INTER- AND INTRASPECIFIC SEROLOGICAL RELATIONSHIPS IN *PELLIA EPIPHYLLA* COMPLEX

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(Received: December 17, 1996. Accepted: July 7, 1997)

ABSTRACT

Antigenic proteins were used as markers for the study of relationships between three liverwort species from *P. epiphylla* complex. It has recently been shown that the electrophoretic phenotypes of this species suggested an amphiploid origin of *P. borealis*. Two sibling species: *P. epiphylla* -species *S* and -species *N* could have probably represented the parental species for *P. borealis*. We examined three clones of *P. borealis* from different localities using immunodiffusion. Then we compared them with *P. epiphylla* species *S* and *N* as well as with the mixture of proteins of *P. epiphylla* *S* and *N* samples. The results indicate that polyploid *P. borealis* shows an identical immunological pattern to that of the mixture of proteins of putative parental species. Only in one case the result resembled much more the pattern of *P. epiphylla* *S* proteins. The sibling species *P. epiphylla* *S* and *N* showed antigenic difference but the nature of the differences requires further studies. Antigenic properties of proteins from *P. epiphylla* *S* and *N* and of their allopolyploid - *P. borealis*, indicated some specificity of the protein spectrum in each of the parental species and intermediate character of proteins in the polyploid forms.

KEY WORDS: *Pellia borealis*, *Pellia epiphylla*, polyploidization, antigen proteins, immunodiffusion.

INTRODUCTION

In bryotaxonomy the origin of tetraploid taxon *P. borealis* represents a still unsolved problem. Almost 90% of liverwort species are diploid in relation to sporophyte (Przywara and Kuta 1995). *Pellia borealis* Lorb. $n = 2x = 18$, was one of the first polyploid form described in this group of plants. Two contrasting theories were tested for solving the way of its polyploidization. On the one hand, some morphological, anatomical and biochemical studies first suggested that polyploidization of *P. epiphylla* $n=x=9$ represents the most probable way of the origin of *P. borealis* (Mendelak, 1971/1972, Szweykowski et al. 1981, Zieliński, 1984). The data supported hypothesis of autopolyploidization, the most frequent way on which polyploids originate among the bryophytes (Smith, 1987). On the other hand, earlier cytological data showed a unique distribution of heterochromatin in each of the 18 chromosomes (Jachimsky, 1935), what may suggests that *P. borealis* is an allopolyploid originated on the basis of interspecific hybridization. Newton (1986) confirmed it using differential staining methods. Each of the *P. borealis* chromosomes shows a unique and individual banding pattern which would not be possible if they were duplicated from chromosomes of one species. The data tend to support the theory of allopolyploidization.

Zieliński (1987) discovered two enzymotypes within *P. epiphylla*, which were recognized as two sibling species, named *P. epiphylla* *S* and *P. epiphylla* *N* (Szweykowski and Odrzykoski, 1990). They show differences in 6 enzymatic loci out of the 17 analysed enzymes, which results in a mean

genetic distance, according to Nei (1973), $D_n = 0.53$. Recent studies conducted on biochemical differentiation of polyploid *P. borealis* suggest that the taxon can represent an allopolyploid species. Analysis of enzymatic phenotypes in the two sibling species of *P. epiphylla* indicate that they are putative parental species of the tetraploid *P. borealis*. Four enzymes loci: NADH-diaphorase 1, NADH-diaphorase 3 (E.C. 1.6.99) mannose-6-phosphate isomerase 1 (E.C. 5.3.1.8.) and aconitase 1 (E.C. E.C. 4.2.1.3.), appear to be fixed heterozygous phenotypes in that polyploid taxon.

Specificity of immunological reactions was frequently used for solving problems in the plant taxonomy, for example in forest genetics (Prus-Głowacki, 1982, 1983) or in genetics of grasses (Smith, 1983; Prus-Głowacki et al. 1971). In the higher plants amphiploid proteins spectra consist of fused proteins spectra of the parental species (Johnson et al. 1967; Prus-Głowacki et al., 1971). Therefore some immunological methods were used to examine the origin of *P. borealis* (Szweykowski et al., 1981a; Prus-Głowacki and Nowak, 1982; Prus-Głowacki and Zieliński, 1987). The first results obtained in such serological analyses using anti-*epiphylla* and anti-*borealis* sera showed close similarity between the two liverworts. However, *P. borealis* and *P. epiphylla* showed proteins which were specific only for one of the species (Szweykowski et al. 1981). Further studies was conducted on the effect of genome duplication in polyploid species *P. borealis*. The content of antigenic proteins in the polyploid form was about 40% higher than that in the diploid *P. epiphylla* species (Prus-Głowacki and Nowak, 1982). This results (i.e. increase in the efficiency of the protein synthesis in the form of higher ploidy)

TABLE 1. Liverwort samples used for analyses

Population	Population number	Localization	Number of chromosomes (n)
<i>Pellia borealis</i>	B1-32455	Rez. Diabli Skok k. Wałcza, woj. pilskie	18
	B2-32456	Sulistrowiczki, Czarna Woda, woj. wrocławskie	18
	B3-32458	Wieczywno Małe, Lipnica, woj. śląskie	18
<i>Pellia epiphylla</i> N	N-34387	Klembów, woj. warszawskie	9
<i>Pellia epiphylla</i> S	S-32479	Rajcza, woj. bielsko-bialskie	9

was consistent with results of other authors, obtained for higher plants (Timko et al. 1980).

In the view of these results we decided to use immunochemical methods to demonstrate serological differences between two sibling species of *P. epiphylla* as well as to detect similarities within *P. epiphylla* complex. We expect that the results might specify more clearly how the polyploid *P. borealis* species originated.

MATERIAL AND METHODS

In the present study we used clones from three populations of *P. borealis* Lorb. (gametophyte chromosome number $n=2x=18$), from one population of *P. epiphylla* N (gametophyte chromosome number $n=x=9$) and one population of *P. epiphylla* S (gametophyte chromosome number $n=x=9$). Each of the studied populations was derived from a single thallus and was considered to represent a clone. Detailed data about this populations (clones) are given in Tab. 1. The plant material used for analyses was cultured for more than one year in a greenhouse under uniform conditions.

Electrophoretic phenotypes were determined for all samples following standard procedure for this two *Pellia* species (Odrzykoski et al., 1996). Diagnostic enzymes markers were: NADH-diaphorase (DIA 1), mannose-6-phosphate isomerase (MPI1) and aconitase 1 (ACO 1). Analysis of the mitotic chromosomes was carried out as described by Newton (1989). Chromosomes were stained in acetic-orceine and hematoxyline. Three to five metaphase plates were studied for every plant.

For serological analyses 1 g of fresh thalli was taken from each population. Proteins were extracted in cold porcelain mortars by grinding the thalli with quartz sand (1 part) supplemented with PVP (1 part to 2 parts of plant material). Extraction of antigens was performed using cold 0.01 M extraction buffer at pH 7.4 according to the ratio: 1 part of plant material to 4 parts of buffer. The composition of buffer was as follows: TRIS – 10.89 g/l, H_3BO_3 – 16.69 g/l, EDTA – 1.12 g/l. The homogenate was centrifuged for 20 min at $-4^\circ C$. For our analyses we used the following extracts: B1, B2, B3, N, S and mixture of extract N and S. Production of the immune sera against antigens of the *P. borealis* and *P. epiphylla* was described by Szweykowski et al. (1981) and Prus-Głowacki et al. (1986). The extracts from studied species of genus *Pellia* were analyzed by immunodiffusion (Ouchterlony, 1967). Immunodiffusion analyses were performed in 1.5% agarose (type A, Pharmacia) in a veronal – acetate buffer at pH 6.75 containing: veronal sodium – 5.886 g/l, sodium acetate – 2.348 g/l, 0.1 M HCl – 260 ml/l.

Following interaction of antigens with the antibodies, the plates were washed in 0.9% NaCl for 48 hours and in distilled water for 1 hour. Next they were dried and stained with Coomassie blue according to standard procedure (Laurell, 1972).

Results of immunodiffusion were interpreted according to the types of serological reactions described by Ouchterlony (1967). This data, described as following: 0 – absence of antigen, 1 – identical antigens, 0.5 – partial identical antigens, were used for calculating Jaccard coefficient of taxonomic similarity (S_{Jac}) according to the Sneath and Sokal formula (1973):

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

where "a" is the number of antigens present in the compared samples, "b" and "c" – numbers of antigens present in one but absent in the second sample and vice versa. Taxonomic distances (D_{Jac}) were calculated on the basis of coefficient of taxonomic similarity:

$$D_{Jac} = 1 - S_{Jac}$$

The taxonomic distances (D_{Jac}) were used to construct a dendrite and a dendrogram to visualize serological similarities of the studied samples.

RESULTS

1. The chromosome number and electrophoretic phenotypes

Tetraploid species *P. borealis* has gametophytic chromosome number of $n=18$ and two diploid species *P. epiphylla* N and S have $n=9$.

Genotypes of the polyploid represented a "fixed heterozygous" electrophoretic phenotype. The pattern of all diploid samples was always two banded for MPI and DIA, three banded for ACO. Sibling species (N and S) of the *P. epiphylla* each showed a simple, one band pattern of all studied alleles (Odrzykoski et al., 1996).

2. Serological analyses

Identification of antigens distinguished in the studied samples is presented in Tab. 2.

At the first stage of the studies, double immunodiffusion was performed using four kinds of anti-borealis serum, in order to compare three samples from the populations of *P. borealis* species from different sites. Two of the mentioned sera

TABLE 2. Results of immunodiffusion analysis of antigenic proteins of *P. borealis* and *P. epiphylla*. Denotation of samples as in Tab. 1.

Samples	Antigen 1	Antigen 2	Antigen 3
B1	0.5	0.5	1
B2	1	1	1
B3	1	1	1
S	0.5	0.5	1
N	1	0	1
S + N	1	1	1

(which we used for further reactions) gave very similar results. Three types of precipitation lines (1, 2, 3) present in all studied samples of *P. borealis* (Fig. 1) could be distinguished. According to the analyses, samples B2 and B3 could be considered as identical, while sample B1 demonstrated dissimilarity in antigenic lines 1 and 2.

The second part of our experiment comprised comparison between the samples of *P. borealis* and *P. epiphylla* N, S as

well as with the mixture of *P. epiphylla* S and N. The reaction of anti-borealis serum with antigens of *P. epiphylla* S and N showed differences in serological patterns (Fig. 1). In the *P. epiphylla* N species two antigenic proteins were observed: 1-very sharp which corresponded to 1 line of *P. borealis* B2 and B3 and weak line 3. In the form S three antigenic proteins were found, resembling those of *P. borealis* B1. Using the anti-epiphylla serum with antigens S and N two precipitation lines (1 and 2) were obtained for the species S and three precipitation lines (1, 2 and 3) for species N (Fig. 2). Immunodiffusion analysis of mixture of samples S with N demonstrated three protein fractions, which were homologous to those obtained for *P. borealis* B2 and B3 and semi-homologous to *P. borealis* B1 (Fig. 3).

Fig. 1. Double immunodiffusion of *Pellia epiphylla* (S, N) and *Pellia borealis* (B1, B2, B3) antigens with anti-borealis serum (b).

Fig. 2. Double immunodiffusion of S and N species of *Pellia epiphylla* with anti-epiphylla serum (e).

Cross reaction of anti-borealis serum with antigen from the species N in double immunodiffusion assay gave the following results: the second antigenic line in the samples of *P. borealis* (except the number B1 where it was very weak) and the third antigenic line in *P. borealis*, *P. epiphylla* S and N were observed. This third fraction was absent in the all studied cases after precipitation of anti-borealis serum with antigen S, while the other antigenic proteins were present (Fig. 4).

On the basis of the obtained data the coefficients of similarity (S_{Jac}) and taxonomic distances (D_{Jac}) were calculated (Tab. 3) as well as the dendrite and dendrogram were constructed (Fig. 5).

Position of the examined samples in the dendrite corresponded to their position in the dendrogram (Fig. 5). They formed two distinct groups. One of them consisted of immunochemically identical samples B2, B3 and of the mixture of proteins of species S and N. However, the species N of *P. epiphylla* linked to this group on the dendrogram, exhibited significant specificity. The second group formed the immunochemically identical samples B1 and S.

Fig. 3. Double immunodiffusion of *Pellia borealis* samples (B1, B2, B3), mixture of S and N samples of *Pellia epiphylla* (S+N) with anti-*borealis* serum (b).

DISCUSSION

The immunological reactions were used to study antigenic differences or similarities of the examined forms. The differences in serological patterns of *P. epiphylla* S and N were significant, which permitted to distinguish them from one another very clearly. This was documented both in reactions with anti-*borealis* and anti-*epiphylla* sera. Some protein fractions, common for the two forms, have been observed in double immunodiffusion tests after absorbtion in gel. The data confirmed the earlier study on genetic heterogenity within *P. epiphylla* in Poland (Zieliński, 1987) and the hypothesis of sibling species in *P. epiphylla* complex postulated by Szweykowski and Odrzykoski (1990).

Double immunodiffusion analyses of *P. borealis* antigens using anti-*borealis* serum has shown an interesting serological relationship within this species. One of the studied sample (B1 from northern Poland) demonstrated a clearer similarity to *P. epiphylla* S (from southern Poland) which was more evident than similarity to other samples of *P. borealis*, which were serologically identical. The same tendency has been observed after precipitation of anti-*borealis* and anti-*epiphylla* sera with the antigen N in the first assay and with antigen S in the second assay. The data seem to point to serological differences within the species of *P. borealis* as our earlier studies (unpublished) on several samples of clones of *P. borealis* made by quantitative immunoelectrophoresis (Fig. 6). Ob-

TABLE 3. Coefficients of serological similarity (S_{Jac}) and serological taxonomic distances (D_{Jac}) for the studied samples of *P. borealis* and *P. epiphylla*. Denotation of samples as in Table 1.

Samples		B1	B2	B3	S	N	S + N	D Jac
B1	S Jac		0.75	0.75	1.0	0.625	0.75	
B2		0.25		1.0	0.75	0.833	1.0	
B3		0.25	0.0		0.75	0.833	1.0	
S		0.0	0.25	0.25		0.625	0.75	
N		0.375	0.167	0.167	0.375		0.833	
S+N		0.25	0.0	0	0.25	0.167

Fig. 4. Cross reaction in double immunodiffusion of *Pellia borealis* (B1, B2, B3) and *Pellia epiphylla* (S,N) antigens with anti-*borealis* serum precipitated with antigen N (b+N) or S (b+S).

Fig. 5. Dendrogram (A) and dendrite (B) based on Jaccard coefficient of taxonomic similarity (S_{Jac}) showing serological similarities within genus *Pellia*.

Fig. 6. Quantitative immunoelectrophoresis of clones of *Pellia borealis* from different sites (anti-borealis serum). II, III, IV – denotations of protein bands; Px – denotations of peroxidase phenotype.

viously, the findings require further detailed studies on more numerous samples of the liverwort species originating from different geographic sites.

The second problem which is worth discussing involves the fact that protein fractions of the species *S* and *N* have been present in the serological patterns of *P. borealis* samples. The results obtained from reaction of precipitation anti-borealis and anti-epiphylla sera with *N* and *S* antigens indicate that the polyploid form contains antigen proteins, which are common for *P. epiphylla* species. This conclusion seems to be confirmed by serological patterns observed in a reaction of mixed *S* and *N* extracts which were identical to B2 and B3 of *P. borealis* and semi-homologous to B1 (Fig. 5). In this aspect our study corresponds to the findings of Odrzykoski et al. (1996) who have suggested that the polyploid *P. borealis* is an allopolyploid species between the two sibling species *S* and *N* of *P. epiphylla*. The authors have postulated a fixed heterozygous phenotype of the polyploid for three enzyme loci: mannose-6-phosphate isomerase 1, NADH-diaphorase 1 and aconitase 1. Alleles of this heterozygous phenotype are present in the sibling species of *P. epiphylla*. On the other hand, variation observed in species of the polyploid suggests three (or more) separate origins of *P. borealis* (Odrzykoski et al. 1996). Ziełński (1987) have supported the argument that *P. borealis* is an autopolyploid derived probably from the species *S*. Prus-Głowacki and Nowak (1982) have shown 40% higher synthesis of antigenic proteins in the polyploid than in the *P. epiphylla*, which has been interpreted as an effect of genome duplication in the way of autopolyploidization. Extensive similarity in the antigenic patterns of species *S* and the one population of the

studied *P. borealis* – B1 (Fig. 5) can support the autopolyploid origin of the liverwort.

Summarising, our results allow for easy differentiation between the two species of *P. epiphylla* using serological tests. Moreover, serological differentiation within polyploid species *P. borealis* was found. Obviously, the findings require additional studies on wider plant material.

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MIĘDZY- I WEWNĄTRZGATUNKOWE ZRÓŻNICOWANIE SEROLOGICZNE KOMPLEKSU *PELLIA EPIPHYLLA*

STRESZCZENIE

W celu zbadania serologicznych zależności pomiędzy gatunkami wątrobowców należącymi do kompleksu *Pellia epiphylla* (n=9) użyto roślinnych białek antygenowych. Z ostatnich doniesień wynika, że elektroforetyczny fenotyp *P. borealis* sugeruje jego amfiploidalne pochodzenie. Dwa gatunki bliźniacze *P. epiphylla* N i S są przypuszczalnymi rodzicami wspomnianego poliploida. Metodą podwójnej immunodyfuzji w żelu agarozowym zbadano pod względem serologicznym trzy klonów *P. borealis* pochodzące z różnych stanowisk w Polsce. Następnie porównano je z gatunkami bliźniaczymi S i N *P. epiphylla* oraz z próbą uzyskaną przez zmieszanie ekstraktów pochodzących z gatunków S i N. Uzyskane wyniki wskazują, że antygenowy wzór próby mieszanej jest identyczny ze wzorem dwóch naturalnych klonów poliploidalnego taksonu *P. borealis*. Jeden z badanych klonów *P. borealis* wykazywał większe podobieństwo antygenowe do *P. epiphylla* S niż do pozostałych poliploidalnych klonów. Ponadto wykazano wyraźne serologiczne różnice pomiędzy gatunkami bliźniaczymi S i N, które pozwalają na ich rozróżnienie.

SŁOWA KLUCZOWE: białka antygenowe, immunodyfuzja, *Pellia borealis*, *Pellia epiphylla*, poliploidyzacja.