Cryptic speciation in liverworts – a case study in the *Aneura pinguis* complex

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Bryophytes are amongst the most ancestral terrestrial plants and often have large distribution ranges across continents. Recent biochemical and molecular studies have suggested that many worldwide morphological species of bryophyte may represent genetically divergent and reproductively isolated cryptic species. We tested the cryptic species hypothesis in the thalloid liverwort *A. pinguis* complex. We applied analyses of chloroplast DNA (cpDNA) sequence variation and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods to discriminate between specimens of *A. pinguis* derived from various habitats in five distant geographical regions in Poland. Of the 19 specimens sequence characterized for the cpDNA tRNA^Leu^ region, seven haplotypes were identified divided into three nonmonophyletic clusters. The application of developed PCR-RFLP markers confirmed the existence of three tRNA^Leu^ types of *A. pinguis* (A–C) within the specimens derived from 21 populations. Sympatric populations of different tRNA^Leu^ types were found in lowland and mountain regions. No clear correlation between stand type and the presence of two tRNA^Leu^ types (A, B) was observed, as both were growing on soil, humus, and rocks. The tRNA^Leu^ type C was found only on humus and its distribution was restricted to low-lying northern populations. The above results indicate that the *A. pinguis* complex is highly differentiated at the molecular level and may represent three cryptic species. © 2007 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2007, 155, 273–282.

ADDITIONAL KEYWORDS: bryophytes – genetic – Hepticae – reproductive isolation.

INTRODUCTION

The accumulation of genetic differences may cause reproductive barriers between populations and, consequently, may lead to the formation of new biological species. Speciation is usually accompanied by differences in biometric traits. However, it is difficult to recognize genetic subdivision within a species with only a limited number of characters available for traditional taxonomic investigations. A very simple morphological and anatomical structure is typical of bryophytes, some of the most ancestral terrestrial plants (Qiu *et al*., 1998; Groth-Malonek *et al*., 2004). Many bryophytes species, including mosses, liverworts, and hornworts, have large distribution ranges, often without morphological differences between specimens derived from different continents. The structural uniformity of such taxa may potentially result from extremely slow morphological evolution or may hide complex patterns of genetic structure. Recent biochemical and molecular studies have provided evidence of genetic divergence unmarked by morphological changes (cryptic speciation) in bryophytes. In liverworts, the first records of cryptic speciation came from *Conocephalum conicum*. Szweykowski & Krzakowa (1979) found two different allozyme types in allopatric populations of the species, with no evidence of recombinant gametophytes in sympatric populations from Poland (Odrzykoski, 1985). Further allozyme studies throughout the...
geographical distribution of *C. conicum* identified six types in total, which were assumed to represent cryptic species (Odrzykoski & Szweykowski, 1991; Akiyama & Hirooka, 1994; Kim, Harada & Yamazaki, 1996; Szweykowski, Bukowska & Odrzykoski, 2005). Similar studies based on analyses of chemical compounds, isozymes, and DNA have led to the discrimination of two cryptic species in *Pellia epiphylla* (Szweykowski et al., 1995; Odrzykoski, Chudzińska & Szweykowski, 1996; Fiedorow & Szweykowska-Kulińska, 1998; Pacak & Szweykowska-Kulińska, 2000), and two cryptic species in *Pellia endivifolia* (Fiedorow et al., 2001). Further examples have been discussed by Shaw (2001) and Odrzykoski (2004).

In mosses, fixed allelic differences at isozyme loci have provided evidence of cryptic speciation in *Plagiomnium cuspidatum*, which is widely distributed in the Northern Hemisphere (Wyatt & Odrzykoski, 1998). Both isozyme (Shaw & Schneider, 1995) and sequence variation of nuclear ribosomal internal transcribed spacer (ITS) regions (Shaw, 2000) identified two cryptic species in *Mielichhoferia elongata*, which is widespread in the Northern Hemisphere. Other molecular studies have provided evidence of cryptic speciation in *Pyrrhobryum mnioides*, disjunct between Austrasialasia and South America (McDaniel & Shaw, 2003), and in the North American and European aquatic moss *Fontinalis antipyretica*, as revealed by nuclear ITS and chloroplast DNA (cpDNA) analysis (Shaw & Allen, 2000).

These well-documented examples of cryptic speciation in liverworts and mosses suggest that genetic subdivision without clear morphological differentiation may be common in bryophytes. In the present study, we tested the cryptic species hypothesis in a liverwort *Aneura pinguis* (L.) Dumort. The genus *Aneura* (Metzgeriales) includes thallose liverworts of very simple morphological structure. *Aneura* is divided into the three subgenera *Aneura* Dumort., *Lobatiriccardia* Mizut. Et Hatt., and *Austroaneura* Schust. Subgenus *Aneura* comprises several species, most of which have been described recently, and some of which are endemic (Furuki, 1991, 1994; Schuster, 1992; Furuki & Long, 1994). *A. pinguis* is a widespread, almost cosmopolitan species and is found in many different microhabitats. It differs from other species of the genus *Aneura* in respect of its narrow unistratose thallus wings, the shape of the thallus margin, and its smooth dorsal surface (Schuster, 1992; Paton, 1999).

In recent years, isozyme studies have indicated the existence of electrophoretic phenotypes discriminating between two groups of *A. pinguis* from southern Poland (Szweykowski & Odrzykoski, 1990). These two isozyme types, called A and B, grow in different habitats, i.e. wet limestone rocks and humus layer overlying the rocks. Further isozyme studies of *A. pinguis* from northern Poland have indicated the existence of an additional type C isozyme (Andrzejewska, 2000). It is found on wet sand on the shore of oligotrophic lakes, growing together with rare liverwort species, including *Haplomitrium hookeri*, *Riccardia incurvata*, *Fossombronia foeloata*, and *Scapania irrigua* (Bączkiewicz & Szweykowski, 2001). The genetic differentiation between these groups, as measured by Nei's genetic distance (Nei, 1972), matched that between different but related species in other groups. As the genetically differentiated specimens also shared different ecological preferences, it was suggested that these three ecotypes might represent different evolutionary lines.

In the present study, cpDNA sequence variation was examined in specimens of *A. pinguis* derived from five geographically distant regions in Poland. Uniparentally inherited, nonrecombining cpDNA with a relatively low mutation rate has been found to be very useful in many phylogenetic studies in lower and higher plants. The tRNA\_Leu gene of the chloroplast genome was applied in our study. This region has been described previously by Szweykowska-Kulińska, Pacak & Jankowiak (2002), and has been used for discrimination between closely related liverwort species. We analysed the nucleotide polymorphism of the gene across populations, and compared our results with the outcomes of isozyme studies conducted on the same plant material. The above approaches were applied to test the predictions of cryptic speciation in *A. pinguis* and to check whether different ecotypes are genetically uniform or, rather, represent a mixture of reproductively isolated species.

**MATERIAL AND METHODS**

**POPULATION SAMPLING**

Ninety specimens of *A. pinguis* were included in the molecular analyses. They were collected in 21 populations from five geographically distant regions in Poland. Twenty specimens originated from Białywiecki National Park, 40 from the Tatry Mountains, ten from Male Pieniny Mountains, 11 from Bieszczadzki National Park, and nine from the Diabli Skok Reserve in Wielkopolska Province. They were collected in various habitats representing different stand types, including humus, soil, and rocks. A distance of at least a few metres separated the specimens derived from one population. A description of the plant material used is presented in Table 1.

**DNA EXTRACTION AND POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION**

Total genomic DNA was extracted from about 50 mg of the green part of the gametophyte using a
Table 1. Populations of the *Aneura pinguis* complex included in the molecular analyses

| No. | Geographical region and locality                      | Latitude and longitude | Stand type          | Specimen | *A. pinguis* type |
|-----|-------------------------------------------------------|------------------------|---------------------|----------|------------------|
| 1   | I. Białowieski National Park Žebra Żubra, track, part 425 | 52°42'N, 23°48'E       | Humus               | BZ2-1, BZ3-1, BZ3-2, BZ4-4, BZ4-5 | B     |
|     |                                                       |                        | Humus               | BZ3-3, BZ4-3          |       |
|     |                                                       |                        | Humus               | BZ3-4              | A     |
| 2   | Forest stand, part 254 Dc                             | 52°46'N, 23°50'E       | Humus               | BZ6-3              | B     |
|     |                                                       |                        | Humus               | BZ5-1, BZ5-2, BZ6-1, BZ7-1, BZ7-2 | C     |
|     |                                                       |                        | Humus               | BZ6-2              | C     |
| 3   | Wysokie Bagno Reserve                                 | 52°41'N, 23°50'E       | Humus               | BZ1-1, BZ1-3, BZ8-1, BZ8-3, BZ8-4 | C     |
|     |                                                       |                        | Humus               | BZ3-3, BZ4-3        |       |
|     |                                                       |                        | Humus               | BZ3-4              | A     |
| 4   | II. Tatrzanski National Park Dolina Jaworzynka        | 49°16'N, 19°59'E       | Humus               | T9-2               | B     |
| 5   | Skupniów Uplaz                                        | 49°15'N, 20°00'E       | Rocks               | T14-1, T15-1       | B     |
|     |                                                       |                        | Humus               | T17-1, T17-2, T17-3, T17-4, T18-1, T20-2 | A     |
|     |                                                       |                        | Rocks               | T13-2, T13-3, T13-4, T16-1, T20-1 | A     |
|     |                                                       |                        | Soil                | T18-2, T21-1, T21-2 | A     |
| 6   | Dolina Białego Potoku                                | 49°16'N, 19°57'E       | Soil                | T1-1               | B     |
|     |                                                       |                        | Rock                | T2-11              | A     |
|     |                                                       |                        | Humus               | T7-4, T8-1, T8-2, T8-4 | A     |
| 7   | Wielka Sucha Dolina                                  | 49°16'N, 19°49'E       | Rocks               | T24-1, T24-2, T24-4, T26-1, T26-5 | A     |
| 8   | Žleb Kozieniecki                                      | 49°16'N, 19°49'E       | Rocks               | T32-5, T34-3, T34-4, T35-1 | A     |
| 9   | Wawóz Kraków                                         | 49°13'N, 19°52'E       | Rocks               | T10-1, T11-1, T11-3 | A     |
| 10  | Dolina Suchej Wody                                    | 49°16'N, 20°02'E       | Humus               | T11-2              | A     |
| 11  | Dolina Pańszczyca                                    | 49°15'N, 20°02'E       | Rocks               | T38-5              | A     |
|     |                                                       |                        | Soil                | T39-1              | A     |
|     |                                                       |                        | Rocks               | T41-1              | A     |
|     |                                                       |                        | Humus               | T44-2              | A     |
| 12  | III. Małe Pieniny                                     | 49°24'N, 20°33'E       | Soil                | P46-1, P46-2, P46-4, P51-1 | A     |
| 13  | Potok Skalskie                                        | 49°23'N, 20°34'E       | Humus               | P47-3, P49-1       | A     |
| 14  | Potok Skalskie                                        | 49°22'N, 20°34'E       | Soil                | P48-1              | A     |
| 15  | Potok Skalskie                                        | 49°23'N, 20°34'E       | Soil                | P56-1              | A     |
|     |                                                       |                        | Humus               | P53-1              | A     |
|     |                                                       |                        | Rocks               | P54-2              | A     |
| 16  | IV. Bieszczadzki National Park Moczarne, Górna Solinka | 49°07'N, 22°29'E       | Humus               | A1-3, A1-4         | B     |
| 17  | Brzeg Górny                                           | 48°08'N, 22°34'E       | Soil                | A2-1, A2-3, A6-1   | B     |
| 18  | Sianki, Potok Niedźwiedź                              | 49°09'N, 22°25'E       | Soil                | A3-1               | B     |
| 19  | Sianki, Potok Niedźwiedź                              | 49°02'N, 22°49'E       | Soil                | A4-2, A4-3         | B     |
| 20  | Dolina Górnej Solinki                                 | 49°08'N, 22°29'E       | Soil                | A11-1, A12-1, A14-1 | B     |
|     | V. Wielkopolska Province                              |                         |                      |                    |       |
| 21  | Diabli Skok Reserve                                   | 53°23'N, 16°35'E       | Humus               | DS4-1, DS4-3, DS4-4 | B     |
|     |                                                       |                        | Humus               | DS2-2, DS3-1, DS3-2, DS3-3, DS3-4, DS5-4 | C     |
modification of a cetyltrimethylammonium bromide (CTAB) protocol described by Doyle & Doyle (1987). PCR amplification of the tRNA<sub>Leu</sub> cpDNA region was carried out in a total volume of 25 µl containing about 20 ng of template DNA, 2.5 mM MgCl₂, 100 µM of each deoxynucleoside triphosphate (dNTP), 1 mM of spermidine (Fiedorow & Szweykowska-Kulińska, 1997), 0.2 µM of each primer, and 0.25 U Taq polymerase, with the respective 1 × PCR buffer (Taq polymerase and 10 × PCR buffer were provided by Fermentas, Lithuania). PCRs followed the cycle profile and primers described by Szweykowska-Kulińska et al. (2002). The PCR products were run on a 1.5% agarose gel (Sambrook, Fritsch & Maniatis, 1989), stained with ethidium bromide, and analysed under ultraviolet light.

**DNA sequencing and analyses of haplotypes**

PCR fragments of approximately 400 nucleotides for tRNA<sub>Leu</sub> regions were purified using a QIAquick PCR Purification Kit (Qiagen). PCR products (approximately 20 ng) were used as a template for a 10-µl sequencing reaction with the Big Dye Terminator DNA Sequencing Kit (Applied Biosystems), and the samples were run on an ABI automated sequencer (ABI 377). SeqManII software was used to edit and assemble the sequence chromatograms to produce alignments based on nucleotide identification from both DNA strands.

Nineteen specimens of *A. pinguis* were sequence characterized for the tRNA<sub>Leu</sub> region. They originated from different habitats from the five geographical regions (Tables 1 and 2). The remaining specimens were genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Three restriction enzymes, including *Hinf*I, *Hae*III, and *Taq*I, were used to identify the main cpDNA haplotypes revealed by sequencing analyses and representing the three clusters in the molecular phylogenetic study. The PCR products (10 µl) were digested at 37 °C overnight (*Hinf*I, *Hae*III) and at 65 °C for 3 h (*Taq*I). After digestion, the samples were separated in an 8% polyacrylamide gel (Sambrook et al., 1989), stained with ethidium bromide, and analysed under ultraviolet light. The results of DNA sequencing and PCR-RFLP analyses were compared with those of multilocus isozyme studies conducted on the same plant material (Bączkiewicz & Buczkowska, 2005).

**Phylogenetic analyses**

The sequences of the tRNA<sub>Leu</sub> region were aligned using the ClustalX program, version 1.81 (Thompson et al., 1997), and multiple alignments were adjusted manually in GeneDoc (Nicholas & Nicholas, 1997). Phylogenetic analyses were inferred with the use of Mega 2.0. Neighbour-joining methods (Saitou & Nei, 1987) with the Kimura two-parameter model were used to construct a phylogenetic tree. The same DNA data set was analysed by the maximum parsimony method using the close neighbour-interchange searching technique and ten replications of randomly added trees. Bootstrap values with 1000 replicates were used to assess the confidence of branching in both data sets (Felsenstein, 1985). The orthologue GenBank DNA sequences of *Noteroaclada confuens* (Accession No. AY463577), *Lobatiriccardia lobata* (Accession No. AY507553), and *Marchantia polymorpha* (Accession No. X04465) were used as outgroups.

**RESULTS**

**Nucleotide polymorphism**

The nucleotide alignments of the tRNA<sub>Leu</sub> gene resulted in a data set of 391 putative homologous sites. Twenty-seven single nucleotide polymorphisms (SNPs), including seven singleton and one nucleotide insertion/deletion (indel), were found amongst the 19 studied sequences (Table 2). Seven haplotypes were found in total (Table 2). The results of molecular phylogenetic analyses of the sequences representing the different haplotypes are presented in Figure 1. The topology of the trees, as well as the bootstrap values for individual nodes, were similar in the neighbour-joining and maximum parsimony methods used to construct the phylogenetic trees. Therefore, the tree obtained from the neighbour-joining method is presented. Three clades in total were identified, including groups of four, two, and one haplotype, respectively. Two haplotypes were located in the same clade with *Noteroaclada confuens* used as outgroup. The comparison of our DNA results with the isozyme studies by Bączkiewicz & Buczkowska (2005) indicated that the specimens representing these two grouped haplotypes (called A1 and A2) could be classified as isozyme electrophoretic phenotype A. Therefore, they were assigned as tRNA<sub>Leu</sub> type A (Table 2). Similarly, the group of four haplotypes (called B1–B4) and the single haplotype C1 were identified as isozyme types B and C, and assigned as tRNA<sub>Leu</sub> type B and tRNA<sub>Leu</sub> type C, respectively. The haplotype A1 could be differentiated from A2 by four nucleotides and one base pair indel. The haplotypes B1–B4 could be differentiated from one another by one to four nucleotide sites. Different *A. pinguis* types were differentiated from one another by eight (B3 vs. C1) to 19 (A1 vs. B4) nucleotide sites (Table 2). The sequences representing different *A. pinguis* types were submitted to the GenBank database under the
Table 2. Polymorphic sites in tRNA\textsubscript{Leu} gene and types of *Aneura pinguis* complex distinguished in phylogenetic analyses. *Noteroclada confluens* was used as outgroup.

| Specimen | tRNA\textsubscript{Leu} of cpDNA | A. pinguis haplotype type |
|----------|---------------------------------|--------------------------|
|          | Nucleotide position             |                          |
|          | 11111222222222222222233333     |                          |
|          | 2344560068900112334555555991135689 |                          |
|          | 98062334150596774690136801487754 |                          |

*Noteroclada confluens*

- Specimen: BZ3-4, P46-4, P47-3, P46-1
- Haplotype: CTGGCTTGCTACCTACGCTATAAAATCGAGC

- Specimen: BZ4-5, BZ3-1
- Haplotype: A. ...TCG...–A.GGGA...C.T

- Specimen: BZ4-4
- Haplotype: A. ...TCG.A.GA.GG.....C.T

- Specimen: BZ3-1
- Haplotype: T.TAA...AAC.A.CG..TGAGG....TAC.T

- Specimen: BZ1-4
- Haplotype: T.TAA...AAC.A.CG..TGAGG....T.C.T

- Specimen: T14-1
- Haplotype: T.TAAT..AAC.A.CGT..TGAGG...TT.CAT

- Specimen: DS3-1, DS3-2, DS2-2, BZ8-3, BZ5-2, BZ8-4
- Haplotype: T.TAAT..AAC...A.CG.T..TGAGG...TT.CAT
following Accession Numbers: DQ272347, haplotype A1; DQ272348, haplotype B1; DQ272349, haplotype C1.

**PCR-RFLP MARKERS OF tRNA\textsubscript{LEU} GENE**

Nucleotide polymorphism of the analysed cpDNA sequences was used to develop PCR-RFLP markers useful for the identification of tRNA\textsubscript{LEU} types A–C. Restriction analyses of this region with the use of the three enzymes \textit{Hae}\textsubscript{III}, \textit{Hinf}\textsubscript{I}, and \textit{Taq}\textsubscript{I} resulted in three, two, and four different haplotypes, respectively (Fig. 2). No other haplotypes were observed amongst the analysed \textit{A. pinguis} specimens. \textit{Hae}\textsubscript{III} and \textit{Taq}\textsubscript{I} enzymes differentiated between all tRNA\textsubscript{LEU} types (A, B, and C), and \textit{Hinf}\textsubscript{I} distinguished type B from the remaining two types. In addition, \textit{Taq}\textsubscript{I} could be applied to identify haplotypes A1 and A2 within \textit{A. pinguis} tRNA\textsubscript{LEU} type A.

**PATTERNS OF GEOGRAPHICAL DISTRIBUTION**

The distribution of the identified tRNA\textsubscript{LEU} types in the studied geographical regions in Poland is presented in Figure 3. tRNA\textsubscript{LEU} types A and B were observed in lowland and mountain populations. Type A was derived from Bialowieski National Park (one specimen), Tatrzanski National Park (36), and Male Pieniny Mountains (all ten specimens from this region). tRNA\textsubscript{LEU} type B was derived from Bialowieski National Park (six), Tatrzanski National Park (four), Bieszczadzki National Park (all 11 specimens from this region), and Diabli Skok Reserve in Wielkopolska Province (three). tRNA\textsubscript{LEU} type C was found only in low-lying populations including Bialowieski National Park (13) and Diabli Skok Reserve (six). Types A and B were found in different populations growing on humus or soil in low-lying forest stands and on a humus layer over basic rocks or on soil in mountain regions. Only type C could be assigned to one stand type, as it was found only on humus (Table 1).

**DISCUSSION**

Nucleotide polymorphism in the chloroplast genome is a good preliminary indicator of species differentiation.
tion at different taxonomic levels. The rate of molecular evolution is several times lower in the chloroplast genome than in nuclear DNA and, in many plant species, including liverworts, cpDNA is inherited in only one of the parental lines without sexual recombination (Birky, 2001; Pacak & Szweykowska-Kulinska, 2003). Because of the low rate of evolution in the chloroplast genome, comparative analysis of several cpDNA regions has been used in many studies to reconstruct the phylogenetic relationships between plant species (Nickrent, Parkinson & Duff, 2000; Gernandt et al., 2005). The nucleotide substitution rate of cpDNA in liverworts seems to be within the range of that in higher plants, although there is evidence of lineage and gene-specific heterogeneity in the rate of cpDNA molecular evolution (Lewis, Mishler & Vilgalys, 1997; Szweykowska-Kulinska et al., 2002). In the rbcL gene, which is one of the most frequent genes used in phylogenetic reconstruction, the total nucleotide substitution rate in ferns was estimated to be about (6.9–9.3) × 10⁻¹¹ nucleotide per site per year (Yatabe, Nishida & Murakami, 1999). Therefore, even low nucleotide polymorphism in cpDNA may indicate high genetic divergence between taxa.

Our study shows nucleotide differentiation in the chloroplast genome of the A. pinguis complex. Molecular phylogenetic analyses of sequence data allowed us to distinguish three A. pinguis tRNAleu types (A–C). Amongst the specimens derived from different geographical regions and analysed using developed PCR-RFLP markers, we observed only the combination of haplotypes specific to the three defined A. pinguis types. The tRNAleu types were differentiated from one another by 8–19 nucleotide sites, and such a level of nucleotide polymorphism is within the range observed between cryptic species in other liverworts. Six nucleotide substitutions and two indels in the ≈800-bp tRNAleu region differentiated between P. epiphylla species N and P. epiphylla species S, two cryptic species showing an allopatric north–south distribution in Poland. The same species were also differentiated by five nucleotide substitutions and one indel in the ≈2300-bp region of the tRNA Asp gene (Pacak & Szweykowska-Kulinska, 2000). Three cryptic species of Asian Conocephalum japonicum were differentiated from each other by 6–10 nucleotides in the analysed 1304-bp rbcL region (Miwa et al., 2003, 2004). Several nucleotide substitutions were found in the psbA gene of cpDNA that discriminated between six putatively cryptic species across the worldwide distribution of Conocephalum conicum (Kim et al., 2001).

Figure 3. Geographical distribution of the identified tRNAleu types of Aneura pinguis (A–C) in locations I–V described in Table 1.

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In addition to the high level of nucleotide polymorphism discovered in the \textit{tRNA} \text{Leu} gene, the molecular phylogenetic analyses show that the \textit{A. pinguis} complex forms a nonmonophyletic group, as \textit{Notorecla confluens} clusters together with \textit{A. pinguis} \textit{tRNA} \text{Leu} type A. The nonmonophyletic character of the group suggests that the \textit{A. pinguis} complex may be represented by different evolutionary lines. The cryptic speciation hypothesis is supported further by the combined results of DNA and isozyme studies. Multilocus isozyme analyses on the same \textit{A. pinguis} specimens as used for the DNA study indicated the existence of three isozyme phenotypes (Bączkiewicz & Buczkowska, 2005). Our study shows that cpDNA and isozyme polymorphism correspond to each other and that \textit{tRNA} \text{Leu} types can be assigned to isozyme phenotypes. The biparentally inherited isozyme markers showed no evidence of recombined genotypes resulting from interbreeding in any of the analysed populations in which phenotypes A–C occurred sympatrically (Bączkiewicz & Buczkowska, 2005). The lack of recombined genotypes indicates that the identified \textit{tRNA} \text{Leu} types may represent three reproductively isolated cryptic species: \textit{A. pinguis} species A, B, and C, respectively. We propose the application of the developed DNA markers for the delimitation of the identified \textit{A. pinguis} types. The markers can also be applied in the analysis of dry specimen collections, which cannot be studied with the use of isozymes.

Our results indicate that three \textit{A. pinguis} types occur in Poland. Furthermore, the genotypes detected in Poland correspond to the genotypes found in neighbouring countries. Type A was observed in populations from south Germany and Slovakia, and types B and C in Lithuania (Bączkiewicz & Buczkowska, 2005). The three isozyme phenotypes showed high genetic differentiation, as measured by Nei's genetic distance (Nei, 1972; Bączkiewicz & Buczkowska, 2005). Thus, the available data indicate that three evolutionary lines of \textit{A. pinguis} types are present in central and eastern Europe. However, an additional fixed difference between \textit{A. pinguis} accessions was found in the Acp (acid phosphatase) locus in two populations from the British Isles. These specimens seem to reflect a more complex pattern of genetic differentiation within the \textit{A. pinguis} complex and, as suggested by Bączkiewicz & Buczkowska (2005), may even represent additional cryptic species D, not present in central and eastern Europe. However, detailed studies on the British Isles are needed to confirm these observations.

An allopatic or nearly allopatic distribution has been observed between some liverwort cryptic species, including \textit{P. epiphylla} species N, distributed in northern Poland, and \textit{P. epiphylla} species S, which occurs mostly in the southern part of Poland (Szweykowski \textit{et al.}, 1995). Our results show that \textit{A. pinguis} type C is restricted to northern low-lying populations, whereas type A has a rather southern distribution. However, different types of \textit{A. pinguis} could not be assigned to a specific stand type, such as, for instance, different taxa from \textit{P. endiviifolia}, which grow either on limestone rocks or on wet soil (Zielinski, 1987). Mixed stands of \textit{tRNA} \text{Leu} types A and B were identified growing sympathetically on humus, wet soil, and rocks. Only type C was found on humus only in low-lying forest stands in two geographical regions in northern Poland. However, this electrophenotype has previously been reported to occur on wet sands of the shores of oligotrophic lakes (Andrzejewska, 2000).

Recent studies have provided evidence of associations between liverworts and fungi that seem to imitate mycorrhizas (Sellesse, 2005). The mycorrhizal symbiosis is a well-described phenomenon in vascular plants, and leads to nutrient exchange between a host plant and soil fungi. The fungi involved in several putative mycorrhiza-like associations with liverworts have been identified and described morphologically (Kottke & Nebel, 2005). The role and benefits of such associations in liverworts can be investigated by \textit{in vitro} cultivation experiments. These studies indicate high host specificity within taxa, including members of the Jungermanniales and Metzgeriales. It was shown that fungi from the achlorophyllous \textit{Cryptothallus mirabilis} (Aneuraceae) do not form normal associations with \textit{A. pinguis}. In addition, different fungal endophytes were found in upland and lowland specimens of \textit{A. pinguis}, which showed high host specificity with no signs of cross-invasion in reciprocal test experiments (Duckett \textit{et al.}, 2004). It seems that the co-evolution of mycorrhiza-like associations in \textit{A. pinguis} and other bryophytes requires detailed investigation. These studies should address the question of whether mycorrhiza-like associations may influence the success of bryophyte propagation by long-range spore dispersal, or contribute to the present-day patterns of biogeographical distribution.

The identification and description of cryptic species are important, but initial, steps to more complex studies concerning the mechanisms of speciation. The disjunctive distribution observed in many bryophytes can promote allopatic speciation by the accumulation of genetic differences between isolated populations. Thus, the sympatric occurrence of cryptic species may be the result of dispersal from isolated populations. However, a wide scale of ecological tolerance and the features of bryophyte sexual reproduction [short-distance sperm dispersal causing highly effective isolation of even neighbouring colonies (Wyatt, 1985; Freitas & Brehm, 2001); self-fertilization, which is likely to occur in species with bisexual gametophytes (Shaw, 2001); and sexual reproduction restricted only
to some parts of their geographical distribution (Heirichs et al., 2004) also indicate the possibility of sympatric speciation. The molecular methods employed in our study proved to be useful in species delimitation, and may be used to advance other approaches to provide more evidence on cryptic speciation in bryophytes. The application of molecular methods, accompanied by detailed biometric, biochemical, and ecological studies, will help us to gain a better understanding of the mechanism behind genetic differentiation and biogeographical distribution in this group of plants.

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