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Application of PCR – RFLP markers for identification of genetically delimited groups of the *Calypogeia fissa* complex (Jungermanniopsida, Calypogeiaceae)

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Abstract: Currently, two subspecies are formally recognized within *Calypogeia fissa*: *C. fissa* subsp. *fissa* occurring in Europe and *C. fissa* subsp. *neogea* known from North America. Genetic studies have revealed a complex structure of this species. Within the European part of distribution, three genetically distinct groups P_S, P_B and G are distinguished. The combination of the SCAR marker Cal04 and PCR-RFLP markers with three restriction enzymes (*Sma*I, *Taq*I and *TspGW*I) allowed the recognition of all groups within the *C. fissa* complex. The *Taq*I enzyme recognizing the restriction sites in the PCR product of SCAR marker Cal04 turned out to be the best marker.

Key words: Bryophyta, liverworts, complex species, *Calypogeia*, PCR-RFLP, SCAR

1. Introduction

Genetic studies of bryophytes demonstrated that classical taxonomic approach based on morphology was able to detect only a part of bryophyte diversity (Heinrichs *et al.* 2009; Kreier *et al.* 2010; Bell *et al.* 2012). The isozyme and molecular taxonomy studies revealed that some bryophyte species, having a wide range of geographic distribution, are, in fact, species complexes composed of several genetically isolated species, in liverworts e.g. *Pellia epiphylla* (Szweykowski *et al.* 1995); *Conocephalum conicum* (Szweykowski *et al.* 2005), *Aneura pinguis* (Wachowiak *et al.* 2007), *Metzgeria furcata*, *M. conjugata* (Fuselier *et al.* 2009); *Frullania tamarisci* (Heinrichs *et al.* 2010), *Ptilidium* (Kreier *et al.* 2010), in mosses e.g. *Rhytidiadelphus* (Vanderpoorten *et. al.* 2003), *Orthotrichum* (Sawicki *et al.* 2012). For this reason, some species described in previous works require revision and re-characterization (Bischler & Boisselier-Dubayle 2000; Schumacker & Váňa 2005; Szweykowski 2006). This problem also applies to the *Calypogeia* Raddi genus.

The genus *Calypogeia* is distributed worldwide, primarily, in subtropical to tropical climates (Bischler 1963; Schuster 1969). In the Holarctis, species richness of *Calypogeia* is much lower, only from 9-13 species are known in different parts of the northern hemisphere, 9 species occur in Europe (Grolle & Long 2000) and North America (Schuster 1969), 13 – in Asia (Japan) (Inoue 1975). However, genetic studies of the genus based on isozyme polymorphism, molecular markers (ISSR, SCAR) and DNA sequences have revealed that some taxonomically accepted species e.g. *C. muelleriana* (Schiffn.) Müll. Frib., *C. sphagnicola* (Parnell & J. Perss.) Warnst. & Loeske and *C. fissa* (L.) Raddi are genetically heterogeneous, each consisting of several cryptic taxa (Buczkowska 2004; Buczkowska & Bączkiewicz 2011; Buczkowska & Dabert 2011; Buczkowska *et al.* 2012a). On the basis of the above studies, it can be assumed that in Holarctic, the number of genetically different taxa of *Calypogeia* genus is underestimated.

Calypogeia fissa occurs in North America, Europe, North Africa and Asia and is regarded as suboceanic-mediterrean, amphiatlantic species (Müller 1951-1958; Schuster 1969; Paton 1999; Damsholt 2002). Currently, two subspecies are formally recognized: *C. fissa* subsp. *fissa* occurring in Europe and *C. fissa* subsp. *neogea* Schust. known from North America (Schuster 1969; Damsholt 2002). Application of isozyme markers has

shown that within the European part of distribution, three genetically distinct groups P_S, P_B and G were distinguished (Buczkowska 2004). The groups P_S and P_B initially were found in Poland and comprised, respectively, small and large plants, whereas the group G was noted in Germany, and it resembles the size of the P_B group (Buczkowska 2004). Further studies indicate that the G group is more frequent in south-western parts of Europe, whereas in Poland, it is very rare (Buczkowska *et al.* 2012a). The most distinct, both in terms of genetic and morphological features, is the P_S group (Buczkowska 2004; Buczkowska *et al.* 2011). Morphological differences between the remaining groups (P_B, G and *C. fissa* subsp. *neogea*) are less clear, thus, their proper identification only on the basis of classical methods is difficult. Despite this, molecular studies support differences between them at the genome level detected by SCAR markers (Buczkowska *et al.* 2012a). Another group, which can be difficult to distinguish from the *C. fissa* complex, is a newly detected taxon – group B of the *C. muelleriana* complex (Buczkowska & Bączkiewicz 2011). The plants differ from *C. fissa* in oil body features; however, because of similar shape of underleaves, the above taxa can be confused. Therefore, another marker useful for identification of plants belonging to these groups, also in case of plants from the herbarium collection, is necessary.

Restriction Fragment Length Polymorphism (PCR-RFLP) is a molecular method that facilitates fast identification of species (Avise 2004). The method allows displaying differences in the DNA sequence directly at the level of PCR products, without costly sequencing. It is a highly reliable and easy method for identifying polymorphism in DNA sequences using restriction enzymes and gel electrophoresis (Avise 2004). Markers based on the PCR-RFLP method have recently been developed for species identification in many taxa, including bryophytes, particular cryptic species of liverworts e.g. *Marchantia* (Boisselier-Dubayle *et al.* 1995), *Pellia* (Szweykowska-Kulińska *et al.* 2002; Pacak & Szweykowska-Kulińska 2003), *Porella* (Jankowiak & Szweykowska-Kulińska 2004), *Aneura* (Wachowiak *et al.* 2007) and mosses e.g. *Rhytidiadelphus* (Vanderpoorten *et. al.* 2003).

In the present study, we use PCR-RFLP method to find a helpful tool for the identification of plants belonging to genetic groups of European *C. fissa* subsp. *fissa* (P_S and P_B) and to North American *C. fissa* ssp. *neogea*. Development of reliable marker will allow in the future examining of material from wider geographic range and phylogeographic study of the *C. fissa* complex.

2. Material and methods

2.1. Plant material

The studied plants were initially determined as belonging to the *C. fissa* complex on the basis of morphological traits and oil body characters according to Schuster (1969) and Szweykowski (2006). Subsequently, particular genetic groups were identified on the basis of isozyme markers according to Buczkowska (2004). In general, 20 samples of *C. fissa* subsp. *fissa* were examined (10 of the P_S group, 10 of P_B) and 6 samples of *C. fissa* subsp. *neogea* from the USA. Moreover, 10 samples of the *C. muelleriana* complex – group B (Buczkowska & Bączkiewicz 2011) were used as a comparison (Table 1).

2.2. DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from fresh plants. Several stems from one sample were ground with steel beads in a Bio-prep-24 Homogenizer for 35 seconds. A standard CTAB procedure (Murray & Thompson

Table 1. Collection sites of the studied samples of *C. fissa* complex and GenBank accession numbers

Sample No.	Locality	Collector	Herbarium No.	GenBank No.		
				ITS2	trnL	Cal04
	C. fissa subsp. *fissa* – group P_S					
1	W Poland, Lubuskie Province, Bogumiłów near Żary, sandy soil on ditch banks	SR	42628	KR909073	KR909061	JX402494
2	W Poland, Lubuskie Province, Bogumiłów near Żary, sandy soil on ditch banks	KB, JS	39199	–	–	–
3	W Poland, Lubuskie Province, Tuplice near Lubsko, sandy soil on ditch banks	KB, JS	39203	KR909074	KR909062	KR909052
4	Central Poland, Wielkopolskie Province, Antonin near Ostrów Wlkp., sandy soil on ditch banks	KB	42225	KR909075	KR909063	JX402495

Sample No.	Locality	Collector	Herbarium No.	GenBank No.		
				ITS2	trnL	Cal04
5	Central Poland, Wielkopolskie Province, Antonin near Ostrów Wlkp., sandy soil on ditch banks	KB	42236	–	–	–
6	Central Poland, Wielkopolskie Province, Antonin near Ostrów Wlkp., sandy soil on ditch banks	KB	42234	–	–	–
7	Central Poland, Wielkopolskie Province, Antonin near Ostrów Wlkp., sandy soil on ditch banks	KB	42227	KR909076	KR909064	KR909053
8	Central Poland, Wielkopolskie Province, Antonin near Ostrów Wlkp., sandy soil on ditch banks	KB	42238	–	–	–
9	Central Poland, Wielkopolskie Province, Antonin near Ostrów Wlkp., sandy soil on ditch banks	KB	42235	–	–	–
10	Central Poland, Wielkopolskie Province, Antonin near Ostrów Wlkp. sandy soil on ditch banks	KB	42629	–	–	–
	C. fissa subsp. *fissa* – group P_B					
11	NW Poland, Pomorskie Province, Lake Małe Sitno near Czarna Dąbrówka, *Carex* tussocks at lakeshore	KB, JS	42345	KR909077	KR909065	JX402498
12	NW Poland, Pomorskie Province, Lake Kamień near Miastko, peaty soil at lakeshore	KB, AB	42205	KR909078	KR909066	KR909054
13	NW Poland, Pomorskie Province, Lake Kamień near Miastko, peaty soil at lakeshore	KB, AB	42200	KR909079	KR909067	KR909055
14	W Poland, Lubuskie Province, Starosiedle forest division, *Carex* tussocks at lakeshore	SR, KB	42302	–	–	–
15	W Poland, Lubuskie Province, Starosiedle forest division, *Carex* tussocks at lakeshore	SR, KB	42277	–	–	–
16	W Poland, Lubuskie Province, Starosiedle forest division, *Carex* tussocks at lakeshore	SR, KB	42275	–	–	–
17	W Poland, Lubuskie Province, Mierków forestry, humus in *Carici elongate-Alnetum*	SR, KB	42298	KR909080	KR909068	JX402499
18	W Poland, Lubuskie Province, Mierków forestry, humus in *Carici elongate-Alnetum*	SR, KB	42299	–	–	–
19	W Poland, Lubuskie Province, Nabłoto forestry, humus in *Carici elongate-Alnetum*	SR, KB	42317	–	–	–
20	W Poland, Lubuskie Province, Rzeczyca river, on wet humus	SR	42630	–	–	–
	C. fissa subsp. *neogea*					
21	North America, North Carolina, the Southern Appalachian, Dry Falls, upper part rocks	BS	42626	KR909084	KR909072	JX402506

Sample No.	Locality	Collector	Herbarium No.	GenBank No.		
				ITS2	trnL	Cal04
22	North America, North Carolina, the Southern Appalachian, Dry Falls, soil in RO Biss	BS	42622	KR909082	KR909070	JX402507
23	North America, North Carolina, the Southern Appalachian, on sandy soil, Duke Herbarium no 1132c	BS	42620	KR909081	KR909069	JX402508
24	North America, North Carolina, the Southern Appalachian, on sandy soil	BS	42625	KR909083	KR909071	JX402509
25	North America, North Carolina, the Southern Appalachian, Nantaha National Forest	BS	42616	–	–	–
26	North America, North Carolina, the Southern Appalachian, Duke Herbarium no 1132b/2	BS	42617	–	–	–
	C. muelleriana complex – group B[1]					
27	NE Poland, Warmińsko-Mazurskie Province, Lake Godle near Ełk	KB, AB	41706	KR909085	KF371571	KR909056
28	NE Poland, Warmińsko-Mazurskie Province, Lake Godle near Ełk	KB, AB	41707	KR909086	KF371570	KR909057
29	NW Poland, Pomorskie Province, Lake Lubygość near Kartuzy	KB, AB	42220	KR909087	KF371568	JX402493
30	NW Poland, Pomorskie Province, Lake Smołowe near Miastko	KB, AB	42285a	KR909088	KF371573	KR909058
31	NW Poland, Pomorskie Province, Lake Smołowe near Miastko	KB, AB	42285b	KR909089	KF371574	KR909059
32	North America, Tunk Lake, west of Cherryfield, woods on S side of road, 60	BA	dhl825	KR909090	KF371575	KR909060
33	North America, Maine, Duke Herbarium no 1174/3	BS	42460	–	–	–
34	North America, North Carolina, Duke Herbarium no 1178f/17	BS	42453	–	–	–
35	North America, North Carolina, Duke Herbarium no 1174/4	BS	42461	–	–	–
36	North America, North Carolina, Eno River area, Duke Herbarium no 1178f/9	BS	42462	–	–	–

Explanations: 1 – the name of the group is consistent with Buczkowska & Bączkiewicz (2011); collectors, AB – A. Bączkiewicz, BA – B.K. Andreas, BS – Blanka Shaw, JS – J. Szweykowski, KB – K. Buczkowska, SR – S. Rosadziński

1980) downscaled to fit 1.5 ml Eppendorf tubes was applied. The isolated DNA was dissolved in TE buffer and stored at -20° C. The quality of the isolated DNA was evaluated by electrophoresis in 0.8% agarose gel and the concentration and purity of DNA samples were determined using the Epoch™ Multi-Volume Spectrophotometer System. Primers used for the amplification and sequencing of the studied regions were according to Pacak & Szweykowska-Kulińska (2003) for cp *trnL* gene intron, Bell *et al.* (2012) – for ITS2 and Buczkowska & Dabert (2011) – for SCAR marker Cal04. PCR amplifications were performed in a total volume of 20 µL containing 2 µL of 10x PCR buffer with Mg^{2+} (Novazym; 25 mmol $Mgcl_2$), 1 µL BSA (0.25 mg/ml), 200 µmol of each dNTP (Novazym), 0.4 µmol of each primer, 1 U of Taq DNA polymerase (Novazym) and

Table 2. Summary of RFLP digestion conditions for each studied enzyme

Stage	*Taq*I	*Sma*I	*TspGW*I
Incubation	60 min., 65°C	60 min., 25°C	>2 h, 70°C
Inactivation	2.1 µL 0.5M EDTA pH 8.0	20 min., 65°C	2.1 µL 0.5M EDTA pH 8.0

1 µL of the DNA solution (about 20 ng). The thermocycling profile was as follows: 4 min of initial denaturation at 94°C, followed by 30 cycles of 60 s at 94°C, 30 s at annealing temperature (58°C for ITS-2 and Cal04 primers, 56°C for *trnL*(cp) primer), and 60 s at 72°C, with a final extension step of 7 min of 72°C. Finally, 5 µL of the amplification products were visualized on 1.5% agarose gel containing ethidium bromide. The agarose gels were analyzed under UV light at a wavelength of 302 nm and documented using the Kodak (1 D v.3.5.4) gel documentation system. Purified PCR products were sequenced in both directions using BigDye 3.1 reagents and an ABI Prism 31 30XL genetic analyzer (Applied Biosystems, Foster City, CA, USA). Chromatograms of DNA sequences were edited and assembled using Sequencher 4.5 (Genecodes Inc.). Contigs were aligned manually with MEGA 6.06 (Tamura *et al.* 2013).

2.3. RFLP digestion

Sequences of ITS2, *trnL* and Cal04 were analyzed using CLC Sequence Viewer software (CLC Bio, Aarhus, Denmark) to detect the restriction sites suitable for the identification of the studied taxa. Three restriction endonucleases, *Sma*I, *TspGW*I and *Taq*I were chosen. For PCR-RFLP analysis, 10 µL of ITS2, *trnL* and Cal 04 PCR product was digested with *Sma*I, *TspGW*I and *Taq*I (EURx, Poland), respectively. Restriction analyses were carried out in a total volume about 66 µL (*Taq*I: High Buffer 10x 5 µL, BSA 100x 0,5 µL, H_2O 50 µL, PCR product 10 µL, *Taq*I enzyme 0,4 µL; *Sma*I: Acet Buffer 10x 5 µL, BSA 100x 0,5 µL, H_2O 50 µL, PCR product 10 µL, *Sma*I enzyme 0,4 µL; *TspGW*I: TspGWI buffer 10x 5 µL, H_2O 50 µL, PCR product 10 µL, *TspGW*I enzyme 1 µL). RFLP digestion followed incubation and inactivation cycles described in Table 2. Restriction fragments were separated on 2% agarose gel, stained with ethidium bromide. Fragment sizes were compared with marker of molecular mass (Nova 100 bp DNA ladder, Novazym).

3. Results

Only one haplotype characteristic for each examined group: P_S, P_B, *C. fissa* subsp. *neogea* and the group B of the *C. muelleriana* complex was detected in sequences of the analyzed DNA regions. Sequences representing each haplotype were submitted to the GenBank database (Table 1). SCAR marker Cal04 distinguished the P_S group of *C. fissa* subsp. *fissa* from all other examined groups directly based on the size of the PCR product. Two fragments were amplified: 380 bp in the P_S group and 310 bp in the remaining groups. Since this marker did not differentiate the P_B group of *C. fissa* subsp. *fissa* with *C. fissa* subsp. *neogea* and the *C. muelleriana* complex – group B, the PCR-RFLP method was applied in order to distinguish them.

Nucleotide polymorphism of the analyzed sequences was the basis for development of PCR-RFLP markers that allowed distinguishing the studied groups. Three restriction enzymes (*Sma*I, *Taq*I and *TspGW*I) were selected to identify the studied groups. RFLP profiles of the enzymes *Sma*I, *Taq*I and *TspGW*I distinguished between the P_B group and *C. fissa* subsp. *neogea.* The same result was observed for pair P_B and the group B of the *C. muelleriana* complex*,* whereas the taxa pair *C. fissa* subsp. *neogea* and the group B of the *C. muelleriana* complex, could be distinguished only on the basis of the *Taq*I enzyme (Table 3, Fig. 1). In the case of the *Sma*I enzyme used for digestion of ITS2 product was observed three fragments in the P_B group (500 bp, 300 bp, 200 bp) and only one fragment in *C. fissa* subsp. *neogea* and in the group B of the *C. muelleriana* complex (500 bp).

The *Taq*I enzyme used for digestion of Cal04 product gave three fragments in the B group of the *C. muelleriana* complex (250 bp, 200 bp, 100 bp), two in the P_B group (250 bp, 100 bp) and also two in *C. fissa* subsp. *neogea* (200 bp, 100 bp). The *TspGW*I enzyme used

Table 3. Summary of PCR-RFLP results for compared pairs of taxa

Compared taxa pair	*Sma*I	*Taq*I	*TspGW*I
P_B – *C. fissa* subsp. *neogea*	+	+	+
P_B – group B of the *C . muelleriana* complex	+	+	+
C. fissa subsp. *neogea* – group B of the *C . muelleriana* complex	-	+	-

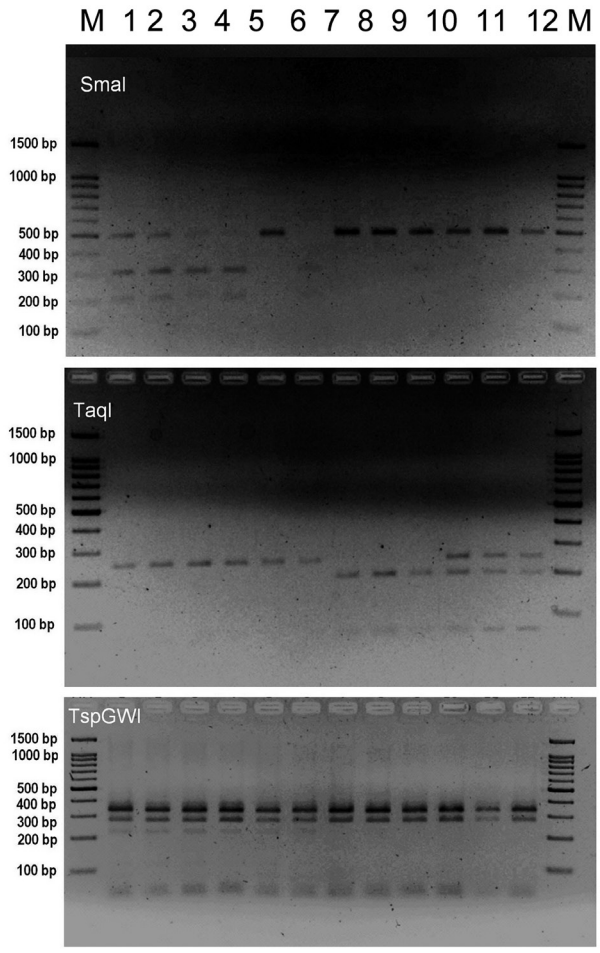

Fig. 1. PCR amplification products of ITS2, Cal04, *trnL* after digested using *Sma*I, *Taq*I, *TspGW*I restriction enzymes, respectively, resolved in 2% agarose gel

Explanations: lines, M – marker of molecular mass (Nova 100 bp DNA ladder, Novazym); 1-6 group PB: 42345, 42205; 42200; 42275, 42302, 42630; 7-9 *C. fissa* subsp. *neogea*: 42626, 42622, 42617; 10-12: *C. muelleriana* complex – group B: 41706, 42220, 42460

for digestion of *trnL*(cp) product gave four products in the P_B group (shorter than 100 bp, 200 bp and two with length about 300 bp). In *C. fissa* subsp. *neogea* and *C. muelleriana* complex – group B, three fragments (two with length about 300 bp and one shorter than 100 bp) were observed (Fig. 1).

The validity of the developed RFLP markers was tested in all studied samples of each group (Table 1). Our results did not show intraspecific polymorphism for *Taq*I and *TspGW*I restriction enzymes and discrimination of tested samples was consistent with their previous identification based on isozyme pattern. It demonstrates that the developed markers were correct and reproducible. One sample of the P_B group was not digested in the case of enzyme *Sma*I used for digestion of ITS2 product; it made it impossible to distinguish the sample from other groups. Thus, the enzyme can not be a good marker.

4. Discussion

Genetic studies of liverworts prove that some species were too broadly defined (Bischler & Boisselier-Dubayle 2000; Schumacker & Váňa 2005). Only on the basis of morphological criteria, it was difficult to find for some species good diagnostic features; as a result such species were interpreted as highly variable (Schuster 1969). These problems also apply to species of the *Calypogeia* genus. Although the *Calypogeia* genus is very characteristic and easy to distinguish, in some cases correct identification of particular species is difficult, especially plants from herbarium collections (Schuster 1969; Szweykowski 2006). Data from studies of isozymes and DNA provided evidence that the *Calypogeia* genus comprises a number of complexes and each consists of several genetically distinct lineages which have been hidden within traditionally defined morphological species (Buczkowska & Bączkiewicz 2011; Buczkowska *et al.* 2012b).

Results of the present study support the hypothesis that *C. fissa* is a complex species consisting of genetically isolated taxa. In previous works, three distinct groups (P_S, P_B and G) within European subspecies of *C. fissa* were detected by isozyme and molecular studies (Buczkowska 2004; Buczkowska *et al.* 2012a).

Genetically delimited groups of the *C. fissa* complex differ also in oil body features (Buczkowska 2004). However, oil bodies are available for analysis only in living plants; they are useless in the case of herbarium material. Our results indicate that markers based on the PCR-RFLP method can be helpful for the identification of newly detected units within the *C. fissa* complex. Application of restriction enzymes allows distinguishing the P_B group of *C. fissa* subsp. *fissa* from North American *C. fissa* subsp. *neogea*. The above taxa of the *C. fissa* complex also differ from the B group of the *C. muelleriana* complex (Buczkowska & Bączkiewicz 2011). The results of PCR-RFLP analysis of *Taq*I and *TspGW*I enzymes are consistent with previous identification of samples based on isozyme pattern, demonstrating that the developed markers are correct and reproducible. Restriction fragments of PCR product of SCAR marker Cal04 produced by *Taq*I enzyme allowed the recognition of three haplotypes corresponding to: P_B groups of *C. fissa* subsp. *fissa*, *C. fissa* subsp. *neogea* and *C. muelleriana* – group B. Since, the enzymes *Taq*I and *TspGW*I recognize restriction sites in DNA regions (intron of *trnL* gene and Cal04), which are easy to amplify even from herbarium material (Jankowiak *et al.* 2005; Buczkowska *et al.* 2012a), these markers can also be used for plant identification from herbarium collections.

Unfortunately, the RFLP marker tested in this study on the basis of nuclear DNA region ITS2 proved to be not fully diagnostic for the studied taxa. The PCR product of one sample (# 14, Table 1) belonging to the P_B group was not cut and the typical profile of restriction fragments for this group was not observed. This may suggest the presence of polymorphism in the ITS2 region within the P_B group resulting in the loss of the restriction site. Thus, RFLP marker based on ITS2 cannot be considered as specific for the P_B group of *C. fissa* complex. The sequence of the nuclear region ITS2 is widely used in phylogenetic analysis and as species-specific markers in bryophytes (e.g. Feldberg *et al.* 2004; Hentschel *et al.* 2007; Fuselier *et al.* 2009). However, some disadvantages of the locus, such as the presence of intra-specific variation or non-homogenized paralogues at different loci in a genome (Chen *et al.* 2010) can cause difficulties when the ITS region is used for development of PCR-RFLP marker. Despite the disadvantages, usefulness of the ITS2 PCR-RFLP marker for species identification was reported by many authors (e.g. Vanderpoorten *et al.* 2003; Yuan *et al.* 2007; Lin *et al.* 2012).

PCR-RFLP analysis of DNA regions from chloroplast and mitochondrial genome has been proved to be useful for separating cryptic species of liverworts e.g. *P. epiphylla* (Pacak & Szweykowska-Kulińska 2003), *A. pinguis* (Wachowiak *et al.* 2007) or species of allopolyploid origin, *P. baueri*, and its haploid parental species *P. platyphylla* (Jankowiak & Szweykowska-Kulińska 2004).

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