic distances between each pair of the other taxa analysed in the present study were less than half as low, within a range characteristic for subspecies or varieties rather than for species (Table 4).

The majority of authors has assumed that *Pinus uliginosa* results from hybridization of *P. sylvestris* and *P. mugo* (Staszkiewicz and Tyszkiewicz 1969, Staszkiewicz and Tyszkiewicz 1972, Krzakowa et al. 1984, Lauranson-Broyer et al. 1997). In this respect, the distribution of alleles in two loci (*Mdh 3* and *6Pgd 2*) appears interesting. The most common allele in locus *Mdh3* (designated as 5) occurred in *P. mugo* with a high (mean 0.96) and in *P. sylvestris* with a low (mean 0.22) frequency, at locus *6Pgd 2* allele 3 with a frequency of 0.98 in *P. mugo* and 0.26 in *P. sylvestris*. These two alleles had intermediate frequencies of 0.61 and 0.68, respectively, in the analysed population of *P. uliginosa* as would be expected for a hybrid. Assuming that *P. uliginosa* is a product of natural hybridization, many of its alleles should be present in the two putative parental species. In fact, except for four alleles, all the others occurred in one or both of the putative parents, although often with different frequencies. Thus, the observed allozyme composition of *P. uliginosa* could be extracted from the allozyme polymorphism available in *P. sylvestris* and *P. mugo*. If *P. uliginosa* is really a hybrid, it should be expected to be intermediate between *P. sylvestris* and *P. mugo* also in genetic distances. The largest genetic distance (D = 0.171) was found between *P. mugo* and *P. sylvestris*. The distance between *P. uliginosa* and its two hypothetic putative parents was half as large and nearly equal (0.065 between *P. uliginosa* and *P. mugo*, and 0.086 between *P. uliginosa* and *P. sylvestris*). There is mounting biochemical and molecular evidence that evolution of at least some conifer species has been affected by the process of hybridization (Wheeler and Guries 1987, Wagner et al. 1987, Szmidt et al. 1988, Wang et al. 1990). The population of *P. uliginosa* from Batorów has also been analysed with respect to isoenzymes by Siedlewska and Prus-Głowacki (1995), and neither confirmed nor excluded the hybrid origin of *P. uliginosa*. Similar to earlier morphological and biochemical evidence (Szwey-

Table 4. Matrix of Nei's genetic distance coefficients between the studied populations (above diagonal) and the taxa (below digonal)

Taxa/populations	*P. sylvestris*			*P. uncinata*		*P. uliginosa*	*P. mugo*	
	1	2	3	4	5	6	7	8
Pinus sylvestris								
1. Sierra	–	0.041	0.022	0.124	0.115	0.117	0.241	0.175
2. Morter		–	0.012	0.065	0.056	0.070	0.178	0.128
3. Szczeliniec		0.025	–	0.069	0.059	0.071	0.177	0.129
P. uncinata								
4. Barranco				–	0.006	0.068	0.089	0.072
5. Tossal		0.081		0.006	–	0.048	0.076	0.054
P.uliginosa								
6. Batorów		0.086		0.058		–	0.085	0.044
P. mugo								
7. Czarnohora A							–	0.044
8. Czarnohora B		0.171		0.073		0.065		–

kowski 1969, Prus-Głowacki and Szweykowski 1983, Siedlewska and Prus-Głowacki 1995), our investigations of *P. uliginosa* from Batorów also suggest closer relationships to *P. mugo* than to *P. sylvestris*, probably as a result of longer contact of *P. uliginosa* with *P. mugo* than with *P. sylvestris*. The relationships between these three taxa are clearly demonstrated by the dendrogram constructed on the basis of Nei's genetic distances (Fig. 2).

Another taxonomic problem concerns *P. uncinata*. Christensen and Dar (1997) include this species in *P. mugo s.l.* as a western subspecies. But our investigations show that *P. uncinata* has an intermediate position between *P. sylvestris* and *P. mugo*, like *P. uliginosa*. The mean genetic distance between *P. uncinata* and *P. mugo* (D = 0.073) is only slightly smaller than the distance between *P. uncinata* and *P. sylvestris* (D = 0.081). The mean genetic distance which separates the investigated populations of *P. uncinata* and *P. mugo* (D = 0.073) is even slightly larger than the distance between *P. mugo* and *P. uliginosa* (D = 0.065). So called *P. uncinata* from the Swiss Alps may be more closely related to *P. mugo* (Neet-Sarqueda 1994) according to one population of *P. mugo* analysed.

If we approve a hypothesis that *P. uliginosa* is probably a taxon of hybrid origin, *P. uncinata* could also be derived from ancient hybridization of *P. sylvestris* and *P. mugo*, as was suggested by Gams (1928/29), and Staszkiewicz and Tyszkiewicz (1972). In that case *P. uncinata* and *P. uliginosa* could have been formed independently as an effect of gene flow between different populations of *P. mugo* and *P. sylvestris* or could have originated at one place and then dispersed in favourable periods of the Pleistocene. However, because of the restricted material we cannot confirm any of the above-described hypotheses. Krzakowa et al. (1984) suggest that the population of *P. uliginosa* from Batorów could be a marginal population of *P. uncinata* colonizing peatbogs. Staszkiewicz and Tyszkiewicz (1972) are of a similar opinion and believe that *P. uliginosa* from Poland is a synonym of *P. uncinata* ssp. *rotundata*. Some results of our study agree with this hypothesis. Among all the mean values of genetic distances calculated by us, the distance between *P. uncinata* and *P. uliginosa* (D = 0.058) has the lowest value (Table 4), in spite of the marked geographical separation of these populations (Fig. 1). Furthermore, two alleles marked as 4 at *Gdh* and *Got 1* were found only in *P. uncinata* and *P. uliginosa*.

It seems that, if *P. uncinata* was formed with participation of *P. sylvestris* genes, the relic populations of this species from Spain should be rather excluded from this process. The genetic distances between population of *P. sylvestris* from Sierra de Guadarrama in Spain and populations of *P. uncinata* from Spanish Pyrenees (Barranco de Vallibierna and Tossal de l'Orri de Rubio) were high (D = 0.124 and D = 0.115 respectively), and are considerably higher than the mean genetic distances between the remaining two populations of *P. sylvestris* (Morter from the Italian Alps and Szczeliniec from the Polish Sudety Mts.) and *P. uliginosa*. The isoenzymatically distinct character of the Spanish populations of *P. sylvestris* was also described by Prus-Głowacki and Stephan (1994). There is an opinion that they originated from the Tertiary period (Mirov 1967), and did not take part in reforestation of Europe after the last glaciation (Prus-Głowacki and Stephan 1994).

Because of the small number of populations studied, our considerations should be treated with caution. Isozyme markers probably can not resolve beyond doubt the suggestion that *P. uliginosa* and *P. uncinata* are the result of an ancient hybridization of *P. mugo* and *P. sylvestris*, since the two putative parental species share most alleles. However, we hope that isoenzymatic investigations on a larger number of populations, connected with DNA analyses, will clarify the phylogenetic relationships between the *Pinus* taxa analysed in this paper. Nevertheless, selection of populations for such future studies will be very important. They should be chosen on the basis of taxa-specific morphological characters and should be spatially well isolated.

We thank J. Kozłowska and M. Ratajczak for technical assistance. Dr. J.M. Montserrat from Institute of Botany from Barcelona (Spain) and Dr. J. Petrovich Didukh from Institute of Botany from Kiev (Ukraine) helped collecting the plant material. Valuable comments from the two anonymous reviewers are also acknowledged.

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