IMMUNOCHEMICAL AND ISOENZYMATIC CHARACTERIZATION OF HYBRIDS FROM CONTROLLED CROSSES BETWEEN *PINUS MONTANA* VAR. *ROSTRATA* AND *PINUS SYLVESTRIS*

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ABSTRACT

Inheritance of antigenic proteins and alloenzymes was studied in controlled crosses between *Pinus montana* var. *rostrata* and *P. sylvestris*. Hybrids exhibited varying degrees of similarity to parents. Some of progenies were similar to *P. montana* var. *rostrata* exhibiting maternal patterns of inheritance, some were similar to *P. sylvestris* showing patroclinal inheritance. The remaining hybrids were intermediate. Analyses of alloenzyme inheritance showed deviation from Mendelian segregation. In some crosses an excess of heterozygotes was observed. Both immunochemical and alloenzyme electrophoresis analyses revealed the presence of „novel" proteins in hybrids not present in the parents. Possible explanations of this phenomenon are discussed.

Key words: *Pinus montana* var. *rostrata, P. sylvestris*, hybrids, antigenic proteins, alloenzymes, inheritance

INTRODUCTION

Two closely related pine species, *Pinus montana* Mill. (taxon of the *P. mugo* complex) and *P. sylvestris* L., occur sympatrically in European mountains from the Pyrenees through the Alps to the Carpathians. Putative hybrids of these two species are found in form of hybrid swarms (MARCET 1967, STASZKIEWICZ & TYSZKIEWICZ 1972). Individuals of these populations are morphologically very variable and in some characteristics intermediate between *P. montana* and *P. sylvestris*, for some traits hybrids resemble one of the parental species.

Usually the hybrid swarms inhabit ecological niches, where „pure species" cannot survive, such as peat bogs and wet soils (SZWEYKOWSKI 1969, CHRISTENSEN 1987a, 1987b, NEET-SARQUEDA *et al.* 1988, STASZKIEWICZ 1993).

Hybridization and introgression are among the most important speciation processes leading to the formation of new taxa (ANDERSON 1949, ANDERSON & STEBBINS 1954, STEBBINS 1959, 1969, LEWONTIN 1974). Therefore, hybrid swarm populations are of great interest for population geneticists and evolutionary biologists. The investigation of hybrids are also interesting from a practical point of view, because hybrids of domesticated animals or cultivated plants often possess useful traits for breeding purposes, such as resistance to diseases or hybrid vigour.

In the case of natural *P. montana* × *P. sylvestris* hybrids, many studies have been conducted on their morphological and anatomical variability, the isoenzymatic, antigenic properties of their proteins, and characteristics of chloroplast DNA (MARCET 1967, CHRISTENSEN 1987B, PRUS-GŁOWACKI & SZWEYKOWSKI 1980, 1983, PRUS-GŁOWACKI *et al.* 1978, 1981, NEET-SARQUEDA *et al.* 1988, BOBOWICZ 1990, FILPPULA *et al.* 1992, SIEDLEWSKA & PRUS-GŁOWACKI 1994).

Depending on the traits examined in these studies, evidence for hybridization between the two species has been documented. However, opinions concerning the frequency of hybridization differ. Some authors are convinced that hybridization is rare, whereas others suggest that hybridization is frequent (CHRISTENSEN 1987 B, FILPPULA *et al.* 1992, STASZKIEWICZ & TYSZKIEWICZ 1972, PRUS-GŁOWACKI & SZWEYKOWSKI 1983, SIEDLEWSKA & PRUS-GŁOWACKI 1994, 1995, DOBRINOV 1965, DOBRINOV & JAGDZIDIS 1971).

It has been proven by means of controlled crosses that hybridization does occur between these two species (DENGLER 1932, STEPHAN & PRUS-GŁOWACKI, in preparation). Therefore, it can be assumed that hybridization is possible and not so rare under natural

conditions.

The existence of hybrids from controlled crosses and the parental trees at the Institute of Forest Genetics at Grosshansdorf offered the unique opportunity for studies on the pattern of inheritance of protein markers as antigenic proteins and isoenzymes to provide a better understanding of the microevolutionary processes in natural hybrid swarm populations.

MATERIALS AND METHODS

Plant material

In Southern Germany individual trees of the mountain pine (*P. mugo* complex) with a straight stem form were collected by the former director of the Institute of Forest Genetics in Grosshansdorf, Professor W. LANGNER, in 1964 and named *P. montana* Mill. var. *rostrata* (Ant.) Hoopes. The pine material of the present study will be referred to under this name, as we are aware of the difficulty in deciding on the taxonomically correct position of this speciments in the complicated *P. mugo* complex (CHRISTENSEN 1987a). The trees were propagated by grafting. Clones were planted in the pine collection of the Institute at Grosshansdorf (northern Germany). Three clones from Ramsau, district Wimbach (Bavaria), (R1, R7, R10), and four clones from Känigseggwald (Bavaria) (K9, K11, K13, K14) of this collection were used as mother trees for controlled crosses with pollen from the two clones Schl 77/1 and Hasl E203 of *P. sylvestris* (Table 1). Controlled crosses were carried out in spring 1977, cones were collected in autumn 1978. The extracted seed was sown in spring 1981 and the seedlings were planted in a field trial with four plants per plot and two repetitions in April 1984. The hybrid families are represented with various numbers of living individuals, which were used for the present investigation. Detailed information about the results of the crossings and the performance of the hybrid families will be presented elsewhere (STEPHAN & PRUS-GŁOWACKI, in preparation).

From each of the parental clones and progenies, winter buds were collected for immunochemical and isoenzymatic analyses.

Method of antisera production and immunodiffusion

Four different antisera containing antibodies against the proteins from the vegetative tissue of parental clones of *P. sylvestris* (Schl 77/1 [S12] and Hasl E203 [S11]) and two clones of *P. montana* var. *rostrata* (K14 and R1) were produced. For obtaining antisera, rabbits of the race 'Giant Belgic' (two animals for one antiserum) were inoculated subcutaneously with 1 ml of crude protein extract once a week during a two month period (8 injections). The titres of the obtained antisera were from 1:128 to 1:256. Before analyses antibodies were isolated and purified by ammonium persulfate to obtain pure γ-globulins from the antisera, according to the procedure described by CLAUSEN (1972). Due to similar immunodiffusion patterns, which were observed with both anti-montana sera, the antibodies against these two clones were mixed together. In the case of anti-sylvestris, sera for investigating of particular crosses, homologous antisera were used. The protein extraction for immunization and immunodiffusion analyses was conducted using the same procedure; 1 g of winter buds without scales were ground in a cold (–10 °C) porcelain mortar with an addition of quartz sand and PVP 40,000 (0.2 g per 1 g of fresh weight of tissue), in a pH 7.4 extraction buffer (Tris: 10.89 g, boric acid: 16.69 g, EDTA: 1.12 g, sodium azide: 0.02 g per 1 liter of H_2O) in the ratio of 1:5 (buds : buffer).

Table 1. Clones of *Pinus montana* var. *rostrata* and *P. sylvestris*, types of crosses and number of investigated hybrids.

P. montana var. *rostrata*	*P. sylvestris*	Number of the cross	Number of tested individuals
Ramsau			
R1	Schl 77/1 (S12)	51	16
R7	Schl 77/1 (S12)	55	8
R10	Schl 77/1 (S12)	56	6
Königseggwald			
K9	Hasl E203 (S11)	57	10
K9	Schl 77/1 (S12)	58	10
K11	Schl 77/1 (S12)	60	11
K13	Hasl E203 (S11)	61	8
K13	Schl 77/1 (S12)	62	8
K14	Hasl E203 (S11)	63	20
K14	Schl 77/1 (S12)	64	19
7	2	10	116

Before extraction, 0.6 ml of 2-mercaptoethanol were added to 100 ml of buffer.

The homogenates were left for 1 h at +4 °C and then centrifugated with 10,000 rpm for 15 min with cooling. Clear supernatants were stored at –20 °C for analyses. Immunodiffusion was performed in 1.5% agarose gel containing a pH 6.7 buffer (sodium barbital 5.88 g, sodium acetate 3.88 g, HCl 5.4 ml 0.1 N in 1000 ml of H_2O). Dry immunodiffusion plates were stained by Coomassie brilliant blue R-250 using the standard procedure. The protein spectrum of each hybrid was compared side by side with the protein spectrum of each parent, and the homology of the particular protein fractions in the hybrid and parents was described according to double immunodiffusion patterns (OUCHTERLONY & NILLSSON 1973).

Similarity coefficients of the hybrids to parental forms and the hybrids to themselves were calculated according to JACCARD's formula (SNEATH & SOKAL 1973).

$$S_{jc} = \frac{a}{a+b+c}$$

where: a = antigens present in OTU1 and OTU2, b = present in OTU1, absent in OTU2, c = present in OTU2, absent in OTU1, (OTU = operational taxonomic unit; individual trees).

The homology of the proteins was denoted: 1.0 – identical antigens; 0.5 – partial identity; 0 – nonidentical protein. From S_{jc} coefficients, ANDERSON's hybridization indices were calculated (ANDERSON 1949).

Isoenzymatic method

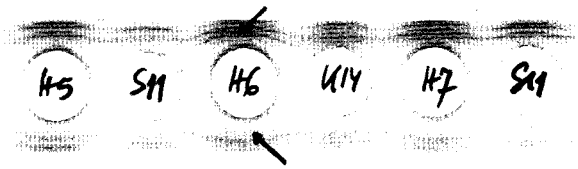
Isoenzymatic analyses were performed in horizontal starch gel electrophoresis as described previously (RUDIN & EKBERG 1978, GULLBERG *et al.* 1982, SZMIDT & YAZDANI 1984, YAZDANI *et al.* 1985). Studies of allozyme inheritance were conducted by comparing the parent genotypes with offspring genotypes. The segregation of isoenzymatic alleles and genotypic frequences were calculated and tested by the chi-square test assuming their distribution followed Mendelian inheritance patterns. The following enzymatic loci were studied: fluorescent esterase (FEST; EC 3.1.1.1), 6-phosphogluconate dehydrogenase (6-PGD; EC 1.1.1.44), shikimate dehydrogenase (2 loci) (SKDH; EC 1.1.1.25), glutamate oxaloacetate transaminase (2 loci) (GOT; EC 2.6.1.1), glutamate dehydrogenase (GDH; EC 1.4.1.2). malate dehydrogenase (2 loci) (MDH; EC 1.1.1.37) and diaphorase (DIA; EC 1.6.4.3). The alleles at particular loci were named according to their frequency from 1 to 4.

RESULTS

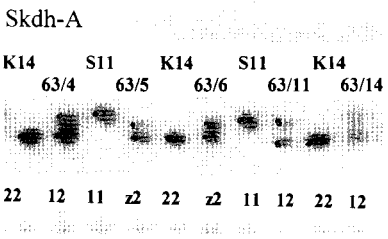
Immunodiffusion

Immunodiffusion analyses of proteins of parental trees and progenies exhibited large variation in the antigen spectra. Two of the crosses no. 57 and no. 58 were left out of from the analysis due to overlapping bands and some difficulties in the recognition antigen homology.

Depending on the antiserum and the extract from particular trees, up to 8 protein fractions can be identified. The degree of similarity based on the homology of proteins between parental forms and particular hybrids showed that there are from 1 to 3 common proteins in the parental species. In the hybrids, proteins derived from the parents and „hybrid" specific protein fractions were observed. These novel proteins were found in 31 hybrids among immunochemicaly 96 studied trees. Among crosses, the frequency of individuals possessing novel protein fractions averaged 32%, except cross 60, where all trees possessed „novel" protein fractions. Figure 1 shows the immunodiffusion plate with the patterns of precipitation lines.



A



B

Figure 1. Immunodiffusion (A) and electrophoretic (B) patterns of antigens and alloenzymes (Skdh) in parents and in hybrids. The arrows indicate with „specific" proteins of the hybrids.

There is no common pattern of inheritance of antigenic proteins among the studied crosses or within the families of the hybrids. The studied trees showed varying degrees of similarity to the parental trees *Pinus montana* or *P. sylvestris*. Figure 2 shows the diagrams of similarity of hybrids to their parents. Some of the

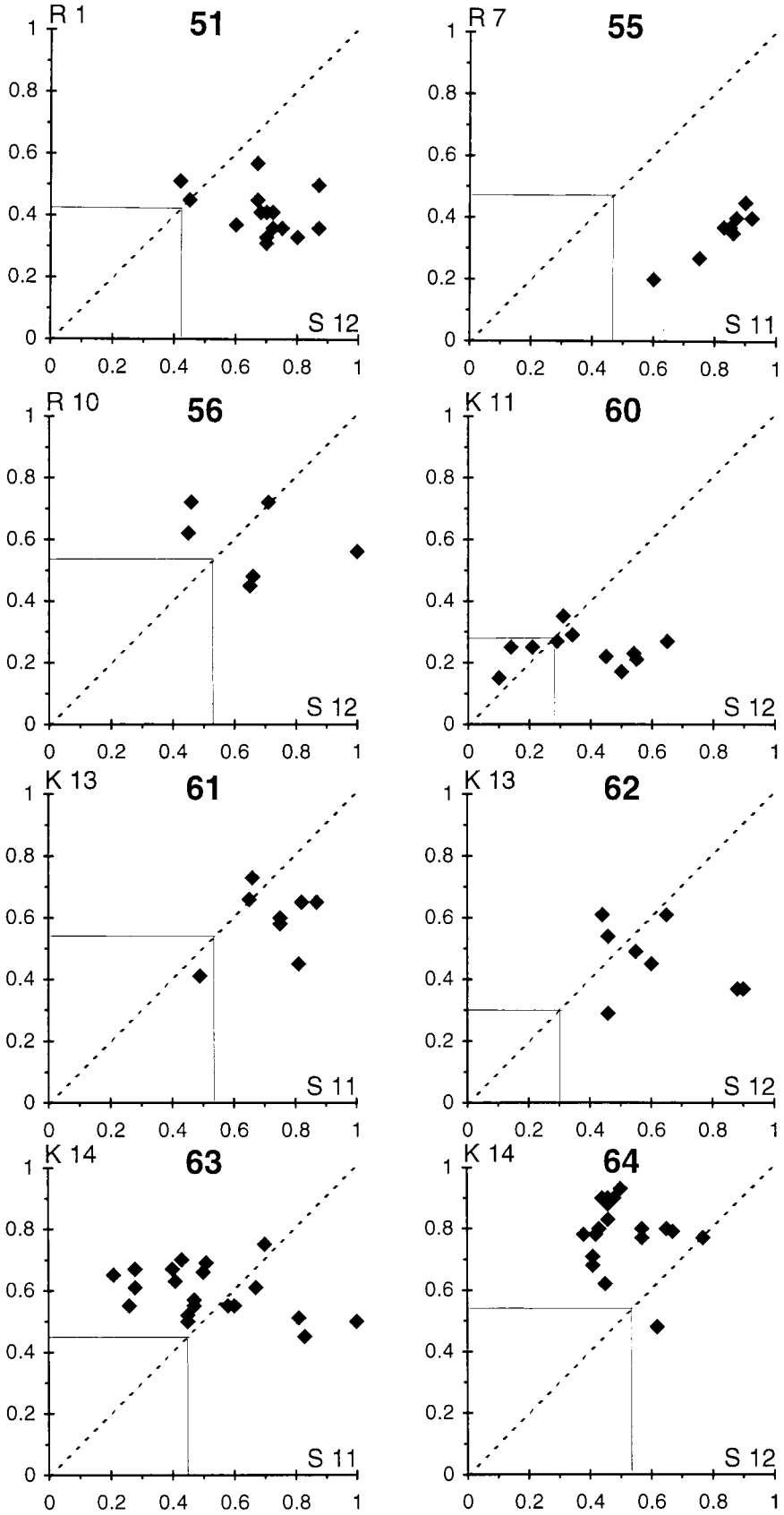


Figure 2. Graphic representation of antigenic similarity coefficients of hybrids to parents *Pinus montana* var. *rostrata* and *P. sylvestris* in particular crosses. Squares show the proteins common to both parents.

Table 2. Isoenzymatic multilocus genotypes of parental clones S11, S12 of *Pinus sylvestris*, and R and K of *P. montana* var. *rostrata*.

Parents	Loci										
	Fest	*6Pgd*	*Got-A*	*Got-B*	*Adh*	*Mdh-A*	*Mdh-C*	*Dia*	*Gdh*	*Skdh-A*	*Skdh-B*
S11	11	12	11	11	12	11	12	13	22	11	11
S12	11	11	13	11	11	11	12	11	12	12	12
R1	12	22	11	12	22	11	22	11	12	23	11
R7	22	22	11	12	12	11	22	11	12	22	11
R10	12	12	14	12	22	11	11	11	11	22	11
K9	11	22	11	12	11	11	22	11	23	22	11
K11	11	22	11	12	22	11	22	11	23	22	11
K13	11	22	13	12	11	11	22	11	13	22	11
K14	11	22	44	11	22	11	12	11	11	22	11

hybrids were more similar to *P. sylvestris*, as for example, the crosses R1 × S12 (no. 51), R7 × S12 (no. 55), K13 × S11 (no. 61), and K13 × S12 (no. 62), or to *P. montana* as *e.g.* K14 × S11 (no. 63) and K14 × S12 (no. 64). The others were intermediate. Figure 3 Illustrates ANDERSON'S hybridization indices based on JACCARD's similarity coefficients for the whole group of hybrids. Most of the hybrids (41 individuals) showed varying degrees of similarity to *P. sylvestris*, some were intermediate (25 individuals), and the rest were more or less similar to *P. montana* (29 individuals). The average values of ANDERSON'S hybridization index for the the group of hybrids resembling *P. montana* was +0.25 (value +1.0 is for the pure species), and the value for hybrids similar to *P. sylvestris* was –0.33 (–1.0 for pure *P. sylvestris*).

Isoenzymes

The isoenzymatic genotypes noted for the parental trees are shown in Table 2. For some of the loci (*Fest, Got–A, Gdh,* and *Dia*) alleles specific to the parents were observed. For example, the allele 3 at the locus Dia was found only in clone S11 (*Pinus sylvestris*) and was not present in the seven *P. montana* clones. The allele 3 at locus *Gdh* was present only in three *P. montana* clones (K9, K11, K13). This situation allows us to follow the mode of inheritance of particular alleles specific for parents in the studied crosses.

Mendelian analyses of inheritance of alleles for eleven loci are shown in Table 3. For all but two loci (*Skdh* and *Gdh*), the alleles segregated according to predictions of the Mendelian model, but in several cases the ratio of observed genotypes significantly deviated from the expected values (Table 3). For locus *Got–A* the deviation is evident for the 11 × 13 crosses where an excess of homozygotes *Got–A* 11 is noted. For *Adh* locus in cross types of 22 × 12 and 11 × 12 the homozygotes 11 and 22 are absent among the progenies. The same is true for locus *Mdh–C* in cross 22 × 12, where the chi-square test revealed a significant deficit of homozygotes of type 22. However, in the case of locus *Fest* cross genotypes 12 × 11, and for locus *Gdh* cross 23 × 22, there were significant deficits of the heterozygotes.

At two loci, *Gdh* and *Skdh–A*, unexpected genotypes were found in some of the crosses. These „novel" genotypes in locus *Gdh* appear as one-banded, faster migrating phenotypes in crosses 51, 55, 58, 61 and 62 (type x). In cross 60 (23 × 12) there are four hybrids with a new band denoted as y. This band is slower migrating than the allele denoted as 3. In the case of shikimate dehydrogenase (*Skdh–A*) there are two „novel", unexpected types of hybrids: z2 with allele z migrating faster than allele 1. In cross 55 only one slower migrating band w was visible, close to the position of allele 2. In both loci (*Gdh, Skdh–A*) these novel proteins were found in 11% and 10% of the hybrids, respectively.

DISCUSSION AND CONCLUSIONS

The studied hybrids *P. montana* var. *rostrata* × *P. sylvestris* showed varying degrees of similarity to the parents when antigenic proteins were used as parent-specific markers. A portion of the progenies (30%) were similar to *P. montana* , representing a matroclinal type of inheritance of antigens. Most of the progenies (43%) exhibited a patroclinal pattern and about 25% of the hybrids were intermediate (Figures 2 and 3). These types of asymmetry in inheritance of proteins were also described for *Alnus incana* × *A. glutinosa* F_1-hybrids from controlled crosses where most of hybrides were similar to the mother species (PRUS-GŁOWACKI & MEJNARTOWICZ 1992). In the present study when *P. sylvestris* S12 clone (Schl 77/1) was used in a crossing,

Table 3. Patterns of inheritance of alloenzymes in controlled crosses of *Pinus montana* var. *rostrata* (M) × *P. sylvestris* (S).

Locus	No. of cross	Parental genotypes M × S	Genotypes observed in hybrids and their numbers (..)
Fest	60, 61, 62, 63, 64, 57, 58	11 × 11	11(86)
	56, 51	12 × 11	11(15), 12(7) *
	55	22 × 11	12(8)
6Pdg	57, 61, 63	22 × 12	12(23), 22(15)
	51,55, 58, 60, 62, 64	22 × 11	12(72)
	56	12 × 11	11(4), 12(2)
Got–A	57, 61	13 × 11	13(10), 11(8)
	51, 55, 58, 60	11 × 13	13(10), 11(35) *
	63	44 × 11	14(20)
	64	44 × 13	14(17), 34(2)
	56	14 × 13	13(3), 14(3), 11(0), 34(0)
	62	13 × 13	11(6), 13(2), 33(0)
Got–B	63, 64	11 × 11	11(39)
	51, 55, 56, 57, 58, 60, 61, 62	12 × 11	11(35), 12(42)
Adh	51, 56, 58, 60, 64	22 × 11	12(59)
	63	22 × 12	12(20), 22(0) *
	57, 61	11 × 12	12(17), 11(0) *
	55	12 × 11	11(4), 12(4)
	62	11 × 11	11(8)
Mdh–A	all crosses	11 × 11	11(116)
Mdh–C	57, 61, 60, 58, 51, 55, 62	22 × 12	12(45), 22(26) *
	63, 64	12 × 12	11(13), 12(20), 22(7)
	56	11 × 12	12(4), 11(2)
Dia	51, 55, 56, 58, 60, 62, 64	11 × 11	11(79)
	63, 57, 61	11 × 13	11(16), 13(22)
Gdh	63	11 × 22	12(20)
	57	23 × 22	22(8), 23(2)
	56, 64	11 × 12	11(15), 12(11)
	61	13 × 22	23(5), 12(0), x(3) *
	60	23 × 12	22(2), 13(3), 12(1), 2y(4) *
	58	23 × 12	22(1), 13(3), 12(3), 23(1), x(2) *
	51, 55	12 × 12	11(8), 12(8), 22(6), x(2) *
	62	13 × 12	11(0), 13(2), 12(2), 23(2), x(2) *
Skdh–A	61	22 × 11	12(8)
	57, 63	22 × 11	12(26), z2(4) *
	56, 62	22 × 12	12(7), 22(7)
	60, 64, 58	22 × 12	12(12), 22(21), z2(8) *
	55	22 × 12	12(6), w(2) *
	51	23 × 12	13(6), 23(4), 22(2), 12(4)
Skdh–B	57, 61, 63	11 × 11	11(38)
	51,55, 56, 58, 60, 62, 64	11 × 12	11(41), 12(46)

* Deviations from Mendelian segregation.(χ^2-test) „Novel" alloenzymatic phenotypes not expected from the cross are denoted by letters. Abbreviations: w = individuals with only one band close to the position of allele 2; x = individuals with only one faster migrating band; y = two-banded individuals with one band migrating faster than allele 3; z = two-banded individuals with one band migrating faster than allele 1.

Table 4. The types of crosses and the number of hybrids with the specific allele* present in only one of the parents. M – *Pinus montana*, S – *P. sylvestris*.

Locus	Crosses	Genotypes of parents M × S	Genotypes of progeny and their number		χ^2-test	Prevailing types of inheritance
Fest	51, 56	12* × 11	12* (7),	11 (15)	***	patroclinal
Got–A	51, 55, 58, 60	11 × 13*	13* (10),	11 (35)	***	matroclinal
	64	4*4 * × 1*3*	1*4*(17),	3*4 *(2)	–	intermediate
Adh	57, 61	11 × 12*	12* (17),	11 (0)	***	patroclinal
Mdh–C	51, 55, 57, 58, 60, 61 62	22 × 12*	12* (45),	22 (26)	***	patroclinal
Gdh	57	23* × 22	23* (2),	22 (8)	–	patroclinal
6Pgd	57, 61, 63	22 × 12*	12* (23),	22 (15)	ns	patroclinal

Abbreviations: *** = significant at $p \leq 0.001$; ns = not significant; – = calculation was not done because of low number of individuals

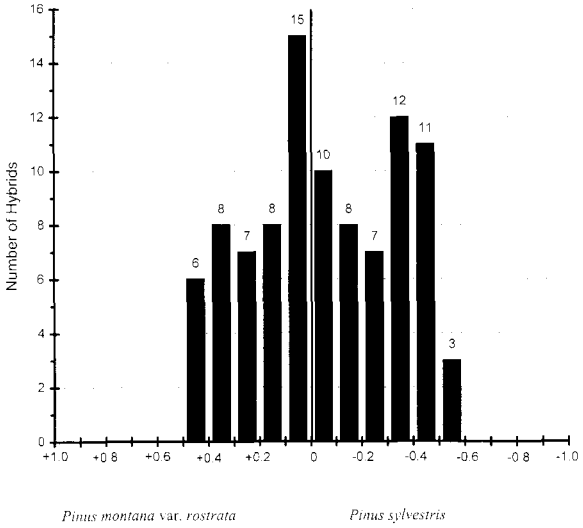


Figure 3. Anderson's hybridization indices for investigated hybrids based on Jaccard's similarity coefficients. The values for the parental taxa are +1.0 for *Pinus montana* var. *rostrata*, and –1.0 for *P. sylvestris*.

most of the hybrids possess a majority of *P. sylvestris* characters. When clone K14 (*P. montana*) was used as the mother, most hybrids showed a matroclinal type of inheritance (Figure 2). Investigations of morphological and anatomical traits of needles of these hybrids also showed the same patterns of inheritance (BOBOWICZ, PRUS-GŁOWACKI & STEPHAN, in preparation). This phenomenon may be explained by an uneven gene expression from both parents, gametic disequilibrium or most probably by gametic or zygotic selection processes. Similar observation, asymmetrically distributed hybrids in a contact zone of small and large populations of *Iris*, in different generations of *Quercus* and *Arctostaphylos* were described also by NASON *et al.* (1992). In the present study we consider the decrease in number or lack of some genotypes from expectations according to Mendelian segregation (Table 4) as evidence for selection.

Mendelian analyses of crosses show an excess of heterozygotes in several loci for some hybrid families (Table 3). However, it is not clear if this phenomenon is connected with better survival rate of heterozygotic progenies or if the pattern could be explained by the other mechanisms mentioned above. LINHART *et al.* (1989) also noted an excess of heterozygosity in progenies of controlled crosses of *Pinus ponderosa* var. *ponderosa* × *P. ponderosa* var. *scopulorum* and DE PHAMPHILIS & WYATT (1990) increase of heterozygosity level in populations of natural hybrides of *Aesculus*.

There is close correlation in the characters of antigenic proteins and isoenzymes in some of the family of progenies. It can be seen in the cases when one of the parents is carrying a specific allele. In Table 4 this kind of crosses are shown. When the majority of progeny in the crosses carry the specific allele from *P. montana*, then they are antigenically similar to this species. If the allele is specific for *P. sylvestris* (Table 4), they immunologically resemble the latter species (Figure 2). One exception is noted for locus *Got–A* in crosses 51, 55, 58 60 and 64, where this kind of correlation is not observed.

Both serological and isoenzymatic studies show a rather interesting phenomenon of the appearance of „novel" proteins in some hybrids that were not observed in the parents. These types of proteins were also detected in allopolyploid *Lolium* × *Festuca*, where the proteins of the hybrids possess immunochemical properties that differ from the parents (PRUS-GŁOWACKI *et al.* 1971). Also in *Alnus glutinosa* × *A. incana* hybrids from controlled crosses, some novel antigens were noted (PRUS-GŁOWACKI & MEJNARTOWICZ 1992). In the case of isoenzymes in two loci, *Skdh* and *Gdh*,

new bands appeared in some hybrids that were unexpected from the cross types. These bands differ in electrophoretic mobility from the allozymes of the parents. The new variants of alloenzymes of *Skdh* and *Gdh* we have observed in our study cannot be explained by the presence of a modifier gene, as it was described for *Mdh* loci in *Picea abies* (BREITENBACH - DORFER & GEBUREK 1995). The frequency of this phenomenon is, in the case of antigens, about 30%, and for isoenzymatic Gdh and Skdh loci about 10 to 12%, respectively. A similar observation for presence of „novel" allozymes was reported by LINHART *et al.* (1989) also for the Skdh locus in crosses of *P. ponderosa* var. *ponderosa* × *P. ponderosa* var. *scopulorum*. The frequency of novel allozymes among hybrids was 7%. A study of hybrid zones in *Aesculus* also revealed few alleles unique to the hybrid zone that were not present in the pure species (DE PAMPHILIS & WYATT 1990).

This kind of polymorphism was also described for chloroplast DNA in hybrids of *P. contorta* × *P. banksiana*, where unusual cpDNA phenotypes were observed in zones of sympatry of the two species (WAGNER *et al.* 1987, GOVINDARAJU *et al.* 1989). For *Pseudotsuga menziesii* hybrids, non parental cpDNA was also described (NEALE *et al.* 1986). For animal hybrid swarms, rare alleles have been described, that were not observed in parental populations (for review see BARTON & HEWITT 1985). A theoretical basis for the explanation of this phenomenon was given by STROBECK & MORGAN (1978), MORGAN & STROBECK (1979). They concluded that intragenic recombination between different existing alleles in a population can create a new allele, especially in crosses between different, but related species. Therefore, intragenic recombination may be responsible for some excess of rare alleles in a natural population and particularly in a hybrid zone. GOLDING & STROBECK (1983) suggested that the process of intragenic recombination can be a significant factor increasing the genetic variability in hybrid populations.

However, the phenomenon of „novel" alleles or antigens could also be explained by post-translational modifications of the proteins instead of the creation of new genetic variants of alleles or antigens. Another explanation for the presence of unique alleles may be an increased mutation rate in hybrids (GOLDING & STROBECK 1983).

Advanced hybridization and introgression together with intragenic recombination can possibly increase the genetic variation of populations as suggested by the present study. This new variation may be the basis for an increased fitness of the hybrid populations, which could colonize a new ecological niche. Hybrids may be better suited to their environmental conditions compared to the parent species. This phenomenon may be observed in an area with natural stands of *P. sylvestris*, *P. mugo* and their putative hybrids in southwestern Poland, where the hybrids are found growing under conditions unfavourable for the parent species. This kind of phenomenon could initiate the process of speciation.

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