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PCR-based molecular markers for identification of taxa from the *Calypogeia fissa* complex (Jungermanniopsida, Calypogeiaceae)

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Abstract: Within *Calypogeia fissa*, two subspecies connected with geographic distribution are formally recognized: *C. fissa* subsp. *fissa* in Europe and *C. fissa* subsp. *neogea* in North America. Isoenzyme studies have shown that the European subspecies is genetically differentiated and composed of three genetically distinct groups P_S, P_B and G. The P_S group has the most distinctive morphological features, but no morphological diagnostic traits have been found for groups P_B and G. The sequence characterized amplified region (SCAR) markers developed on the basis of ISSR markers, applied in the study, allowed the delimitation of all groups distinguished in Europe within the *C. fissa* complex (P_S, P_B and G). The markers also revealed genetic differences between the European and American subspecies. Five primer pairs (Cal01, Cal03-Cal06) of the six pairs studied are useful as the diagnostic tool for the identification of particular groups from the *C. fissa* complex. The examined SCAR markers showed that the P_S group of *C. fissa* subsp. *fissa* was the most distinct; it differed from both groups P_B and G as well as from *C. fissa* subsp. *neogea*. All plants determined on the basis of diagnostic isozyme loci as the P_S group amplified a longer product (380 bp) of the Cal04 primer pair than the rest of studied groups and yielded no amplification products in Cal03, Cal05 and Cal06 primers. The primer pair Cal03 distinguished the plants of the P_B group from the remaining groups, since only the P_B group generated a PCR product of about 290 bp. The genetic differences between all four studied groups of the *C. fissa* complex were supported by DNA sequences of the SCAR marker Cal04.

Key words: Bryophyta, liverworts, *Calypogeia*, molecular markers, SCAR, DNA sequence

1. Introduction

Genetic studies of the *Calypogeia* Raddi genus revealed that some species described last century on the basis of morphological criteria are genetically differentiated and include previously unrecognized species e.g. *C. muelleriana* (Schiffn.) Müll. Frib. (Buczkowska 2010; Buczkowska & Bączkiewicz 2011) or *C. sphagnicola* (Parnell & J.Perss.) Warnst. & Loeske (Buczkowska *et al.* 2012a). *Calypogeia fissa* (L.) Raddi is probably one of such complex species. It is a suboceanic-mediterranean, amphiatlantic species reported from North America, Europe, North Africa and Asia (Müller 1951-1958; Schuster 1969; Paton 1999; Damsholt 2002). Currently, two subspecies connected with geographic distribution are formally recognized within *C. fissa*: *C. fissa* subsp. *fissa* in Europe (Schuster 1969; Damsholt 2002) and *C. fissa* subsp. *neogea* Schust. described by Schuster (1969) from North America. In Poland, *C. fissa* is rare and occurs mainly in western regions of the country (Koła 1989; Szweykowski 2006).

Isoenzyme studies showed that the European subspecies was differentiated and composed of three genetically distinct groups which were tentatively called: P_S, P_B and G (Buczkowska 2004). The P_S, P_B groups were initially found in Poland and comprised, respectively, small and bigger plants, whereas group G was recorded in Germany. Further studies showed that all the groups occur also in other parts of Europe (Buczkowska *et al.* 2011). The P_S group was genetically most distinct, whereas the genetic distance between groups P_B and G was lower. Differences in oil body characters and habitat requirements were also revealed between them (Buczkowska 2004). The P_S group had

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the most distinctive morphological features, but no morphological diagnostic traits were found so far for P_B and G groups (Buczkowska *et al.* 2011). Preliminary studies of the intron sequence of chloroplast *trn*G gene, commonly applied in molecular taxonomy of bryophytes (Szweykowska-Kulińska *et al.* 2002; Pacak & Szweykowska-Kulińska 2003; Shaw *et al.* 2003; Fuselier *et al.* 2009), confirmed genetic differentiation within the *C. fissa* complex initially detected by isozyme markers (Buczkowska *et al.* unpbl.). However, sequencing is too expensive method to be used for the identification of large numbers of samples. On the other hand, isozyme markers and oil body features are useless in the case of herbarium material. Therefore, other diagnostic markers for the groups of *C. fissa* complex, suitable also for dried specimens, would be helpful.

Molecular biology, besides sequencing strategy (Stech & Quandt 2010), offers also more straightforward PCR-based genetic markers, which can be applied in bryophyte taxonomy e.g. RFLP (Boisselier-Dubayle *et al.* 1995; Pacak & Szweykowska-Kulińska 2003), RAPD, ISSR, ISJ (Hassel & Gunnarsson 2003; Vanderpoorten *et al.* 2003; Bączkiewicz *et al.* 2008; Sawicki & Szczecińska 2011). On the basis of RAPDs or ISSRs, more specific markers – SCARs (sequence characterized amplified region) can be also derived by cloning and sequencing of selected PCR products (Paran & Michelmore 1993; Melotto *et al.* 1996). The SCARs combine simplicity of application and reliability of the obtained results and, therefore, can be regarded as good gel-based markers. These markers allow fast determination of a large number of samples without sequencing, directly at the level of PCR products. The SCAR markers have been developed for identification of several plant species e.g. *Leucaena* (Bailey *et al.* 2004), *Bambusa* (Das *et al.* 2005), *Pinus* (Mehes *et al.* 2007) or *Nepenthes* (Anuniwat *et al.* 2009). In bryophyte studies, SCAR markers were found to be useful for species delimitation and phylogeny of *Sphagnum* (Shaw *et al.* 2003, 2005, 2008), for sex determination in *Pseudocalliergon trifarium* moss (Korpelainen *et al.* 2008) and for the identification of some *Calypogeia* species (Buczkowska & Dabert 2011).

The aim of the present study was to develop specific SCAR markers for the identification of taxa distinguished within the *C. fissa* complex, which could be also applied for plants from herbarium collections.

2. Materials and methods

2.1. Plant material

Plants used in the present study originated from different regions of Europe. The studied plants were initially determined as *C. fissa* on the basis of morphological traits according to Müller (1951-1958) and Schuster (1969). The living plants were identified on the basis of isozyme markers and oil body characters as *C. fissa* group P_S, P_B and G according to Buczkowska (2004). Fixed alleles in three isozyme loci (*Est*-1, *Aat* and *Gdh*) were diagnostic for individual groups, for P_S: *Est*-1 allele 1, *Aat* allele 2, *Gdh* allele 1, for P_B: *Est*-1 allele 2, *Aat* allele 2, *Gdh* allele 12 and for G: *Est*-1 allele 2, *Aat* allele 1, *Gdh* allele 12. The P_S group comprised small plants (shoots width ranged from 922 to 1780 μm) with large oil bodies (5-7 × 8-12 to 17 μm exceptionally 19 μm) formed of 2-6 (rarely 7-10) large, clear-cut globules. Plants identified as groups P_B and G were bigger (shoots width 1600-3900 μm) and had smaller oil bodies (3-5 × 8-10 (12) μm), but they differed in structure and distribution of oil bodies in leaf cells. Oil bodies of the P_B group were formed of larger and well-marked globules than in the G group. In the G group, oil bodies were present in all cells, whereas in the P_B group, they were absent in single live cells (Buczkowska 2004; Buczkowska *et al.* 2011).

The samples from herbarium collections were determined based on the intron sequence of chloroplast *trn*G gene. The length of sequenced introns was 656 bp in the P_S and 658 bp in P_B and G groups. The P_S group differed from the P_B and G groups with respect to 17 and 16 substitutions, respectively. Two fixed nucleotide differences were found between the P_B and G groups (Buczkowska *et al.* unpubl.).

In general 50 samples of *C. fissa* complex were examined: 18 of the P_S group, 18 of P_B, 9 of the G group and 5 samples of *C. fissa* subsp. *neogea* from the USA, including herbarium specimen from Schuster's collection (determined by Schuster), which was used for comparison (Table 1).

2.2. DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from fresh or dried material. For fresh samples, several stems from one sample were ground with steel beads in a Bioprep-24 Homogenizer for 35 seconds. A standard CTAB procedure (Murray & Thompson 1980) downscaled to fit 1.5 ml Eppendorf tubes was applied. For extraction of DNA from herbarium samples, a Novabeads Plant DNA Kit (Novazym, Poznań, Poland) was used. The isolated DNA was dissolved in TE buffer and stored at –20ºC. 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 NanoDrop ND-1000 Spectrophotometer.

SCAR markers used in the study were developed on the basis of ISSR markers. Six primer pairs were tested, three (Cal01, Cal02, Cal03) described in the previous paper (Buczkowska & Dabert 2011), and three (Cal04,

Table 1. Collection sites of the studied samples of *C. fissa* complex and GenBank accession numbers. The groups were delineated by isozyme markers and/or DNA sequences; samples marked with an asterisk were from herbarium collections

Sample No.	Locality	Collector	Collection year	Herbarium No.	Group	GenBank No. Cal04
	C. fissa subsp. *fissa*					
1	W Poland, Lubuskie Province, Bogumiłów near Żary, sandy soil on ditch banks	SR	2011	POZW 42628	P_S	JX402494
2	W Poland, Lubuskie Province, Bogumiłów near Żary, sandy soil on ditch banks	KB, JS	2000	POZW 39191*	P_S	–
3	W Poland, Lubuskie Province, Bogumiłów near Żary, sandy soil on ditch banks	KB, JS	2000	POZW 39199	P_S	–
4	W Poland, Lubuskie Province, Bogumiłów near Żary, sandy soil on ditch banks	KB, JS	2000	POZW 39194	P_S	–
5	W Poland, Lubuskie Province, Tuplice near Lubsko, sandy soil on ditch banks	KB, JS	2000	POZW 39203	P_S	–
6	Central Poland, Wielkopolska Province, Antonin near Ostrów Wlkp., sandy soil on ditch banks	KB	2009	POZW 42225	P_S	JX402495
7	Central Poland, Wielkopolska Province, Antonin near Ostrów Wlkp., sandy soil on ditch banks	KB	2009	POZW 42236	P_S	–
8	Central Poland, Wielkopolska Province, Antonin near Ostrów Wlkp., sandy soil on ditch banks	KB	2009	POZW 42234	P_S	–
9	Central Poland, Wielkopolska Province, Antonin near Ostrów Wlkp., sandy soil on ditch banks	KB	2009	POZW 42227	P_S	–
10	Central Poland, Wielkopolska Province, Antonin near Ostrów Wlkp., sandy soil on ditch banks	KB	2009	POZW 42238	P_S	–
11	Central Poland, Wielkopolska Province, Antonin near Ostrów Wlkp., sandy soil on ditch banks	KB	2009	POZW 42235	P_S	–
12	Central Poland, Wielkopolska Province, Antonin near Ostrów Wlkp. sandy soil on ditch banks	KB	2011	POZW 42629	P_S	–
13	Germany, Schwarzwald, sandy soil in forest	IM	2010	POZW 42428	P_S	–
14	United Kingom, South Lancashire, in damp sandstone crevice of disused quarry wall	DAC	2012	DC 1419	P_S	–
15	Portugal, Beira Litoral, Luso, National Park of Mata de Buçaco, Cruz Alta, 433 m a.s.l.	AS-V	2010	S-V 31461*	P_S	–
16	Azores Archipelago, São Miguel Island, east part of island, Furnas, 280 m a.s.l.	AS-V	2009	S-V 29323*	P_S	–
17	Canary Islands, La Palma, Cumbre Nueva, Pista de Los Lomos, 1150 m a.s.l.	AS-V	2005	S-V 24667*	P_S	JX402496
18	Czech Republic, Krnalovice, Babi hora 500 m a.s.l.	JD	1988	OP 171908*	P_S	JX402497
19	NW Poland, Pomorskie Province, Lake Małe Sitno near Czarna Dąbrówka, *Carex* tussocks at the lakeshore	KB, AB	2009	POZW 42345	P_B	JX402498
20	NW Poland, Pomorskie Province, Lake Kamień near Miastko, peaty soil at lakeshore	KB, AB	2009	POZW 42205	P_B	–
21	NW Poland, Pomorskie Province, Lake Kamień near Miastko, peaty soil at the lakeshore	KB, AB	2009	POZW 42200	P_B	–
22	W Poland, Lubuskie Province, Starosiedle forest division, *Carex* tussocks at the lakeshore	SR, KB	2009	POZW 42302	P_B	–
23	W Poland, Lubuskie Province, Starosiedle forest division, *Carex* tussocks at the lakeshore	SR, KB	2009	POZW 42277	P_B	–
24	W Poland, Lubuskie Province, Starosiedle forest division, *Carex* tussocks at the lakeshore	SR, KB	2009	POZW 42275	P_B	–
25	W Poland, Lubuskie Province, Mierków forestry, humus in *Carici elongate-Alnetum*	SR, KB	2009	POZW 42298	P_B	JX402499
26	W Poland, Lubuskie Province, Mierków forestry, humus in *Carici elongate-Alnetum*	SR, KB	2009	POZW 42299	P_B	–
27	W Poland, Lubuskie Province, Nabłoto forestry, humus in *Carici elongate-Alnetum*	SR, KB	2009	POZW 42317	P_B	–
28	W Poland, Lubuskie Province, Rzeczyca river, on wet	SR	2011	POZW 42630	P_B	–

Sample No.	Locality	Collector	Collection year	Herbarium No.	Group	GenBank No. Cal04
29	NW Poland, Pomorskie Province, Lake Duże Katarzynki near Lipnica, sandy bank	KB, JS, HB	1994	POZW 35690*	P_B	–
30	NW Poland, Pomorskie Province, Lake Duże Katarzynki near Lipnica, on wet sand at the lakeshore	KB, JS, HB	1994	POZW 35692*	P_B	–
31	W Poland, Lubuskie Province, Lake Płytkie near Brody, *Carex* tussocks at the lakeshore	KB, JS	2000	POZW 39210	P_B	–
32	W Poland, Lubuskie Province, Tuplice near Lubsko, sandy soil on ditch banks	KB, JS	2000	POZW 39208	P_B	–
33	Holland, Maarssen, 2 km North of Utrecht, Molenpolder, on wet peat	JS, RG, HG	1993	POZW 34147*	P_B	JX402500
34	Holland, Maarssen, 2 km North of Utrecht, Molenpolder, on wet peat	JS	1993	POZW 34148*	P_B	–
35	United Kingom, North Devon, at the base of *Molinia* tussock in coastal mire	DAC	2012	DC 1426	P_B	–
36	United Kingom, West Cornwall with Scilly, amongst *Sphagnum subnitens* plants in heathland	DAC	2012	DC 1461	P_B	JX402501
37	Germany, Bonn – Bad Godesberg, Annaberger Bachtal, pine forest	AS, DQ	2000	POZW 39074*	G	JX402502
38	Germany, pine forest near Bonn	AS, DQ	2000	POZW 39076*	G	
39	W Poland, Lubuskie Province, Nabłotno forestry, humus in *Carici elongate-Alnetum*	SR	2011	POZW 42437	G	JX402503
40	Georgia, Ajaria, Batumi, in Horto Botanico, pars Australiae.	AA	1961	OP 110668*	G	–
41	Czech Republic, Krnov, Hájek in the Květnice Mts. 450m.	JD	1976	OP 150890*	G	JX402504
42	Germany, Bayern, Alpenvorland, between Rieden-Beckstetten and Frankenhofener lake, 652 m a.s.l.	AS-V	2010	S-V 31656*	G	–
43	Germany, Baden-Württemberg, western Bodenseegebiet, Schienerberg-Nordabhang above Bankholzen, Öde Halde north from Langenmoos, 620 m a.s.l.	AS-V	2006	S-V 25448*	G	–
44	Atlantic Islands, Madeira, road from Encumeada-Pass to Pico Ruivo, 1150 m a.s.l.	AS-V	2006	S-V 25699*	G	JX402505
45	Canary Islands, La Palma, near Los Sauces, road to Casa del Monte, 520 m a.s.l.	AS-V	2005	S-V 24778*	G	
	C. fissa subsp. *neogea*					
46	North America, North Carolina, the Southern Appalachian, on sandy soil	BS	2010	POZW 42626		JX402506
47	North America, North Carolina, the Southern Appalachian, on humus	BS	2010	POZW 42622		JX402507
48	North America, North Carolina, the Southern Appalachian, 1132c, on sandy soil	BS	2010	POZW 42620		JX402508
49	North America, North Carolina, the Southern Appalachian, on sandy soil	BS	2010	POZW 42625		JX402509
50	West Virginia, Cranberry Glades, W. of Mill Point, Pocahontas Co., ca 3500-3600 ft.	R. Schuster	1963	F 61206*		JX402510

Explanations: Collectors, AA – A.L. et I.I. Abramovi, AB – A. Bączkiewicz, AS – A. Sloga, AS-V – A. Schäfer-Verwimp, BS – Blanka Shaw, DAC – Des A. Callaghan, DQ – D. Quandt, HB – H. Barczak, HG – H. Greven, IM – Iwona Melosik, JD – J. Duda, JS – J. Szweykowski, KB – K. Buczkowska, RG – R. Gradstein, SR – S. Rosadziński; Herbaria: AS-V – Herb. Schäfer-Verwimp., OP – Musei Silesiensis Opava, POZW – Herbarium of Adam Mickiewicz University, DC – Herb. D. A. Callaghan, F – Field Museum of Natural History

Cal05, Cal06) newly designed (Table 2). The new primer pairs were designed based on three (JX402493, JX402511, JX402512) from among several different sequences obtained by cloning species-specific DNA product generated for the new species detected within the *C. muelleriana* complex by primer ISSR 807 (Buczkowska & Dabert 2011).

PCR amplifications using specific SCAR primers were performed in 20 µL reaction volumes containing 2 µL of 10× 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 1 µL of the DNA solution (about 20 ng). The thermocycling profile

Table 2. Sequences of primers used in the present study

SCA Rmarker	Primer sequence (5'-3')	Primer direction	T_a [°C]	References	Primer design based on GenBank Acc. no
Cal01	GTCTCTTTCTTTCTGTCATTTGC	F	62	Buczkowska & Dabert 2011	JF414728
Cal01	ATTTGTACGTATGATTTTGCAGAAG	R			
Cal02	CACGAAAGATATAATAACGGCAAG	F	64	Buczkowska & Dabert 2011	JF414729
Cal02	TTAGGGCGTCATACCTGCTCC	R			
Cal03	TTAGAGTTTAGGGTGAATTGTAGG	F	62	Buczkowska & Dabert 2011	JF414730
Cal03	CGGGAGATTGATGAGCCAAA	R			
Cal04	GAGTTTATCGACGGGAAGAG	F	62	designed in the present study	JX402493
Cal04	AACGAAATAATGGCTCACAGAA	R			
Cal05	GCAAGCAAGCAGGAACTAGAA	F	64	designed in the present study	JX402511
Cal05	AGGGGACAAGGGACGCAAC	R			
Cal06	ACTGAATGAATGAATGGAATGA	F	60	designed in the present study	JX402512
Cal06	GGGACGAGTGATGAGAGAACA	R			

Explanation: T_a – annealing temperature

was as follows: 4 min of initial denaturation at 94ºC, followed by 30 cycles of 60 s at 94ºC, 30 s at annealing temperature (60-64 ºC, depending on primer, see Table 2), and 60 s at 72ºC, with a final extension step of 7 min of 72ºC. Finally 10 µl of the amplification products were visualized on 1.5% agarose gel containing ethidium bromide. The agarose gels were analysed under UV light at a wavelength of 302 nm and documented using the Kodak (1D v. 3.5.4) gel documentation system.

Purified PCR products of one SCAR marker (Cal04) were sequenced in both directions using BigDye 3.1 reagents and an ABI Prism 3130XL 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 4.1 (Tamura *et al.* 2007).

3. Results

The designed SCAR markers made it possible to distinguish three groups of European subspecies of *C. fissa* subsp. *fissa* (P_S, P_B and G) detected at first by isozyme analysis. The SCAR markers also revealed differences between European and American subspecies: *C. fissa* subsp. *fissa* and *C. fissa* subsp. *neogea*. The P_S group of *C. fissa* subsp. *fissa* was the most distinct, differences in genomes of the P_S group and both groups – P_B and G as well as of *C. fissa* subsp. *neogea* were revealed in most of the SCAR markers studied.

Five primer pairs (Cal01, Cal03-Cal06) of the six studied were found to be useful markers in *C. fissa* complex (Table 3). The Cal02 primer pair did not discriminate the studied groups. The Cal01 primer pair allowed differentiation of plants of European *C. fissa* subsp. *fissa* from American *C. fissa* subsp. *neogea*. One product of the same length (about 225 bp) was amplified in all European groups (P_S, P_B and G), but did not amplify in the studied samples from the USA (Fig. 1). Primer pair Cal03 amplified a single band of about 290 bp only for the P_B group, but yielded no amplification product in P_S and G groups of *C. fissa* subsp. *fissa* as well as in *C. fissa* subsp. *neogea* (Fig. 1). Primer pair Cal04

Table 3. SCAR markers amplification products in the groups of *C. fissa* complex

SCAR marker	PCR product size (bp)	*C. fissa* subsp. *fissa*			*C. fissa* subsp. *neogea*
		group P_S	group P_B	group G	
Cal01	225	+	+	+	
	no product				+
Cal02	600	+	+	+	+
Cal03	290		+		
	no product	+		+	+
Cal04	310		+	+	+
	380	+			
Cal05	300		+	+	+
	no product	+			
Cal06	390		+	+	+
	no product	+			

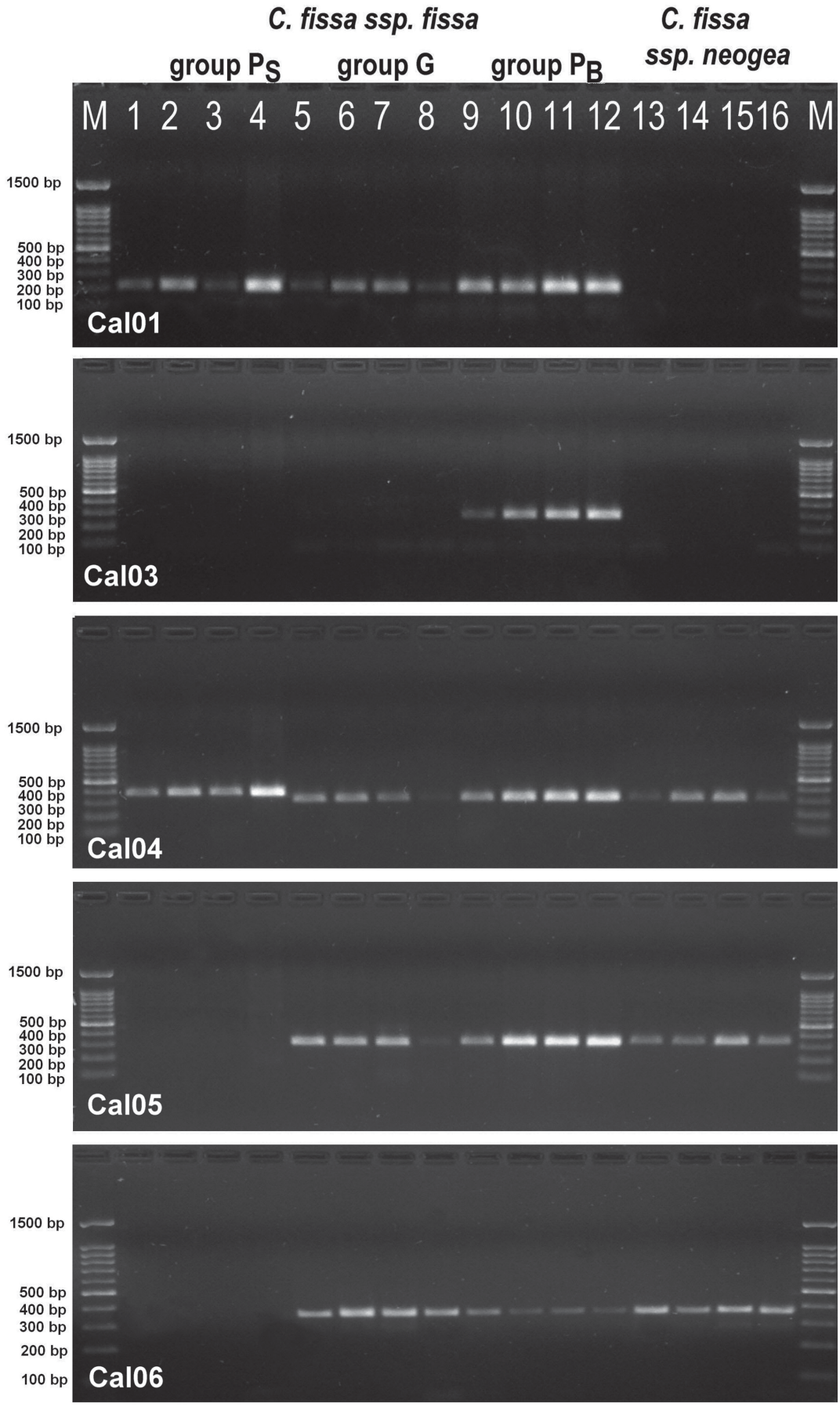

Fig. 1. Products of PCR amplification of the *C. fissa* complex using specific SCAR markers resolved in 1.5% agarose gel

Explanations: lines, M – marker of molecular mass (Nova 100 bp DNA ladder, Novazym); 1-4 group P_S: 42628, 42225, 24667*, 171908*; 5-8 group G: 42437, 39074*, 24778*, 150890*; 9-12 group P_B: 34147*, 42298, 1461, 42345; 13-16: *C. fissa* subsp. *neogea* 42626, 42622, 42620, 42625; *Samples from a herbarium collection

Table 4. Fixed nucleotide differences in the sequence of SCAR marker Cal04 among the studied groups of *C. fissa* complex

	C. fissa subsp. *fissa*		
	group P_S	group P_B	group G
C. fissa subsp. *fissa* P_B	6 s, 2 indel		
C. fissa subsp. *fissa* G	6 s, 2 indel	7 s	
C. fissa subsp. *neogea*	4 s, 2 indel	5 s	4 s

amplified two fragments: one of 380 bp in the P_S group, the second of 310 bp in P_B, G and in the samples from the USA (Fig. 1). Next two primer pairs: Cal05 and Cal06 yielded a single amplification product, respectively of 300 and 390 bp for P_B, G and *C. fissa* subsp. *neogea*, but did not amplify in the P_S group (Fig. 1). The Cal02 primer pair gave a single amplification product of about 600 bp in all examined samples of *C. fissa* complex.

Specificity of the designed SCAR primer pairs was tested by screening samples that were previously identified on the basis of the isozyme pattern and *trn*G sequence. All the SCAR markers showed fully consistent results for all examined samples in each group. All samples preliminary classified as belonging to the particular group of the *C. fissa* complex generated products characteristic for the group.

The SCAR marker Cal04, which amplified in all groups of the *C. fissa* complex, was used for sequencing. Sequences of this DNA region support the above results and revealed differences between all four studied groups of the *C. fissa* complex. The length of the readable sequence of the Cal04 was 377 bp in the P_S group and 312 bp in the remaining groups of *C. fissa* subsp. *fissa* and in *C. fissa* subsp. *neogea*. In this DNA region, 4-8 fixed nucleotide differences (substitutions and indels – insertions/deletions) were found between the studied groups of *C. fissa* complex. The highest number of differences were observed in case of pairs P_B-G (7 substitutions) and P_S-P_B (6 substitutions and 2 indels). *C. fissa* subsp. *neogea* differed from the P_S and G groups in 4, and from the P_B group – in 5 sites (Table 4). Sequence obtained from the sample determined by Schuster as *C. fissa* subsp. *neogea* (F 61200a) was the same as sequences of other samples of *C. fissa* from the USA.

4. Discussion

The SCAR markers, developed previously to distinguish *C. muelleriana* and the new species separated recently from the *C. muelleriana* complex (Buczkowska & Dabert 2011), can be also employed as a good diagnostic tool for groups detected in the European subspecies of *C. fissa*. The SCAR markers supported the hypothesis arrived at on the basis of isozyme results that *C. fissa* in Europe was a species complex comprising three genetically distinct taxa provisionally referred to as P_S, P_B and G groups (Buczkowska 2004). Results of the present study revealed also genetic differences between all groups detected in Europe and the American subspecies – *C. fissa* subsp. *neogea*. The genetic differences between all four studied groups of the *C. fissa* complex were supported by DNA sequences of the SCAR marker Cal04.

The specificity and reproducibility of the developed SCAR markers in discriminating the groups of the *C. fissa* complex was verified by analyzing samples that were previously identified by the isozyme pattern, oil body characters (Buczkowska 2004) and intron sequence of chloroplast *trn*G gene (Buczkowska *et al.* unpubl.). A strict correlation between all the examined markers was observed. However, isozymes and oil bodies were unusable in case of the dried samples. Therefore, the designed species-specific SCAR markers provided a useful tool for fast and unambiguous identification of a large number of samples without sequencing, directly based on the size or presence/absence of the PCR product.

Among the groups distinguished within European subspecies the P_S group was the most distinct both genetically (Buczkowska 2004) and morphologically (Buczkowska *et al.* 2011). Its high genetic distinctness from the P_B and G groups as well as from *C. fissa* subsp. *neogea* was additionally confirmed by the SCAR markers. All plants determined based on diagnostic isozyme loci as the P_S group amplified longer product (380 bp) of the Cal04 primer pair and yielded no amplification products in Cal03, Cal05 and Cal06 primers. The plants of the P_S group were characterized by small shoots (922-1780 μm) with leaves almost as long as wide or wider than long and large oil bodies composed of big and clear-cut globules (Buczkowska 2004; Buczkowska *et al.* 2011).

The plants identified by isozyme markers as the P_B group generated the product of about 290 bp using the primer pair Cal03; it differed in this respect from the remaining groups where lack of amplification was observed. The plants of the P_B group were bigger (shoots ranged from 1600-3900 μm), had usually longer than wider leaves and smaller oil bodies than plants of the P_S group (Buczkowska 2004; Buczkowska *et al.* 2011).

The P_B and G groups of the European subspecies were morphologically very similar and their proper recognition based on biometrical features was not possible (Buczkowska *et al.* 2011). However, the present molecular studies confirmed differences in genomes

of these two groups, which were revealed both in the presence/absence of the PCR product of the SCAR marker Cal03 and 7 substitution in the SCAR Cal04 sequence. Both groups differed also in the sequence of the *trn*G intron (Buczkowska *et al.* unpubl.). Thus, the classification functions of discriminant analysis computed formerly for the P_B group based on morphological characters (Buczkowska *et al.* 2011) was not sufficient for successful delimitation of plants of the P_B and G groups. For this reason, some specimens of the G group (samples 40-45, see Table 1) classified previously on the basis of classification functions only, were wrongly determined as the P_B group. Detailed morphological studies are necessary to make sure if plants of both groups can also be identified on the basis of morphological criteria. At present, the developed SCAR markers have been proven to be an efficient and available tool for quick identification of all studied groups of the *C. fissa* complex.

Two SCAR primer pairs (Cal03, Cal04) were enough to distinguish three groups of *C. fissa* subsp. *fissa* (P_S, P_B and G), whereas Cal01 differentiated the European plants from *C. fissa* subsp. *neogea*. No differences were found in the case of *C. fissa* complex in the primer pair Cal02, which was very good for distinguishing other *Calypogeia* species (Buczkowska & Dabert 2011). Unfortunately, none of the primer pairs examined so far allowed separation the P_B group of the *C. fissa* complex from the new species of *C. muelleriana* complex (Buczkowska & Bączkiewicz 2011) and the LC group of *C. sphagnicola* (Buczkowska *et al.* 2012b). However, the present study confirmed the usefulness of the markers for the identification of units distinguished within complex species.

The higher length (21-24 bp) and high annealing temperature (>60°C) of the SCAR primers make the PCR reaction more specific, reproducible and stable. Moreover, the PCR products of the diagnostic SCAR markers are short (<400bp) increasing the ability for their amplification in the PCR reaction also from herbarium samples in which DNA can be more degraded. In the present studies, amplification products of the SCAR marker Cal04 (312 bp) were obtained from 50-year old samples. However, as it was shown by Jankowiak *at al.* (2005) and Lister *et al.* (2008), the method of sample drying is more important for successful amplification of DNA than is the age of the sample.

SCAR markers, at first, were developed in search of markers linked with particular genes in cultivated plants (Paran & Michelmore 1993; Melotto *et al.* 1996). Subsequently, the SCAR-based approach was proven to be of great value also for distinguishing difficult to identify species e.g. *Pinus strobus* and *P. monticola* (Mehes *et al.* 2007), *Sinocalycanthus chinensis* from its closely related species (Ye *et al.* 2006), toxic and non-toxic genotypes of *Jatrophora curcas* (Basha & Sujatha 2007), for identification of *Flammulina velutipes* cultivars (Su *et al.* 2008) or detection of pathogens e.g. *Tilletia controversa* (Gao *et al.* 2010). In bryophyte studies, SCAR-based approach is not so widely applied as in the case of crop species. However, three SCAR loci (RAPDa, RAPDb and RAPDf) were found to be highly informative for species delimitation and phylogeny of *Sphagnum* (Shaw *et al.* 2003, 2005, 2008). Such markers were also developed and successfully used for gender determination in *Pseudocalliergon trifarium* moss (Korpelainen *et al.* 2008; Hedenäs *et al.* 2010). Our studies showed that SCAR markers could also be useful for the separation of difficult to identify species of liverworts. Three previously designed SCAR markers (Cal01, Cal02 and Cal03) permit delimitation of almost all European *Calypogeia* species, including newly distinguished taxa within *C. muelleriana* (Buczkowska & Dabert 2011) and *C. sphagnicola* (Buczkowska *et al.* 2012b). Thus, the SCARs can be proposed as the alternative markers for species identification in relation to methods based on sequencing, which are applied in molecular taxonomy of bryophytes (Szweykowska-Kulińska *et al.* 2002; Heinrichs *et al.* 2010; Kreier *et al.* 2010; Liu *et al.* 2010; Stech & Quandt 2010). However, further studies are needed to evaluate the usefulness of such kind of markers in other liverwort species. Unfortunately, to the best of our knowledge, there are no published reports on the development of SCAR markers in liverworts except for the *Calypogeia* genus.

On the basis of the results of the present studies some preliminary conclusions regarding geographic distribution of particular groups of *C. fissa* subsp. *fissa* in Europe can be drawn. The P_B group occurs mainly in the north-western part of Europe, it was found so far in Holland, southern parts of the United Kingdom, western and north-western parts of Poland, whereas the G group is more frequent in south-western parts of Europe. Samples determined as the G group came from Portugal, Atlantic Islands, the Czech Republic, south-western parts of Germany and Poland as well as from Georgia. The P_S group seems to be more widespread. However, more specimens from a wider range of geographic distribution need to be analyzed before a reliable conclusion can be drawn on the geographic distribution of the groups distinguished by genetic methods within *C. fissa* subsp. *fissa* in Europe.

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