

UNIwersytet IM. ADAMA MICKIEWICZA W POZNANIU
SERIA BIOLOGIA NR 72

Variability and Evolution – New Perspectives

*Professor Jerzy Szweykowski
in memoriam*

Editor WIESŁAW PRUS-GŁOWACKI

Co-editor EWA M. PAWLACZYK



POZNAŃ 2005

EVOLUTIONARY DIVERGENCE WITHIN *PELLIA ENDIVIIFOLIA* (DICKS.) DUM. FROM POLAND

Kornelia Polok¹, Jakub Sawicki², Katarzyna Kubiak¹,
Monika Szczecińska², Karol Korzekwa¹, Kamil Szandar¹,
Roman Zieliński¹

¹Department of Genetics, University of Warmia and Mazury in Olsztyn
Plac Łódzki 3, 10-967 Olsztyn, Poland, e-mail: rzielin@moskit.uwm.edu.pl

²Department of Botany and Nature Protection, University of Warmia and Mazury in Olsztyn
Plac Łódzki 1, 10-967 Olsztyn, Poland

ABSTRACT. Three molecular forms of *Pellia endiviifolia* were identified in the Polish territory based on semi-specific markers ISJ, that amplify nuclear DNA on the exon-intron junctions. Two of them corresponded to early sibling species identified by isozymic markers and called the typical and water form of *P. endiviifolia*. The third molecular form appeared to be typical of well-heads. Nei's genetic similarity between the typical and water form of *P. endiviifolia*, calculated on the base of ISJ loci was very low, with I value of 0.183 whereas between them and the well-headed form the I coefficient was equal to 0.182 and 0.330, respectively. Significant differences among those molecular forms were observed for most of morphological characteristics studied after several months of culture. Three molecular forms were also easily distinguishable in their natural environment. Taking into consideration the molecular and morphological diversity, the evolutionary status of the well-headed form as well as two early sibling species of *P. endiviifolia* can correspond to well defined biological species.

KEY WORDS: *Pellia endiviifolia*, sibling species, DNA markers, molecular diversity

INTRODUCTION

The liverworts of the genus *Pellia* are represented in the European flora by two dioecious and two monoecious species. The former include *P. neesiana* and *P. endiviifolia*, n=9 while the latter include *P. epiphylla*, n=9 and *P. borealis*, n=18. However, the data available have shown their high morphological plasticity that made species identification difficult. The usefulness of peroxidase isoenzymes for species differentiation (Fig. 1) on a larger scale has been reported since the early eighties of the 20th century (Krzakowa 1977, 1981, Szweykowski et al. 1981, Zieliński 1986, 1987a, Szweykowski et al. 1995). Further data on variation of enzymatic loci had enabled the detection of two sibling species of *P. epiphylla*, later called the S (South) and N (North)

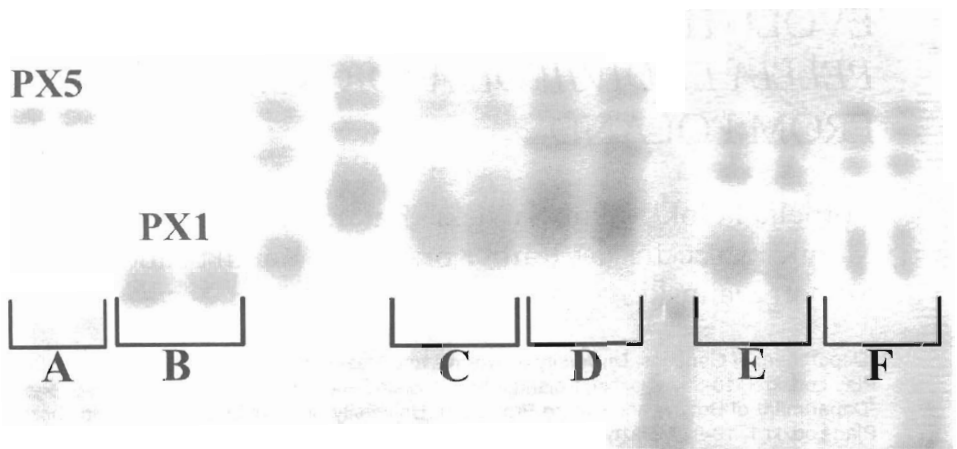


Fig. 1. Peroxidase patterns in *Pellia* sp.

A – *P. endiviifolia*, water form, B – *P. endiviifolia*, typical form, C – *P. borealis*, D – *P. neesiana*, E – *P. epiphylla* S, F – *P. epiphylla* N

form based upon their geographical distribution in Poland as well as two sibling species of *P. endiviifolia* named the water and typical form (Zieliński 1987a). *P. endiviifolia* sibling species have different alleles in 10 enzymatic loci and they are distinguished easily on the base of PX pattern. The fast migrating band of PX (PX5) is observed in the water form while the band with slower mobility (PX1) is characteristic of the typical form. The first DNA data supporting the genetic differentiation of *P. endiviifolia* were provided by Polok (2000). Using RAPD markers, she estimated Nei's genetic identity at the level 0.79 that corresponds to species at the early stage of divergence.

In the present studies, the DNA markers amplifying the exon-intron junctions (ISJ – Intron Splice Junction) were used for comparison of *P. endiviifolia* sibling species. However, although the differences on the DNA level are very informative for evolutionary considerations, they are of little use for taxonomic purposes. Therefore the analysis of morphological characteristics was undertaken to find morphological markers enabling the distinction of species by taxonomists.

MATERIAL AND METHODS

A total of 144 samples of *Pellia* sp. representing 19 lowland sites and 85 samples representing 16 sites in the Tatra Mountains were collected in 2002–2003. The samples were identified both using peroxidase isoenzymes

and chromosome counting. Each sample was divided into two equal parts, and one half was cultured and then was subjected to molecular and morphological analyses. The remaining part was frozen and deposited in the Department of Genetics. Out of this collection, 55 samples representing all *P. endiviifolia* populations were analysed (Tab. 1).

Table 1. *Pellia endiviifolia* samples from Poland examined in this study

Abbreviation	Collection site	N° of samples
Lowland stations		
P-S	Szczecin, Szmaragdowe Lake	5
P-BTP	Tuchola forest, Piekło	5
P-DS	Diabli Skok Reserve near Walcz	9
P-ZS	Stążka spring, Tuchola Landscape	4
P-WEL	Welski Landscape	4
P-WEZ	Welski Landscape, south well-head	2
The Tatra Mountain stations		
P-ST	Strążyska Valley	6
P-KM	Koryciska Małe	2
P-K	Kościeliska Valley	4
P-ŁP	Łysa Polana	5
P-PST	Siwiańskie Turnie	5

A modified version of CTAB method (Murray and Thompson 1980) was used for the isolation of *Pellia* DNA. Briefly, liquid nitrogen-ground thalli were thoroughly mixed with 3 ml preheated CTAB isolation buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl and 2% β -mercaptoethanol) and incubated at 55°C for 1 h. After three chloroform extractions, the DNA was precipitated and dissolved in sterile, deionized H₂O. The purity of DNA samples was assessed spectrophotometrically and averaged 90-95%. Two 18-nucleotide (ISJ2 - 5'ACTTACCTGAGGCGCCAC3', ISJ4- 5'GTCGGCGGACAGGTAAGT3') primers and a 10-nucleotide primer (ISJ3 -5'TGCAGGTCAG3') that amplify DNA fragments on the exon-intron junctions, were chosen for the analysis. The PCR reaction was conducted in a volume of 20 μ l containing 20 mM (NH₄)SO₄, 50 mM Tris-HCl (pH 9.0 at 25°C), 2 mM MgCl₂, 1 x concentrated Enhancer with betaine (Epicentre Technology), 200 μ M each dATP, dGTP, dCTP, dTTP, 0.3 μ M primer, 1 unit of Taq polymerase and 80 ng of template DNA. The reaction was processed at 94°C for 3 min. followed by 45 cycles at 94°C for 1 min., 48°C (18-mers) or 36°C (10-mers) for 1 min., and 72°C for 2.5 min., with a final extension step of 72°C for 5 min. After PCR, samples were loaded on a 1.2% agarose gel containing 0.5 μ g/ml ethidium bromide and separated in 1 x TBE buffer at 100 V constant power. Gels were ob-

served in UV light, 312 nm and photographed. All bands that could be reliably read were treated as single dominant loci and scored either present (1) or absent (0) across all genotypes. Estimates of similarity were based on the number of shared amplification products (Nei and Li 1979).

The morphological analysis was carried on thalli cultured for 6 months. Two kinds of culture were applied: the growth chamber and greenhouse culture. In the growth chamber thalli were kept in high humidity, in plastic boxes covered with plastic wrap while in the greenhouse the samples were grown in the conditions simulating the natural environment. Six morpho-anatomical characters dealing with length, width and area of both basal and marginal cells were measured on the base of 5 thalli per a sample and ten measurements per thallus. The samples were grown in a complete random design and the data were analysed by one-way ANOVA with LSD test.

RESULTS

Out of 229 samples, 55 were identified as *P. endiviifolia* based on peroxidase patterns. Furthermore, the samples collected from 4 sites (P-S, P-BTP, P-ST and P-KM) showed the PX1 phenotype specific of Zieliński's typical form (Tab. 2). The PX5 phenotype, i.e. specific of Zieliński's water form, was observed in 3 populations collected in the Tatra Mountains (P-K, P-ŁP and P-PST) and a lowland population originated from the spring of the River Stążka (P-ZS). The peroxidase phenotypes of the population from the Diabli Skok Reserve (P-DS), and two populations from the Welski Landscape (P-WEL and P-WEZ) were difficult to assess. There was no expression of peroxidases immediately after collecting while after several months of culture either PX1 or PX1 and PX5 simultaneously were observed. However, the classification of these samples to *P. endiviifolia* was supported by the similarity of their karyotypes, particularly the presence of a small chromosome.

The DNA of all *P. endiviifolia* samples was amplified with ISJ primers. In total 35 bands were detected and almost all of them were polymorphic (97%). The *P. endiviifolia* samples were clearly divided into three distinct groups (A, B, C) on the base of their ISJ patterns. Each group can be described by the presence of unique ISJ genotypes (Fig. 2). A lot of amplified fragments were restricted to a single group. There were 2 bands (5.7%) specific to group A, 9 bands (25.7%) specific to group B and 6 bands (17.1%) to group C. It is interesting to note that the general consensus was found between DNA patterns, peroxidase patterns and habitats (Tab. 2). All samples of group A had the PX1 phenotype. They were collected from the alkaline

Table 2. Ecological and molecular diversity of *Pellia endiviifolia* from Poland

Abbreviation	Description of collection site	PX pattern	ISJ type	Zieliński's classification	Classification proposed in this paper
P-S	Soil surface of banks of a lake, chalk excavation	PX1	A	typical form	land form
P-BTP	Soil surface	PX1	A	typical form	land form
P-ST	Soil surface, banks of a stream	PX1	A	typical form	land form
P-KM	Stony banks of the calcium stream	PX1	A	typical form	land form
P-ZS	In water and floating on the surface, springs	PX5	B	water form	water form
P-K	In a stream (30 cm deep) and "cabbage heads" floating on the surface	PX5	B	water form	water form
P-ŁP	In a stream (20 cm deep) and "cabbage heads" on the surface	PX5	B	water form	water form
P-PST	In a stream and "cabbage heads" on the surface	PX5	B	water form	water form
P-DS	On the wet soil surface near a well-head	PX1/ PX5	C	–	well-headed form
P-WEL	On the wet soil surface near a well-head	PX1/ PX5	C	–	well-headed form
P-WEZ	On the wet soil surface near a well-head	PX1/ PX5	C	–	well-headed form

soil surface, with pH ranging from 7.4 to 7.7 (Fig. 3a). The samples of group B were characterised by the PX5 phenotype while PX pattern of group C was unstable. Both groups (B and C) occurred in humid, wet stands and, stagnant or spring water with similar pH. However, the thalli of the group B were always floating on the surface of rather deep (20-30 cm), stagnant water, and usually formed structures alike "cabbage heads" (Fig. 3b) while the thalli of group C were growing on the surface of muddy soil with dripping water, in the vicinity of well-heads (Fig. 3c).

Estimates of Nei's genetic identities among three groups of *P. endiviifolia* samples were very low, ranging from 0.183 between the A and B group and 0.182 between the A and C group thus indicated a significant degree of their differentiation. The coefficient of similarity between B and C was 0.330, that is twice as high as for pairs A-B and A-C, but still the value was very low justifying their separation from each other.

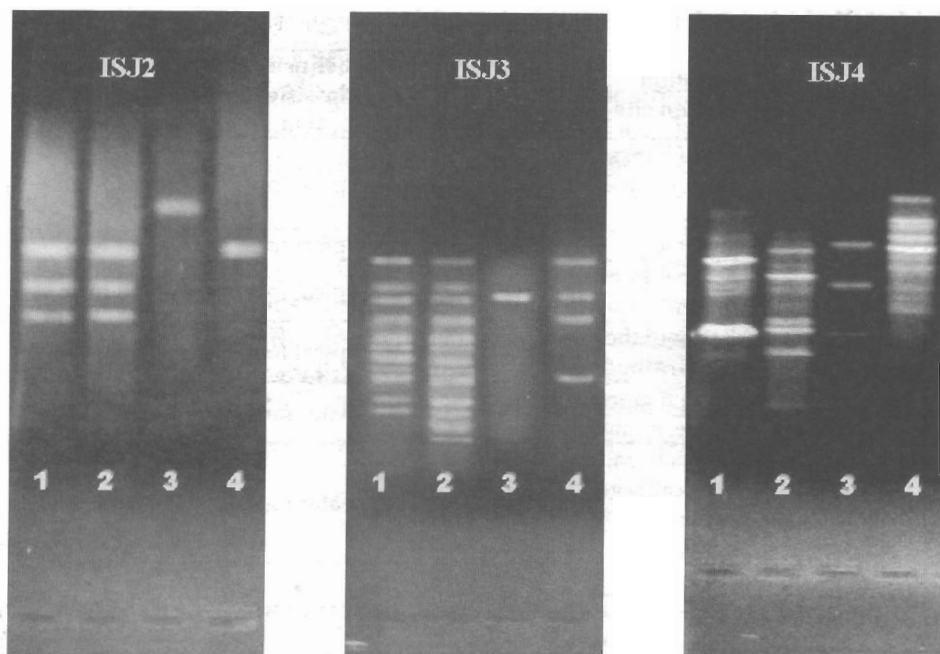


Fig. 2. Amplification products generated from DNA of *P. endiviifolia* using ISJ primers. 1-2 – B group, 3 – A group, 4 – C group

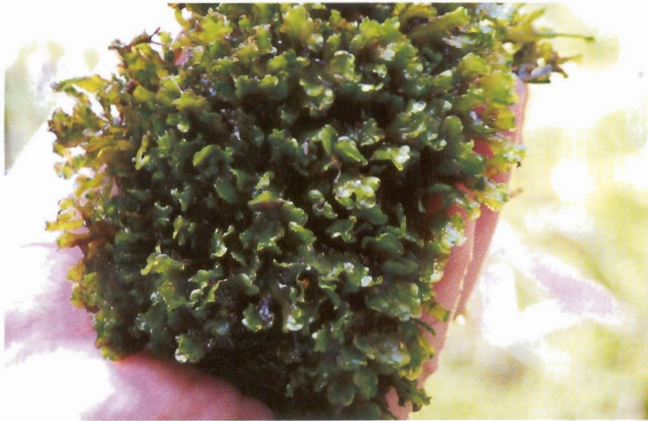
The analysis of morphological characteristics of thalli fully supported the molecular differentiation of *P. endiviifolia* into three distinct evolutionary groups. The samples of group A growing in the growth chamber differed statistically from group B in 67% of studied characters and in 33% of characters in comparison with group C (Fig. 4). The similar differences (33%) were found between forms B and C. The distinct character of three molecular groups of *P. endiviifolia* was much more pronounced in the greenhouse (Fig. 5). The significant differences were found between group A and B, A and C as well as between B and C. The respective values were 83%, 67% and 83%. The highest differences were observed for length and area of basal cells, but no differences were found for marginal cell width.

DISCUSSION

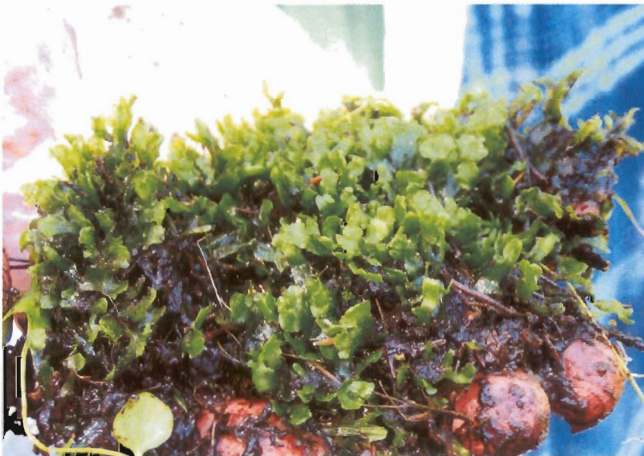
The present studies have demonstrated clearly that *P. endiviifolia* is differentiated into three groups. The molecular diversity correlates well with analyses of thallus morphology as well as ecological preferences of three



A group



B group



C group

Fig. 3. Morphology of *P. endiviifolia* in natural environment

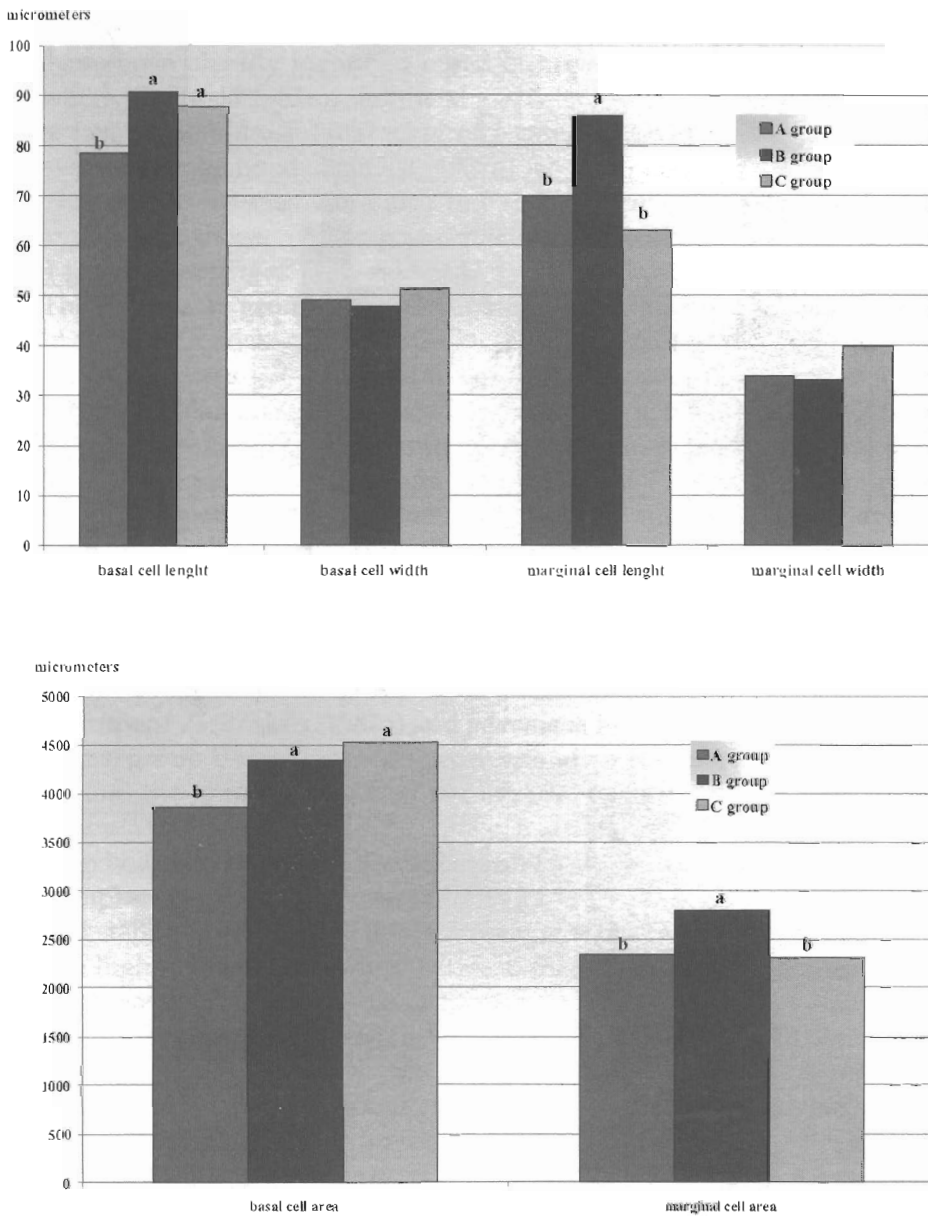


Fig. 4. Comparison of thallus morphology of 3 molecular groups of *P. endiviifolia* after 6 months of culture in the growth chamber (Different letters mean the significant differences at $p=0.05$)

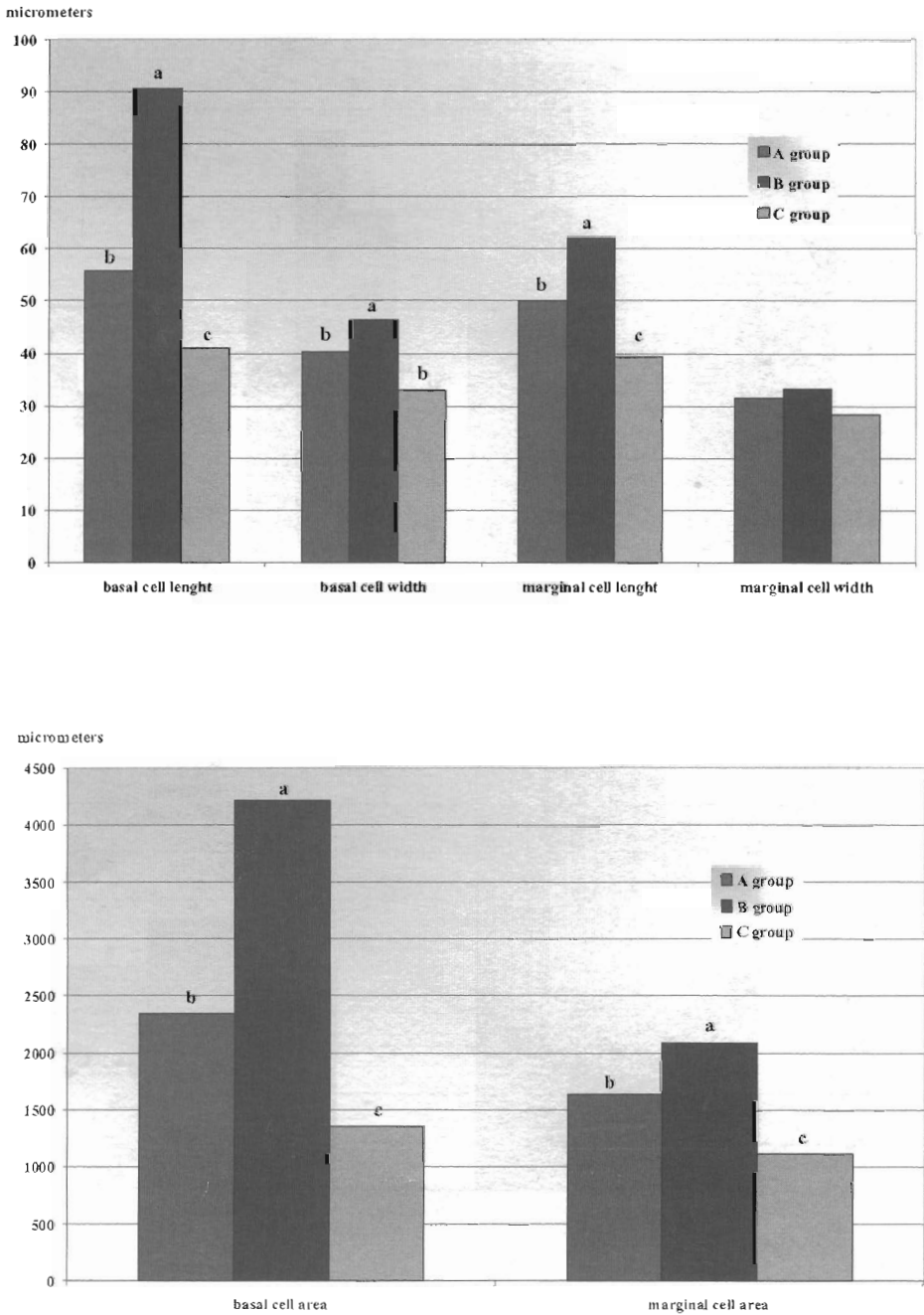


Fig. 5. Comparison of thallus morphology of 3 molecular groups of *P. endiviifolia* after 6 months of culture in the greenhouse (Different letters mean the significant differences at $p=0.05$)

forms. The intraspecific variation of *P. endiviifolia* revealed in the present studies is in agreement with previous data. Out of three observed forms, two have been already identified based on isoenzymes and morphological characters (Zieliński 1987a), and they have been described as the typical and water form. The almost equal level of I value (0.231) between the typical and water form inclined Zieliński (1987a) to classify them into sibling species. The sibling species were also found in *P. epiphylla* (Zieliński 1987a), *Conocephalum conicum* (Odrzykoski 1985, Odrzykoski and Szweykowski 1991) and *Aneura pinguis* (Szweykowski and Odrzykoski 1990).

The molecular groups A and B observed in the present work correspond well to Zieliński's sibling species. The third molecular group (C) has not been discovered yet. The level of genetic similarities among three *P. endiviifolia* molecular groups estimated on the base of ISJ loci, ranging from 0.183-0.330, may again reflect a split between them. The very low value of genetic similarities between groups A, B, and C of *P. endiviifolia*, the presence of group specific DNA markers together with morphological diagnostic characters justifying their elevation to independent biological species. In addition, each group lives in a specific habitat where it is visually recognised. This enables easy classification of any sample to one of the three groups before molecular analysis. On the base of visual appearance and habitat we propose to call group A as a land form, corresponding to the typical form of Zieliński's (1987a) and *fabroniana* in a lot of taxonomical classifications, group B as a water form corresponding to Zieliński's water form (1987a) and the group C as a well-headed form. The land and water forms are common in the whole Polish territory. It also seems that the well-headed form can be found both in mountainous and lowland regions. Although the first samples were collected from 3 lowland stations, later on, 2 additional lowland stations and a mountainous station were identified.

The highest genetic similarity between the water and well headed form raises the possibility that these two forms simultaneously evolved from the land form, followed by spatial isolation and local adaptation, leading to fragmentation into different species. It seems that the taxonomic description of all identified *P. endiviifolia* forms and conferment of botanical names will be a matter of time as it was in case of *Conocephalum conicum*. The isozyme differences were found between two cryptic species from Poland that correlated with morphological differentiation. This enabled to identify one of them as the nomenclature type while the other has been described as a new, not yet recognised, morphological species (Buczowska and Odrzykoski 2004).

Interesting to note is that the divergence among the well-headed (C) and land (A) *P. endiviifolia* forms was found only on the DNA level without clear differences in enzymatic loci neither in peroxidases nor in the other

isoenzymes. The peroxidase isoenzymes appeared to be diagnostic markers enabling identification of all *Pellia* sp. including sibling species (S and N) of *P. epiphylla* as well as *P. endiviifolia* (land – A and water – B). The distinct PX phenotype was also observed in *P. endiviifolia* from Japan which can be also regarded as a new sibling species (Zieliński 1987a). Among 13 enzymatic loci studied, specific alleles were found in 9–10 loci. Nei's genetic identity between Japanese and Polish forms ranged from 0.231 to 0.307. The similar isozymic differences, including peroxidases, were observed in *P. endiviifolia* from the USA. Further morphological and cytological analyses brought about the separation of a new taxon, *P. megaspora* (Schuster 1981). However, the identification of the well-headed form with the help of peroxidases was much more troublesome but still not impossible. This form was characterised by unstable peroxidases pattern with the lack of bands specific to the land (A) and water form (B), although the pattern specific to the land form could appear after several months of culture. Sometimes the bands of the land and water form were observed simultaneously. Out of liverworts of the genus *Pellia*, the changes in a pattern of peroxidase expression are only known in *P. endiviifolia* (Zieliński 1987b). It could not be ruled out that the lack of the correlation between DNA and isoenzyme variation and difficulties in interpretation of PX pattern increased the genetic identity between the land and water form ($I=0.79$) ascribable by RAPD (Polok 2000). At that stage of research both forms were analysed together, and therefore it was impossible to clarify significant differences between the genetic similarity estimated on the base of isoenzymes by Zieliński (1987a) and RAPD markers (0.79).

On the other hand, the question about the lack of enzymatic diagnostic markers in the well-headed form and lots of DNA markers, is still opened. This result is even more surprising when taking into consideration the relatively low genetic similarity between the well-headed form and the other *P. endiviifolia* forms based on ISJ markers (0.182 and 0.330). Such a low I value is rather a feature of well defined biological species. The similar disparity is also observed between well-defined pine species, *Pinus sylvestris* and dwarf pine, *P. mugo*. Despite extensive research conducted for around 30 years (Odrzykoski 2002), no diagnostic enzymatic marker has been found while a huge number of markers was easy to reveal on the DNA level (Zieliński and Polok 2005). Although further experimentation would be required, it could not be excluded that the genes encoding enzymatic loci are conservative in closely related taxa. It can be also pointed out that DNA markers provide a degree of resolution unavailable with isoenzymes, thus it is likely that with more and more wide application of molecular methods to bryology, new taxa will be identified in many liverworts in the near future.

CONCLUSIONS

1. Three biological taxa of *P. endiviifolia*, the land, water and well-headed forms were identified on the base of molecular and morphological analysis.
2. The DNA markers proved to be more effective than isozymes in taxonomic studies of liverworts.

Acknowledgements

This work was supported by the European Community Grant: Marie Curie Host Fellowships for the Transfer of Knowledge, GenCrop. Contract No MTKD-CT-2004-509834.

References

- Buczowska K., Odrzykoski I.J. 2004. Morfometryczne różnice pomiędzy dwoma europejskimi gatunkami kryptycznymi *Conocephalum conicum* sensu lato. In: *Materiały z konferencji Zmienność i Ewolucja, 16-18 czerwca 2004*. Poznań, p. 35.
- Krzakowa M. 1977. Isoenzymes as markers of inter- and intra-specific differentiation in hepatics. *Bryophyt. Bibliotheca* 13: 427-434.
- Krzakowa M. 1981. Evolution and speciation in *Pellia*, with special reference to the *Pellia megaspora* – *endiviifolia* complex (Metzgeriales), IV. Isoenzyme investigations. *J. Bryol.* 11: 447-450.
- Murray M., Thompson W. 1980. The isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8: 4321-4325.
- Nei M., Li W. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci.* 76: 5269-5273.
- Odrzykoski I.J. 1985. *Conocephalum conicum* as a group of sibling species. *Abstracta Botanica* 9: 1-28.
- Odrzykoski I.J. 2002. *Badania nad zmiennością genetyczną kosodrzewiny (Pinus mugo) z wykorzystaniem markerów biochemicznych i molekularnych*. Seria Biologia nr 67. Adam Mickiewicz University Press. Poznań, pp. 1-135.
- Odrzykoski I.J., Szweykowski J. 1991. Genetic differentiation without concordant morphological divergence in the thallose liverwort *Conocephalum conicum*. *Plant Syst. Evol.* 178: 135-151.
- Polok K. 2000. Comparison of two *Pellia endiviifolia* sibling species on the base of RAPD and ISJ markers. *International Conference on Genetic Diversity and Taxonomy of Bryophytes, Poznań 11-12 October 2000*.
- Schuster R.M. 1981. Evolution and speciation in *Pellia*, with special reference to the *Pellia megaspora* – *endiviifolia* complex (Metzgeriales), I. Taxonomy and distribution. *J. Bryol.* 11: 411-431.
- Szweykowski J., Odrzykoski I.J. 1990. Chemical differentiation of *Aneura pinguis* (L.) Dum. (*Hepaticae, Aneuraceae*) in Poland and some comments on application of enzymatic markers in bryology. In: Zinsmeister H.D., Mues R. *Bryophytes. Their chemistry and chemical taxonomy*. Oxford Sci. Publications. Clarendon Press. Oxford.

- Szweykowski J., Zieliński R., Mendelak M. 1981. Variation of peroxidase isoenzymes in Central European taxa of the liverwort genus *Pellia*. *Bull. Acad. Pol. Sci., Ser. Sci., Nat.* (II) 29 (1-2): 9-19.
- Szweykowski J., Zieliński R., Odrzykoski I., Buczkowska K. 1995. Geographic distribution of *Pellia* ssp. (*Hepaticae*, *Metzgeriales*) in Poland based on electrophoretic identification. *Acta Soc. Bot. Pol.* 64(1): 59-70.
- Zieliński R. 1986. Cross-fertilization in the monoecious *Pellia borealis*, n=18, and spatial distribution of two peroxidase genotypes. *Heredity* 56: 299-304.
- Zieliński R. 1987a. Genetic variation of the liverwort genus *Pellia* with special reference to Central European territory. *Rozprawy i Studia Uniwersytetu Szczecińskiego* 24: 1-297.
- Zieliński R. 1987b. Interpretation of electrophoretic patterns in population genetics of bryophytes. *Lindbergia* 12: 87-96.
- Zieliński R., Polok K. 2005. Molecular evolution and taxonomy of plants. In: W. Prus-Głowacki (ed.). *Variability and Evolution*. Adam Mickiewicz University Press. Poznań, pp. 37-56.