PHYLOGENETIC STUDIES OF LIVERWORTS FROM THE GENUS *PELLIA* USING A NEW TYPE OF A MOLECULAR MARKER

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ABSTRACT

In order to analyse taxonomically liverworts from the genus *Pellia* a new type of molecular marker was applied using two kinds of primers. The first one is a typical RAPD primer whereas the second one is a universal primer whose nucleotide sequence is based on a consensus sequence common to all tRNA molecules. Using these markers phenetic and phylogenetic trees were constructed for all the species of the genus *Pellia*, using the liverwort species *Aneura pinguis* as the outgroup. Additionally, it was demonstrated that the allopolyploid species *P. borealis*, which originated as a result of the hybridization of two parent species: *P. epiphylla-species S* and *P. epiphylla-species N*, is more closely related to *P. epiphylla-species N*. This is because both: mitochondria and chloroplasts in *P. borealis* are from *P. epiphylla-species N*.

KEY WORDS: liverworts, allopolyploid, bootstrap analysis, molecular evolution, phylogeny.

INTRODUCTION

The genus *Pellia* is represented in Poland by two diploid, monoecious species: *P. epiphylla-species S* (n=9), *P. epiphylla-species N* (n=9) (cryptic species), one tetraploid monoecious species *P. borealis* (n=18) and three diploid, dioecious species: *P. neesiana* (n=9), *P. endiviifolia-species A* (n=9), *P. endiviifolia-species B* (n=9) (the last two are cryptic species). One of these species, *P. borealis*, is an allopolyploid that originated after hybridization and duplication of chromosome sets of two cryptic species: *Pellia epiphylla-species N* and *Pellia epiphylla-species S* (Odrzykoski 1996; Fiedorow 2001a).

The PCR technique has been widely used in taxonomic studies of genus *Pellia* (Fiedorow 1998; Fiedorow 2001a; Fiedorow 2001b; Pacak 1998; Pacak 2000). Two approaches have been adopted in this research. The first one was the analysis of particular DNA fragments, eg. a sequence of nuclear tandems of tRNA^{Leu}_{CAA} genes (Fiedorow 1998; Fiedorow 2001a); the second one was the RAPD analysis method (Pacak 1998). The RAPD technique is relatively fast and cheap, but in some cases quite inaccurate. It was

used to study the relationships between the three species: *P. epiphylla-species S*, *P. borealis*, *P. epiphylla-species N*. Computer data analysis methods showed close relations of *P. epiphylla-species S* and *P. borealis* (the Camin-Sokal parsimony and the compatibility method – CLIQUE program, tree no 2), while others showed close relations of *P. epiphylla-species N* and *P. borealis* (among others: Fitch-Margoliash, UPGMA method, Wagner parsimony) (Pacak 1998). This can be explained by the fact that *P. borealis* is a hybrid species. However, in the light of the recent studies, which show that both chloroplasts and mitochondria in the species *P. borealis* derive from *P. epiphylla-species N* (Pacak 2000), the result obtained with the RAPD technique is quite inaccurate. Determination of the nucleotide sequence of a given DNA fragment allows to place organisms precisely on a tree presenting taxonomic relationships. Such an analysis is much more time-consuming and, frequently, further DNA fragments need to be analyzed in order to verify a given result. Moreover, the obtained information concerns usually a DNA sequence from only one genome: nuclear, chloroplast or mitochondrial, which may influence the results of analysis in the case of hybrid species. The new molecular marker we postulate – from now on called RAPD-tRNA – allows to combine on one hand the advantages of the RAPD technique (analysis rate, information based on total DNA of organisms), and on the other hand we can obtain precise information from more defined DNA

Abbreviations

N – any nucleotide; OTU – Operational Taxonomic Unit; PAGE – polyacrylamid gel electrophoresis; ψ – pseudouridine; R – purine; TBE – Tris-Borate-EDTA buffer; Y – pyrimidine

fragments. In the PCR reaction we used two different primers: the first one is a typical 10-nucleotide RAPD primer; the second one is a universal primer based on a conservative nucleotide sequence of a particular part of all tRNA genes.

Using tRNA genes as the place of anchoring of one of the primers in the PCR reaction, we based on a well-known phenomenon concerning the organization of tRNA genes in eucaryotic genomes. In the case of the nuclear genome, they can be dispersed and/or organized in tandem arrays. For example, most tRNA nuclear genes in yeast are scattered, eg. 8 tRNA^{Tyr} genes occurring in this genome are localized in 6 different chromosomes. Occasionally, in yeast there are small gene clusters, eg. tRNA^{Arg}₃ and tRNA^{Asp} separated by an intergenic fragment 10 bp long (Szweykowska-Kulińska 1987). Clusters of tRNA genes can be found also in yellow lupin, in which a nuclear DNA fragment 7.8 kb long contains three tandemwise repeated units. Each unit contains a gene and a pseudogene of tRNA^{Gly}_{GCC} (Nuc 1997).

We know complete chloroplast and mitochondrial genome from liverwort *Marchantia polymorpha* (Ohyama 1996). tRNA genes in chloroplasts are localized all over the whole genome. They often occur individually or in clusters, most often as pairs of two genes, eg.: tRNA^{Trp}, tRNA^{Pro}, or in groups of three, eg.: tRNA^{Asp}, tRNA^{Tyr}, tRNA^{Glu}. The same happens in the mitochondrial genome. In *M. polymorpha* some tRNA genes can appear as single copies, some can also form clusters consisting of even 4 tRNA genes, eg.: tRNA^{Arg}, tRNA^{Tyr}, tRNA^{Arg}, tRNA^{Gly}.

A dispersed type of tRNA genes organization in all three compartments allows to use fragments of tRNA genes as primers in PCR reactions. There is a high probability of amplification of DNA fragments that are representative for three genomes (nuclear, chloroplast and mitochondrial ones). The aim of this study was to determine the taxonomic relationships in *Pellia*, to compare of the obtained results with earlier data and to determinate of relations between the three species: *P. epiphylla*-species *S*, *P. borealis* and *P. epiphylla*-species *N*.

MATERIALS AND METHODS

In vitro culture of liverworts

Apical parts of liverworts thalli were sterilised for 15 min in 0.5% sodium hypochlorite. Sterilized sections of thallus were put on a sterile solid medium containing per one liter: NH₄NO₃ – 0.12 g, KH₂PO₄ – 0.7g, MgSO₄×7 H₂O – 0.246 g, CaCl₂ – 0.02 g, FeCl₃ – 0.03 g, sucrose 10 g, agar – 10 g. The agar medium was adjusted to pH 5.0 (Lukavsky 1991). For the *in vitro* culture of liverworts we used 1/2× Murashige & Skoog medium pH 5.6 (Murashige 1962).

Total genomic DNA isolation

Genomic DNA was extracted using the Dneasy Plant Mini Kit (QIAGEN) procedure. Total DNA was isolated from 100 mg of plant tissue.

Polymerase chain reaction (PCR)

The PCR mixture contained the following components for 10 µl reactions: 7.5 ng of total DNA; 0.5 µM RAPD primer, 0.5 µM oligotRNA primer, 1× QIAGEN buffer,

1 mM spermidine (Fiedorow 1997); 200 µM of each dNTP; 0.25 unit of Taq DNA polymerase (QIAGEN). The reaction was performed always for two different populations of each liverwort species.

PCR was initiated by denaturation at 95°C for 5 min and followed by 50 cycles: denaturation 92°C – 1 min, annealing 35°C – 2 min, elongation 72°C – 2 min.

The reactions were ended by elongation at 72°C for 5 min.

Primers used:

RAPD primers, oligotRNA:

P2) 5'-TGCACACTGA-3', 5'-RGTTTCRANNN-3'

P3) 5'-TGGTGACTGA-3'

P4) 5'-TGGTCACTGT-3'

P5) 5'-GTCCCGACGA-3'

P6) 5'-CTCGTTTGGG-3'

Sequences of 10-nucleotide RAPD primers were taken from: (Klein-Lankhorst 1991; Hosaka 1994). In the case of the oligotRNA primer the sequence RGTTTCRA is a conservative nucleotide sequence derived from the fragment of TψC arm present in almost all tRNA genes (Fig. 1).

Fig. 1. tRNA structure.

Bold letters depict conservative nucleotides, boxed sequence shows the tRNA fragment that served for oligotRNA primer construction, anticodon is marked by asterix (Szweykowska-Kulińska 1987).

The PCR products were separated using native 8% PAGE in 1×TBE. DNA was stained with ethidium bromide

TABLE 1. Pairwise distance matrix calculated by modified Nei & Li method.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.
1.	0.0000	0.0362	0.0365	0.0466	0.0468	0.0396	0.0396	0.0512	0.0475	0.0505	0.0512	0.0505	0.0570
2.		0.0000	0.0035	0.0326	0.0286	0.0320	0.0320	0.0280	0.0275	0.0292	0.0341	0.0312	0.0272
3.			0.0000	0.0255	0.0205	0.0288	0.0288	0.0211	0.0221	0.0241	0.0290	0.0268	0.0215
4.				0.0000	0.0094	0.0546	0.0546	0.0454	0.0468	0.0317	0.0302	0.0295	0.0272
5.					0.0000	0.0348	0.0325	0.0305	0.0315	0.0267	0.0288	0.0299	0.0281
6.						0.0000	0.0007	0.0280	0.0310	0.0390	0.0367	0.0312	0.0272
7.							0.0000	0.0263	0.0310	0.0361	0.0341	0.0292	0.0239
8.								0.0000	0.0018	0.0129	0.0121	0.0169	0.0186
9.									0.0000	0.0132	0.0118	0.0162	0.0178
10.										0.0000	0.0028	0.0043	0.0064
11.											0.0000	0.0028	0.0046
12.												0.0000	0.0022
13.													0.0000

Transition/transversion ratio=2.0
Numbers describe appropriate OTU analyzed (see the appendix).

(0,5 µg/ml). The number of RAPD-tRNA fragments for each tested individual was used to construct the data matrix. The characters were coded in the binary system [0.1] where „0” means no particular DNA fragment, „1” means the presence of a particular DNA fragment. To construct and test trees we used PHYLIP v.3.573c and v.3.6a2 (Felsenstein 1995, 2001). The software Tree View v.1.6.6 (Page, 1996) was used to draw the trees. Two types of tree constructing methods were used. The first one applied algorithms using the distance data – the UPGMA cluster analysis, the Fitch-Margoliash and the Neighbor-Joining methods. The pairwise distances were calculated by the modified Nei & Li method (Felsenstein 2001) (Table 1) and the Yule & Kendall algorithm (Armstrong 1994) (data not presented). The second one applied the analysis of raw data

[0.1] performed by Dollo and Polymorphism parsimonies algorithms (programs in the PHYLIP package). The species *Aneura pinguis* was used as an outgroup.

RESULTS AND DISCUSSION

Total genomic DNA isolated from: *A. pinguis*, *P. endiviifolia*-species A, *P. endiviifolia*-species B, *P. neesiana*, *P. epiphylla*-species S, *P. borealis* and *P. epiphylla*-species N were used as templates for PCR reactions using the following combinations of primers: P2, P3, P4, P5, P6 (respectively) + oligotRNA. Figure 2 presents an example of electrophoretic separation of PCR amplification products derived from the P3 + oligotRNA primers.

Fig. 2. An example of electrophoretic separation of PCR amplification products derived from the P3 + oligotRNA primers. M-Marker GeneRuler 100 bp DNA Ladder MBI Fermentas (200 bp., 300 bp., 400 bp., 500 bp., 600 bp., 700 bp., 800 bp., 900 bp., 1031 bp.). 1 – *Aneura pinguis* 38016; 2 – *P. endiviifolia*-species A 38609; 3 – *P. endiviifolia*-species A 37905; 4 – *P. endiviifolia*-species B 37422; 5 – *P. endiviifolia*-species B 38724; 6 – *P. neesiana* 37976; 7 – *P. neesiana* 37969; 8 – *P. epiphylla*-species S 32816; 9 – *P. epiphylla*-species S 38446; 10 – *P. borealis* 36928; 11 – *P. borealis* 35822; 12 – *P. epiphylla*-species N 37729; 13 – *P. epiphylla*-species N 37730.

Fig. 3. The trees for *A. pinguis*, *P. endiviifolia*-species A, *P. endiviifolia*-species B, *P. neesiana*, *P. epiphylla*-species S, *P. borealis*, *P. epiphylla*-species N obtained using both distance (calculated using Nei & Li method), and character state matrices and different algorithms: A) The phenogram (d1) derived from UPGMA method. The same topology obtained when distance matrix created using Yule & Kendall algorithm, was used. B) The consensus tree (d2) for UPGMA method. C) The tree based on similarity obtained using Fitch-Margoliash algorithm. D) The consensus tree (d2) for Fitch-Margoliash method. E) The dendrogram (d1) derived from Neighbor-Joining algorithm. F) The most parsimonius tree (d1) obtained using Dollo parsimony method, one most parsimonius tree-235 steps. The same topology was obtained when Polymorphism parsimony method was used G), one most parsimonius tree-287 steps. In the case of E), F), G) trees, bootstrap values (percentage) of 100 replications derived for the consensus tree (d2) were laid on (d1) trees. Bootstrap values are shown near the nodes.

Fig. 3. cont., see next page.

Fig. 3. cont.

Altogether 211 DNA fragments were obtained (211 characters) and have been scored. Using the matrix of binary coded characters we calculated the pairwise distances (Table 1 for modified Nei & Li algorithm).

It results from the size of the distance that populations of the species *P. endiviifolia*-species A (distance value:

0.0362-0.0365), and then populations of *P. neesiana* (0.0396) should be the closest to the outgroup, i.e. the species *A. pinguis*. Comparatively, the distance between *A. pinguis* and populations of *P. endiviifolia*-species B is: 0.0466-0.0468. However, a relatively short distance between *P. endiviifolia*-species A and *P. endiviifolia*-species B (0.0205-0.0326), results in that both these species are grouped together on the obtained phenetic and phylogenetic trees and are localized farther from *A. pinguis* than *P. neesiana*. The highest distance value in relation to *A. pinguis* occurs in the case of *P. epiphylla*-species N (0.0505-0.0570). The analysis of the distance matrices brings additional information on the relation between particular populations of a given species. It should be noted that the distance between populations of *P. neesiana* is 0.0007 (0.000548 according to the Yule & Kendall method-data not shown). This is much less than the distance counted for the same *P. neesiana* populations with the use of the RAPD technique (value 0.150838, Yule & Kendall method, Pacak 1998). This value (0.0007) does not differ much from the distance size between other populations of the same species in contrast to the differences in values of distances between populations counted according to the RAPD analysis. This result shows that the application of the new marker allows to obtain more precise results. Phenetic trees were obtained using the distance matrix counted according to the modified Nei & Li method with the following algorithms: UPGMA (Fig. 3A, 3B), Fitch-Margoliash (Fig. 3C, 3D), Neighbor-Joining (Fig. 3E). In the case of methods based on parsimony i.e. the Dollo method (Fig. 3F) and Polymorphism (3G) binary matrix [0.1] was used directly in analysis. Each of the above-mentioned methods was used to obtain two categories of trees. The first tree, from now on referred to as the basic tree (d1), is based directly on obtained molecular characters, presented in the

form of the binary matrix [0.1], or the distance matrix. The second one is the consensus tree (d2) of the bootstrap test (conducted on the basis of 100 replications). It should be remembered that only d1 trees can be used in the phenetic comparison and phylogenetic reconstruction analysis. Type d2 trees show only the certainty of analyzed d1 trees. All trees obtained by means of the mentioned methods exhibit a similar topology. The used methods confirmed the separate status of all the postulated (on the ground of isoenzymatic research) cryptic species: *P. endiviifolia*-species A, *P. endiviifolia*-species B, *P. epiphylla*-species S, *P. epiphylla*-species N (Zieliński 1987; Szweykowski 1990; Szweykowski 1995). In every performed analysis *P. epiphylla*-species N is always grouped near *P. borealis*; each time the species *P. epiphylla*-species S is added to these species (Fig. 3A, 3C, 3E, 3F, 3G). In the light of the latest results (Pacak 2000), which shows that both the chloroplast and the mitochondrial genomes in *P. borealis* derived from *P. epiphylla*-species N, this result becomes quite easily comprehensible (the same result was achieved by the analysis performed using: Camin-Sokal parsimony, Wagner parsimony and the compatibility (CLIQUE) method – data not shown). Relatively lower results of the bootstrap test for some methods (Fig. 3B, 3D, 3E, 3F) on the node leading to the *P. borealis* populations, can suggest that a given program could not decide which of the examined populations of *P. epiphylla*-species N are closer related with the tested populations of *P. borealis*.

The species *P. endiviifolia*-species A and *P. endiviifolia*-species B are also grouped together, close to the group containing *P. epiphylla*-species S, *P. borealis* and *P. epiphylla*-species N. Surprising though is the localization of *P. neesiana* in comparison to the previous results showing its close affinity with the group: *P. epiphylla*-species S, *P. borealis* and *P. epiphylla*-species N (Szweykowski 1981; Fiedorow 2001a). This species is clearly situated on phenetic and phylogenetic trees far from the liverworts *P. epiphylla*-species S, *P. borealis* and *P. epiphylla*-species N and close to the outgroup *A. pinguis* (Fig. 3A, 3C, 3E, 3F, 3G). This may prove that it is necessary to verify the data concerning taxonomic relations of *P. neesiana* in relation to other species of liverworts from the genus *Pellia*. However, this result is undermined by very low values of the bootstrap test (Hillis 1993) for nodes leading to the *P. endiviifolia*-species A and *P. endiviifolia*-species B and to other liverwort species from the genus *Pellia*. The described nodes are represented by bootstrap values between 31% and 42% for distance-based analysis methods: UPGMA, Fitch & Margoliash and Neighbor-Joining. The very low certainty of this result is clearly shown on the bootstrap consensus tree (d2) of the Fitch & Margoliash method (Fig. 3D). The topology of the mentioned tree is inconsistent with the Fitch & Margoliash basic (d1) tree (Fig. 3C), because *P. neesiana* species is localized there near the group *P. epiphylla*-species S, *P. borealis* and *P. epiphylla*-species N. Nevertheless, the postulated position of *P. neesiana* as the most distant *Pellia* genus species finds strong support in the results of the Dollo and Polymorphism parsimony analysis (Fig. 3F, 3G). In this case bootstrap values of the discussed node, which situate *P. neesiana* near the outgroup, are relatively high, between 79.8–82%. Significantly, the Dollo parsimony is recognized as the method especially reliable in DNA fragments data analysis.

Summing up, the results of using the new molecular marker RAPD-tRNA allow to perform a phylogenetic analysis of liverworts from the genus *Pellia*, confirm the existence of cryptic species and show interrelations which would not be seen in case of the RAPD method (close similarity of *P. borealis* and *P. epiphylla*-species N). The latter example suggests that the new method should be used for analysis of hybrid species. The results show that the taxonomic position of the species *P. neesiana* within the genus *Pellia* is different from what had been accepted basing on previous research. They suggest that additional studies on this species should be performed.

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BADANIA TAKSONOMICZNE WĄTROBOWCÓW Z RODZAJU *PELLIA*, PRZY WYKORZYSTANIU NOWEGO TYPU MARKERA MOLEKULARNEGO

STRESZCZENIE

W celu analizy taksonomicznej wątrobowców z rodzaju *Pellia* wykorzystano nowy typ markera molekularnego stosując dwa rodzaje starterów. Pierwszy jest typowym starterem RAPD, drugi zaś jest starterem uniwersalnym, którego sekwencja nukleotydowa bazuje na wspólnej dla wszystkich cząsteczek tRNA sekwencji trzonu i pętli T ψ C. Stosując te markery skonstruowano drzewa fenetyczne i filogenetyczne dla wszystkich gatunków z rodzaju *Pellia*, stosując jako grupę zewnętrzną gatunek wątrobowca *Aneura pinguis*. Dodatkowo wykazano, że allopoliploidalny gatunek *P. borealis*, powstały wskutek hybrydyzacji dwóch gatunków rodzicielskich: *P. epiphylla*-species S i *P. epiphylla*-species N, jest bliżej spokrewniony z gatunkiem *P. epiphylla*-species N. Spowodowane to jest faktem, że w gatunku *P. borealis* zarówno mitochondria jak i chloroplasty pochodzą od gatunku *P. epiphylla*-species N.

SŁOWA KLUCZOWE: wątrobowce, allopoliploid, analiza bootstrap, ewolucja molekularna.

Appendix

List of samples, with their collection numbers (No), voucher number No* (in herbarium POZW), C = collector initials.

No	Herb. No*	Locality	Voivodship	Year	C.
<i>Aneura pinguis</i> , n=10					
1.	38016	Bieszczady Mts, Wołkowe Berdo 650 m.	Podkarpackie	96	J.S.
<i>Pellia endiviifolia</i> -species A, n=9					
2.	38609	Beskid Wysoki Mts, Babia Góra Mt, 1260 m, Szumiąca Woda stream	Małopolskie	98	J.S., A.B.
3.	37905	Bieszczady Mts, Nasieczyński stream, 680 m	Podkarpackie	96	J.S.
<i>Pellia endiviifolia</i> -species B, n=9					
4.	37422	Kopanina, SW part of Poznań	Wielkopolskie	96	K.B.
5.	38724	Tatry Zachodnie Mts, Ścieżka nad Reglami pass	Małopolskie	98	K.B.
<i>Pellia neesiana</i> , n=9					
6.	37976	Bieszczady Mts, Sianki, tributary of San river	Podkarpackie	96	J.S. et soc.
7.	37969	Bieszczady, Beniowa, Sychłowy stream	Podkarpackie	96	J.S. et soc.
<i>Pellia epiphylla</i> -species S, n=9					
8.	32816	Bieszczady Mts, Tworylczyk stream	Podkarpackie	92	J.S. et soc.
9.	38446	Tatry Mts, Dolina Olczyńska	Małopolskie	96	I.O.
<i>Pellia borealis</i> , n=18					
10.	36928	E from Łódź stream	Pomorskie	94	J.S. et soc.
11.	35822	Wieczywno, N bank of Małe Wieczywno Lake	Pomorskie	94	J.S. et soc.
<i>Pellia epiphylla</i> -species N, n=9					
12.	37729	E bank of Małe Płociczne Lake	Pomorskie	96	K.B.
13.	37730	S bank of Małe Płociczne Lake	Pomorskie	96	K.B.

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