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ISOZYME AND DNA MARKERS REVEAL A NEW GENETICALLY DISTINCT TAXON OF *CALYPOGEIA SPHAGNICOLA* (JUNGERMANNIOPSIADA, CALYPOGEIACEAE)

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Abstract. Genetic studies of *Calypogeia sphagnicola* (Arnell & J. Perss.) Warnst. & Loeske revealed that besides *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* (Warnst.) Schust., recently recognized as separate species, a third genetically distinct group of plants connected with *Sphagnum* communities can be distinguished in the *Calypogeia* genus in Poland. The new group differs from *C. sphagnicola* f. *sphagnicola* in 4 loci and from *C. sphagnicola* f. *paludosa* in 6 loci. The distinctness of the new group was supported by sequence analysis of four chloroplast loci (*rpo*C1, *trn*H-*psb*A, *trn*G, *trn*L) and species-specific SCAR markers. Three SCAR markers (Cal01, Cal02 and Cal03) distinguished the new *Calypogeia* group both from two *C. sphagnicola* forms as well as from other European *Calypogeia* species. The new *C. sphagnicola* is very rare, so far it was found in only one locality in the Tatra National Park, where it occurs in a swamp by the Sichlański stream, in the Las Capowski forest.

Key words: Liverworts, *Calypogeia*, chloroplast genome, isozyme pattern, SCAR markers

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INTRODUCTION

Complementing classical taxonomy of liverworts, based on morphology, with characters inferred from genetic markers revealed numerous examples of species speciation and gave evidence that the number of liverwort species is still underestimated (Szweykowski *et al.* 2005; Bączkiewicz *et al.* 2008; Heinrichs *et al.* 2010; Kreier *et al.* 2010). It is currently widely acknowledged that some species described in old works need to be reassessed and characterized again (Bischler & Boisselier-Dubayle 2000; Schumacker & Váňa 2005). This opinion concerns e.g. the genus *Calypogeia* Raddi, considered by many taxonomists as one of the most difficult groups of liverworts (Schuster 1969; Szweykowski 2006).

The genus *Calypogeia* has a wide geographic distribution and comprises about 90 described species (Schuster 1969). Nine species of the genus are known from Europe: *C. azurea* Stotler & Crotz, *C. integristipula* Steph., *C. neesiana* (Massal. & Carestia) Müll. Frib., *C. suecica* (Arnell & J. Perss.) Müll. Frib., *C. muelleriana* (Schiffn.) Müll. Frib., *C. sphagnicola* (Arnell & J. Perss.) Warnst. & Loeske, *C. fissa* (L.) Raddi, *C. arguta* Nees & Mont. and *C. azorica* Bischl. (Grolle & Long, 2000). Recently, genetic studies in the genus *Calypogeia* revealed the presence of collections genetically distinct from the well-known and accepted species, which probably represent new taxa. Genetically distinct groups of plants were, for instance, recognized within the complexes of *C. fissa* (Buczkowska 2004a), *C. muelleriana* (Buczkowska & Bączkiewicz 2011) and *C. sphagnicola* (Buczkowska *et al.* 2012). Subsequent biometric studies based on genetically identified material enable to find morphological

diagnostic characters for the newly detected taxa (Buczkowska 2010, Buczkowska *et al.* 2011). Thus, the number of *Calypogeia* species in Europe is probably higher than presently known.

Recently, two forms of *C. sphagnicola* distinguished by Schuster (1969), *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* (Warnst.) Schust., were recognized by Buczkowska *et al.* (2012) as reproductively isolated species. *C. sphagnicola* f. *sphagnicola* is haploid, whereas *C. sphagnicola* f. *paludosa* – a diploid species. The haploid species corresponds to the syntype specimen of *C. sphagnicola* (Arnell 1902), but the final name of the diploid species has been not determined yet (Buczkowska *et al.* 2012). Extended sampling and further studies of *C. sphagnicola* s.l. suggest that besides the above two species, another genetically distinct group could be distinguished among the plants whose occurrence is closely connected with peat bogs. Plants of this group have colorless oil bodies and never produce gemmae. However, morphological variation within the group was observed to correlate with growing conditions. Plants creeping between the stems of *Sphagnum* spp. best correspond to *C. sphagnicola* f. *paludosa* but usually are much larger, whereas plants growing in more dense patches are more similar to *C. muelleriana* or *C. azurea* in size and facies, especially with regard to the leaf shape. Thus, the question arises, whether another species could be hidden within the *Calypogeia* genus. In the present study, we use isozyme markers, DNA sequences of chloroplast genome and three SCAR markers developed on the basis of ISSR method (Buczkowska & Dabert 2011) to explain genetic differences between the recently discovered plants and two recently distinguished taxa of *C. sphagnicola*: *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa*, and to find useful markers for their identification.

Materials and methods

Plant material

The new group of *C. sphagnicola* was found in only one population at the foot of the Tatra Mts. It occurs in a swamp, in the Las Capowski forest. Plants were initially identified as *C. sphagnicola* on the basis of morphological traits according to Müller (1951–1958), Buczkowska (2004b) and Szweykowski (2006). Each sample was divided into two parts: one was deposited as a voucher in the POZW Herbarium, while the other was used for isozyme analyses and a greenhouse culture. A total of 160 gametophytes (shoots) from the following 26 samples: 39779, 41138, 41141, 41144, 41145, 41146, 41159, 41160, 41161, 41162, 41163, 41165, 41379, 41392, 41389, 41391, 41692, 41701, 41704, 41699, 41166, 41700, 41702, 41693, 41694, 41698 were examined (5–8 shoots from each sample) by isozyme analysis. Samples representing groups identified on the basis of isozyme pattern were used for DNA extraction: six samples of the new group from the Las Capowski population, eight samples of *C. sphagnicola* f. *sphagnicola* and six samples of *C. sphagnicola* f. *paludosa*. The examined plants were additionally compared with samples determined genetically in the previous studies (Buczkowska *et al.* 2012) as *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* (Table 1). Moreover, three samples of *C. azurea* and two samples of *Tritomaria quinquedentata* (Huds.) H. Buch used in the previous study were taken as an outgroup in DNA analysis. To obtain plant material free from contamination for DNA extraction, *in vitro* cultures of the studied species were established according to Buczkowska *et al.* (2006) and Buczkowska & Dabert (2011). Since the final taxonomical conclusion of the recently distinguished species within *C. sphagnicola* complex has not yet been done, the previous taxonomical names was maintained in the present paper. The names are used as follows: *C. sphagnicola* f. *sphagnicola* for the haoplid species, *C. sphagnicola* f. *paludosa* for the diploid species, and the LC group for the new taxon detected in the present paper.

Isozyme analysis

Isozyme analysis of seven enzyme systems: GOT, GDH, EST, PGI, MDH, PGD and PGM was conducted according to Buczkowska *et al.* (2012). In the enzymes with multiple loci, the fastest migrating bands were numbered as 1 and the slower as 2 (i.e. *Est*-1, *Est*-2). Alleles were labeled according to Buczkowska *et al.* (2004), Buczkowska (2004a), Buczkowska *et al.* (2012), and newly detected alleles were denoted with subsequent numbers.

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from fresh material. Several stems from one sample were ground with silica

Table 1. Localities of the samples of *Calypogeia sphagnicola* s.l. used for DNA studies and GenBank accession numbers. [1]Numbers correspond to vouchers of POZW Herbarium. [2]Sequence new in the present study are marked by bold.

Locality	No. of samples[1]	Accession number[2]			
		*trn*L	*trn*G	*rpo*C1	*trn*H-*psb*A
C. sphagnicola group LC					
S Poland, Rów Zakiopiański at N base of Tatra Mts, Las Capowski forest, swamp at Sichlański stream, 971 m a.s.l.	41166	**JQ658799**	**JQ658782**	**JQ951993**	**JQ951996**
	41700	**JQ658800**	**JQ658783**	**JQ951994**	**JQ951997**
	41702	**JQ658801**	**JQ658784**	**JQ951995**	**JQ951998**
	41693	–	–	–	–
	41694	–	–	–	–
	41698	–	–	–	–
C. sphagnicola f. ***paludosa***					
S Poland, Tatra Mts, E slope of Mt. Żółta Turnia, *Sphagnum-Polytrichum* hummocks, 1687 m a.s.l.	41174	**JQ658795**	**JQ658778**	JF831169	JF776829
S Poland, Tatra Mts, peat bog Toporowy Staw Wyżni, 1110 m a.s.l.	41148	**JQ658796**	**JQ658779**	JF831171	JF776831
S Poland, Tatra Mts, Pańszczyca Valley, peat bog Wielka Pańszczycka Młaka, 1274 m a.s.l.	42277	**JQ658797**	**JQ658780**	JF831170	JF776830
S Poland, Tatra Mts, N slope of Mt Ornak, *Sphagnum-Polytrichum* hummocks, 1700 m a.s.l.	41722	**JQ658798**	**JQ658781**	JF831172	JF776832
S Poland, Izerskie Mts, peat bog reserve Torfowiska Doliny Izery, 825 m a.s.l.	41178	–	–	–	–
SE Poland, Bieszczady Mts, Lutowiska, peat bog Tarnawa Wyżnia, 670 m a.s.l.	41147	–	–	–	–
C. sphagnicola f. ***sphagnicola***					
NW Poland, Pomorskie Province, peat mat in the littoral zone of Lake Wałachy near Wdzydze	42284	**JQ658790**	**JQ658773**	JF831177	JF776837
NW Poland, Pomorskie Province, peat mat in the littoral zone of Lake Małe Katarzynki near Borowy Młyn	42245	**JQ658791**	**JQ658774**	JF831176	JF776836
NW Poland, Pomorskie Province, Kościerzyna, Krwawe Doły peat bog near Lake Chądzie	42351	**JQ658792**	**JQ658775**	JF831180	JF776840
	42262	–	–	–	–
	42263	–	–	–	–
NW Poland, Pomorskie Province, Lake Czyste peat bog near Płocice	42266	**JQ658793**	**JQ658776**	JF831178	JF776838
	42283	–	–	–	–
NE Poland, Warmińsko-Mazurskie Province, peat mat in the littoral zone of Godle lake near Ełk	41711	**JQ658794**	**JQ658777**	JF831179	JF776839
C. azurea					
S Poland, Tatra Mts, Sucha Woda Valley, Psia Trawka meadow, 1183 m a.s.l.	41746	**JQ658804**	**JQ658787**	JF831183	JF776843
SE Poland, Bieszczady Mts, W slope of Mt Rozsypaniec Wołosacki, 1215 m a.s.l.	41949	**JQ658803**	**JQ658786**	JF831181	JF776841
NE Poland, Jez. Godle lake near Ełk	41748	**JQ658802**	**JQ658785**	JF831182	JF776842
Tritomaria quinquedentata					
S Poland, Tatra Mts, Jaworzynka Valley, 1338 m a.s.l. (TQ705)	41479	**JQ658805**	**JQ658788**	JF831190	JF776850
S Poland, Tatra Mts, Miętusia Valley, 1037 m a.s.l. (TQ1007)	41204	**JQ658806**	**JQ658789**	JF831191	JF776851

beads in a FastPrep tissue disruptor for 20 seconds and subsequently processed using the DNeasy® Plant Mini Kit (Qiagen), following the manufacturer's protocol. Extracted DNA samples were stored at –20°C. Primer for the amplification and sequencing of *trn*H-*psb*A spacer (psbAF and trnHR) were according to Sang *et al.* (1997), *rpo*C1 (LP1F and LP5R) from website of Royal Botanical Garden in Kew (www.kew.org./barcoding/protocols.html), introns of *trn*G and *trn*L (primers A and B) from Pacak & Szweykowska-Kulińska (2003). PCR amplification was carried out as described by Buczkowska *et al.* (2012). Purified PCR products were sequenced in both directions using BigDye 3.1 Terminator Cycle Kit (Applied Biosystems). Sequencing products were separated and detected using an ABI Prism 3130 Automated DNA Sequencer (Applied Biosystems).

PCR amplifications of three specific SCAR markers (Cal01, Cal02 and Cal03) were carried out according to Buczkowska & Dabert (2011).

DATA ANALYSIS

Electropherograms of DNA sequences were edited and assembled using Sequencher 4.5 (Genecodes Inc.). The assembled sequences were aligned manually with BioEdit 7 (Hall 1999). Regions of incomplete data (i.e., at the beginning and end of sequences) were excluded from the analyses. Gaps were excluded from phylogenetic analyses. Phylogenetic analyses were conducted using maximum parsimony (MP) as implemented in MEGA 4.1 (Tamura *et al.* 2007). For parsimony analyses, we applied branch and bound search as implemented in MEGA 4.1. Statistical significance of clades within inferred trees was evaluated using the bootstrap method (Felsenstein 1985) with 2000 replicates. Incongruence between the *rpo*C1, *trn*H-*psb*A, *trn*G and *trn*L data was assessed by comparing clade support on the consensus MP tree. For example, if species A was included in clade A with significant bootstrap support based on interference in the *rpo*C1 region, but resolved as a member of clade B with significant support based on the *trn*H-*psb*A region, the phylogenetic trees based on these loci were considered incongruent. To identify incongruence in phylogenetic signal, we used the 70% bootstrap criterion. Since incongruence was not observed, datasets of all analyzed DNA regions were combined for subsequent phylogenetic analyses. As another measure of distinctiveness, the number of fixed nucleotide differences among taxa was estimated for all pairwise combinations of species using the Sites program (Hey & Wakeley 1997).

MORPHOLOGICAL ANALYSIS

Five stems from 10 samples of the LC group were measured and biometrical analysis of 47 morphometric traits was performed according to the method described by Buczkowska (2004b). Descriptive statistics (means, minimums and maximums) were computed to evaluate the range of morphological variation of the studied plants. Means computed for the LC group were compared with means obtained for *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* in the previous study (Buczkowska *et al*. 2009). The significance of the difference between means was tested by variance analysis (ANOVA) and post-hoc Scheffé test. Statistical analyses were performed using STATISTICA 10.0 for Windows.

RESULTS

ISOZYME ANALYSIS

Nine isozyme loci were detected in the seven studied enzymes. Their analysis revealed that the studied plants from the Las Capowski population form a genetically distinct group (provisionally called LC), which differs both from *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* in four and six loci, respectively (Fig. 1). Three of the studied loci (*Pgd*, *Pgm* and *Est*-2) are diagnostic for each of the studied group, whereas two (*Got*, *Gdh*) allow to distinguish only *C. sphagnicola* f. *paludosa* from the two remaining groups (*C. sphagnicola* f. *sphagnicola* and the group LC). As no variation was observed among samples within a particular group, these isozyme loci may be regarded as good diagnostic markers. Genotypes in the six loci are correlated and composed of three multienzyme phenotypes (MEP) characteristic for each group: MEP 1 is characteristic for *C. sphagnicola* f. *paludosa*, MEP 2 for

→

Fig. 1. Isozyme phenotypes of *Calypogeia sphagnicola*. Alleles were labeled according to Buczkowska *et al.* (2004, 2012), Buczkowska (2004a), and new detected alleles were denoted with subsequent numbers (*Pgd* allele 6, *Pgm* allele 6, *Est*-2 allele 3). MEP – multienzyme phenotypes characteristic for: *C. sphagnicola* f. *paludosa* MEP 1, *C. sphagnicola* f. *sphagnicola* MEP 2, the LC group MEP 3.

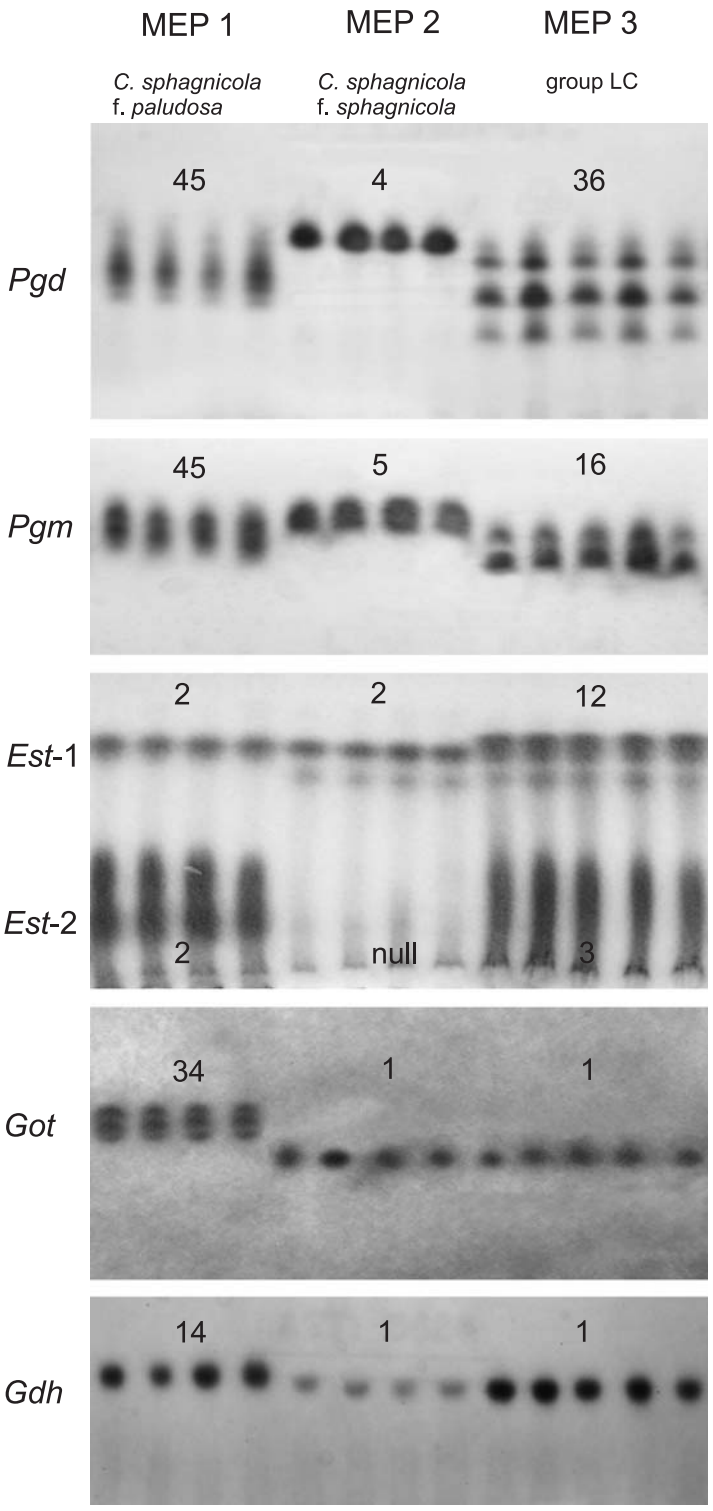
MEP 1
MEP 2
MEP 3
C. sphagnicola f. paludosa
C. sphagnicola f. sphagnicola
group LC
Pgd
45
4
36
Pgm
45
5
16
Est-1
2
2
12
Est-2
2
null
3
Got
34
1
1
Gdh
14
1
1

Table 2. Fixed nucleotide differences in chloroplast regions among studied groups of *Calypogeia sphagnicola* s.l.

Taxa pair	*rpo*C1	*trn*H-*psb*A	*trn*G	*trn*L	total
LC – *C. sphagnicola* f. *paludosa*	10	3	3	1	17
LC – *C. sphagnicola* f. *sphagnicola*	6	4	12	4	26
C. sphagnicola f. *paludosa* – *C. sphagnicola* f. *sphagnicola*	3	5	15	5	28

Table 3. Numbers of specific substitutions (s) and indels (i or d) for each studied group of *Calypogeia sphagnicola* s.l.

Taxa	*rpo*C1	*trn*H-*psb*A	*trn*G	*trn*L
C. sphagnicola f. *sphagnicola*	0	3s	1d	4s
C. sphagnicola f. *paludosa*	3s	2s	2s	1s
LC	1s	1s	0	0

C. sphagnicola f. *sphagnicola* and MEP 3 for the group LC (Fig. 1). Polymorphism within the group LC was detected in two loci (*Mdh*-2 and *Pgi*). Two *Pgi* alleles were present in this group, shared with both *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa*: a frequent allele 1 (0.979) and a rare allele 2 (0.021). Three alleles were detected in *Mdh*-2: allele 4 (the most frequent – 0.762), allele 1 – 0.188 and allele 5 – 0.050. No polymorphism was observed in locus *Mdh*-1.

DNA SEQUENCING

The length of the *trn*H-*psb*A spacer was 222 bp in the outgroup taxon *T. quinquedentata* and 225 bp in all samples of *C. sphagnicola* and *C. azurea*. The length of the sequenced *rpo*C1 was 641 bp in all analyzed specimens. The length of *trn*G intron ranged from 625 bp in *T. quinquedentata* to 645 – 658 bp in *Calypogeia* species, and *trn*L intron from 290 bp in *T. quinquedentata* to 297 bp in *Calypogeia* species. The LC group differs both from *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* in all examined chloroplast regions. In the non-coding regions (*trn*H-*psb*A, *trn*G and *trn*L) more fixed nucleotide differences were found between the LC group and *C. sphagnicola* f. *sphagnicola*, whereas in the coding *rpo*C1 between LC and *C. sphagnicola* f. *paludosa* (Table 2). Each of the studied *C. sphagnicola* groups had specific, distinguishing mutations. Eight specific mutations oc-

Table 4. SCAR markers products of the LC group and other *Calypogeia* species obtained by Buczkowska & Dabert (2011). *C. sp. nov.* – new species of the *C. muelleriana* complex.

	PCR product size (bp)	LC group	*C. sph.* f. *pal.*	*C. sph.* f. *sph.*	*C. suecica*	*C. azurea*	*C. integristipula*	*C. neesiana*	*C. muelleriana*	*C. fissa* P_S	*C. fissa* P_B	*C. sp. nov.*
Cal01	200			+	+			+				
	225	+	+			+	+	+	+	+	+	+
Cal02	360						+		+			
	520							+				
	560		+	+								
	600	+	+		+	+				+	+	+
Cal03	290	+							+		+	+
	no product		+	+	+	+	+	+		+		

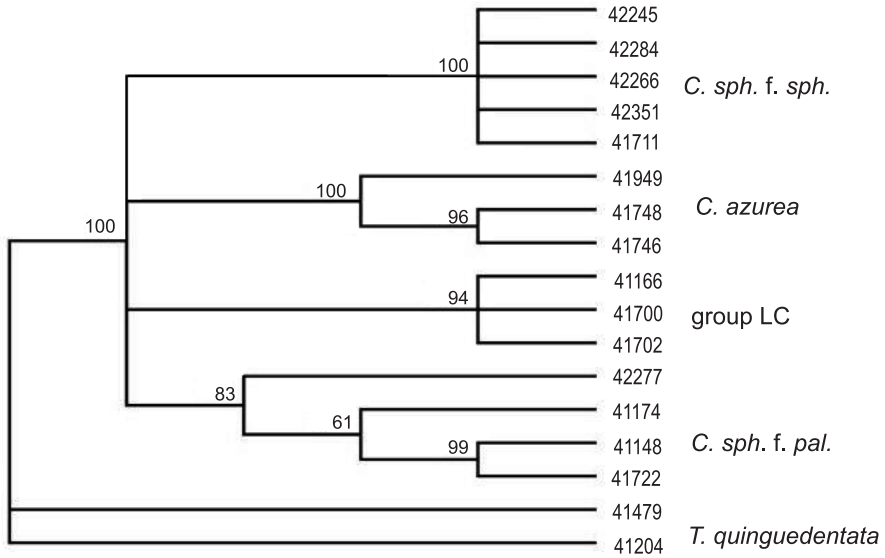

Fig. 2. Strict consensus tree from 233 most parsimonious trees based on combined analysis of all studied chloroplast sequences. Bootstrap values are given above branches.

curred in each of the previously recognized species *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa*, and two in the LC group, one in each *trn*H-*psb*A and *rpo*C1 (Table 3). A maximum parsimony (MP) analysis resulted in 233 most parsimonious trees. In the strict consensus tree all samples of *C. sphagnicola* are resolved in three well-supported (BSP 83–100%) monophyletic lineages, two previously recognized species *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* and the LC group. The groups of *C. sphagnicola* are places in a polytomy together with *C. azurea* with BST of 100% (Fig. 2).

SCAR MARKERS

The group LC can be distinguished from *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* by SCAR markers. Using the Cal02 primer pair, two products of different size (600 bp and 560 bp) were amplified in *C. sphagnicola* f. *paludosa*, whereas only one product was present in *C. sphagnicola* f. *sphagnicola* and in the LC group, 560 and 600 bp, respectively (Fig. 3, Table 4). Primers Cal01 yielded two fragments: one of 200 bp in *C. sphagnicola* f. *sphagnicola*, the second of 225 bp in *C. sphagnicola* f. *paludosa* and in the LC group (Fig. 3). Third primer pair (Cal03) amplified a single band of about 290 bp only for the group LC, but yielded no amplification product in *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* (Fig. 3).

MORPHOMETRY

The descriptive statistics for the LC group were compared with means for *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* (Table 5). The analysis of variance (ANOVA) and post-hoc Scheffé test showed that the LC group differs statistically significantly from both *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* in respect of 30 and 29 analyzed traits, respectively (Table 5). The greatest differences between the LC group and the two species recently distinguished within *C. sphagnicola* were found in the features connected with the size of leaf (23–29) and underleaf (8, 9, 13, 14) as well as characters of stem: length of internodes (43 – 45) and length of internodes cells (35, 37, 39). In general, plants of the LC group are biggest (width of shoots range from 2200 – 2790 μm, on average 2545.0 μm) and have

Table 5. Descriptive statistics for 47 quantitative characters of the LC group and results of the post-hoc Scheffe test: ns – not significant, * – $p \leq 0.05$, ** – $p \leq 0.01$. Mean for *Calypogeia sphagnicola* f. *paludosa* (PAL) and *C. sphagnicola* f. *sphagnicola* (SPH) Buczkowska *et al.* (2009) were used for comparison. N = number of samples measured.

No.	Character	LC			PAL	SPH	Post-hoc Scheffé test		
		N = 10 mean	min	max	N = 18 mean	N = 15 mean	LC-PAL	LC-SPH	PAL-SPH
1	Width of cells of rhizoid initial field in underleaf	20.9	18.3	22.2	26.2	23.8	**	*	*
2	Length of cells of rhizoid initial field in underleaf	27.1	22.2	33.8	28.3	26.3	ns	ns	ns
3	Width of cells in underleaf lobe	31.2	25.1	35.7	33.7	30.8	ns	ns	*
4	Length of cells in underleaf lobe	56.9	51.2	63.4	41.7	39.1	**	**	ns
5	Width of cells in underleaf middle	30.0	22.2	35.5	31.7	29.3	ns	ns	*
6	Length of cells in underleaf middle	53.1	43.6	61.6	41.8	33.8	**	**	**
7	Number of cells between the sinus and the base of underleaf	2.6	2.0	3.4	3.2	2.3	*	ns	**
8	Width of underleaf	632.6	526.8	824.8	529.6	411.3	*	**	**
9	Length of the whole underleaf	558.0	422.1	752.7	457.5	374.7	*	**	*
10	Length of underleaf to the base of rhizoid initial field	394.4	306.0	463.1	431.4	362.3	ns	ns	*
11	Underleaf sinus depth	190.3	124.3	213.9	194.4	183.6	ns	ns	*
12	Ratio of width to height of underleaf – 8/9	1.2	0.9	1.4	1.2	1.1	ns	ns	ns
13	'Measure' of underleaf decurrence – 9/10	1.4	1.2	1.6	1.1	1.0	**	**	ns
14	Ratio of width of underleaf to width of stem – 8/47	1.8	1.6	2.2	2.4	2.2	**	*	*
15	Width of marginal cells in dorsal part of leaf	35.1	31.0	39.4	38.0	36.6	ns	ns	ns
16	Length of marginal cells in dorsal part of leaf	28.7	24.5	30.6	29.8	28.5	ns	ns	ns
17	Width of median cells in leaf	37.3	29.6	45.3	40.7	37.5	ns	ns	ns
18	Length of median cells in leaf	55.5	46.3	70.0	47.4	46.8	*	*	ns
19	Width of cells at ventral leaf base	35.3	27.9	41.3	39.4	39.0	ns	ns	ns
20	Length of cells at ventral leaf base	64.5	48.6	82.7	58.8	54.7	ns	*	ns
21	Width of 2nd row marginal cells in dorsal part of leaf	34.4	32.0	36.4	33.7	34.9	ns	ns	ns
22	Length of 2nd row marginal cells in dorsal part of leaf	32.5	27.4	38.3	35.9	34.6	ns	ns	ns
23	Length of leaf	1328.1	1156.5	1555.8	914.6	757.3	**	**	*
24	Width of leaf	1458.1	1117.7	1725.9	1068.4	750.1	**	**	**
25	Height of dorsal part of leaf	515.7	465.4	596.5	377.4	273.8	**	**	**
26	Distance from apex (a) to ventral base of leaf (c)	1746.3	1368.1	1990.3	1144.1	903.9	**	**	*
27	Length of the 1st coordinate	1147.2	846.8	1384.3	651.4	487.1	**	**	**
28	Length of the 2nd coordinate	1098.2	765.5	1328.4	621.2	454.4	**	**	**
29	Length of the 3rd coordinate	1069.2	732.6	1266.1	618.7	440.1	**		**
30	Ratio of length to width of leaf – 23/24	0.9	0.9	1.0	0.9	1.0	ns	*	**
31	Ratio of leaf length to distance from apex (a) to ventral leaf base (c) – 23/26	0.8	0.7	0.8	0.8	0.8	ns	ns	ns
32	Ratio of distance a-c to the 1st coordinate – 26/27	1.5	1.4	1.7	1.8	1.9	**	**	*
33	Ratio of length of leaf dorsal part to width of leaf 25/24	0.4	0.3	0.4	0.4	0.4	ns	ns	ns

Table 5. *Continued.*

No.	Character	LC			PAL	SPH	Post-hoc Scheffé test		
		N = 10 mean	min	max	N = 18 mean	N = 15 mean	LC-PAL	LC-SPH	PAL-SPH
34	Width of stem cells in the 4th internode	23.8	22.1	26.4	26.7	25.2	*	ns	*
35	Length of stem cells in the 4th internode	87.4	80.4	96.5	71.8	56.3	*	**	*
36	Width of stem cells in the 5th internode	24.3	22.5	26.6	26.3	24.9	*	ns	*
37	Length of stem cells in the 5th internode	88.4	81.1	97.0	71.4	58.2	*	**	*
38	Width of stem cells in the 6th internode	24.6	22.8	26.9	26.2	24.5	ns	ns	*
39	Length of stem cells in the 6th internode	88.9	82.3	98.1	66.9	60.9	**	**	ns
40	Number of stem cells in the 4th internode	10.7	8.0	12.0	9.4	8.3	ns	*	ns
41	Number of stem cells in the 5th internode	11.8	11.0	13.0	9.7	8.7	*	*	ns
42	Number of stem cells in the 6th internode	12.6	11.0	14.0	9.9	8.9	*	**	ns
43	Length of the 4th internode	1221.8	917.2	1445.0	755.4	538.6	**	**	*
44	Length of the 5th internode	1284.7	1001.3	1510.4	772.7	587.4	**	**	*
45	Length of the 6th internode	1290.1	1011.2	1523.1	769.5	609.0	**	**	*
46	Width of the whole plant	2545.0	2199.5	2788.6	1514.7	1177.7	**	**	**
47	Width of stem (without leaves)	348.5	307.9	383.5	224.6	194.8	**	**	*

longest internodes (segment between underleaves). The examined groups did not differ statistically significant in respect of leaf cells size (15–17, 19–22) and underleaf shape (10–12). Plants of the LC group had colorless oil bodies and never produced gemmae.

Discussion

Previous genetic studies of the genus *Calypogeia* revealed presence of distinct units which probably represent taxa unrecognized so far (Buczkowska 2004a; Buczkowska & Bączkiewicz 2011). The present study showed that besides *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa*, which recently were proved to be two distinct species (Buczkowska *et al.* 2012), a third genetically divergent group connected with *Sphagnum* communities can be distinguished in the *Calypogeia* genus in Poland. The newly detected group, provisionally called the LC group, differs from *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* in four and six isozyme loci, respectively. Isozyme phenotypes in three loci: *Pgd*, *Est*-2 and *Pgm* detected in the group LC were unique, they had not been found so far in any of the formerly studied species of the genus *Calypogeia*. Isozyme loci have already been recognized as good diagnostic markers in many liverwort studies (e.g. Szweykowski & Krzakowa 1979; Boisselier-Dubayle *et al.* 1998; Bączkiewicz & Buczkowska 2005). Differences observed in isozyme pattern clearly indicate that the LC group represents another genetically distinct unit of the *Calypogeia* genus.

The distinctness of the LC group from both forms of *C. sphagnicola* was further supported by sequence analysis. The studied chloroplast regions are commonly applied in molecular taxonomy and phylogenetic studies of bryophytes (Szweykowska-Kulińska *et al.* 2002; Wilson *et al.* 2007; Kreier *et al.* 2010; Sawicki *et al.* 2010). Two of the studied regions (*rpo*C1 and *trn*H-*psb*A) are proposed as potential candidates for plastid barcoding loci in mosses and liverworts (Hollingsworth *et al.* 2009; Liu *et al.* 2010). According to our results, all the examined regions amplified well in *Calypogeia* and can be regarded as good DNA markers for this genus. Based on *rpo*C1 and *trn*H-*psb*A sequences, two forms of *C. sphagnicola* were earlier proved to be distinct species (Buczkowska *et al.* 2012). The results of the present study suggest a closer affinity of the LC group to *C. sphagnicola* f. *paludosa* than to *C. sphagnicola* f. *sphagnicola*. Moreover, preliminary larger-scale molecular studies indicated that the LC group differs genetically also from other European species

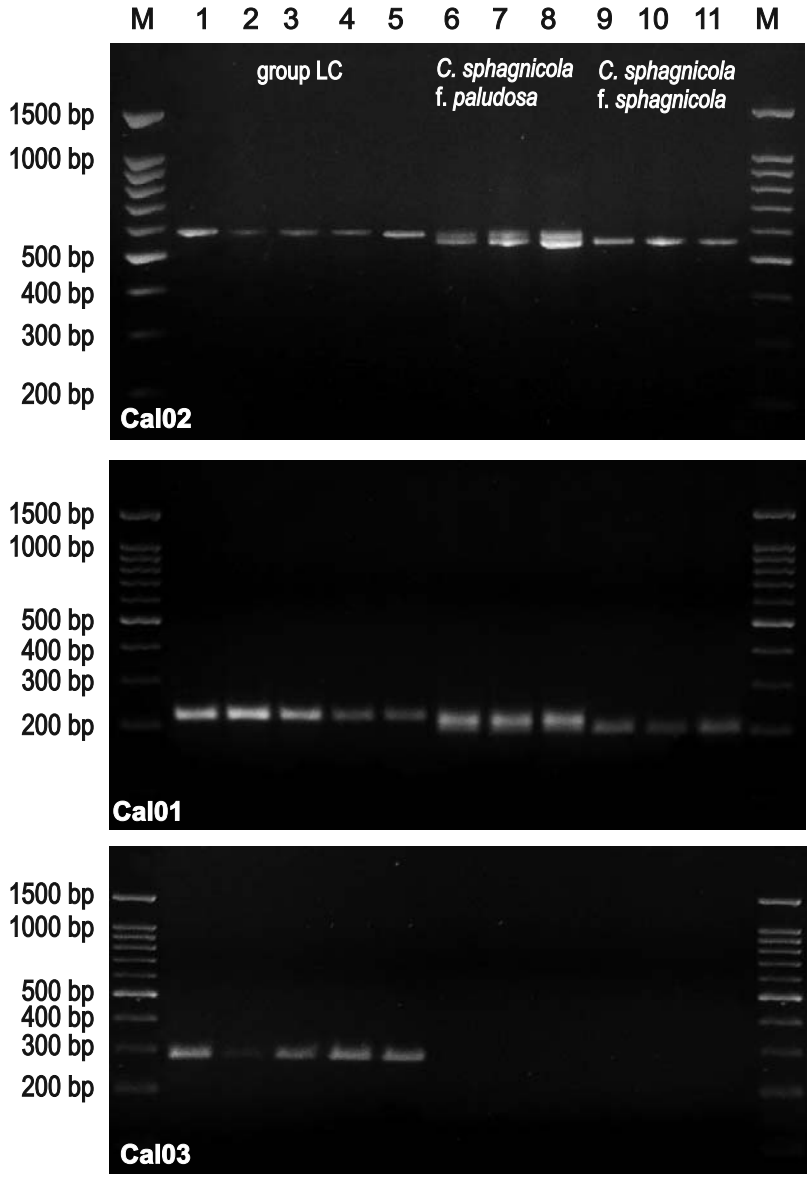

Fig. 3. Products of PCR amplification of the *C. sphagnicola* complex using specific SCAR primers (Cal02, Cal01, Cal03) resolved in 1.5% agarose gel. Lines M – marker of molecular mass (Nova 100 bp DNA ladder, Novazym). 1–5 – group LC: samples 41166, 41700, 41702, 41694; 41698; 6–8 – *C. sphagnicola* f. *paludosa*: 41148, 41178, 42277; 9–11 – *C. sphagnicola* f. *sphagnicola*: 42245, 41711, 42262.

of the genus *Calypogeia* (Buczkowska *et al.* unpbl.). The group LC has two specific mutations allowing its identification, both found in the barcode loci (*rpo*C1 and *trn*H-*psb*A). The number of fixed nucleotide differences between the LC group and *C. sphagnicola* f. *sphagnicola* was over 1.5 times higher than between the LC group and *C. sphagnicola* f. *paludosa*. The highest number of the fixed nucleotide differences between LC and *C. sphagnicola* f. *paludosa* occurred in the coding

region *rpo*C1, and it was comparable to the number of differences detected for the pair of accepted species *C. suecica* and *C. azurea* (Buczkowska *et al.* 2012). Similar level of molecular differentiation as in other liverwort species was detected between the examined groups of *C. sphagnicola* in the non-coding regions. In the *trn*H-*psb*A spacer the same number of mutations was found for species pair *C. integristipula* and *C. neesiana* (Buczkowska *et al.* 2012) and for species of the genus *Orthophyllum* (Sawicki *et al.* 2010). Differences detected in the introns of *trn*L and *trn*G genes were comparable with reported for other liverwort species (Szweykowska-Kulińska *et al.* 2002; Pacak & Szweykowska-Kulińska 2003). In both regions the LC group differs also from other *Calypogeia* species (Buczkowska *et al.*, unpbl.).

The SCAR markers, developed previously for complex of *C. muelleriana* (Buczkowska & Dabert 2011), can also be employed as a good diagnostic tool for the LC group. Two SCAR primer pairs (Cal02, Cal03) distinguished the LC group from both species of *C. sphagnicola*, and primer Cal01 differentiated LC from *C. sphagnicola* f. *sphagnicola*. The species-specific SCAR markers allow a fast and cost-effective determination of a large number of samples. Applying the set of all SCAR markers studied (Cal01, Cal02 and Cal03) allows to separate the LC group from other European *Calypogeia* species, including genotype P_S distinguished in the *C. fissa* complex (Table 4). The LC group has the same Cal01 and Cal02 products as *C. azurea*, *C. suecica*, both genotypes of the *C. fissa* complex (P_S and P_B) and *C. sp. nov.* (new species from the *C. muelleriana* complex), but *C. azurea*, *C. suecica* and P_S of *C. fissa* complex did not yield amplification in Cal03 primer pair (Buczkowska & Dabert 2011). Unfortunately, none of these primer pairs allows separation of the LC group from the genotype P_B of the *C. fissa* complex and the new species from *C. muelleriana* complex, two taxa formerly detected by isozyme pattern (Buczkowska 2004a; Buczkowska & Bączkiewicz 2011). For these taxa new SCAR markers have to be develop based on ISSR analysis.

The *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* in Poland have an allopatric pattern of geographic distribution. Plants defined as *C. sphagnicola* f. *sphagnicola* occur exclusively in the lowlands of northern part of the country, on raised peat bogs. Plants classified as *C. sphagnicola* f. *paludosa* were found only in southern Poland (in the mountains). They are very rare at lower elevations, growing on peat bogs, but frequent in the subalpine zone, growing on *Sphagnum-Polytrichum* hummocks, in the upper part of north-facing slopes (Szweykowski & Buczkowska 1996; Szweykowski 2006; Szweykowski & Klama 2010; Buczkowska *et al.* 2012). The group LC described in the present study occurs also in the southern part of the country. However, it seems to be very rare as among 131 samples studied so far from 35 populations collected from peat bogs in different regions of Poland and over 300 samples of other *Calypogeia* species (Buczkowska 2004a; Buczkowska *et al.* 2004, 2009, 2012; Buczkowska & Bączkiewicz 2011) it was found in only one locality. The site of the LC group is situated in the Sub-Tatra Furrow (Rów Zakopiański) at the foot of the Tatra Mts. The plants occur in a swamp by the Sichlański stream in the Las Capowski forest. The Las Capowski is situated just at the NE edge of the Tatra National Park, in partly protected reserve near Murzasichle village (Mirek 1996). The swamp is surrounded by poor spruce forest *Plagiothecio-Piceetum* (Mirek, personal communication). In view of the presence of the unique *Calypogeia* taxon, this place deserves special protection to save the plants. The site seems to be interesting also regarding other liverworts species. *Bazzania trilobata* (L.) Gray originating from the Las Capowski population had the highest, among 10 populations studied, genetic variation inferred from ISSR markers. Moreover, *B. trilobata*, which is commonly regarded as a sterile species, was fertile in the local population and had individuals with archegonia (Buczkowska *et al.* 2010).

Plants determined as the group LC have colorless oil bodies and never have gemmae. They display a growth type typical for *C. sphagnicola*, i.e., creeping between the stems of *Sphagnum* spp. over hummocks and in wet places among them. These plants morphologically correspond to

C. sphagnicola f. *paludosa*, have a similar shape of lateral leaves, which are decurrent at base. However, plants of the LC group are bigger, their shoots reach up to about 2800 μm in width, whereas in *C. sphagnicola* f. *paludosa* up to 2190 μm (Buczkowska *et al.* 2009). The plants growing on humus formed more dense patches and had more vigorous stems with less decurrent, almost heart-shaped (more or less as wide as long) lateral leaves. Morphometric studies showed that the LC group differs statistically significantly from both previously recognized species of the *C. sphagnicola* complex (Buczkowska *et al.* 2012) in respect of 30 quantitative traits. However, the quantitative traits are less reliable, therefore, species delimitation on the morphological disparities only may be difficult. Therefore, until some good diagnostic traits will be found, the group LC has to be rather treated as a cryptic species of *C. sphagnicola*.

Many species were so far described in the *Calypogeia* genus, some of them were later considered synonyms of particular species (Müller 1951–1958; Schuster 1969). Thus, it is conceivable that the taxon detected in the present study based on genetic markers was already described. Thus, before final taxonomy conclusion it has to be checked if one of the several published names may fit the plants discussed above.

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