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Studies on phenolic acids variation in Central European *Pinus* species

1. Five Polish populations of *Pinus mugo* Turra and some related forms

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Abstract

Phenolic acids were investigated in the needles of eight different pine populations, five of which were *Pinus mugo*, two — *P. silvestris* and one a critical form described by Neumann as *P. uliginosa*. Twenty different phenolic acids were detected. Six were discovered in pine needles for the first time (salicylic acid, gentisic acid, γ-resorcylic acid, o-hydroxyphenylacetic acid, β-resorcylic acid, p-hydroxyphenylacetic acid). Each of the investigated taxons was shown to exhibit a characteristic spectrum of phenolic acids. The critical form from the locus classicus of *Pinus uliginosa* is of intermediate character.

Key words: phenolic acids, populations, Pinus

INTRODUCTION

Phenolic acids have been studied intensively in the wood of many gymnosperms (see Niemann 1979 for references). However, needles were neglected in this respect, and only recently some papers dealing with this problem have been published (Niemann 1979). Results show that needle phenolic acids are much more diversified than those of wood (Niemann 1979). This is a very important observation, especially for the chemotaxonomic evaluation of critical taxa.

Our paper deals with phenolic acids in needles of eight different pine

populations. Five of them are typical *Pinus mugo* growing in the Tatra Mts. in southern Poland. Two populations belong to *Pinus sylvestris* and one to a critical form described by Neumann (1838) as *Pinus uliginosa* Neumann. This taxon occurs on many mountain peat bogs in the Sudety Mts. and in the western part of Czechoslovakia; the studied population being the locus classicus of *P. uliginosa* Neumann. According to recent studies (Szweykowski 1969, Szweykowski and Bobowicz 1977, Prus-Głowacki et al. 1978, Prus-Głowacki and Szweykowski 1979, 1980) this form is of intermediate character between *Pinus mugo* and *P. sylvestris*.

MATERIAL AND METHODS

Needles collected from separate trees growing in natural populations were dried in a desiccator at +37 C (Table 1) and 100 g samples of dried and powdered needles were extracted twice with 700 cm^3 of boiling methanol for 30 min. under a reflux condenser. Combined extracts were evaporated to dryness in a vacuum evaporator. The residue

Table 1

Populations samples for the phenolic studies

Population	Locality	Number of trees studied
1. *Pinus uliginosa*	Torfowisko Batorowskie, peat bog, Kotlina Kłodzka Valley, Sudety Mts. Lime-free area cretaceous sandstone, elevation ca. 700 m. 1982.10.14	pooled sample from 10 trees
2. *Pinus mugo*	mountain slope between lakes "Czerwony Staw Gąsienicowy" and "Kurtkowiec" and "Dolina Stawów Gąsienicowych" Valley. Tatra Mts. Lime-free area, elevation 1675 m. 1982.09.30	pooled sample from 30 different bushes
3. *Pinus mugo*	mountain slope under the "Przełęcz Kondracka" Tatra Mts. Lime-free area, elevation 1750 m. 1982.09.26	„
4. *Pinus mugo*	over limestone rocks in "Niżnia Swistówka" Valley (Dolina Małej Łąki Valley) elevation 1600 m. Tatra Mts. 1982.09.26	„
5. *Pinus mugo*	peat bog at "Toporowy Staw Wyżni" lake. Lime-free area, elevation 1450 m. Tatra Mts. 1982.09.25	„
6. *Pinus mugo*	at the "Morskie Oko" lake, lime-free area, elevation 1400 m. Tatra Mts. 1982.09.12	„
7. *Pinus sylvestris*	pine forest in the vicinity of Strzeszynek 10 km. W. from Poznań. 1982.12.20	pooled sample from 10 trees
8. *Pinus sylvestris*	pine forest by Janów Lubelski, 80 km. N. from Lublin. 1982.09.1	pooled sample from 2 trees

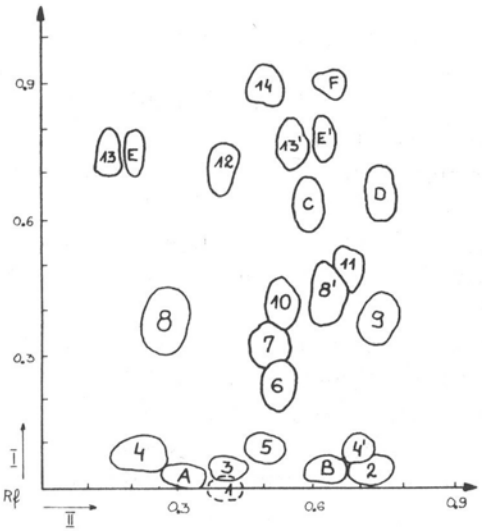

Fig. 1. A two-dimensional paper chromatogram of phenolic acids from the needles of pine species (*Pinus sylvestris*, *P. uliginosa* and *P. mugo*). Number and staining of spots are given in Table 2. The cis isomers are marked with an apostrophe

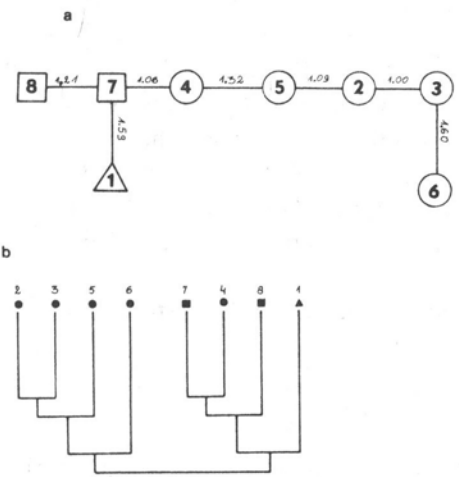

Fig. 2. **a** — Dendrite based on shortest taxonomic distances between populations on the grounds of quantitative and qualitative occurrence of phenolic acids. Lengths of lines connecting the population symbols (cricles with numerals standing for populations of *P. mugo*, squares — *P. sylvestris*, triangle — *P. uliginosa*) are proportional to the real taxonomic distances. **b** — Dendrogram based on results of cluster analysis on the grounds of quantitative and qualitative occurrence of phenolic acids. Explanations as in part a

was resuspended in 150 cm^3 of hot water and left for 12 hours. Then the solution was filtered and lipids were removed by extracting twice with petroleum ether. The water solution was extracted with ethyl ether and the obtained extract was mixed with a saturated solution of $NaHCO_3$ in

water. The pH of the solution was adjusted to about 3.0 with diluted hydrochloric acid and free phenolic acids were extracted again with ethyl ether. The obtained extract was dehydrated with anhydrous Na_2SO_4, evaporated and the residue redissolved in 2 cm^3 of ethyl alcohol. The obtained solution was used for chromatography as fraction A. The remaining water solution was adjusted to 1 N with HCl and hydrolised for 30 min. under a reflux condenser on a boiling water bath. Free phenolic acids were then extracted as in the case of fraction A. The obtained solution was used for chromatography as fraction B.

Two-dimensional paper chromatography was used. The solutions of phenolic acids were applied with a Hamilton's syringe, 0.05 cm^3 each, on Whatman 1 (29 × 29 cm) paper. The solvents were according to Griffiths (1957) and Smith (1958) for direction I: benzene-acetic acid-water (6:7:3) and for direction II: sodium formate-formic acid-water (10:1:200). Chromatograms were examined both under UV ligth (ca. 366 nm. with and without ammonia vapour) and under daylight after spraying with (1) diazotized sulfanilic acid in 10 per cent Na_2CO_3 solution (Bary et al. 1950), with (2) diazotized p-nitroaniline (Randerath 1962) and/or with (3) a 2 per cent water solution of $FeCl_3$. The substances were identified by comparison with a chromatogram of a mixture of known phenolic acids on the basis of R_f values, fluorescence in UV light and colour reactions with a developer (Table 2). The amounts of individual phenolic acids were evaluated considering the size of the respective spots and the intensity of colour reactions. The summarised chromatogram is depicted in Fig. 1.

The data matrix (Table 3) was used to calculate the similarity coefficients and taxonomic distances between respective populations. We used two different kinds of association coefficients:

A. Simple matching coefficient (S_n Sneath and Sokal 1973):

$$S_{m_{kl}} = \frac{a+d}{a+b+c+d},$$

where: a = number of phenolic acids common to both populations compared, b = number of phenolic acids present in population k but absent from population l, c = number of phenolic acids present in population l but absent from population k, d = number of phenolic acids absent from both populations compared.

B. similarity coefficient of Jaccard (S_j Sneath and Sokal 1973):

$$S_{j_{k,l}} = \frac{a}{a+b+c},$$

the meanings of symbols a, b and c are the same as in the former case.

Table 2

Phenolic acids spots after two-dimensional chromatography (Fig. 1) in UV light and after spraying with various reagents

Spot number	Phenolic acids	UV	UV + NH_3	2% $FeCl_3$	dSa	dNa
1	gallic	—	absorbing	grey	light-brown	green-red
2	homoprotocatechuic	—	—	blue	orange	violet
3	protocatechuic	—	absorbing	brown-blue	brown	red-brown
4	caffeic	blue	blue	brown-green	brown	brown
5	gentisic	blue	yellow	navy-blue	grey	grey-green
6	γ-rezorcylic	absorbing	absorbing	blue	yellow-brown	brown-violet
7	p-hydroxybenzoic	—	—	—	yellow	red
8	p-cumaric	—	blue	—	red	brown-blue
9	p-hydroxyphenylacetic	—	—	—	pink	violet
10	β-rezorcylic	—	violet	brown-violet	yellow-brown	red-brown
11	o-hydroxyphenylacetic	—	—	—	orange	violet
12	vanillic	—	—	brown	orange	violet
13	ferulic	blue	blue	—	violet	blue
14	salicylic	blue	blue	violet	yellow	orange
15	a	yellow-green	yellow-green	violet-brown	brown	yellow-brown
16	b	—	—	—	red	violet-brown
17	c	—	—	—	pink	violet
18	d	—	—	—	pink	light-violet
19	e	yellow	pink-yellow	—	pink	violet-red
20	f	—	—	—	pink	brown-blue

Developing reagents: dSa — diazotized sulfanilic acid in 10% Na_2CO_3, dNa — diazotized p-nitroaniline.

Table 3

The occurence of phenolic acids

Species populations \ Phenolic acids		gallic		homoprotocatechuic		protocatechuic		caffeic		gentisic		?-rezorcylic		p-hydroxybenzoic	
Fractions		A	B	A	B	A	B	A	B	A	B	A	B	A	B
Pinus uliginosa	1	0	0	0	1	1	1	1	1	1	1	0	3	3	2
Pinus mugo	2	0	0	3	0	1	1	1	1	1	1	2	3	3	2
	3	0	0.5	3	0	1	1	1	1	1	1	1	2	3	1
	4	0	0.5	3	0	2	2	1	1	0	2	2	3	2	2
	5	0	0	3	1	1	1	1	0	0	1	2	3	2	1
	6	0.5	0	3	0	2	1	1	1	2	1	2	2	3	1
P. sylvestris	7	0	0.5	2	0	2	2	1	1	1	2	2	2	2	1
	8	0	0.5	2	0	1	2	1	2	2	2	1	3	3	3

Explanation: 0 — the compound is absent, 0.5 — traces, 1 — small amount, 2 — medium amount, 3 —

For constructing dendrites (Wrocław diagrams) and for calculation of dendrograms the similarity coefficients were converted into distance measures according to the formulas:

C. For the simple matching coefficient

$$d'_{k,l} = (1 - S_{m_{k,l}})^{\frac{1}{2}}.$$

D. For Jaccard's coefficient

$$D_{k,l} = 1 - S_{ik,l}.$$

As can be seen from formulas A and B — the two coefficients used differ in the evaluation of negative matches. The simple matching coefficient takes them into account, as the „*d*" occurs both in the numerator and in the denominator of S_m. On the other hand, the negative matches are disregarded in the Jaccard's similarity coefficient. This rather basic difference should be kept in mind when interpreting the results of our calculations. In addition, the distance measures obtained from conversion of both association coefficients also differ: that based on the simple matching

in investigated pine species

p-cumaric		p-hydroxyphenylacetic		β-rezorcylic		o-hydroxyphenylacetic		vanillic		ferulic		salicylic		a		b		c		d		e		f	
A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
2	2	1	2	1	2	0	0	3	2	0	2	1	1	3	2	1	3	0	0	2	0	0	2	1	2
3	2	0	1	2	2	0	0	3	2	2	2	0	0	3	2	3	1	3	0	2	0	1	1	2	0
3	3	1	2	2	2	0	0	2	2	1	3	1	0	3	2	2	1	3	1	3	1	2	1	2	0
3	2	2	2	2	2	0	0	3	3	2	3	1	0	3	3	2	3	0	0	3	0	1	0	0	0
2	2	1	2	1	1	0	0	3	2	2	3	0	0	3	2	2	3	3	0	2	0	2	1	1	0
3	2	2	2	1	1	2	0	3	2	3	3	3	2	3	3	3	1	2	1	0	0	2	2	2	0
3	2	2	2	2	3	0	0	3	3	2	2	1	1	3	3	2	3	0	0	0	0	0	0	0	0
3	2	2	2	1	2	1	2	3	3	2	3	2	2	2	2	2	3	0	0	0	0	0	0	0	0

large amount

coefficient ($d'_{k,l}$) is equivalent to the Euclidean distance, on the other hand the $D_{k,l}$ distance measure is nonmetric (in the sense of Sneath and Sokal 1973).

Both similarity coefficients used (and, of course, the distance measures obtained from their conversion) are based on the presence/absence of a particular compound. Thus the information on quantitative differences is lost in their case. As our chromatograms were evaluated quantitatively, we decided to calculate taxonomic distances (Euclidean) between particular populations directly (i.e. without calculation of similarity coefficients):

$$d_{k,l} = \left[\frac{\sum_{i=1}^{n} (X_{i,k} - X_{i,l})^2}{n} \right]^{\frac{1}{2}},$$

where $X_{i,k}$ and $X_{i,l}$ are states of character i in populations k and l respectively, and n is the number of characters scored. We scored the amount of a particular phenolic acid as follows: 0 — the compound is absent, 0.5 — traces, 1 — small amount, 2 — medium amount, 3 — large amount.

As in the cases of coefficients $d'_{k,l}$ and $D_{k,l}$, dendrites (Wrocław diagram) based on the shortest distances, and dendrograms (calculated

according to the UPGMA — unweighted pair group method using the arithmetic averages — procedure, see Sneath and Sokal (1973) were constructed.

All of the mathematical calculations were done in 4 variants. 3 of them on qualitative variability and one on quantitative+qualitative variability:

1. The quantitative and qualitative variant is shown on Figs. 2a, b.
2. Fraction A (Table 3) free phenolic acids — Figs. 3a, b (S_m), c, d (S_j) — trees from population no. 8 treated together. Figs. 4a, b (S_n); c,d (S_j) — trees from populations no. 8 treated separately.
3. Fraction B (Table 3) linked phenolic acids — Figs. 5a, b (S_m); c, d (S_j).
4. Fraction A+B (Table 3) free+linked phenolic acids — Figs. 6a, b (S_m); c, d (S_j).

RESULTS AND DISCUSSION

Twenty different phenolic acids were detected in the needle samples of the studied pine populations. We succeeded in the identification of 14

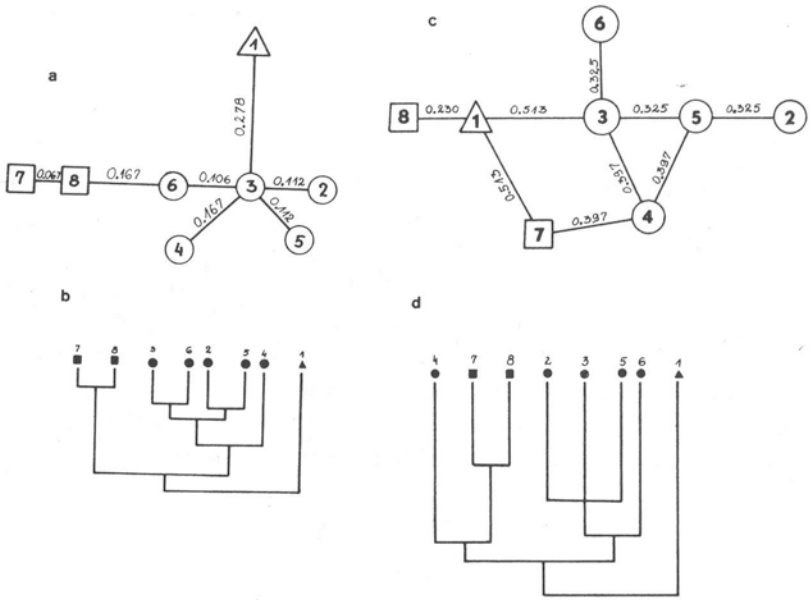

Fig. 3. **a** — Dendrite based on shortest taxonomic distances between populations on the grounds of qualitative occurrence of free phenolic acids (S_n). Explanations as in Fig. 2a. **b** — Dendrogram based on results of cluster analysis on the grounds of qualitative occurrence of free phenolic acids (S_m). Explanations as in Fig. 2a. **c** — Dendrite based on shortest taxonomic distances between populations on the grounds of qualitative occurrence of free phenolic acids (S_j). Explanations as in Fig. 2a. **d** — Dendrogram based on results of cluster analysis on the grounds of qualitative occurrence of free phenolic acids (S_j). Explanations as in Fig. 2a

of them; the rest were named *a* through *f* — their identification is under way. From these 20 different compounds, 6 were discovered in pine needles for the first time. These were: salicylic acid, gentisic acid, β-resorcylic acid, p-hydroxyphenylacetic acid, γ-resorcylic acid and o-hydroxyphenylacetic acid. The last compound was present only in one population of *Pinus mugo* and in one of *P. silvestris*. As can be seen from Table 3, there

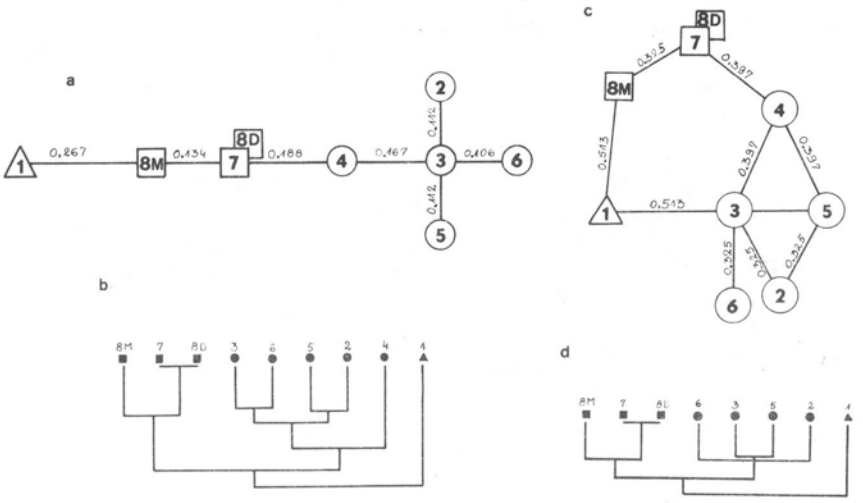

Fig. 4. **a** — Dendrite based on shortest taxonomic distances between populations on the grounds of qualitative occurrence of free phenolic acids (S_n); (8D and 8M — *P. sylvestris* with large and small spot respectively). Explanations as in Fig. 2a. **b** — Dendrogram based on results of cluster analysis on the grounds of qualitative occurrence of free phenolic acids (S_n); (8D and 8M — *P. sylvestris* with large and small spot respectively). Explanations as in Fig. 2a. **c** — Dendrite based on shortest taxonomic distances between populations on the grounds of qualitative occurrence of free phenolic acids (S_j); (8D and 8M — *P. sylvestris* with large and small spot respectively). Explanations as in Fig. 2a. **d** — Dendrogram based on results of cluster analysis on the grunds of qualitative occurrence of free phenolic acids (S_j); (8D and 8M — *P. sylvestris* with large and small spot respectively). Explanations as in Fig. 2a

are both quantitative and qualitative differences between the populations studied. However, the differences are not large enough, and what is more important, they are not significant either between conspecific populations or between those belonging to two different species. This somewhat unexpected result is best shown on the dendrite constructed on the basis of taxonomic distances based on quantitative evaluation of our data ($d_{k,l}$ see Fig. 2a). Even the longest distances between populations 3 and 6 belonging to *Pinus mugo* and between population number 7 (*Pinus sylvestris*) and population number 1 („*P. uliginosa*") are not significantly different ($d \pm 2\sigma = 0.8092 \pm 1.7525$) and do not allow the division of (the „cutting" of)

the diagram into groups which could be considered significantly different.

However, two groups can be discovered by clustering the data as is shown on the dendrogram (Fig. 2b). Four of the five populations belonging to *Pinus mugo* make up one group (nos. 2, 3, 5, 6), the second group is comprised of both populations of *Pinus sylvestris*, the population from the locus classicus of *Pinus uliginosa* Neumann and one population of *Pinus mugo* (no. 4). Interestingly, this last population is the only one of *P. mugo* that was collected on limestone rocks while the rest of them grew on acidic substrates in lime-free areas (Table 1).

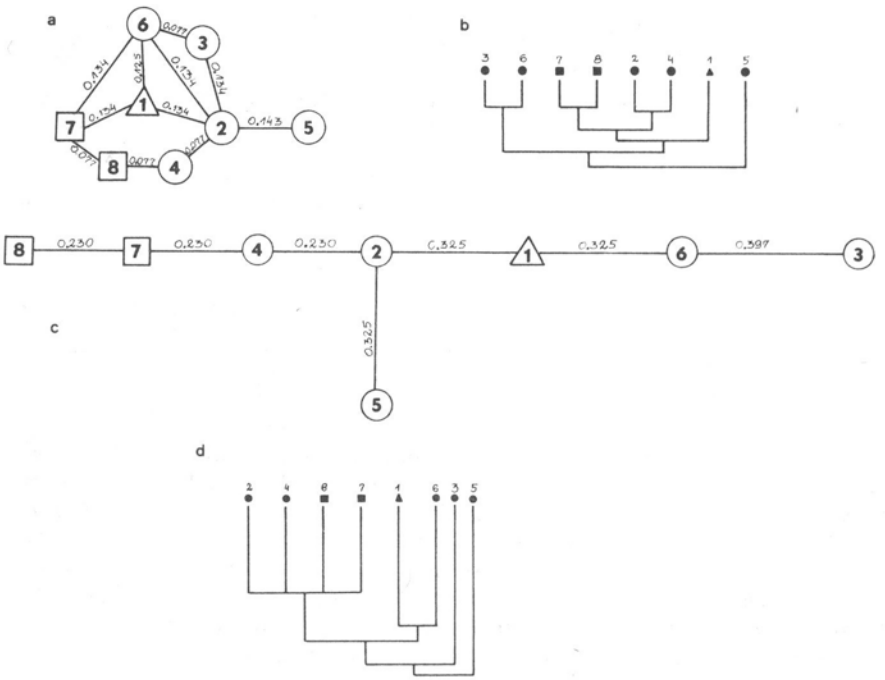

Fig. 5. **a** — Dendrite based on shortest taxonomic distances between populations on the grounds of qualitative occurrence of linked phenolic acids (S_n). Explanations as in Fig. 2a. **b** — Dendrogram based on results of cluster analysis on the grounds of qualitative occurrence of linked phenolic acids (S_n). Explanations as in Fig. 2a. **c** — Dendrite based on shortest taxonomic distances between populations on the grounds of qualitative occurrence of linked phenolic acids (S_j). Explanations as in Fig. 2a. **d** — Dendrogram based on results of cluster analysis on the grounds of qualitative occurrence of linked phenolic acids (S_j). Explanations as in Fig. 2a

The critical population from *P. ulignosa* locus classicus is chemically isolated. It is separated from the most similar population (no. 7, belonging to *Pinus sylvestris*) by one of the longest distances. However, it belongs

chemically to the group of *P. sylvestris* rather than to *P. mugo* (Fig. 2). This is also an interesting result as morphologically and anatomically (at least as needles and cones are concerned — Szweykowski 1969, Szweykowski and Bobowicz 1977) it resembles *Pinus mugo* rather closely.

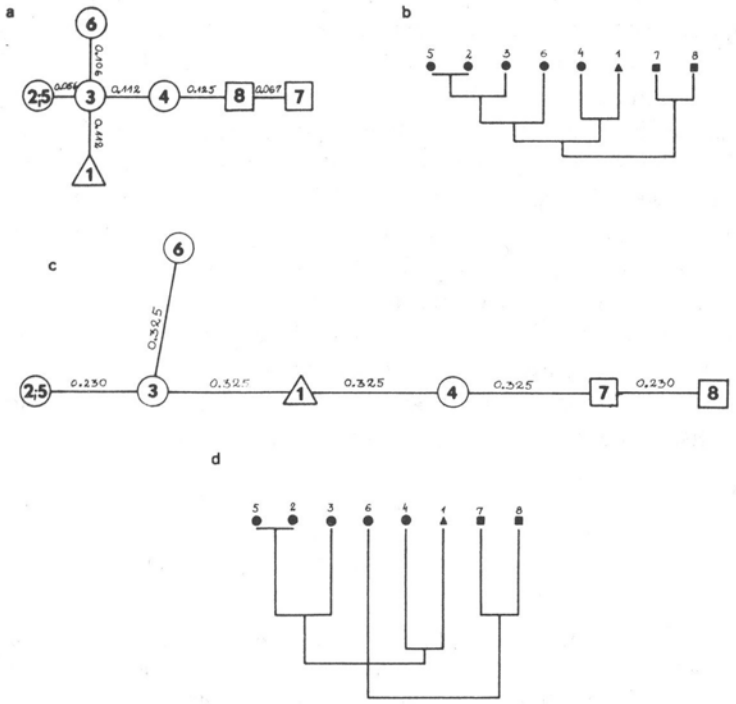

Fig. 6. **a** — Dendrite based on shortest taxonomic distances between populations on the grounds of qualitative occurrence of free and linked phenolic acids treated together (S_n). Explanations as in Fig. 2a. **b** — Dendrogram based on results of cluster analysis on the grounds of qualitative occurrence of free and linked phenolic acids treated together (S_n). Explanations as in Fig. 2a. **c** — Dendrite based on shortest taxonomic distances between populations on the grounds of qualitative occurrence of free and linked phenolic acids treated together (S_j). Explanations as in Fig. 2a. **d** — Dendrogram based on results of cluster analysis on the grounds of qualitative occurrence of free and linked phenolic acids treated together (S_j). Explanations as in Fig. 2a

Some additional information can be obtained from diagrams (Figs. 3-6) where results of ordering and clustering are based on similarity coefficients. As far as the position of the critical *„Pinus uliginosa"* population is concerned (population no. 1 in all diagrams), it joined either *P. mugo* (Fig. 3a, 5c, 6a-d) or *P. sylvestris* (Fig. 4a) or took an intermediate position (e. Figs. 5a, 3c, 4c). The changing position of this population may be taken as evidence of its intermediate character: it resembles more

P. sylvestris or *P. mugo* depending on the traits under study. This is a confirmation of our previous results (Szweykowski 1969, Szweykowski and Bobowicz 1977, Prus-Głowacki and Szweykowski 1980).

The two populations of *Pinus sylvestris* are always joined together. We have shown (Szweykowski and Urbaniak 1982) that the Polish populations of *P. sylvestris* are polymorphic with respect to a group of phenolic substances. There exist trees of two chromatographic types, with a large yellow spot or with a yellow spot in the same place on the chromatogram but a small one. The population sample no. 8 (*P. sylvestris* from Janów Lubelski) consisted of two trees: one of them showed a large (D), the second (M) a small spot. In one run of the analyses, the two trees were assayed separately. The results are shown on Fig. 4. The tree with a large spot (D) resembles the other population of *P. sylvestris* more closely than does the tree with a small spot (M) (they grew side by side in one population). This result is consistent with the geographic distribution of the two types. Trees with a large spot prevail in North Polish populations (Szweykowski and Urbaniak 1982) and population no. 7 (from Strzeszynek by Poznań) grew in a region where a North-Polish type of pine population structure is found.

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Badania nad zmiennością kwasów fenolowych w gatunkach Pinus środkowej Europy. 1. Pięć polskich populacji Pinus mugo Turra i formy pokrewne

Streszczenie

Określono skład kwasów fenolowych w igłach 8 różnych populacji sosny. Pięć z nich to *Pinus mugo*, dwie — *P. sylvestris* oraz jedna to forma krytyczna opisana przez Neumanna jako *Pinus uliginosa*. Wykryto 20 kwasów fenolowych. Kwasy: salicylowy, gentyzowy, β-rezorcylowy, γ-rezorcylowy, o-hydroksyfenylooctowy i p-hydroksyfenylooctowy stwierdzono po raz pierwszy w igłach sosnowych. Każdy z badanych taksonów ma charakterystyczne dla siebie spektrum kwasów fenolowych. Forma krytyczna z locus classicus — *Pinus uliginosa* wykazuje cechy pośrednie między *P. mugo* i *P. sylvestris*.