BIOCHEMICAL CHARACTERIZATION OF TWO SIBLING SPECIES IN THE LIVERWORT *PELLIA EPIPHYLLA* (L.) CORDA (HEPATICAEE, PELLIAEEAE)

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ABSTRACT

Two sibling species in the *Pellia epiphylla* (L.) Corda (n=9) complex, viz. *Pellia epiphylla* sp. N and *Pellia epiphylla* sp. S were compared in their protein concentration and in the dynamics and also in relative activity of some enzymes: malate dehydrogenase (MDH), esterase (EST), peroxidase (PX) and aspartate aminotransferase (GOT). Serological properties of their antigens were studied as well. Activity of malate dehydrogenase, esterase and aspartate aminotransferase was significantly higher in clons of *Pellia epiphylla* N than *Pellia epiphylla* S.

Some differences in soluble protein concentration and in the quantities of the AI to AIV antigens between the two sibling species were detected.

KEY WORDS: *Hepaticae*, *Pellia epiphylla* (n=9) complex, sibling species, biochemical variation.

INTRODUCTION

Electrophoretic research on various populations of *Pellia epiphylla* (n=9) collected in Poland resulted in the rather unexpected discovery of the complex nature of this species. First, Szweykowski et al. (1981) stated that there are at least three different peroxidase phenotypes in Polish populations of this species; they were called PX1a, PX2a and PX3. Further studies by Zieliński (1984, 1987a, 1987b) revealed, that the differences between the phenotypes PX1a and PX2a, as contrasted with the phenotype PX3, are of such a magnitude (different alleles fixed in 9 out of 20 loci studied) that PX1a and PX2a should be treated as representing two distinct species. The species were called, for the time being, *Pellia epiphylla* sp. S, with two different peroxidase phenotypes (i.e. PX1a and PX2a), and *Pellia epiphylla* sp. N, having only one, at least in Poland, peroxidase phenotype (PX3). As all attempts to find morphological or anatomical differences between PX1a and PX2a individuals have failed so far, they fall into the category of sibling species. This was one of the very first reports on the existence of sibling species in *Bryophytes*.

The two species differ in their geographical distributions (Szweykowski et al. 1995); also some karyological differences were observed (Zieliński 1987a, Chudzińska, unpublished data). The aim of the present paper was a comparison of the two species in some biochemical aspects: protein concentration in extracts of the thalli, activity of selected enzymes and some serological characters were studied.

MATERIAL AND METHODS

Nine samples of *Pellia epiphylla* sp. S and 5 samples of *Pellia epiphylla* sp. N, collected in various places in Poland, were studied (Table 1). Individual thalli were planted into plastic pots filled with peat. After several months of cultiva-

TABLE 1. List of the samples of *Pellia epiphylla* used in the study

Clone no.	Place of collection
<i>Pellia epiphylla</i> sp. S	
94	Boczań, Tatra Mts
178	Boczań, Tatra Mts
272	Boczań, Tatra Mts
175	Zbocze Wysockiego, Tatra Mts
180	Zbocze Wysockiego, Tatra Mts
273	Zbocze Wysockiego, Tatra Mts
87	Wisła Czarne, Barania Góra Mts
146	Beretki, Bieszczady Mts
214	Wejherowo
<i>Pellia epiphylla</i> sp. N	
205	Pestkownica near Kuźnica Żelichowska
192	Kampinos near Warszawa
203	Milomłyn near Ostróda
165	Wamowo, Wolin Isl
235	Klembów near Tłuszcz

tion these thalli formed colonies of the genetically identical plants. The electrophoretic phenotypes of peroxidase (PX1a, PX2a and PX3) were identified in each clone according to the method described by Zielinski (1984, 1987a). Moreover, chromosome numbers were checked in all the clones. The number of chromosomes $n=9$ was detected in the all samples as described earlier (Zielinski 1987a).

One gram of fresh thalli was taken from each clone and cleaned from remains of peat and other debris. For protein extraction the thalli were ground in a cooled to -16°C mortar, in the 0.1 M. extraction buffer (Tris- H_3BO_3 -EDTA), pH 7.4. (1 part of thalli: 4 parts of extraction buffer). The homogenate was centrifuged at 4°C and the supernatants were used for further analyses.

The extracts were analysed for protein content by the Lowry method. Variation in the activity of some enzymes was studied. Each kind of analyses were done in triplicate for single clone and mean value for the data were calculated. Serological analyses (immunodiffusion, simple immuno-electrophoresis, tandem – and rocket immunoelectrophoresis) were carried out as well.

Enzymatic activity analyses

The analysis of activity of the enzymes was performed according to the methods of Worthington (1972) with some described below minor modifications. The enzymes activity were measured every 10 or 20 minutes, on the basis of colorimetric reactions at 40°C .

The analysis of selected enzymes aimed to reveal if the two sibling species from *Pellia epiphylla* complex differ in these properties.

As a measure of enzymes activity the increase of the absorbance in time was applied. For particular enzyme the research of dependence of effect of enzymatic reaction upon the time was performed. The results were presented as histograms drawn for the value where the curve of absorbance reached the maximum, but was still proportional and linear to factor of time of reaction.

1. Malate dehydrogenase (MDH-E.C.1.1.1.37). Five ml of staining mixture were put into each of the 28 test-tubes used. The staining mixture contained glycine buffer (1.5 g of glycine, 196 ml of H_2O adjusted with NaOH to pH 8.7), 140 mg of L-malic acid, 10 mg of nitro blue tetrazolium and 10 mg of NADH₂. The test tubes were placed in ice and 50 μl of thalli extract was added to each of them except the tube for blank test.. The results were read out at 625 nm.
2. Esterase (EST-E.C.3.1.1.6). Esterase activity was estimated for the wave length 540 nm. The staining mixture consisted of 150 ml phosphate buffer 0.1 M (pH 6.0), 150 mg of alpha-naphtyl acetate, 75 mg of Fast Blue RR salt dissolved in 2 ml of di-methylformamide.
3. Peroxidase (PX-E.C.1.11.1.7). Enzymatic activity was measured, for the wave length 450 nm. The staining mixture consisted of 75 mg of 3-amino-9-ethyl-carbasole, 3 ml of di-methylformamide, 3 ml of 0.1% MgCl_2 and 8 ml of 3% H_2O_2 , all dissolved in acetate buffer 0.1 M (pH 5.0). Because of the extremely high activity of this enzyme the amount of extract used for the analyses was reduced to 20 μl .
4. Aspartate aminotransferase (GOT-E.C.2.6.11). Analysis of the enzyme activity was performed for the wave length 550 nm. The staining mixture consisted of 340 mg aspartic acid, 150 mg alpha-ketoglutaric acid, 150 mg Fast Blue BB salt, 4 mg of pyridoxal-5-phosphate.

The absorbance values from enzyme activity estimations were recalculated per mg of total soluble proteins.

Serological analyses

Serological analyses were carried out as described in the previous papers (Szweykowski et al. 1981, Prus-Głowacki and Nowak 1982, Prus-Głowacki and Nowak-Bzowy 1986).

Antiserum against proteins of *Pellia borealis* (a tetraploid species of $n=18$), with the titer of 1:128, was additionally concentrated to the half of its previous volume with aquacid. Comparison of the relative amount of particular antigens was performed by rocket immunoelectrophoresis and estimation of the rocket height, which correlated with the quantity of a given antigen in the sample studied.

RESULTS

Total soluble protein content

The average contents of protein in extracts of *Pellia epiphylla* sp. *S* and *Pellia epiphylla* sp. *N*, were, respectively, 17.69 mg/ml and 14.74 mg/ml (Table 2).

TABLE 2. Concentration of proteins in the clones of *Pellia epiphylla* sp. *S* and *Pellia epiphylla* sp. *N*, in mg per ml of extract and in mg per gram of fresh weight

Clone no.	mg/ml	SD	R	mg/g f.w.
<i>Pellia epiphylla</i> sp. <i>S</i>				
94	15.20			60.80
272	15.85			63.40
273	18.00			72.00
214	15.30			61.20
178	21.00			84.00
175	15.50			62.00
180	18.10			72.40
146	21.30			85.20
87	19.00			76.00
	x: 17.69	2.51	4.30	x: 70.10
<i>Pellia epiphylla</i> sp. <i>N</i>				
192	16.10			64.40
205	15.90			63.60
203	18.10			72.40
165	12.80			51.20
235	10.80			43.20
	x: 14.74	6.75	7.30	x: 59.00

Notwithstanding the extensive variation in protein concentrations between samples (standard deviations for *Pellia epiphylla* sp. *S* and *Pellia epiphylla* sp. *N* were 2.51), however these differences were not statistically significant. The protein concentration, calculated in mg/g of fresh thalli, was also higher for the former species ($S = 70.1$ mg/g) in comparison with the latter one ($N = 59.0$ mg/g). Considerable differences in this parameter were detected between individual samples.

Enzymes activity

The results of enzyme activity are presented in Fig. 1. The MDH activity measured after and 50 minutes, was about 4 ti-

Fig. 1. Comparison of the activity of malate dehydrogenase (MDH), esterase (EST), peroxidase (PX) and aspartate transferase (GOT) in various clons of *Pellia epiphylla* N (open bars) and *Pellia epiphylla* S (black bars). Clons numbers: 1-205, 2-192, 3-175, 4-94 and 5-273. E – value of absorbance.

mes lower in extracts of *Pellia epiphylla* sp. S than in extracts of species. *Pellia epiphylla* N (Fig. 1). The mean absorbance values were 0.0051 and 0.0195 respectively.

An almost threefold lower esterase activity was observed after 15 min. in *Pellia epiphylla* sp. S, as compared with *Pellia epiphylla* sp. N. The mean absorbance values were 0.0060 in extract from clons *Pellia epiphylla* S and 0.0180 in *P. epiphylla* N.

The peroxidase activity does not differ significantly among the studied clons of *Pellia* sp. However clon no. 205 (*P. epiphylla* N) posses the highest activity of peroxidase among the studied samples.

Aspartate aminotransferase exhibited also similar differences in activity between the two species: after 10 min. The mean absorbance values amounted to 0.018 in *Pellia epiphylla* sp. S and 0.023 in the other species – *Pellia epiphylla* N.

TABLE 3. Relative amounts of common antigens AI to AIV in the studied clones of *Pellia epiphylla* sp. S and *Pellia epiphylla* sp. N, presented as hights of immunoelectrophoretic rockets (in mm)

Clone no.	AI	AII	AIII	AIV
<i>Pellia epiphylla</i> sp. S				
175	18.8	11.6	3.5	1.1
87	16.3	3.2	5.8	0.8
94	2.4	8.3	5.8	0.1
273	13.6	20.4	5.6	0.2
272	–	15.8	5.0	0.1
146	21.5	10.0	4.4	0.5
214	–	11.9	4.5	0.1
178	–	2.9	3.1	1.2
180	–	5.0	3.7	1.1
Average	14.5	9.9	4.6	0.6
<i>Pellia epiphylla</i> sp. N				
192	8.3	1.4	5.8	0.3
205	9.3	5.0	5.7	0.3
203	11.2	8.9	4.0	0.6
165	8.9	1.4	3.8	0.1
Average	9.4	4.2	4.8	0.3

The three enzymes (MDH, GOT and EST) manifested the differences between the two species: At the similar average protein concentration in the extracts (16.2 and 16.0 mg/ml, respectively), the average activity of the studied enzymes was lower for *Pellia epiphylla* sp. S than for the *Pellia epiphylla* sp. N).

Serological analyses

Presence of common four antigens, denoted AI to AIV, was detected. Amounts of the antigens I and II were higher in *Pellia epiphylla* sp. S than in the N species (Table 3). Because of the considerable inter-clone variation in the amounts of individual antigens, no significant differences could be disclosed between the sibling species. Nevertheless, serological properties of proteins in *Pellia epiphylla* sp. S clones allowed to distinguish two types of patterns typical for the species. In one of the patterns, exemplified by clones no. 175, 87, 273, 146, high amounts of antigen I were present while in the other (clones no. 94, 272, 214, 178 and 180) the antigen was absent or present in very low amounts. A significant inter-clone variation was noted also in the amounts of antigen II (Table 3, Fig. 2).

Fig. 2. Rocket immunoelectrophoresis of electrophoretic phenotypes (PX1, PX2, PX3) of *Pellia epiphylla* complex proteins. Antiserum against proteins of *Pellia borealis* – natural poliploid of *Pellia epiphylla*. Numbers denote individual clone. Roman numbers – I to IV denote individual antigens.

The general serological patterns differed between *P. epiphylla* sp. *S* and *P. epiphylla* sp. *N* clones. In addition, some serological differences were also detected between clones of *P. epiphylla* sp. *N* (Fig. 2).

DISCUSSION

Sibling species were discovered in liverworts by identifying their electrophoretic phenotypes in the following *Bryophyte* species: *Pellia epiphylla* (L.) Corda (Szweykowski et al. 1981, Zieliński 1984, 1987a, 1987b), *Pellia endiviifolia* (Dicks.) Dum. (Zieliński 1987a), *Conocephalum conicum* (L.) (Szweykowski, Krzakowa 1979, Krzakowa, Szweykowski 1977, Szweykowski et al. 1981, Odrzykoski 1985, 1986, 1987, Odrzykoski, Szweykowski 1991), *Riccia dictyospora* Howe (Dewey 1989) and *Aneura pinguis* (L.) Dum. (Szweykowski, Odrzykoski 1990). In all the mentioned cases, sibling species have been different in their multilocus electrophoretic phenotypes to such an extent that the values of their diversity, measured by Nei's genetic distance (Nei 1972), is equal to or even exceeded those observed between generally accepted standards for the so called good species in various groups of organisms, particularly in flowering plants (Odrzykoski and Szweykowski 1991).

The results presented in this paper shows that the sibling species in *Pellia epiphylla* complex differ profoundly in the activity of the studied enzymes and serological properties of proteins. Thus, the differences between them must have also the physiological and ecological basis. Moreover, as it has been shown by Szweykowski et al. (1995) the studied sibling species exhibit also allopatric geographical distributions in Poland. The search for morphological differences in *Pellia* (Zieliński 1987a) have given, however, equivocal results. In contrast, in case of other sibling species in *Bryophytes*, such studies have been successful (Buczowska, Szweykowski in preparation). Taking all these circumstances into account we can conclude that the existence of sibling species in liverworts is a well grounded phenomenon; the classical taxonomy should take this fact into consideration.

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BIOCHEMICZNA CHARAKTERYSTYKA DWÓCH GATUNKÓW BLIŹNIACZYCH U WĄTROBOWCA *PELLIA EPIPHYLLA* (L.) CORDA (HEPATICAE, PELLIACEAE)

STRESZCZENIE

Dwa gatunki bliźniacze w kompleksie *Pellia epiphylla* (L.) Corda n=9, to jest *P. epiphylla* sp. *N* i *Pellia epiphylla* sp. *S* porównano pod względem zawartości białka w plechach i aktywności czterech enzymów (dehydrogenazy jabłczanowej MDH, esterazy EST, peroksydazy PX, aminotransferazy asparaginianowej GOT). Badano także właściwości antygenowe białek u obu taksonów. Aktywność badanych enzymów była znacząco wyższa u *P. epiphylla* N. Wykryto również różnice w zawartości białek rozpuszczalnych jak również w ilości homologicznych antygenów u badanych gatunków bliźniaczych.

SŁOWA KLUCZOWE: wątrobowce, kompleks *Pellia epiphylla* n=9, gatunki bliźniacze, zmienność biochemiczna.