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Chromatographic analysis of phenol compounds in six natural populations of *Anthyllis vulneraria* (L.)

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Abstract

Thin-layer chromatography was used to study the phenol composition in individual plants from six natural populations of *Anthyllis* collected from three distinct geographic regions of Poland.
The results showed a variability of the phenols in the examined populations. The populations from Wielkopolska region proved to be most variable, showing the greatest number of phenols. The lowest number of the phenols studies was found in the Tatry populations. Each population showed its own particular spectrum of phenolic compounds. It was found that the populations originating from similar habitats showed more common spots than those from different regions. Populations from the Tatra region were found to differ most from the rest.

INTRODUCTION

Anthyllis in Poland belongs to the group of common plants. It occurs both on the lowland and in the mountains. In Europe it is considered as a highly variable genus as evidenced by its taxonomy. Rothmaler (1966) distinguishes 6 species: *A. vulneraria* (L.) *A. maritima* (Schweigg), *A. carpatica* (Pantoček), *A. macrocephala* (Wenderoth), *A. alpestris* (Kit), *A. dilleni* (Schult ex Loud), while Hegi (1964) notes only two species: *A. montana* (L.) and *A. vulneraria* (L.) with numerous varieties. Tutin (1968) mentions as many as 19 species. Jalas (1957) who studied *Anthyllis* in Belgium explains this diversity by the fact that the presently existing populations originate from one large hybrid swarm occurring on the territory of Europe after the last glaciation era.

The aim of the investigation was to study the variability of composition of phenol compounds within 6 *Anthyllis vulneraria* s. l. popula-

tions and to characterise them from this aspect. Phenol compounds are suitable for studies of this kind since large numbers of them occur in particular plants and they are detectable by a relatively simple method. The results can be easily elaborated by mathematical methods.

MATERIAL AND METHODS

Two *Anthyllis* populations were chosen from each of three geographical regions of Poland: the Baltic coast — Chłopy (II), Mielno (III), the Tatra National Park — Kalatówki (IV), Skupniów Upłaz (VI) and Wielkopolska Lowland — Rożnowo (VII), Międzychód (IX). From each

Table 1

Site of origin of natural *Anthyllis populations* from three geographical regions of Poland

Population	Origin
II — Chłopy	Chłopy, distr. Koszalin, White dune
III — Mielno	Mielno, distr. Koszalin, White dune
IV — Kalatówki	Tatra, Dolina Kalatówek, ca. 1300 a.s.l.
VI — Skupniów Upłaz	Tatra, N. slope of Skupniów Upłaz ca. 1360 a.s.l.
VII — Rożnowo	Wielkopolska lowland, distr. Poznań, ditch slopes along railway track
IX — Międzychód	Wielkopolska lowland, distr. Gorzów Wkp., old gavel pit

wild-growing population seeds were collected in autumn and sown on an experimental plot. The experiments were run in a system of random blocks in 3 replications. Each replication within one population included 25 plants. The scheme of the experiment is shown below.

VI III VII IX IV II — first replication
IV IX II VI II VII — second replication
II VI VII IV IX III — third replication.

For examination 30 individuals were chosen randomly from each population (10 for each replication).

From each randomly chosen plant 10 leaves were collected, dried at room temperature, comminuted and subjected to extraction as follows: 1 g of dry leaves was infused with 20 ml methanol with 1 ml of 6 N hydrochloric acid and heated in a vessel under reflux for 15 min. After 2 h the whole was filtered and the sediment remaining on the funnel was washed with a small amount of methanol. The methanol extract was evaporated to dryness, and the sediment obtained was infused with boiling water and left to cool to room temperature (for

ca. 1 h). Then the aqueous solution of phenols was purified on a 1.2× 10 cm column with polyamide (Woelm) according to Borkowski (1973). The column, after placing the sample on it, was washed with water and then the phenols were eluted with 25 ml of methanol. After each partition the column with polyamide was regenerated with 25 ml of 1 N sodium base. The methanol extract (0.02 ml) was used for colorimetric determination of the amount of phenolic compounds with Folin's reagent (Lowry, 1951). The aim of these determinations was to make possible adjustment of each sample, before placing it on the chromatographic plate, to an equal concentration.

Fig. 1. Distribution of the investigated populations on the territory of Poland

The phenol compounds were separated in two directions on chromatographic plates (Merck) of 20×20 cm dimensions coated with silica gel 60 F-254 on which the 0.01 ml samples were placed. The chromatograms in both directions were developed on a 15 cm length.

Developers
1-st direction — toluene: ethyl formate: 80% formic acid (5 : 4 : 1)
2nd direction — ethyl acetate: n-propanol: 25% ammonia (3 : 5 : 2)
(Opieńska-Blauth, 1971)

From each sample 3 chromatograms were developed which after drying of plates of phenols were successively identified in normal and UV light, and in UV after saturation of the gel with ammonia vapours and spraying the plates with diazotised sulphanilic acid. From the three replications with each sample a "chromatogram" was prepared for a single plant. On it the dispersion of spots was marked and the

R_f calculated. Finally, for each population synthetic chromatograms were prepared. The results thus obtained were subjected to mathematic analysis.

Variability within each population was calculated by the formula

$$PI = \sum_{i=1}^{n} \frac{q_i(1-q_i)}{n}, \quad \text{(Marshall, Jain — 1969)}$$

where

PI — polymorphism index

m — total number of spots in population,

q_i — frequency of occurrence of i-th spot in population.

In order to compare the particular populations the coefficient of similarity was calculated by means of the formula

$$\frac{a+n}{a+n+d} \quad \text{(Natarella, Sink — 1974)}$$

where a — number of spots common to the compared populations,

n — number of spots absent in both compared populations (but occurring in the remaining ones),

d — number of spots distinguishing both compared populations.

On the basis of the value of the similarity coefficient a dendrite and a dendrogramme were plotted. In plotting of the former the reciprocal of the similarity coefficient was taken as measure of the taxonomical distance.

RESULTS

The chromatographic analyses demonstrated that, within the 6 examined populations, there were 88 different phenol compounds, which were detected by several methods described earlier. Below a list is given of the spots revealed in the specified way:

1. Spots visible only in UW — 1, 22, 23, 24, 25, 29, 30, 31, 41, 50, 51, 66, 69, 86, 87.
2. Spots visible in UV after saturation of the gel with ammonia vapours — 67, 70, 79.
3. Spots visible both in UV and in UV after saturation of gel with ammonia vapours — 17, 19, 20, 21, 27, 28, 32, 33, 35, 36, 37, 39, 40, 42, 43, 44, 45, 46, 47, 48, 49, 52, 59, 60, 62, 63, 64, 65, 71, 72, 73, 74, 75, 81, 85, 88.
4. Spots detected after spraying the plates with diazotised sulphanilic acid — 3, 5, 8, 9, 10, 11, 12, 13, 14, 16, 26, 56, 57, 58, 61, 68.

5. Spots visible with the naked eye — 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 26, 53, 54, 56, 61, 76, 77, 78, 80, 83, 84.
6. Spots detected by all treatments — 3, 8, 10, 11, 14, 16, 26, 56, 57 61.

As seen from this list most spots which developed colour after spraying of the plates with diazotised sulphanilic acid could be noticed with the naked eye.

Analyses of the particular plants made it possible to prepare synthetic chromatograms for each of the studied populations. It was found on their basis that the populations had a characteristic spectrum and a definite number of the separated compounds (Fig. 2). Of all those examined, the populations from Wielkopolska region exhibited the largest number of detectable compounds. The poorest in phenol compounds were the populations from the Tatra National Park, and those from the coast had an intermediate number (Table 2).

Table 2

Mean number of spots per individual and total number of spots in six *Anthyllis populations*

Populations	Mean No. of spots per individual	No. of spots in population
II — Chłopy	10	56
III — Mielno	16	69
IV — Kalatówki	12	53
VI — Skupniów Upłaz	8	43
VII — Rożnowo	21	73
IX — Międzychód	22	75

Table 3

Polymorphism index values for the studied populations

Populations	II	III	IV	VI	VII	IX
Polymorphism index	0.113	0.128	0.137	0.095	0.150	0.156

The values of the polymorphism index rose with the increase in the number of spots on the synthetic chromatograms. This made it possible to determine quantitatively the variability within each population (Table 3). The populations from the Wielkopolska lowland showed the highest variability (highest values of the polymorphism index), whereas that from Skupniów Upłaz was more homogeneous. This is confirmed by the data from Table 3 and Fig. 2. Further evidence of the high intrapopulation variability is found in the comparison of the mean number of spots per individual with the number of spots on

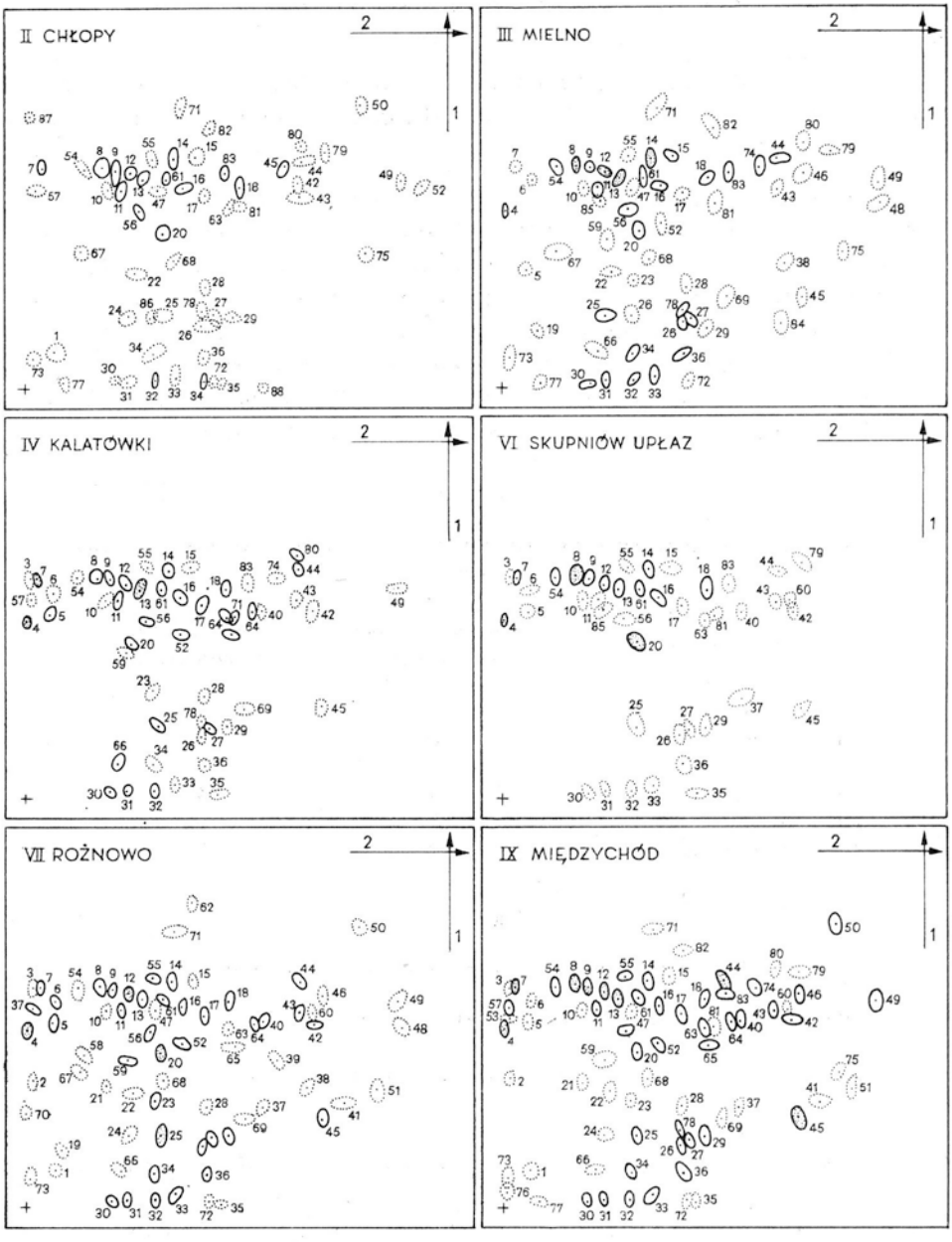

Fig. 2. Synthetic chromatograms for 6 *Anthyllis* populations

Dashed line represents sporadically appearing spots of 0—25% frequency, the continuous line indicates a frequency of 21—70%, a continuous line and dotted surface refer to characteristic spots of 71—100% frequency

Table 4

Frequency of occurrence of given spot in population
(frequency of individuals giving this spot) in per cents

Spot No.	Populations					
	II	III	IV	VI	VII	IX
1	13.8	0	0	0	3.3	13.3
2	0	0	0	0	16.7	10.7
3	0	0	3.4	3.3	20.0	6.7
4	55.2	63.3	72.4	86.7	96.7	93,3
5	0	6.7	27.6	3.3	30.0	10.0
6	0	3.3	3.4	3.3	30.0	3.3
7	55.2	20.0	72.4	36.7	63.3	86.7
8	41.4	73.3	55.2	76.7	66.7	76.7
9	38.0	63.3	44.8	46.7	66.7	73.3
10	10.3	13.3	10.0	3.3	13.3	3.3
11	34.4	30.0	31.0	3.3	30.0	33.3
12	48.3	80.0	58.6	63.3	90.0	86.7
13	31.0	90.0	75.9	73.3	83.3	90.0
14	34.4	76.7	44.8	36.7	63.3	50.0
15	13.8	36.7	13.8	3.3	13.3	20.0
16	31.0	70.0	41.4	33.3	50.0	53.3
17	17.2	3.3	55.2	10.0	56.7	46.7
18	27.6	56.7	24.1	30.0	36.7	36.7
19	0	3.3	0	0	20.0	0
20	44.8	63.3	69.0	100.0	76.7	53.3
21	0	0	0	0	13.3	3.3
22	3.4	6.7	0	0	13.3	3.3
23	0	6.7	3.4	0	30.0	20.0
24	3.4	20.0	0	0	13.3	13.3
25	20.7	60.0	38.0	6.7	73.3	66.7
26	6.9	36.7	13.8	3.3	46.7	40.0
27	3.4	60.0	34.4	3.3	56.7	43.3
28	3.4	6.7	3.4	0	16.7	3.3
29	3.4	13.3	6.9	10.0	43.3	36.7
30	17.2	56.7	24.1	16.7	30.0	33.3
31	6.9	66.7	31.0	6.7	30.0	23.3
32	24.1	33.3	24.1	10.0	36.7	36.7
33	17.2	40.0	13.8	10.0	50.0	33.3
34	3.4	33.3	13.8	0	33.3	46.7
35	17.2	0	17.2	3.3	20.0	13.3
36	6.9	43.3	3.4	6.7	40.0	23.3
37	0	0	0	3.3	6.7	6.7
38	0	3.3	0	0	3.3	0
39	0	0	0	0	6.7	0
40	0	0	10.3	6.7	36.7	33.3
41	0	0	0	0	3.3	6.7
42	3.4	0	10.3	10.0	50.0	50.0
43	13.8	3.3	13.8	3.3	60.0	66.7
44	10.3	36.7	31.0	6.7	70.0	73.3
45	0	13.3	17.2	10.0	70.0	73.3
46	0	3.3	0	0	10.0	30.0
47	10.3	6.7	0	0	10.0	13.3
48	0	3.3	0	0	10.0	0
49	6.9	3.3	3.4	0	6.7	23.3
50	3.4	0	0	0	3.3	23.3
51	0	0	0	0	3.3	3.3
52	13.8	3.3	27.6	0	40.0	26.7
53	0	0	0	0	13.3	3.3
54	3.4	46.7	10.3	30.0	13.3	23.3
55	6.9	20.0	13.8	10.0	30.0	23.3
56	31.0	36.7	38.0	6.7	26.7	33.3
57	17.2	0	13.8	0	23.3	40.0
58	0	0	0	0	13.3	0
59	0	3.3	3.4	0	23.3	6.7
60	0	0	0	10.0	20.0	20.0
61	27.6	66.7	44.8	40.0	36.7	36.7
62	0	0	0	0	3.3	0
63	3.4	0	24.1	3.3	16.7	40.0
64	0	0	20.7	0	26.7	46.7
65	0	0	6.9	0	10.0	30.0
66	0	3.3	6.9	0	6.7	13.3
67	3.4	3.3	0	0	10.0	0
68	3.4	20.0	0	0	10.0	16.7
69	0	3.3	3.4	0	6.7	3.3
70	0	0	0	0	3.3	0
71	3.4	3.3	0	0	3.3	13.3
72	10.3	6.7	0	0	3.3	16.7
73	3.4	3.3	0	0	6.7	16.7
74	14.8	33.3	10.3	0	0	40.0
75	3.4	6.7	0	0	0	3.3
76	0	0	0	0	0	16.7
77	6.9	6.7	0	0	0	3.3
78	10.3	26.7	10.3	0	0	40.0
79	6.9	6.7	0	3.3	0	10.0
80	6.9	10.0	31.0	0	0	16.7
81	10.3	3.3	24.1	3.3	0	20.0
82	3.4	6.7	0	0	0	3.3
83	24.1	26.7	10.3	3.3	0	23.3
84	0	3.3	0	0	0	0
85	0	3.3	0	6.7	0	0
86	3.4	3.3	0	0	0	0
87	3.4	0	0	0	0	0
88	6.9	0	0	0	0	0

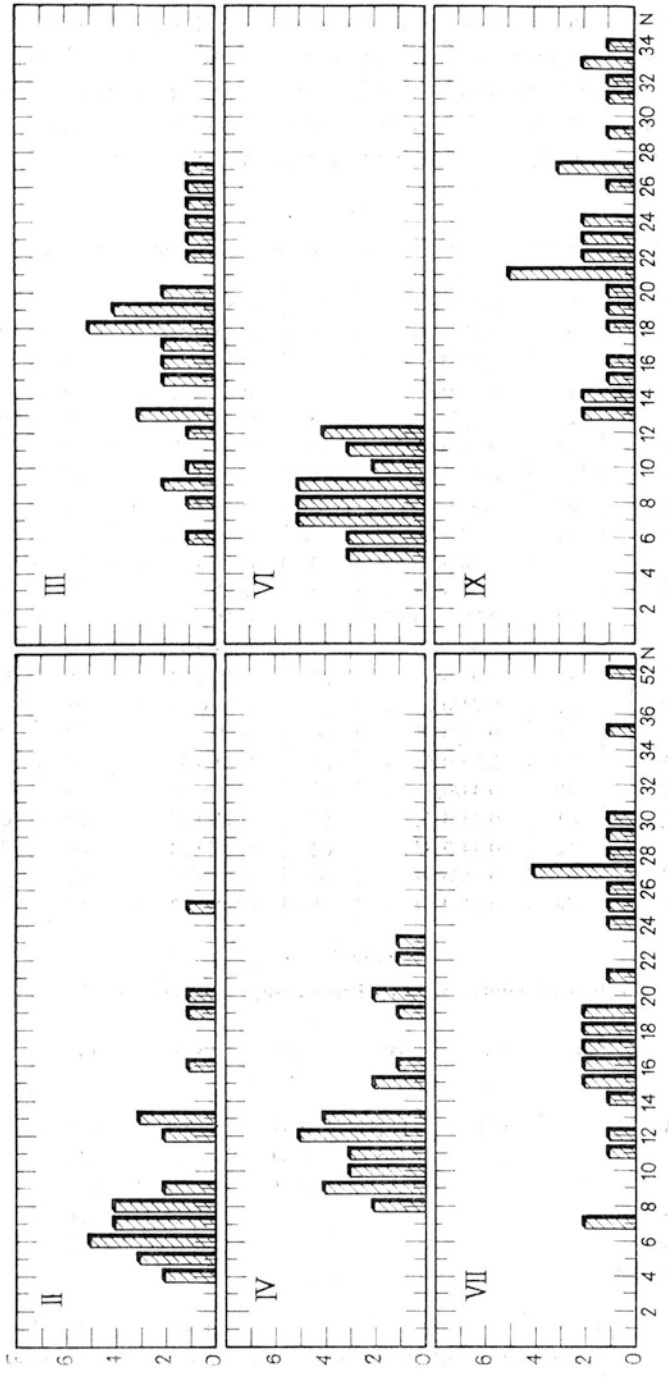

Fig. 3. Distribution of spot frequency for the populations
N — number of single spots on chromatograms, F — number of chromatograms with a definite spot number

the synthetic chromatogram. The mean number of spots on the chromatograms of single plants are listed in Table 2. The wide differences between the plants are stressed by the fact that from the 88 distinguished compounds only 31 were common to all populations. Moreover, many spots were detected which appeared only sporadically that is from one or two individuals in the population (Fig. 3, Table 4). This caused rather wide differences between the populations.

Table 5

R_f values for all the spots detected on the chromatogram

1	0.09/0.07	23	0.25/0.30	45	0.57/0.69	67	0.32/0.12
2	0.30/0.02	24	0.17/0.24	46	0.50/0.69	68	0.30/0.34
3	0.51/0.02	25	0.17/0.31	47	0.46/0.80	69	0.21/0.51
4	0.41/0.01	26	0.15/0.41	48	0.43/0.87	70	0.22/0.00
5	0.43/0.06	27	0.16/0.43	49	0.49/0.86	71	0.65/0.35
6	0.48/0.07	28	0.24/0.42	50	0.66/0.77	72	0.02/0.43
7	0.51/0.03	29	0.17/0.47	51	0.28/0.81	73	0.07/0.01
8	0.52/0.17	30	0.01/0.20	52	0.38/0.36	74	0.51/0.59
9	0.51/0.20	31	0.02/0.24	53	0.44/0.02	75	0.32/0.78
10	0.46/0.19	32	0.02/0.30	54	0.51/0.12	76	0.04/0.01
11	0.46/0.22	33	0.03/0.35	55	0.54/0.29	77	0.01/0.09
12	0.50/0.24	34	0.08/0.30	56	0.41/0.29	78	0.18/0.41
13	0.49/0.27	35	0.01/0.45	57	0.46/0.02	79	0.55/0.69
14	0.53/0.34	36	0.08/0.42	58	0.36/0.14	80	0.56/0.63
15	0.54/0.39	37	0.24/0.55	59	0.34/0.24	81	0.42/0.49
16	0.47/0.36	38	0.29/0.65	60	0.46/0.66	82	0.60/0.42
17	0.45/0.41	39	0.35/0.58	61	0.49/0.32	83	0.50/0.52
18	0.49/0.47	40	0.44/0.55	62	0.71/0.39	84	0.15/0.64
19	0.13/0.08	41	0.25/0.74	63	0.42/0.47	85	0.44/0.23
20	0.36/0.31	42	0.44/0.67	64	0.44/0.53	86	0.17/0.29
21	0.29/0.19	43	0.46/0.63	65	0.38/0.48	87	0.63/0.00
22	0.27/0.25	44	0.53/0.64	66	0.09/0.22	88	0.00/0.55

Table 6

Values of similarity coefficient between populations

	II	III	IV	VI	VII	IX
II	×	0.77	0.67	0.65	0.58	0.72
III		×	0.68	0.60	0.63	0.72
IV			×	0.81	0.66	0.66
VI				×	0.57	0.61
VII					×	0.83
IX						×

In spite of the differences between the plants, the populations originating from the same region had on the synthetic chromatograms more common spots than did the populations from different areas. This was confirmed by the values of the similarity coefficient (Table 5).

On the basis of these data three groups of populations could be distinguished: coastal, Wielkopolska and mountain ones. This is illustrated by the dendrite and dendrogram (Figs 4 and 5). The mountain popula-

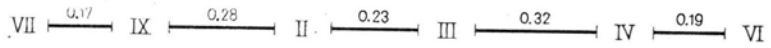

Fig. 4. Dendrite illustrating the position of the populations

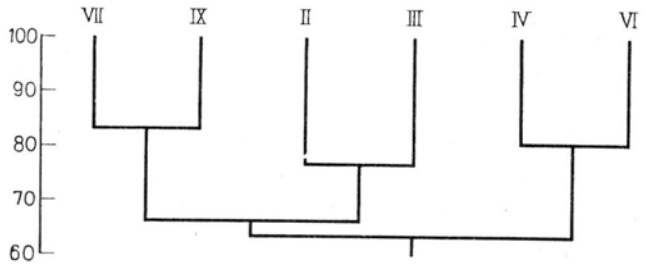

Fig. 5. Dendrogramme showing the similarities between the populations and population groups

tions are most distant both from the Wielkopolska and the coastal ones. The divergence of these populations is still more evident, owing to the fact that they do not exhibit the spots 22, 67, 68, 7, 73 noted for the coastal and Wielkopolska populations. The latter populations exhibited 7 common spots which occurred only for them: 21, 24, 47, 68, 71, 72, 73. The Wielkopolska and mountain populations had only one spot in common which did not appear for the coastal ones (spot 40), and the coastal and mountain populations had no spot which would occur solely for them.

DISCUSSION

The morphological methods applied in taxonomy do not give a full picture of the variability. Therefore numerous authors have tried to analyse the chemical characters. Chromatographic methods were used for this purpose. Among numerous studies the paper by Hegnauer (1970) may be mentioned here. This author described some alkaloid-free lupin races which could not be distinguished by way of morphological methods of analysis.

Couderc and Gonnet (1972) investigated subspecies and species of *Anthyllis*, analysing flavone aglicones in natural populations occurring in France. According to the results obtained they divided the examined species and subspecies into groups of variable and unvariable

ones. The results of their studies were not in agreement with those of examination of morphological characters. They interpreted this fact as being due to the high genetic variability supposed to arise by way of mutation. This, however, is not concordant with the assumption of Jalas (1957) that the presently existing species of *Anthyllis* arose mainly by way of crosses.

There exists within each species a higher or lower variability as demonstrated by many investigators. Among others the studies of Brehm (1965) on the flavonoid composition in *Tragopogon* confirm the existence of a high individual variability. This author clims that this variability depends on the heredity system and results from introgression. The studies of Weimarck (1970) on *Hierochloë* also demonstrated a high individual variability within each examined species.

In the present paper the existence has been demonstrated of a high individual variability within the studied *Anthyllis* populations. Since the experiments were conducted in uniform cultivation conditions, it would seem that the observed variability is largely genetically conditioned. In spite of the existing high variability, each of the populations was characteristic as regards the spectrum of the separated compounds. On the basis of the chromatograms the particular populations can be distinguished mainly according to the characteristic spots. For the groups of populations classified according to their origin characteristic spots were also established.

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Chromatograficzne analizy związków fenolowych u sześciu naturalnych populacji Anthyllis vulneraria L.

Streszczenie

Metodą chromatografii cienkowarstwowej badano skład związków fenolowych u pojedynczych osobników sześciu naturalnych populacji *Anthyllis* z trzech regionów geograficznych Polski.

Wyniki przeprowadzonych analiz wykazały, że populacje były zmienne ze względu na skład badanych związków. Najbardziej zmiennymi były populacje wielkopolskie, u których wykryto największą liczbę plam. Populacje tatrzańskie były najuboższe w badane związki.

Każda populacja miała swoiste spektrum rozdzielonych związków. Mimo tego i mimo dużego zróżnicowania roślin populacje pochodzące z podobnych siedlisk miały więcej plam wspólnych niż populacje z różnych regionów. Najbardziej odległymi były populacje górskie.