ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. XLVIII, nr 2: 217—238
1979

Studies on antigenic differences in needle proteins of *Pinus sylvestris* L., *P. mugo* Turra, *P. uliginosa* Neumann and *P. nigra* Arnold

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(Received: December 15, 1978)

Abstract

By means of serological methods (double immunodiffusion, immunoabsortion and quantitative immunoprecipitation), using three kinds of antisera: anti-sylvestris, antimugo and antiuliginosa authors performed a comparison of antigenic properties of proteins from needles of *P. sylvestris*, *P. mugo*, *P. uliginosa* and *P. nigra*. Proteins characteristic for the species *P. sylvestris*, and *P. uliginosa* were found. On the basis of the results obtained it was established, that the most distinct species are *P. mugo* and *P. sylvestris*, *P. uliginosa* is antigenically different from the two taxa but is showing greater similarity to *P. mugo* than to *P. sylvestris*. *P. nigra* proteins are different from proteins *P. sylvestris*, *P. mugo*, and *P. uliginosa*. They show however, some antigenic similarity to *P. sylvestris* proteins.

INTRODUCTION

To understand better the genetic structure of a complex hybrid swarm population that arose in postglacial period by hybridisation of *P. mugo*, *P. sylvestris* and, possibly, *P. uliginosa* * and is growing recently on a great peat-bog system near village Zieleniec in Góry Bystrzyckie Mountains (S. W. Poland, Sudetes) at the elevation of ca. 720 m., as detailed as possible characteristic of variability of putative parent species is necessary. Among others, also in immunochemical studies are performed (Prus-Głowacki, Szweykowski, 1978). The results published in this paper supplement those studies mentioned above by identification of precipitation lines by means of immunodiffusion. We performed, also, quantitative precipitation and immuno-

* Taxonomic status of the forms called *Pinus uliginosa* Neumann was discussed, a.o., by Szweykowski (1969).

absorption. In addition, a fourth species, *Pinus nigra* Arnold, which hybridises in South Europe with *P. sylvestris* was taken into consideration.

MATERIAL AND METHODS

Proteins from needles of 16 individuals of each taxon i.e. *P. sylvestris*, *P. mugo*, *P. uliginosa* and *P. nigra* were extracted as follows: 1 g of needles of each individual were ground in a mortar with PVP, Tris--boric acid — EDTA buffer pH 7.4 (Rudin 1973). Ratio needles: buffer : PVP were 1 : 4 : 1. Extraction was performed for 1 hour in temperature about 4°C. The extract was centrifugated for 15 min. by 2000 × g.

For analysis 0.5 ml of protein solutions from each individual of particular taxa was mixed together to get a random sample.

Immunodiffusion analyses according to Ouchterlony were made in barbital-sodium acetate — HCl buffer pH 6.75, 0.135 M in 1.5% agarose, using 50 or 100 µl of antisera. After immunodiffusion plates were washed, dried and stained with amido black 10 b for visualization of general proteins.

The staining for enzymes activity was done according to methods described by Uriel (Clausen 1971) with some modifications. Staining for α-esterase activity was done in mixture: phosphate buffer 0.1 pH 6.0 50 ml, α-naphtyl acetate 1.0 mg/ml and Fast Blue RR Salt 0.5 mg/ml.

Peroxidases were stained by 3-amino-9-ethyl carbasole 1 mg/ml in acetate buffer 0.1 M pH 5.0 and 0.03% H_2O_2. Malate dehydrogenase, in 5 mg/ml L-malic acid monosodium salt, 0.01 mg/ml PMS, 0.1 mg/ml MTT, 1 mg/ml NaCN, 0.05 mg/ml NAD, Tris-HCl buffer pH 7.6 0.2 M.

Quantitative immunoprecipitation test was performed using 500 µl of antigens (concentration of proteins 8.6 mg/ml) and 500 µl of serum for antisera S1, S2 and S3, and the same amount of antigens and antiserum (concentration of proteins 10.5 mg/ml) for combining serum (SZ). The determination of protein amount in the extracts and immunoprecipitatins was done by Lowry's method (Prus-Głowacki, Sulinowski, Nowacki, 1971).

Immunoabsorbtion analysis was done according to procedure described by Clausen (1971) using 0.5 ml of extract and 1.0 ml of coresponding antiserum. In the study three kinds of antisera against needles proteins were used: S1 — against *P. sylvestris*, S2 — against *P. mugo* and S3 — against *P. uliginosa*. Antigens for immunisations were prepared as described above, but proteins after extraction were dialysed against phosphate buffer 0.2 M (pH 7.7) over night in 4°C and than

concentrated by using of aquacide to the protein concentration 8—10 mg/ml. The extracts for injections were made freshly for each injection. Rabbits were immunised weekly as long as titre of antiserum reached the level of at least 1 : 32 (12 to 16 weeks), and then blood was collected. Antisera were obtained in usual way.

Results of immunodiffusion were interpreted according to types of antigen-antibody reactions described by Ouchterlony (1967).

On the basis of immunoprecipitation studies, cluster analysis was performed. As characters, the particular precipitation lines were used. In the case of proteins stained according to their enzymatic activity (i.e. esterases, peroxidases and malate dehydrogenase) the intensity of precipitation was also considered. To do this, the appearance of lines was coded in four states depending on amount of their intensity: 0 — no band, 1 — very feeble line, 2 — distinct line, 3 — strong line, and 4 very strong line.

On the basis of data matrix thus obtained, the Euclidean distances between the four taxa were computed according to the formula:

$$D_{j,k}=\left[\sum_{i=1}^{n}\cdot(x_{ij}-x_{ik})^2\right]^{\frac{1}{2}}$$

(Sneath, Sokal, 1963) where $D_{j,k}$ denotes the taxonomic distance between objects j and k, x_{ij} or x_{ik} character i in j or k object.

In the case of general protein (stained with amido black), the intensity of precipitation reaction was not considered and a similarity coefficient between the taxa studied was computed according to formula:

$$S_{j,k}=\frac{a+d}{a+b+c+d}$$

(Sokal, Michener, 1967), where $S_{j,k}$ is the similarity coefficient between taxa j and k, and the letters a through d denote presence or absence of particular lines according to the scheme:

		taxon k: +	taxon k: —
taxon j	+	a	b
	—	c	d

"+" — line present
"—" — line absent

The taxonomic distance was then computed as $D_{j,k} = 1—S_{j,k}$. In the both cases graphs (minimum spanning trees) were constructed on the basis of taxonomic distance matrices.

RESULTS

Proteins stained with amido-black (general protein)

Line patterns obtained with the three antisera differed somewhat and will be dealt with separately. Fig. 1. 1. antiserum (Anti-*P. syl-*

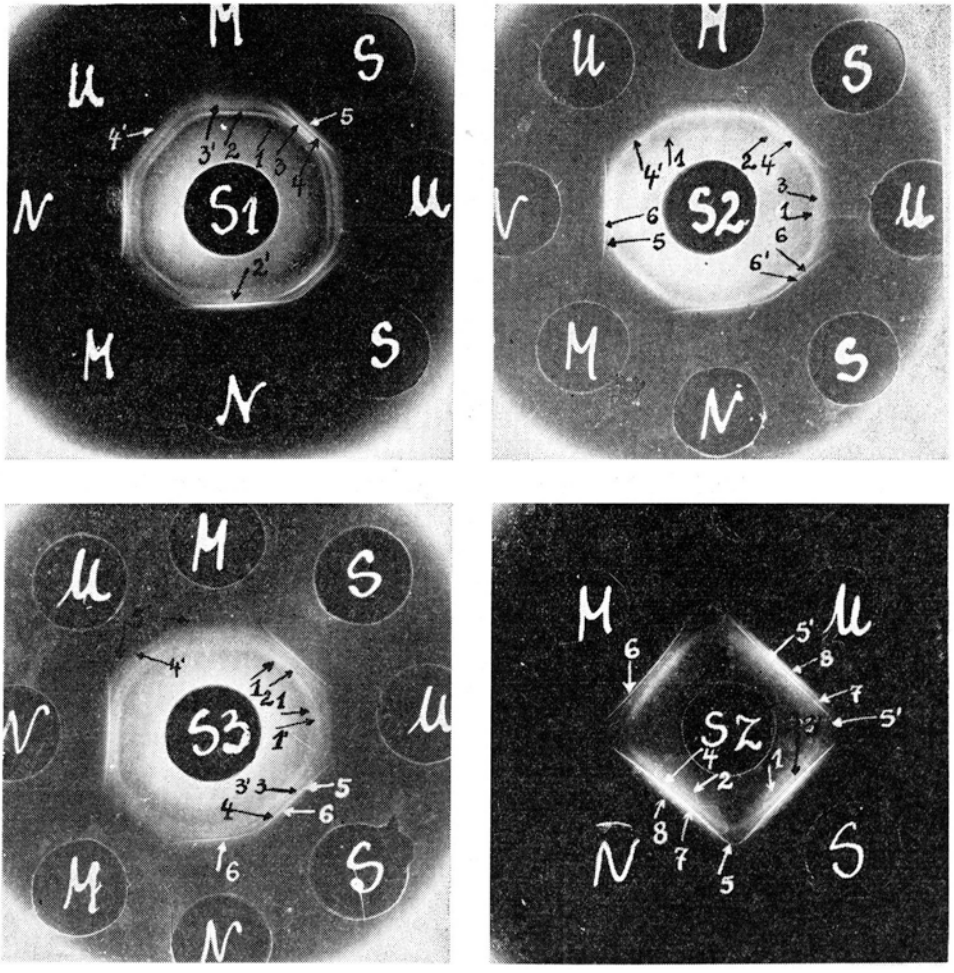

Fig. 1. Double immunodiffusion of needle proteins of *P. sylvestris* (S), *P. mugo* (M), *P. uliginosa* (U) and *P. nigra* (N)

S1 — serum against needle proteins of *P. sylvestris*, S2 — serum against needle proteins of *P. mugo*, S3 — serum against proteins of *P. uliginosa*, SZ — combined mixed serum (S1 + S2 + + S3). Stained with amido-black

vestris). 9 precipitation lines were obtained with this serum. Only one of them (4′) is restricted in its occurence to *P. uliginosa*. Other lines appear in two or even more taxa. The details can be seen in the

Table 1

Results of immunodiffusion with different antisera. Protein stained with amido-black. Crosses give the intensity of precipitin bands

Line no / Antigen	Proteins stained with amido-black															
	S1 antiserum (Antisylvestris)								SZ antiserum (Antimugo)							
	1	2	2′	3	3′	4	4′	5	1	2	3	4	4′	5	6	6′
P. sylvestris	++	+++	0	++++	0	++++	0	+	+++	++	+	+++	0	+	++	+
P. uliginosa	0	++	+	++	++	+	++	0	+++	0	++	++	++	+	+	0
P. mugo	0	++++	0	+	++	0	0	0	+++	0	++	+++	0	+	0	0
P. nigra	++	+	+	+	+	++++	0	+	+++	0	+++	++++	0	+	+++	0

Line no / Antigen	S3 antiserum (Antiuliginosa)										SZ−S1+S2+S3 antiserum (Combinied serum)										
	1	1′	2	3	3′	4	4′	5	5′	6	1	2	3	3′	4	4′	5	5′	6	7	8
P. sylvestris	+	0	+++	++	++	++	0	+++	0	+	+++	0	++	++	0	++	++++	0	0	++	++
P. uliginosa	++	+	+	0	0	+	+	0	+	0	+	0	0	0	++	0	0	+++	0	++	+++
P. mugo	++	0	++	+	0	+	0	0	+	+	+	0	0	0	++++	0	0	0	+++	0	0
P. nigra	++	0	+++	++	0	++	0	+++	0	0	+	++	+++	0	+++	0	++++	0	0	+++	++

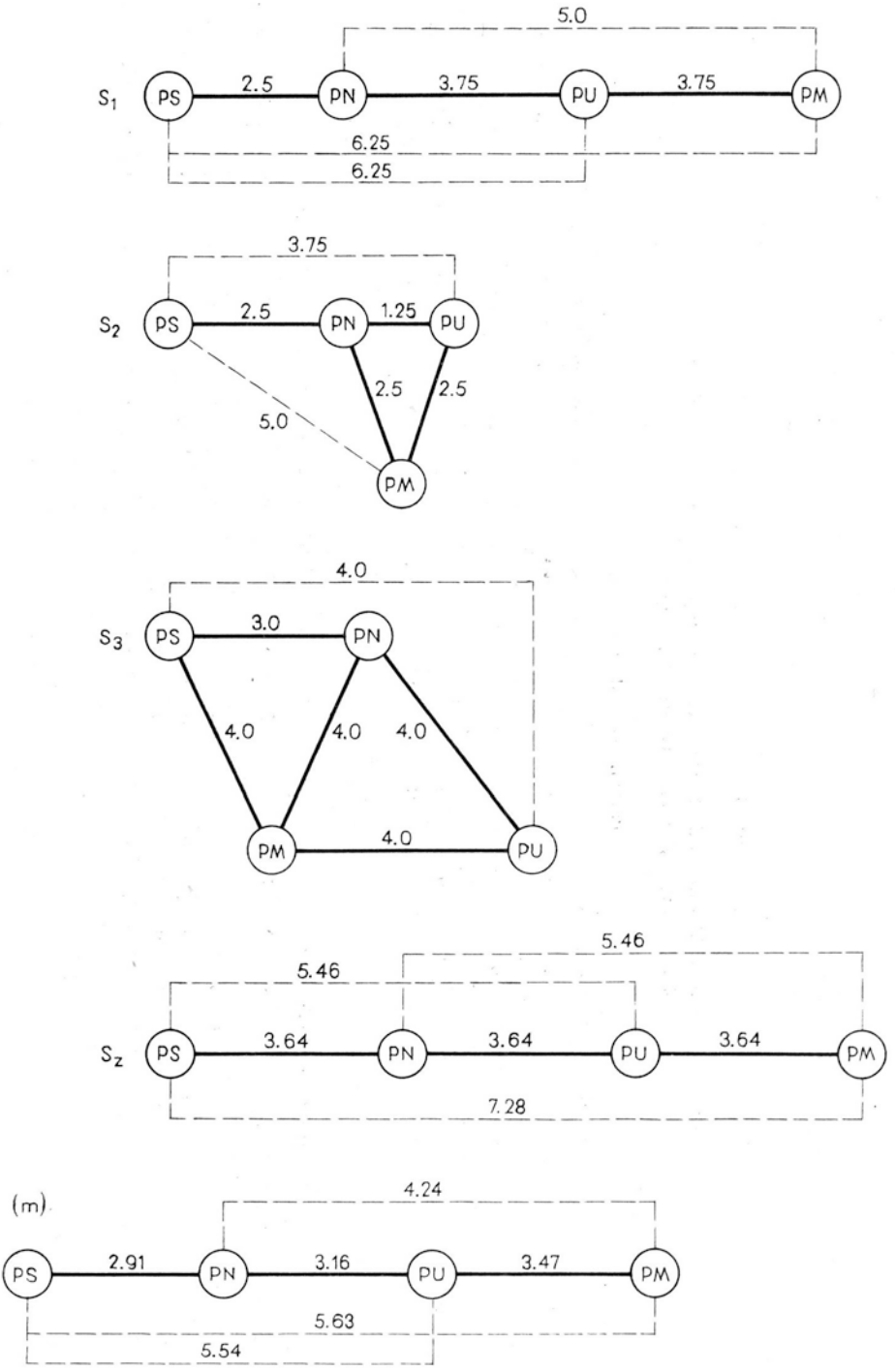

Fig. 2. Diagrams showing the serological similarity of *P. sylvestris* (PS), *P. mugo* (PM), *P. uliginosa* (PU) and *P. nigra* (PN) on the basis of proteins stained with amido-black. Designation of sera as in the Fig. 1

m — average value from S1, S2, S3 and SZ sera

table 1. Lines with primed numbers (2′, 3′, 4′) are partially identical antigenically with respective lines without "primes". 2. antiserum (anti-*P. mugo*) gave 8 precipitation lines. Two of the m(2and 6′) are restricted to *P. sylvestris,* and four (1, 3, 4, 5) occur in all four taxa studied. Again, one band (4′) is characteristic for *P. uliginosa* only, see also table 1. 3. antiserum (anti-*P. uliginosa*) gave 10 precipitation lines which are, in general, weaker than in the two preceding ones. Two of the lines occur in *P. uliginosa* and one in *P. sylvestris* only. Table 1 gives the results of precipitation reaction of this antiserum.

The immunodiffusion analysis was performed also with a mixture of the three antisera mentioned above (1 : 1 : 1). Besides the characteristic bands for *P. nigra* (2), *P. sylvestris* (3′ and 4′) and *P. uliginosa* (5′) there is also one band restricted to *Pinus mugo* (6). This is interesting because this band was not present in analyses performed separately with particular sera. Table 1 gives the results of this analysis, and the graph (Fig. 2) summarises the similarities (as the Euclidean distances).

Immunoabsorption test

The results of immunoabsorption studies are shown in the table 2. Unfortunately, a strong capacity of *Pinus sylvestris* and *P. nigra* to develop a hyperreaction (see discussion for details) makes the results in many respect inconsistent. The most probable (and, as it seems to us, most reliable) results can be summarised as follows:

1. All proteins of *P. mugo* are present in *P. sylvestris* (reaction 14, table 2) but the last species differ from *P. mugo* in having two precipitation lines absent in the dwarf pine (reaction 5, table 2). It seems that protein set of *Pinus mugo* is simply poorer than that of *P. sylvestris.*

2. There is one protein (or protein group) that is present in *P. uliginosa* and not in *P. sylvestris* (reaction 28, table 2). On the other side, the last species (i.e. *P. sylvestris)* differ from the first one in having two precipitation lines absent in *P. uliginosa* (reaction 9, table 2). At the same time the protein sets of *P. mugo* and *P. uliginosa,* as revealed by their immunological properties, are practically the same. The last statement is based on two reciprocal reactions (22 and 32, table 2). The first of them (22) shows that there are no *P. mugo* proteins that would be absent in *P. uliginosa;* the second reaction (32) shows, on the contrary, that there are no *P. uliginosa* proteins not present in *P. mugo.* Unfortunately, this conclusion is not compatible with results of reaction 4 (Table 2).

3. *Pinus sylvestris* is, relatively, well positively characterised by its immunological properties. It has two proteins (or protein groups)

Table 2

Results of immunoabsorption tests. For details see text

Immunoabsorption						Immunodiffusion of absorbed antigens and sera								
Antigen (species)		Antibody (sera)	Absorbed sera	Precipitate	Absorbed antigens	No reaction	Antiserum anti-sylvestris S′	Precipitin line	No reaction	Antiserum antimugo M′	Precipitin line	No reaction	Antiserum antiuliginosa U′	Precipitin line
S	+	S′ →	$-S_{S'}$	S_S	$-S_S$	1	$-S_{SS'}$	0	13	$-S_{SM'}$	0	25	$-S_{SU'}$	0
M	+	S′ →	$-M_{S'}$	S_M	$-S_M$	2	$-S_{MS'}$	0	14	$-S_{MM'}$	0	26	$-S_{MU'}$	0
N	+	S′ →	$-N_{S'}$	S_N	$-S_N$	3	$-S_{NS'}$	1	15	$-S_{NM'}$	2	27	$-S_{NU'}$	2
U	+	S′ →	$-U_{S'}$	S_U	$-S_U$	4	$-S_{US'}$	0	16	$-S_{UM'}$	1	28	$-S_{UU'}$	1
S	+	M′ →	$-S_{M'}$	M_S	$-M_S$	5	$-M_{SS'}$	2	17	$-M_{SM'}$	1	29	$-M_{SU'}$	2
M	+	M′ →	$-M_{M'}$	M_M	$-M_M$	6	$-M_{MS'}$	0	18	$-M_{MM'}$	0	30	$-M_{MU'}$	0
N	+	M′ →	$-N_{M'}$	M_N	$-M_N$	7	$-M_{NS'}$	3	19	$-M_{NM'}$	3	31	$-M_{NU'}$	3
U	+	M′ →	$-U_{M'}$	M_U	$-M_U$	8	$-M_{US'}$	2	20	$-M_{UM'}$	0	32	$-M_{UU'}$	0
S	+	U′ →	$-S_{U'}$	U_S	$-U_S$	9	$-U_{SS'}$	2	21	$-U_{SM'}$	2	33	$-U_{SU'}$	2
M	+	U′ →	$-M_{U'}$	U_M	$-U_M$	10	$-U_{MS'}$	0	22	$-U_{MM'}$	0	34	$-U_{MU'}$	0
N	+	U′ →	$-N_{U'}$	U_N	$-U_N$	11	$-U_{NS'}$	2	23	$-U_{NM'}$	2	35	$-U_{NU'}$	2
U	+	U′ →	$-U_{U'}$	U_U	$-U_U$	12	$-U_{US'}$	0	24	$-U_{UM'}$	0	36	$-U_{UU'}$	0

Table 3

Results of immunodiffusion. Proteins stained on peroxidase activity. Crosses give the intensity of precipitin bands

Species \ Line no.	Peroxidases														
	Antisylvestris sera (S1)				Antimugo sera (S2)		Antiuliginosa sera (S3)				Combined sera SZ (S1+S2+S3)				
	1	1′	2	3	1	2	1	2	3	4	1	1′	2	3	4
P. sylvestris	+++	0	+	++	++	++	+	++	++	++	++	0	0	+++	0
P. uliginosa	0	0	0	+++	0	++++	+	0	+	+++	++	0	+++	+++	0
P. mugo	+	+	0	++	0	++	+	0	0	++	0	++	0	+++	++
P. nigra	++	0	++	++++	0	++++	+	0	++	++	++	0	0	+++	0

Table 4

Results of immunodiffusion. Proteins stained on α-esterase activity. Crosses gives the intensity of precipitin bands

Species \ Line no.	Esterases																	
	Antisylvestris sera (S1)					Antimugo sera (S2)				Antiuliginosa sera (S3)			Combined sera SZ (S1+S2+S3)				
	1	2	3	3′	4	1	2	3	3′	1	2	2′	1	2	2′	3	3′
P. sylvestris	++	+	+	0	++	+	+++	0	+	++	++	0	+++	+++	0	++	++
P. uliginosa	0	++++	++++	++	+++	+++	++	++	0	+	++	+	++	+++	0	++++	0
P. mugo	0	+++	+++	+++	0	++++	++++	+++	0	++	++	++	++++	0	++	++	0
P. nigra	+	++	++	0	+++	++	++++	0	+	++	++	0	++	++	0	+++	0

absent in *P. mugo*, and, similarly, also two proteins (or protein sets) absent in *P. uliginosa*. It cannot be said whether these are the same proteins or not.

4. Proteins of *P. nigra* could have been said to be rather different from all the three species described above were it not for the strong hyperreactivity of that species. However, we did not have the *P. nigra* antiserum and, therefore, the immunological characteristics of this species is far from being complete.

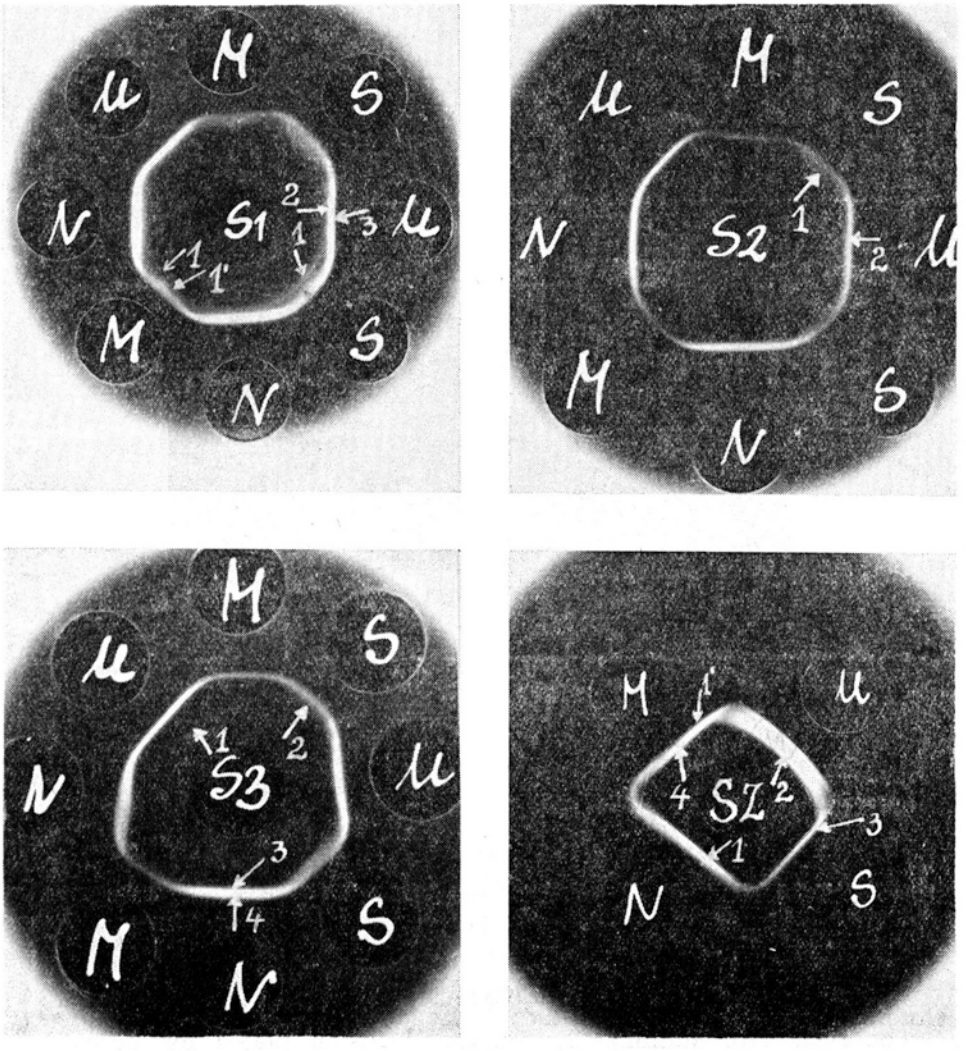

Fig. 3. Double immunodiffusion plates stained for peroxidase activity. The rest of signs as in the Fig. 1

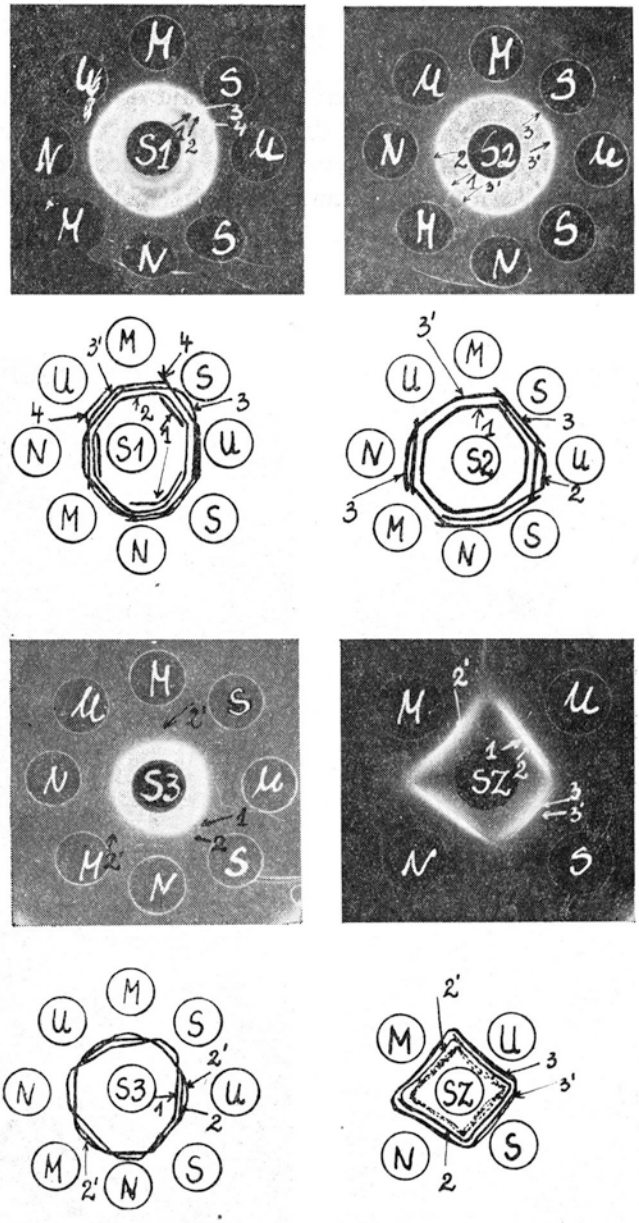

Fig. 4. Double immunodiffusion plates stained for α-esterase activity. The rest of signs as in the Fig. 1

Enzymes

The results of staining the immunodiffusion plates for peroxidase activity can be seen on the fig. 3, and in the table 3; for α-esterase activity — fig. 4 and table 4; and for MDH activity — fig. 5 and

Table 5

Results of immunodiffusion. Proteins stained on malate dehydrogenase activity. Crosses give the intensity of precipitin bands

Line no. / Species	Malate dehydrogenase				
	Antisylvestris sera (S1)	Antimugo sera (S2)	Antiuliginosa sera (S3)		Combined sera SZ (S1+S2+S3)
	1	1	1	2	1
P. sylvestris	+++	+++	+++	+++	++
P. uliginosa	+++	++	++	+	++
P. mugo	++	++	+++	0	++
P. nigra	+++	++	+++	++	++

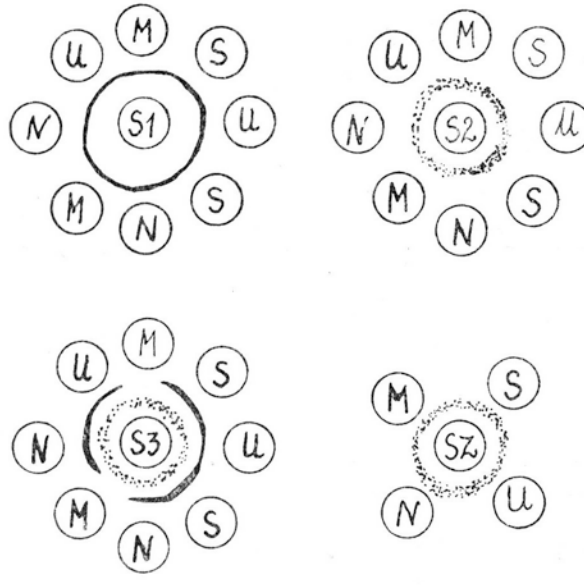

Fig. 5. Double immunodiffusion plates stained for malate dehydrogenase activity. The rest of signs as in the Fig. 1

table 5. The similarities based on enzymatic activities are shown on graphs 6, 7 and 8. Cyclogramms of fig. 9 and 10 picture the immunological phenotypes for general proteins (9) and all the enzymes studied (10).

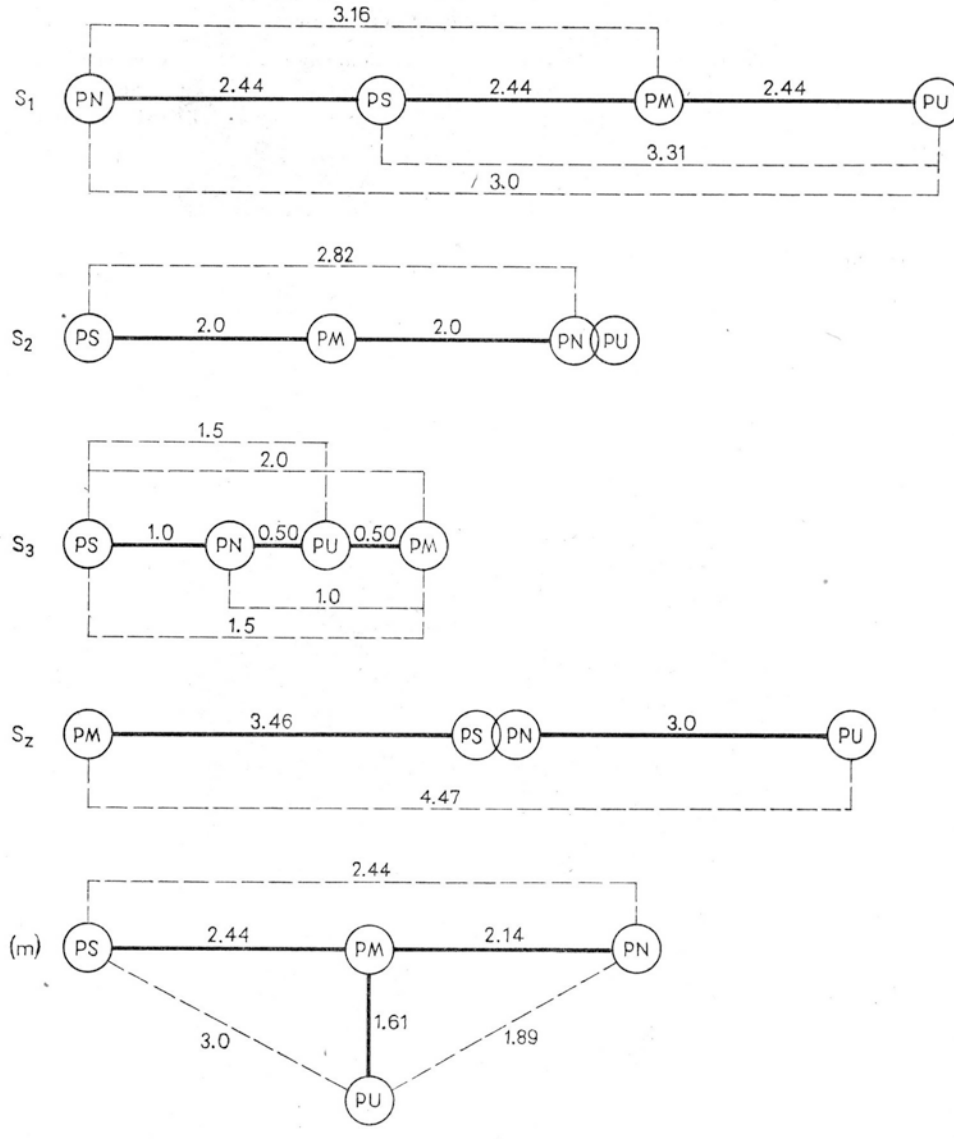

Fig. 6. Diagrams showing the serological similarity of *P. sylvestris* (PS), *P. mugo* (PM), *P. uliginosa* (PU) and *P. nigra* (PN) on the basis of peroxidases. The rest of signs as in the Fig. 1

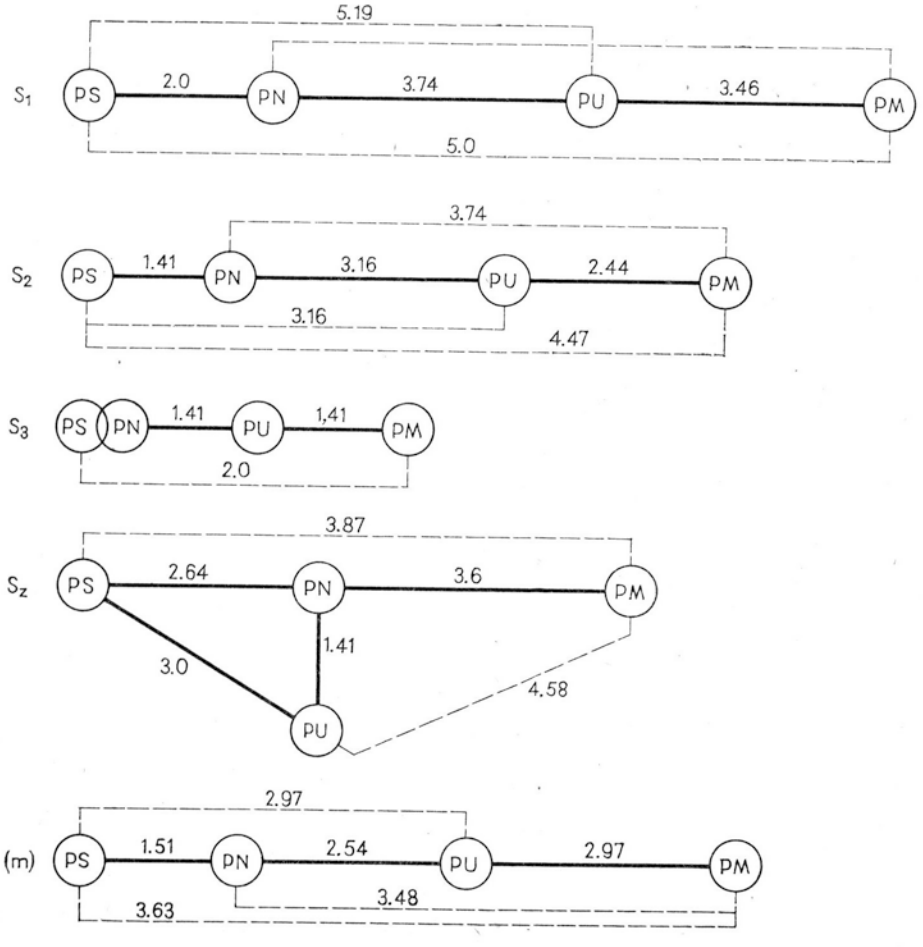

Fig. 7. Diagrams showing the serological similarity of *P. sylvestris* (PS), *P. mugo* (PM), *P. uliginosa* (PU) and *P. nigra* (PN) on the basis of α-esterases. The rest of signs as in the Fig. 1

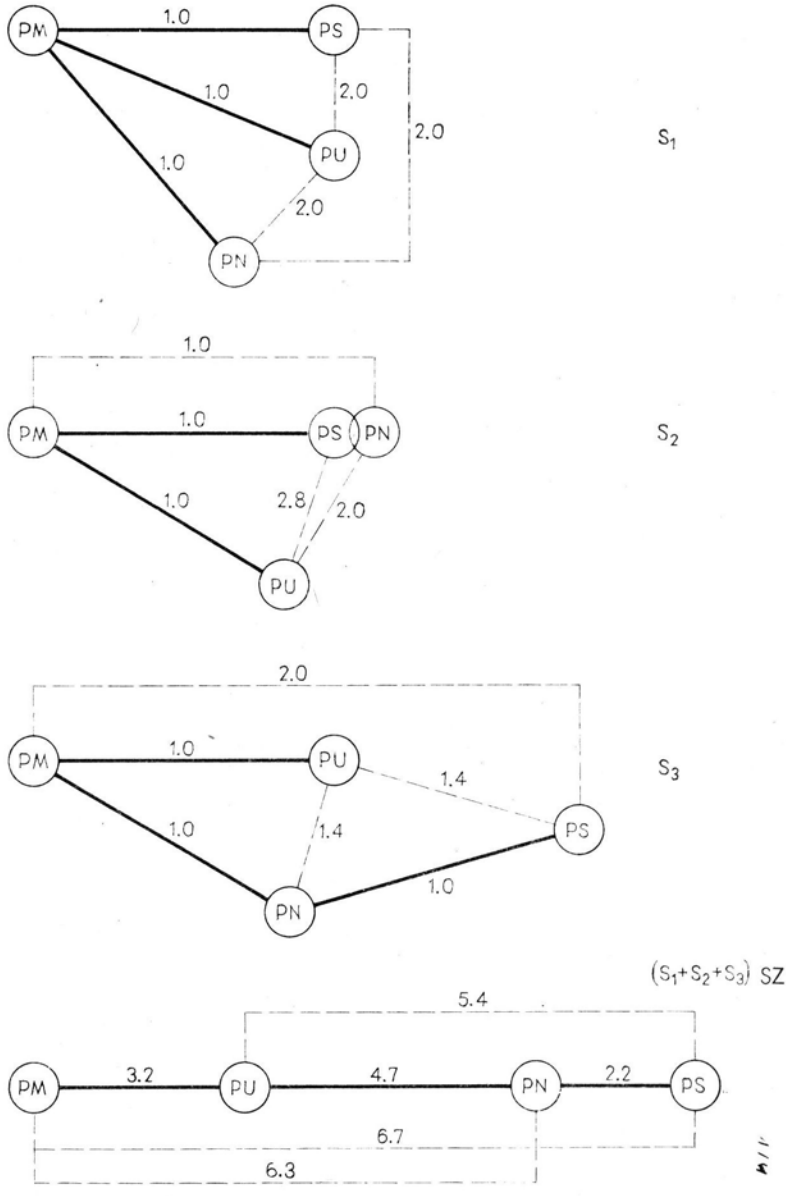

Fig. 8. Diagrams showing the serological similarity of *P. sylvestris* (PS), *P. mugo* (PM), *P. uliginosa* (PU) and *P. nigra* (PN) on the basis of malate dehydrogenase. The rest of signs as in the Fig. 1

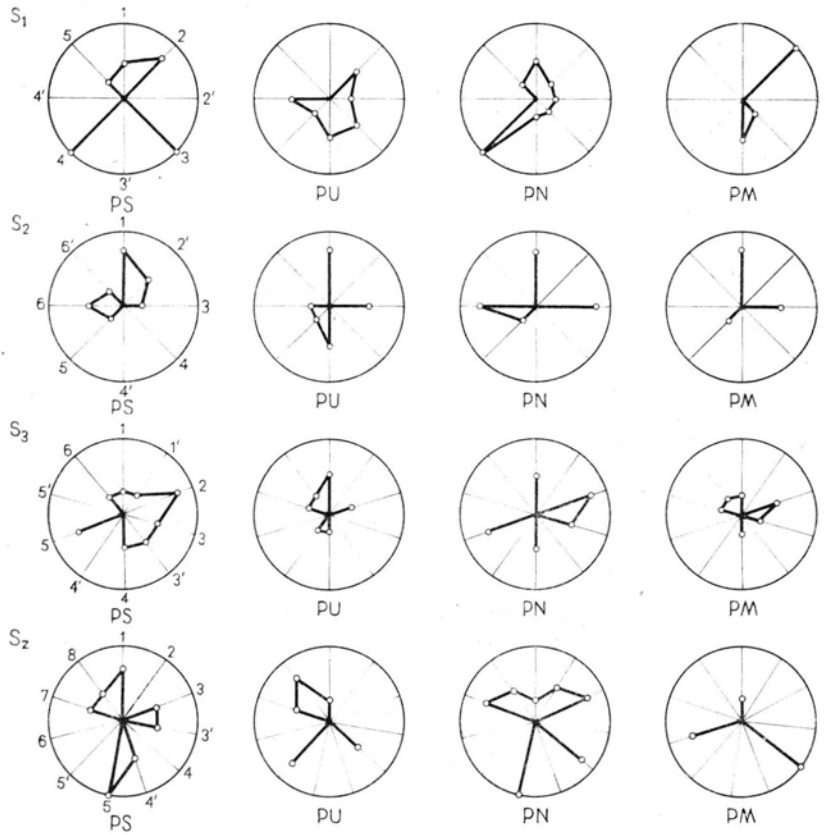

Fig. 9. Cyclograms showing the serological characters of each species investigated. Proteins stained with amido-black. Numbers denote particular precipitin bands. Radii are scaled according to the strength of precipitin reaction. The rest of signs as in the Fig. 1

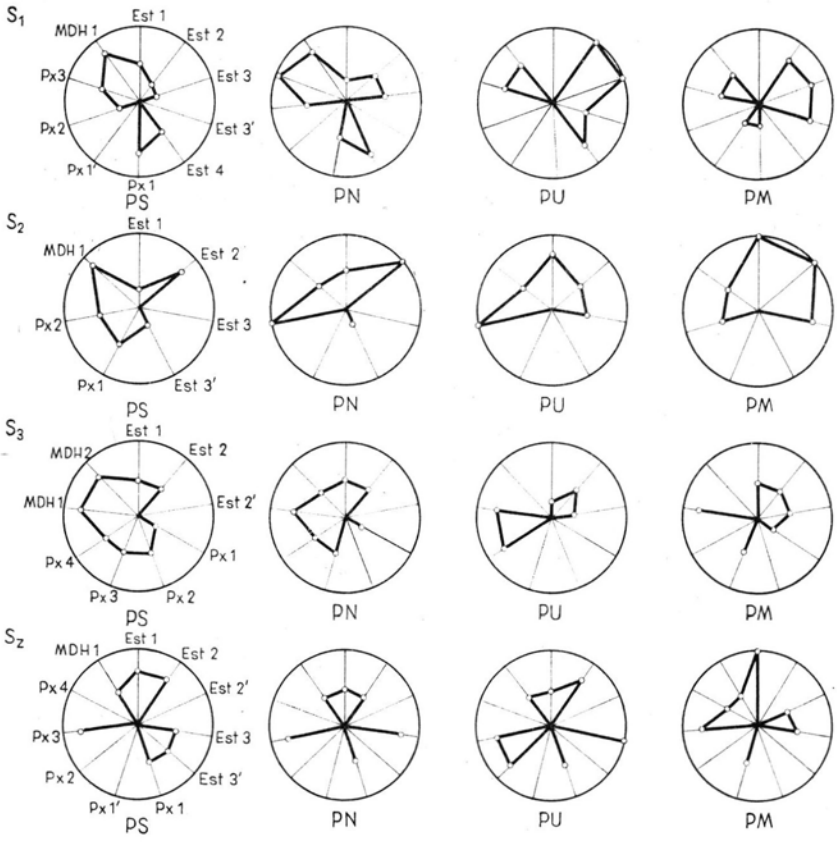

Fig. 10. Cyclogrames showing the enzyme patterns of each species investigated. EST — bands with esterase activity PX — with peroxidase activity MDH — with malate dehydrogenase activity. The rest of signs as in the Fig. 1

Quantitative immunoprecipitation

Results of that sort of analyses are shown in the table 6 and on graphs (Fig. 11). They confirm, to some extent, the results obtained using the absorption method. It is especially important to stress the great amount of precipitation (proteins with the same antigenic properties) in *Pinus uliginosa* × *Pinus mugo* comparison. This shows, again, the rather substantial immunological similarity of these two taxa.

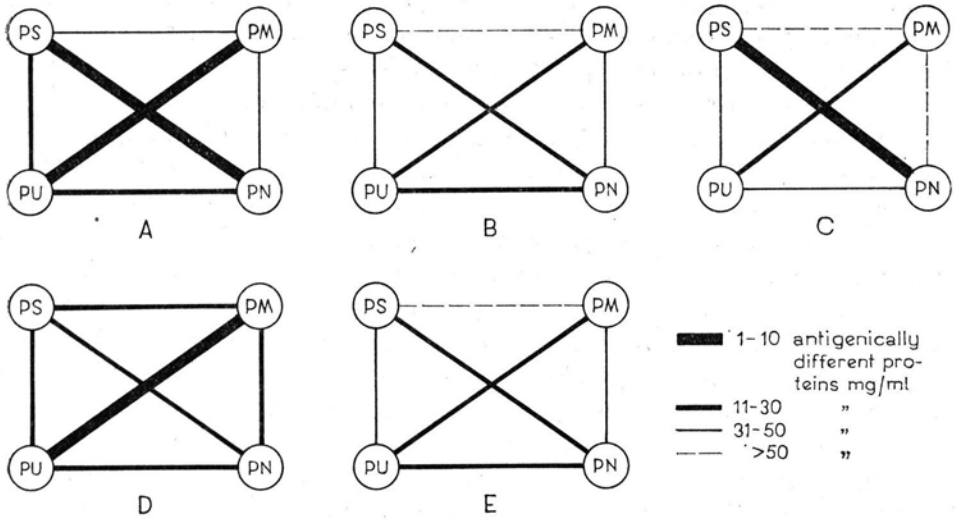

Fig. 11. Diagrams showing the antigenic similarity between *P. sylvestris* (PS), *P. mugo* (PM), *P. uliginosa* (PU) and *P. nigra* (PN) constructed on the basis of immunoprecipitation test

A — S1 serum, B — S2 serum, C — S3 serum, D — SZ serum, E — average value from S1, S2, S3 and SZ sera

DISCUSSION

Three different methods (immunodiffusion, immunoabsorption and quantitative immunoprecipitation) used for characterization of three closely related pines *(P. sylvestris, P. mugo* and *P. uliginosa)* and, in addition, *P. nigra,* gave inconsistent results in some instances. For instance, the immunoabsorption method showed no differences between the antigen sets of *Pinus mugo* and *P. uliginosa* when tested by the cross reaction (reactions no 20, 34, Table 2) and, at the same time, revealed presence of at least one protein characteristic for *P. uliginosa,* i.e. absent in *P. mugo.* The immunodiffusiograms of absorbed sera proved, on the contrary, that both species in question have some proteins specific for them. It seems to us that this inconcistence might have arisen on account of a very small amount of these characteristic proteins in *P. mugo.* The dilution of respective antigens was, possibly, to high to allow, after addition of antibodies, for the precipitation to appear. The second phenomenon that might be responsible for the mentioned inconsistence is the high degree of similarity of proteins in the species studied, especially in *P. mugo, P. uliginosa* and *P. sylvestris.* The results of immunodiffusion performed point out that the specific proteins show nearly always the partial antigenic identity with proteins present

in all (or most) species studied. Such partially identical proteins may be visualised by immunodiffusion method but not by immunoabsorption: they are probably precipitated and disposed of at the very moment of the first addition of antigen to antiserum.

The second category of inconsistencies comprises results of some homologous (reference) reactions as compared with the results of respective heterologous (cross) reactions. E. g. when antibodies of *P. uliginosa* are immunoabsorbed by antigens of *P. sylvestris* then after addition of *P. uliginosa* antiserum there should be no reaction. This is not the case. On the contrary, the precipitation is very strong and two different precipitation lines are formed. Such phenomenon is rather well known as so called hyperreaction. The hyperreaction is present especially frequently in cases when two immunologically similar taxa (or individuals) are compared. The mechanism of such reaction is not known, there is a considerable diversity of opinions in this respect (Simon, 1969; Lee, Fairbrothers, 1969; Anioł, 1976; Smith, Frey, 1970). In our material, especially *Pinus sylvestris* and *P. nigra* show a high degree of antigenic reactivity and in the case of these two species the presence of hyperreaction is highly probable.

The presence of the described inconsequencies shows that for a detailed serological characterization of closely related taxa more precise methods should be used. This is especially important in our case of a hybrid swarm of two closely related species where the serology should help to recognize and to interprete the particular hybrids.

In spite of all those difficulties the results of our study show the great similarity of *Pinus uliginosa* and *P. mugo*. When, however, these two species are compared to *P. sylvestris*, *P. uliginosa* turns to be more closely related (serologically) to that species. Thus, this forgotten taxon is serologically intermediate between monocormic tress *P. sylvestris* and polycormic shrubby *P. mugo*, *P. nigra* is most different of the four taxa studied but tends to cluster most frequently with *P. sylvestris* — its serological similarity to *P. mugo* is practically none.

It should be stressed, however, that the presented results are based on samples of only one population per species studied, and this fact should be taken into consideration when interpreting our results. It should be not forgotten that, with possibly one exception of *P. uliginosa*, all the remained species which were studied in our laboratory are geographically differentiated in many races. For a general characteristic of the taxa in question more samples from different populations belonging to different races should be studied. We are going to continue that kind of work in the next future.

The results presented above are in good agreement with the morpho-anatomical characteristics of needles and cones of the same

taxa based on a considerably greater number of populations (Szweykowski, 1969; Szweykowski, Bobowicz, 1978). Also the results obtained from the serological comparison of many individuals belonging to the mentioned taxa (i.e. *Pinus sylvestris, P. mugo* and *P. uliginosa)* point in the same direction.

Acknowledgments. The receipt of the grant no. 09.7.3.2.1. from the Polish Academy of Sciences is gratefully acknowledged. Thanks are due also to M. Sc. J. Sadowski for preparation of antisera.

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Badania nad zróżnicowaniem antygenowym białek igieł Pinus sylvestris L., P. mugo Turra, P. uliginosa Neumann i P. nigra Arnold

Streszczenie

Za pomocą metod serologicznych (immunodyfuzja podwójna, immunoabsorpcja i precypitacja ilościowa) przy użyciu trzech surowic odpornościowych: antisylvestris, antimugo i antiuliginosa dokonano porównania właściwości antygenowych białek igieł *Pinus sylvestris, P. mugo, P. uliginosa* i *P. nigra*. Wykazano u *P. sylvestris* i *P. uliginosa* istnienie białek dla tych gatunków specyficznych. Na podstawie uzyskanych wyników stwierdzono, iż taksonami najbardziej odległymi są *P. mugo* i *P. sylvestris*. *P. uliginosa* różni się antygenowo od tych dwóch taksonów, wykazuje jednak większe podobieństwa do *Pinus mugo* niż do *Pinus sylvestris*. Białka *P. nigra* różnią się od białek trzech pozostałych gatunków sosen. Wykazują one jednak pewne podobieństwo serologiczne do białek *P. sylvestris*.