Plant Syst Evol (2012) 298:549–560
DOI 10.1007/s00606-011-0565-5

ORIGINAL ARTICLE

Allopolyploid speciation of *Calypogeia sphagnicola* (Jungermanniopsida, Calypogeiaceae) based on isozyme and DNA markers

Katarzyna Buczkowska · Jakub Sawicki · Monika Szczecińska · Henryk Klama · Alina Bączkiewicz

Received: 16 May 2011 / Accepted: 6 November 2011 / Published online: 2 December 2011
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Abstract *Calypogeia sphagnicola* is one of nine species of the genus *Calypogeia* known in Europe. Occurrence of the species is closely connected with peat bogs. Nowadays, two forms of this species are distinguished—*C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa*. The results of the present study, based on two classes of markers—isozymes and sequences of chloroplast genom (*trn*H-*psb*A, *rpo*C1)—unanimously support the genetic differentiation within the taxon and show that the present-day forms represent genetically distinct species. Phylogenetic analysis resolved two lineages that correspond with the present-day forms with high bootstrap support, which differ in ploidy level: *C. sphagnicola* f. *sphagnicola* is haploid, whereas *C. sphagnicola* f. *paludosa* is a diploid form. Allopolyploid origin of the diploid form was revealed by the isozyme pattern. Nei's genetic distance between the two present-day forms of *C. sphagnicola* was 0.472. The forms in Poland have an allopatric pattern of geographic distribution: *C. sphagnicola* f. *sphagnicola* occurs exclusively in the lowlands of the northern part of the country on raised peat bogs, whereas *C. sphagnicola* f. *paludosa* is found only in the mountains of southern Poland, mainly in the subalpine zone, where it grows on *Sphagnum-Polytrichum* hummocks on the upper part of north-facing slopes. Plants regarded in this study as *C. sphagnicola* f. *sphagnicola* morphologically correspond to the syntype specimen of *C. sphagnicola*.

Keywords Liverworts · *Calypogeia* · Isozymes · *rpoC*1 · *trn*H-*psb*A · Species speciation

Introduction

Liverwort taxonomists have at their disposal only a limited number of good diagnostic qualitative traits because of the reduced, compared to angiosperms, morphology of the gametophyte, which dominates in the life cycle of bryophytes. The overwhelming majority of features available for study are quantitative (meristic) traits that have a continuous phenotypic range. Quantitative traits are often polygenic and may also be significantly influenced by environmental factors; hence, in some cases, diagnostic differences between species can be overlooked. For this reason, some species described in the last century on the basis of morphological criteria were too broadly defined and included some unrecognized species, e.g., *Conocephalum conicum* (L.) Dumort. (Szweykowski et al. 2005). Recently, classical taxonomic traits based on morphology have been supplemented with genetic markers that, in contrast with meristic traits, are not influenced by environmental changes; therefore, they are treated as an important source of taxonomic information. In liverworts, taxonomy based on genetic markers, e.g., isozymes (among others, Szweykowski and Krzakowa 1979; Bischler and Boisselier-Dubayle 2000) and molecular markers (Boisselier-Dubayle et al. 1995;

K. Buczkowska (✉) · A. Bączkiewicz
Department of Genetics, Institute of Experimental Biology,
Adam Mickiewicz University, ul. Umultowska 89,
61-614 Poznan, Poland
e-mail: androsac@amu.edu.pl

J. Sawicki · M. Szczecińska
Department of Botany and Nature Protection,
University of Warmia and Mazury in Olsztyn, Plac Łódzki 1,
10-728 Olsztyn, Poland

H. Klama
Institute of Environmental Protection and Engineering,
University of Bielsko-Biała, Willowa 2,
43-309 Bielsko-Biała, Poland

Szweykowska-Kulińska et al. 2002; Wachowiak et al. 2007; Heinrichs et al. 2010; Kreier et al. 2010), allows to define species more precisely than was possible on the basis of classical methods only. The application of isozyme and molecular markers in studies on the genus *Calypogeia* Raddi revealed the presence of plants genetically distinct from the well-known and accepted species occurring in Europe. These plants probably represent new taxa, unrecognized until now. Two genetically distinct groups were recognized within the complex of *C. fissa* (L.) Raddi (Buczkowska 2004a) and *C. muelleriana* (Schiffn.) Müll.Frib. (Buczkowska and Bączkiewicz 2011; Buczkowska and Dabert 2011). *Calypogeia sphagnicola* (Arn. & Perss.) Warnst. and Loeske seems to be another example of a liverwort species defined too broadly.

Calypogeia is a large genus of leafy liverworts of wide geographic distribution with about 90 described species (Schuster 1969). The genus is one of the most difficult groups of liverworts (Schuster 1969; Szweykowski 2006). The delimitation of *Calypogeia* species has often been difficult because of the lack of distinctive morphological characters, and the presence of environmentally induced modifications and atypical forms. Moreover, some European species of *Calypogeia* have a wide Holarctic distribution, and taxonomic controversies may also arise from heterogeneity and the possibility of hidden genetic differentiation within the geographic range (Buczkowska 2004b). According to Schuster (1969), direct adaptation of the Flora compiled for one geographic region to the material from another region can cause difficulties.

Calypogeia sphagnicola is one of nine species of the genus *Calypogeia* known so far in Europe (Grolle and Long 2000). The species has a bipolar pattern of geographic distribution. In the northern hemisphere it is widely distributed in northern and central Europe, the northern part of North America and in Asia (Turkey and Japan), whereas it is rare in the antipodal regions (Schuster 1969; Damsholt 2002). It is a monoecious, frequently fertile species, largely restricted to peat bogs, where it creeps over *Sphagnum* spp. hummocks and among them. The occurrence of *C. sphagnicola* in peat bogs is a diagnostic feature for this species (Schuster 1969). Initially, two different species were described whose occurrence was connected with peat bogs: *C. sphagnicola* (Arnell 1902) and *C. paludosa* (Warnstorf 1906). However, Müller (1913–1916) reduced the second one to the synonymy of *C. sphagnicola*. In spite of narrow environmental requirements, *C. sphagnicoala* shows a considerable morphological variation, caused mainly by the amount of light and moisture available (Schuster 1969; Damsholt 2002). The three most evident phenotypes were described by Schuster (1969) as forms of this species: *C. sphagnicola* f. *sphagnicola*—the typical form, characterized by the small size of plants [0.5–1.8 (2) mm wide], the presence of gemmiparous shoots and oil bodies composed of 1–4 (5–6) globules; *C. sphagnicola* f. *paludosa* (Warnst.) Schust.—distinguished by larger plants (2.0–2.9 mm wide) with oil bodies formed of more numerous segments (7–12), never with gemmae; *C. sphagnicola* f. *bidenticulata* Schust.—the form characterized by moderately vigorous plants (1.5–2.0 mm wide) without gemmae and usually with bidentate leaves with oil bodies of 6–12 (16) segments, limited in occurrence to North America.

In Europe, two forms—*C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa*—were reported from Nordic countries by Damsholt (2002) and from Poland by Szweykowski (2006). Szweykowski (2006) pointed out differences in geographic distribution between both forms: the typical form is widespread in the lowlands of the northern part of Poland, where it is restricted to raised peat bogs, whereas it is very rare in the mountain regions. On the contrary, *C. sphagnicola* f. *paludosa* occurs mainly in the mountains of southern part of the country, where it is bound to *Sphagnum-Polytrichum* hummocks and very rarely occurs in the lowlands. Preliminary isozyme and biometrical studies showed two distinct groups of genotypes correlated with the presence or absence of gemmae, and suggested that the above forms of *C. sphagnicola* can represent genetically distinct species (Buczkowska et al. 2009).

Irrespective of geographic region, only one chromosome number ($n = 18$) is known for *C. sphagnicola* (Müller 1951–1958; Newton 1973; Inoue 1976). The same chromosome number was recorded in Poland (Buczkowska et al. 2004); however, differences in geographic distribution of *C. sphagnicola* forms noted by Szweykowski (2006) indicate that only one form (*C. sphagnicola* f. *paludosa*) was analyzed. In most cases it is not clear which form the chromosome counts refer to in the above-cited authors, but at least in Poland it seems likely that only *C. sphagnicola* f. *paludosa* has been assessed.

In the present study, we use two classes of markers: isozymes and DNA sequences to assess the level of genetic diversity between two forms: *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa*. Our aim is to verify the hypothesis that both forms represent separate species and to develop markers that could be useful for identification of plants belonging to each species, not only on the basis of fresh material but also material from herbarium collections. Additionally, flow cytometry was applied to estimate the ploidy level of the examined forms.

Materials and methods

Plant material

Samples of the studied species were collected from different regions of Poland. In total, 59 samples from 16

populations were examined by means of isozyme analysis: 31 samples of *C. sphagnicola* f. *sphagnicola* and 28 samples of *C. sphagnicola* f. *paludosa* (Table 1). Plants were initially identified on the basis of morphological traits and oil body characters according to Szweykowski (2006). Plants classified as *C. sphagnicola* f. *sphagnicola* were small, had gemmiparous shoots and oil bodies mostly undivided or formed of 2–3 (4) segments, whereas plants classified as *C. sphagnicola* f. *paludosa* were bigger, had no gemmae and more segmented (5–10) oil bodies. Each sample was divided into two parts: one was deposited as a voucher in the POZW Herbarium, whereas the other was used for analyses. A total of 350 gametophytes (shoots) were examined (4–7 shoots from each sample) in isozyme analysis. Moreover, 28 samples of *C. azurea* Stotler & Crotz, the same as used by Buczkowska and Baczkiewicz (2011), were included in the isozyme study as reference species.

Next, the plants identified on the basis of isozyme pattern were used for DNA extraction. A total of 21 samples were studied: 5 of *C. sphagnicola* f. *sphagnicola*, 4 of *C. sphagnicola* f. *paludosa* and 3 samples each from the other *Calypogeia* species: *C. azurea*, *C. neesiana* (Massal. & Carestia) Müll. Frib., *C. integristipula* Steph. and *C. suecica* (Arnell & J.Perss.) Müll.Frib. Two samples of *Tritomaria quinquedentata* (Huds.) H. Buch were used as an outgroup in DNA analysis (Table 2).

Isozyme analysis

Electrophoretic separation of isozymes was conducted according to the procedure described in detail by Wendel and Weeden (1989). Crude cell extract was prepared by homogenization of one stem in 40 µl of extraction buffer (Gottlieb 1981). Isozymes were separated in 10% starch gel slabs using three buffer systems: Tris-citrate pH 8.2/lithium-borate pH 8.3 (GOT, GDH, EST, PGI), morpholine-citrate pH 6.1 in dilution of electrode buffer 1:14 (MDH, PGD) and Tris-histidine pH 7.0 (PGM, ME). In the enzymes with multiple loci, the fastest migrating bands were numbered as 1 and the slower as 2 (i.e., *Mdh*-1, *Mdh*-2). Alleles were labeled according to Buczkowska et al.

Table 1 Localities of the populations of *C. sphagnicola* used for isozyme studies

Population no.	Locality	POZW no. of studied samples[a]
C. sphagnicola f. *sphagnicola*		
1	NW Poland, Pomorskie Province, Duże Sitno peat bog	42270, 42286, 42343, 42346, 42348
2	NW Poland, Pomorskie Province, peat mat in the littoral zone of Lake Wałachy near Wdzydze	42243, 42244, 42282, 42284, 42287
3	NW Poland, Pomorskie Province, peat mat in the littoral zone of Lake Małe Katarzynki near Borowy Młyn	42337, 42261, 42245, 42344
4	NW Poland, Pomorskie Province, Kościerzyna, Krwawe Doły peat bog near Lake Chądzie	42267, 42263, 42269, CS1402
5	NE Poland, Podlaskie Province, Wigierski National Park, peat mat in the littoral zone of Lake Sucharek near Suwałki	30944, 30913
6	NW Poland, Pomorskie Province, Lake Czyste peat bog near Płocice	42283, 42342, 42266
7	NE Poland, Warmińsko-Mazurskie Province, peat mat in the littoral zone of Godle lake near Ełk	41711
8	NE Poland, Podlaskie Province, peat bog around Lake Druce near Sejny	30910, 31026, 32433, 32434
9	NE Poland, Warmińsko-Mazurskie Province, Mechacz Wielki peat bog reserve near Gołdap	32431, 32430, 32429
C. sphagnicola f. *paludosa*		
10	SE Poland, Bieszczady Mts, Lutowiska, peat bog Tarnawa Wyżnia, 670 m a.s.l.	34837, 34839, 34843
11	S Poland, Tatra Mts, E slope of Mt. Żółta Turnia, sphagnum-polytrichum hummocks, 1,687 m a.s.l.	41168, 41170, 41172, 41174
12	S Poland, Tatra Mts, peat bog Toporowy Staw Wyżni, 1,110 m a.s.l.	41142, 41148, 41171, 41723, 41726
13	S Poland, Tatra Mts, Pańszczyca Valley, peat bog Wielka Pańszczycka Młaka, 1,274 m a.s.l.	41137, 41173, 41174, 41175, 41176, 42277
14	S Poland, Izerskie Mts, peat bog reserve Torfowiska Doliny Izery, 825 m a.s.l.	35110, 35115, 35187
15	S Poland, Tatra Mts, N slope of Mt Ornak, sphagnum-polytrichum hummocks, 1,700 m a.s.l.	41722a, 41722b, 41722c, 41722d
16	S Poland, Beskid Żywiecki Mts, Mt Babia Góra, sphagnum-polytrichum hummocks, 1,560 m a.s.l.	39412, 39503, 39022

[a] The vouchers are deposited in the POZW Herbarium

Table 2 Localities of the samples of *Calypogeia* species used for DNA studies and GenBank accession numbers

Locality	POZW no. of studied samples[a]	Accession no.	
		*rpo*C1	*trn*H-*psb*A
C. sphagnicola f. *sphagnicola*			
NW Poland, Pomorskie Province, peat mat in the littoral zone of Lake Wałachy near Wdzydze	42284*	JF831177	JF776837
NW Poland, Pomorskie Province, peat mat in the littoral zone of Lake Małe Katarzynki near Borowy Młyn	42245	JF831176	JF776836
NW Poland, Pomorskie Province, Kościerzyna, Krwawe Doły peat bog near Lake Chądzie	CS1402*	JF831180	JF776840
NW Poland, Pomorskie Province, Lake Czyste peat bog near Płocice	42266	JF831178	JF776838
NE Poland, Warmińsko-Mazurskie Province, peat mat in the littoral zone of Godle lake near Ełk	41711*	JF831179	JF776839
C. sphagnicola f. *paludosa*			
S Poland, Tatra Mts, E slope of Mt. Żółta Turnia, sphagnum-polytrichum hummocks, 1,687 m a.s.l.	41174	JF831169	JF776829
S Poland, Tatra Mts, peat bog Toporowy Staw Wyżni, 1,110 m a.s.l.	41148*	JF831171	JF776831
S Poland, Tatra Mts, Pańszczyca Valley, peat bog Wielka Pańszczycka Młaka, 1,274 m a.s.l.	42277*	JF831170	JF776830
S Poland, Tatra Mts, N slope of Mt Ornak, sphagnum-polytrichum hummocks, 1,700 m a.s.l.	41722d*	JF831172	JF776832
C. azurea			
S Poland, Tatra Mts, Sucha Woda Valley, Psia Trawka meadow, 1,183 m a.s.l.	41746	JF831183	JF776843
SE Poland, Bieszczady Mts, W slope of Mt Rozsypaniec Wołosacki, 1,215 m a.s.l.	41949	JF831181	JF776841
NE Poland, Jez. Godle lake near Ełk	41748	JF831182	JF776842
C. integristipula			
S Poland, Tatra Mts, Sucha Woda Valley, Psia Trawka meadow, 1,180 m a.s.l.	CI1401	JF831187	JF776847
NE Poland, Warmińsko-Mazurskie Province, Mechacz Wielki peat bog	41730	JF831188	JF776848
SE Poland, Bieszczady Mts, W slope of Mt Rozsypaniec Wołosacki, 1,214 m a.s.l.	41928	JF831189	JF776849
C. neesiana			
S Poland, Tatra Mts, N slope of Mt Ornak, sphagnum-polytrichum hummocks, 1,680 m a.s.l.	41731	JF831184	JF776844
NE Poland, Warmińsko-Mazurskie Province, Mechacz Wielki peat bog	41735	JF831185	JF776845
SE Poland, Bieszczady Mts, W slope of Mt Rozsypaniec Wołosacki, 1204 m a.s.l.	41927	JF831186	JF776846
C. suecica			
S Poland, Beskid Żywiecki Mts, Mt Babia Góra, 1,190 m a.s.l.	39500	JF831175	JF776835
S Poland, Tatra Mts, stream near Lake Toporowy Staw Wyżni, 1,110 m a.s.l.	41727	JF831174	JF776834
SE Poland, Bieszczady Mts, Górna Solinka Valley, 772 m a.s.l.	41936	JF831173	JF776833
Tritomaria quinquedentata			
S Poland, Tatra Mts, Jaworzynka Valley, 1,338 m a.s.l.	TQ705	JF831190	JF776850
S Poland, Tatra Mts, Miętusia Valley, 1,037 m a.s.l.	TQ1007	JF831191	JF776851

[a] The vouchers are deposited in the POZW Herbarium; * samples used for flow cytometry

(2004) and Buczkowska (2004a), and new detected alleles were denoted with subsequent numbers.

DNA extraction, PCR amplification and sequencing of chloroplast regions

Total genomic DNA was extracted from fresh material. Single stems were ground with silica beads in a FastPrep tissue disruptor for 20 s and subsequently processed using the DNeasy® Plant Mini Kit (Qiagen), following the manufacturer's protocol. Extracted DNA samples were stored at −20°C. For the amplification and sequencing of *trn*H-*psb*A and *rpo*C1, we used the primers of Sang et al. (1997) and from the Royal Botanical Garden in Kew website. The chloroplast regions were amplified in a volume of 25 µl containing 20 mM $(NH_4)SO_4$, 50 mM Tris–HCl (pH 9.0 at 25°C), 1.5 mM $MgCl_2$, 1 µl BSA, 200 µM each, dATP, dGTP, dCTP, dTTP, 1.0 µM of each primer,

1 unit of *Taq* polymerase (Novazym, Poznań, Poland) and 1 ml of DNA solution. The reaction was processed at 94°C for 1 min followed by 30 cycles at 94°C for 1 min., 56°C for 1 min. and 72°C for 1.5 min., with a final extension step of 72°C for 5 min. Finally, 5 µl of the amplification products was visualized on 1.5% agarose gel with ethidium bromide staining. Purified PCR products were sequenced in both directions using the ABI BigDye 3.1 Terminator Cycle Kit (Applied Biosystems) and were then visualized using an ABI Prism 3130 Automated DNA Sequencer (Applied Biosystems).

Flow cytometry

Several (about 20–30) stems (gametophytes) were ground in a mortar with 2-ml nuclei isolation buffer NIM-DAPI (NPE Systems, Pembroke Pines, Fl) and 100 µl DNA reference calibrator. The suspension with nuclei was filtered through a 30-µm nylon filter. The fluorescence of the nuclei for each sample was measured after 10 min of incubation using Cell Lab Quanta SC flow cytometer, equipped with a mercury arc lamp. Chicken red blood cells (CRBC) were used as internal standard, because this standard has a convenient amount of DNA related to liverworts. DNA content was measured assuming that CRBC contained 2.33 pg DNA/2C (Baird et al. 1994). Data were analyzed by means of the Quanta SC software. Three different samples of each form of *C. sphagnicola* were measured.

Data analysis

For isozyme data allele frequencies, the percentage of polymorphic loci (P) and mean numbers of alleles per locus (A) were calculated for each form. For estimation of genetic variation, gene diversity statistics (Nei 1973, 1978) were calculated. Nei's (1978) genetic distance (D) was estimated between the populations of each form and between the forms. The resulting distance matrix was used to infer an UPGMA dendrogram. Analyses were performed using GenAlEx 6.3 (Peakall and Smouse 2005). The dendrogram was constructed using Mega 4.1 (Tamura et al. 2007). It is difficult to calculate the genetic distance between species of different ploidy levels. In order to compute the distance between the two studied forms, genotypes of the haploid form were treated as homozygotes of the diploid data format, and codominant options of GenAlEx were used.

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 ambiguous alignment and incomplete data (i.e., at the beginning and end of sequences) were excluded from the analyses. Gaps were excluded from all phylogenetic analyses. Phylogenetic analyses were conducted using maximum parsimony (MP) as implemented in MEGA 4.1. 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 2,000 replicates.

Incongruence between the *rpo*C1 and *trn*H-*psb*A 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 the phylogenetic signal, we used the 70% bootstrap criterion. Since incongruence was not observed, the *rpo*C1 and *trn*H-*psb*A data sets 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 and Wakeley 1997).

Results

Isozyme analysis

The ten putative loci in seven enzyme systems were consistently resolved in the studied populations of *C. sphagnicola*. The examined forms: *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* differ in respect to seven (*Got*, *Est*-1, *Est*-2, *Gdh*, *Pgd*, *Pgm* and *Me*) out of ten studied isozyme loci. The following alleles were diagnostic for *C. sphagnicola* f. *sphagnicola*: *Got* allele 1, *Gdh* allele 1, *Pgd*-2 allele 4, *Pgm* allele 5 and *Me* phenotype 1, and for *C. sphagnicola* f. *paludosa*: *Got* alleles 34, *Est*-2 allele 2, *Gdh* alleles 14, *Pgd*-2 alleles 45, *Pgm* alleles 45 and *Me* phenotype 2 (Fig. 1; Table 3). All isozyme phenotypes observed in typical form were single-banded, whereas in *C. sphagnicola* f. *paludosa*, four loci—*Got*, *Pgd*-2, *Gdh* and *Pgm*—showed a fixed heterozygous pattern (Fig. 1). Genetic variation within forms was low; two polymorphic loci (*Mdh*-2, *Pgi*) with two or three alleles were detected, respectively, in *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa*. The gene diversity (H_T) based on allelic frequencies of all loci over all populations was 0.049 for *C. sphagnicola* f. *sphagnicola* and 0.048 *C. sphagnicola* f. *paludosa*. The mean number of alleles per locus was 1.20 and 1.30, respectively.

Nei's genetic distance between *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* was equal to 0.472 and is lower than distances among each of the forms

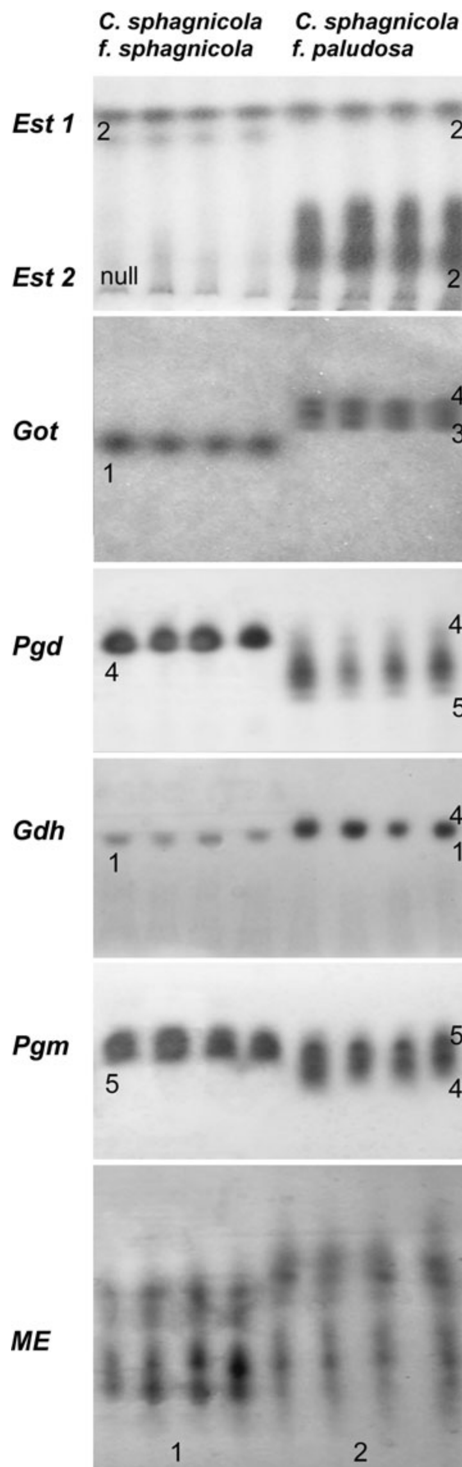

Fig. 1 Isozyme phenotypes of *C. spagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa*. Alleles were labeled according to Buczkowska et al. (2004) and Buczkowska (2004a). Newly detected alleles are denoted with the subsequent numbers

and *C. azurea*, the species used as a reference group (0.869 and 0.922, respectively). Genetic distances among populations within *C. sphagnicola* f. *sphagnicola* lay in the range of 0.000–0.098, with a mean equal to 0.022, and within *C. sphagnicola* f. *paludosa* between 0.000 and 0.063, with a mean value equal to 0.017, similarly, within *C. azurea* (0.000–0.094, with a mean of 0.038) (Fig. 2). The UPGMA dendrogram based on Nei's genetic distances shows the grouping of studied populations of *C. sphagnicola* into two distinct clusters, the first including *C. sphagnicola* f. *sphagnicola*, and the second populations of *C. sphagnicola* f. *paludosa*, both clearly separated from *C. azurea* (Fig. 3). Differences observed in the isozyme pattern clearly indicate that *C. sphagnicola* comprises two genetically distinct entities.

Table 3 Allele frequencies in two studied forms of *C. sphagnicola*

Locus	Allele	SPH	PAL
Est-1	2	1.000	1.000
Est-2	Null	1.000	0.000
	2	0.000	1.000
Got	1	1.000	0.000
	3	0.000	0.500
	4	0.000	0.500
Pgd-2	4	1.000	0.500
	5	0.000	0.500
Gdh	1	1.000	0.500
	4	0.000	0.500
Pgm	4	0.000	0.500
	5	1.000	0.500
Mdh-1	1	1.000	1.000
Mdh-2	1	0.808	0.874
	4	0.192	0.126
Pgi	1	0.013	0.025
	2	0.894	0.859
	3	0.093	0.116
*Me**	1	1.000	0.000
	2	0.000	1.000
N		151	199

N number of analyzed shoots, *SPH C. sphagnicola* f. *sphagnicola*, *PAL C. sphagnicola* f. *paludosa*. Alleles were labeled according to Buczkowska et al. (2004) and Buczkowska (2004a); new detected alleles are denoted with the subsequent numbers. * In *Me* phenotypes were scored

Flow cytometry

The DNA content of *C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa* was determined (Fig. 4). Haploid species *C. suecica* ($n = 9$) was used as a reference species. Nuclei DNA content of both forms was calculated using a comparative ratio: $MC_{sample} \times 2.33/MC_{standard}$, where MC indicates the mean channel of fluorescence. MC in *C. sphagnicola* f. *sphagnicola* was 110, in *C. sphagnicola*

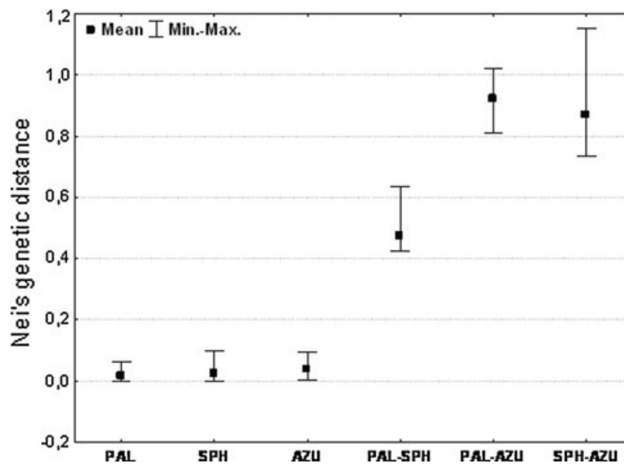

Fig. 2 Diagram showing the range of Nei's (1978) genetic distance based on isozyme data among populations of *C. sphagnicola* f. *paludosa*, *C. spagnicola* f. *sphagnicola* and *C. azurea* and between each examined pair of species

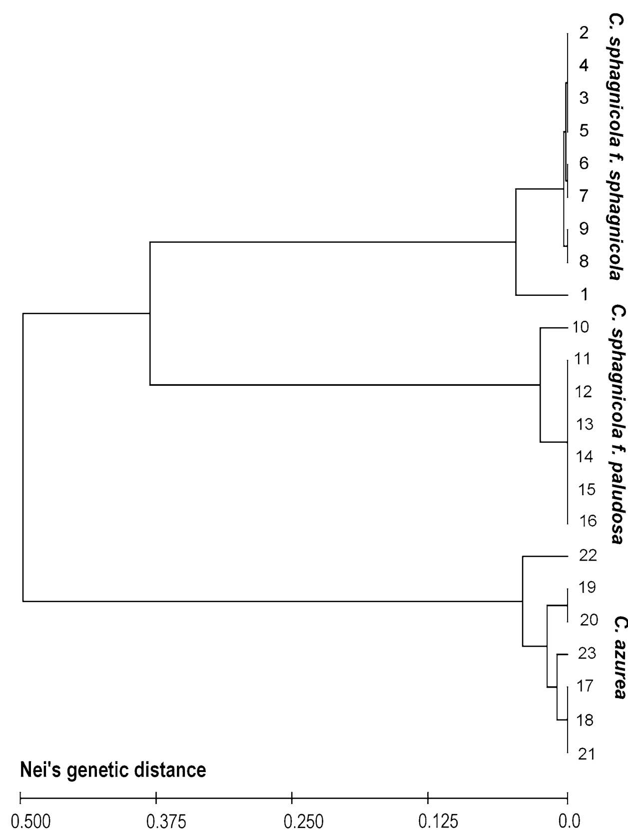

Fig. 3 UPGMA dendrogram of the studied populations of *C. spagnicola* f. *sphagnicola, C. sphagnicola* f. *paludosa* and *C. azurea* based on isozyme data

f. *paludosa* 181.6 and in *C. suecica* 119.1. The CRBC mean channel was 404, 376 and 420, respectively. The DNA content in examined samples was 0.634 pg/nucleus in *C. sphagnicola* f. *sphagnicola*, 1.125 pg/nucleus in *C. sphagnicola* f. *paludosa* and 0.661 pg/nucleus in *C. suecica*. The measurements were repeated three times for each sample, and the SD was 0.002, 0.005 and 0.003, respectively. The nuclear DNA content indicates that examined forms differ in ploidy level: *C. sphagnicola* f. *sphagnicola* is haploid, and *C. sphagnicola* f. *paludosa* is a diploid form.

DNA sequencing

The length of the *trn*H-*psb*A spacer ranged from 222 bp in the outgroup taxon *Tritomaria* to 225 bp in the most samples of *Calypogeia*. Infraspecific length variation was noted in *C. sphagnicola* f. *paludosa* (224–225 bp), *C. suecica* (224–225 bp) and *C. integristipula* (224–225 bp). No substitutions were observed in the *trn*H-*psb*A sequences at the intraspecific level. The length of the sequenced *rpo*C1 region was constant across all analyzed specimens (641 bp). Among analyzed species, the highest intraspecific variation was observed in *C. sphagnicola* f. *paludosa* (11 substitutions). Much lower variation was found in *C. integristipula* (4 substitutions), *C. azurea* (2) and *C. neesiana* (2).

The total length of the aligned *trn*H-*psb*A spacer was 230 base pairs, including indels, with 52 variable and parsimony informative sites. The aligned *rpo*C1 data set contained 641 base pairs. The *rpo*C1 data set contained 108 parsimony informative characters and 9 variable, but uninformative characters. A maximum parsimony (MP) analysis resulted in 125 most parsimonious trees [length 191, consistency index (CI) 0.9066, retention index (RI) 0.9393]. The strict consensus tree with bootstrap values for supported nodes is presented in Fig. 5. The genus *Calypogeia* clade was well supported with bootstrap values of 99%. Because each species forms a well-supported clade with bootstrap support ranging from 81% in case of *C. sphagnicola* f. *sphagnicola* to 99% in *C. azurea*, *C. integristipula*, *C. nessiana* and *C. suecica*, the analyzed data set does not provide much information about genetic relationships among species. Only two species form a common clade, *C. integristipula* and *C. neesiana*, with bootstrap support 87%.

The non-coding *trn*H-*psb*A spacer revealed fixed nucleotide differences for every pair of species. Their number for a pair of *Calypogeia* taxa ranged from 3 (*C. sphagnicola* and *C. integristipula*, *C. integristipula* and *C. neesiana*) to 7 (*C. sphagnicola* f. *paludosa* and *C. neesiana*, *C. sphagnicola* f. *paludosa* and *C. suecica*). The numbers of fixed nucleotide differences between the studied *Calypogeia* species and outgroup species of *Tritomaria* were significantly higher (45–48). The number of nucleotide pairwise differences in the coding *rpo*C1 region between the studied *Calypogeia* species ranged from 2 (*C. azurea*

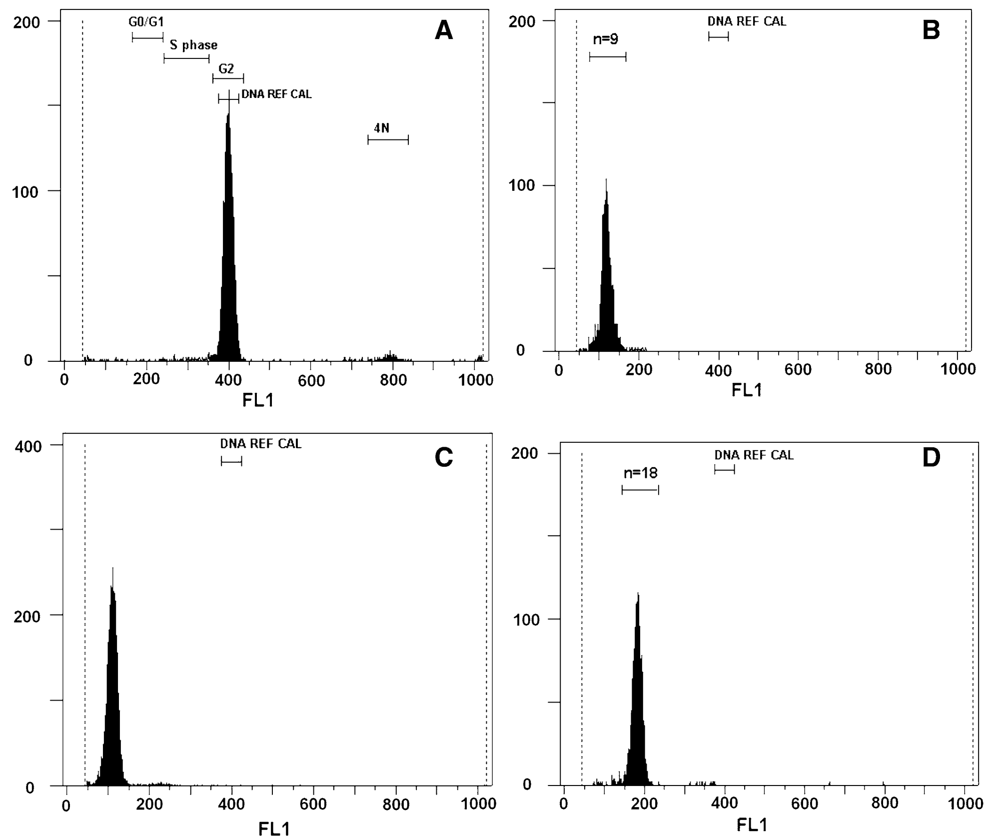

Fig. 4 Histograms of relative DNA content obtained after a flow cytometric analysis of DAPI-stained nuclei of: **a** internal standard, **b** *C. suecica*, **c** *C. spagnicola* f. *sphagnicola* and **d** *C. sphagnicola* f. *paludosa*

and *C. sphagnicola* f. *sphagnicola*) to 21 (*C. neesiana* and *C. suecica*). *Calypogeia* species differ from the outgroup *Tritomaria* in 78–86 sites depending on species (Table 4). Between the studied forms of *C. sphagnicola,* five fixed nucleotide differences in *trn*H-*psb*A and three in the *rpo*C1 region were found.

Discussion

Complementing classical taxonomy of liverworts, based on morphology, with characters inferred from genetic markers has revealed numerous examples of earlier unrecognized species, suggesting that the number of liverwort species is still underestimated (e.g., Shaw 2001; Bączkiewicz et al. 2008; Heinrichs et al. 2009, 2010). Results of the present study show that *C. sphagnicola* is another example of a liverwort species that was too broadly defined on the basis of morphological criteria.

Our results support the hypothesis that the two examined forms of *C. sphagnicola* represent genetically distinct species. Congruence of isozyme, flow cytometry and DNA sequence data strongly suggests that the two forms of *C. sphagnicola* should be recognized as distinct species. The genetic distance between these two forms ($D = 0.472$) estimated on the basis of ten isozyme loci is comparable, or

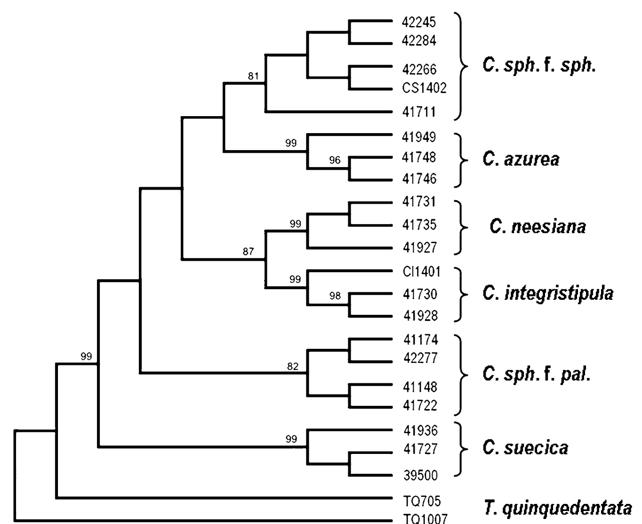

Fig. 5 Strict consensus of 191 most parsimonious trees based on combined analysis of *rpo*C1 and *trn*H-*psb*A sequences. Bootstrap values above 70% are given above branches

even higher than the values found by Boisselier-Dubayle and Bischler (1998) between haploid and diploid cytotypes of *Corsinia coriandrina* (Sprengel) Lindberg ($I = 0.667$) or between liverwort of an allopolyploid origin, *Porella baueri* (Schiffn.) C. Jens., and its two parental species: *P. platyphylla* L.Pfeiff. ($I = 0.802$) and *P. cordeana* (Hüb.)

Table 4 Fixed nucleotide differences among studied *Calypogeia* species (*trn*H-*psb*A above and *rpo*C1 below)

	T. quinquedentata	*C. integristipula*	*C. suecica*	*C. sph.* f. *sphagnicola*	*C. neesiana*	*C. sph.* f. *paludosa*	*C. azurea*
T. quinquedentata	–	47	46	47	48	45	48
C. integristipula	86	–	5	3	3	6	5
C. suecica	80	18	–	4	6	7	4
C. sphagnicola f. sphagnicola	79	16	7	–	4	**5**	4
C. neesiana	85	19	21	16	–	7	6
C. sphagnicola f. paludosa	78	15	6	**3**	17	–	5
C. azurea	80	18	9	2	18	5	–

Bold indicates the number of differences between the studied form of *C. sphagnicola*

Moore ($I = 0.738$; Boisselier-Dubayle et al. 1998a); the above values of genetic identities are the equivalent of genetic distance 0.405, 0.221 and 0.304, respectively (according to formula $D = -\ln I$).

Fixed alleles diagnostic for each form were found in seven loci, among others, in *Got*, *Est* and *Gdh*, which had already been recognized as good diagnostic markers for *Calypogeia* species (Buczkowska et al. 2004). These loci have been acknowledged as diagnostic markers in many studies (e.g., Boisselier-Dubayle et al. 1998a; Bączkiewicz and Buczkowska 2005). Isozyme patterns of *Got* and *Est*-1 observed in the present study in the form *C. sphagnicola* f. *paludosa* were the same as previously detected for *C. sphagnicola* (Buczkowska et al. 2004), whereas isozyme phenotypes revealed in the form *C. sphagnicola* f. *sphagnicola* have not been found so far in any of the formerly studied *Calypogeia* species. Unfortunately, a previous isozyme study did not reveal genetic diversity of *C. sphagnicola*, because all samples originated from mountain populations, and thus only one form (*C. sphagnicola* f. *paludosa*) was analyzed. The present study confirms the allopatric distribution of both forms in Poland as noted by Szweykowski (2006). Plants with genotypes characteristic of *C. sphagnicola* f. *sphagnicola* occur exclusively in the northern part of the country on raised peat bogs and peat mats in the littoral zone of dystrophic lakes. Plants classified as *C. sphagnicola* f. *paludosa* were found only in southern Poland (in the mountains). They are very rare at lower attitudes, growing on peat bogs, but frequent in the subalpine zone, growing on *Sphagnum-Polytrichum* hummocks in the upper part of north-facing slopes. No mixed samples occurred in any population.

Strong evidence for genetic distinctness of the studied forms of *C. sphagnicola* was provided by the ploidy level. Nuclear DNA content of *C. sphagnicola* f. *sphagnicola* was about two times lower compared to *C. sphagnicola* f. *paludosa*, whose chromosome number was previously determined as $n = 18$. *C. sphagnicola* f. *sphagnicola* had a similar DNA content to *C. suecica*, a species with the haploid ($n = 9$) chromosome number (Buczkowska et al. 2004). This result indicates that the haploid condition separates *C. sphagnicola* f. *sphagnicola* from the diploid *C. sphagnicola* f. *paludosa*. The different ploidy level leads to reproductive isolation; thus, the haploid and diploid cytotypes should be treated as distinct species. The ploidy level of both *C. sphagnicola* forms was in an agreement with their isozyme pattern. In the diploid *C. sphagnicola* f. *paludosa* at four (*Got*, *Gdh*, *Pgd* and *Pgm*) of the ten studied loci, fixed heterozygosity was observed, whereas in the typical form all isozyme loci had single-banded phenotypes. The results confirmed the allopolyploid origin of *C. sphagnicola* f. *paludosa* was initially suggested only on the basis of the *Got* isozyme (Buczkowska et al. 2004). A homogeneous pattern of fixed heterozygosity in all analyzed populations suggests that the allopolyploid species most probably has arisen once, in one hybridization event. Alleles in some isozyme loci indicated that *C. sphagnicola* f. *sphagnicola* could be one of the supposed parental species; however, not all alleles of the haploid form correspond to the alleles with the fixed heterozygosity of the polyploid. Except for *Me*, whose multi-banded pattern is difficult to interpret genetically, one allele (*Got* allele 1) is clearly in conflict with the hypothesis of *C. sphagnicola* f. *sphagnicola* as the supposed parental species. The second progenitor of the diploid form of *C. sphagnicola* could not be indicated at the moment because not all European species (*C. arguta* and *C. azorica*) have been examined yet. Moreover, present-day geographic distribution of the progenitor and derivatives species could not overlap, as was shown in the case of another polyploid liverwort, *Reboulia hemisphaerica* (Boisselier-Dubayle et al. 1998b). Thus, answering the question about the progenitors of the allopolyploid form of *C. sphagnicola* requires further studies of species from a wider range of geographic distribution since the genus comprises about 90 described species (Schuster 1969). An additional argument supporting the hypothesis that the haploid form could be one of the putative parental species comes from the results of SCAR

markers (sequence characterized amplified region), which were previously developed for *Calypogeia* species on the basis of the ISSR method (Buczkowska and Dabert 2011). Two products of different sizes were amplified by the markers using the Cal02 primer pair in *C. sphagnicola* f. *paludosa* (600 and 560 bp), whereas in *C. sphagnicola* f. *sphagnicola* only one product of 560 bp was present. Polyploidization is considered one of the most important mechanisms of species speciation in plants—between 30 and 70% of angiosperm, 4.6–10.3% of liverworts and 6.4–18.6% of moss species have a polyploid origin (Stebbins 1971; Såstad 2005). Isozyme and molecular data provide evidence that allopolyploid species might be more frequent in liverworts than was previously presumed (e.g., Boisselier-Dubayle and Bischler 1998; Boisselier-Dubayle et al. 1998a, b; Fiedorow et al. 2001; Buczkowska et al. 2004). Data concerning the ploidy level in bryophytes are still incomplete as chromosome counting in some species is very difficult. Flow cytometry has proven to be helpful for the determination of the ploidy level in bryophyte species (Śliwińska et al. 2000; Ricca et al. 2008; Temsch et al. 2010).

Further evidence supporting species rank for the present-day forms of *C. sphagnicola* arose from a phylogenetic analysis based on chloroplast *rpo*C1 and *trn*H-*psb*A regions. *C. sphagnicola* f. *sphagnicola* is clearly separated from *C. sphagnicola* f. *paludosa* with high bootstrap support. Moreover, both forms are distinct from other examined species of the genus *Calypogeia*. Samples of *C. sphagnicola* were grouped into two distinct clades, which conform with the results inferred from isozyme markers. The phylogenetic analysis revealed a closer relationship between *C. sphagnicola* f. *sphagnicola* and *C. azurea* than between the present-day forms of *C. sphagnicola*. Noteworthily, *C. sphagnicola* f. *paludosa* formed a separate, well-supported clade. It suggests that the chloroplasts of *C. sphagnicola* f. *paludosa* were not inherited from *C. sphagnicola* f. *sphagnicola*, but rather from the other unknown parental taxon. The number of fixed nucleotide differences between the forms in the analyzed sequences is comparable to the number of differences between each of the forms and *C. azurea* or *C. integristipula*, which are well-known and accepted species. In the noncoding region *trn*H-*psb*A, five fixed differences were detected between the studied forms; the same number of mutations was noted between the pairs of unequivocally different species: *C. integristipula* and *C. azurea*, *C. integristipula* and *C. suecica*, and *C. azurea* and *C. suecica*. The similar number of nucleotide differences in this chloroplast region was reported by Sawicki et al. (2010) for moss species from the subgenus *Phaneroporum* Delogne and the genus *Zygodon* Hook. & Taylor or by Hedderson and Zander (2007) for the species *Triquetrella* Müll.Hal. Fixed nucleotide differences between the studied forms were also detected in the coding region *rpo*C1; however, the number of mutations was lower than between other pairs of accepted species of *Calypogeia*. The chloroplast *rpo*C1 and *trn*H-*psb*A sequences are commonly applied in molecular taxonomy and phylogenetic studies of bryophytes (Wilson et al. 2007). These chloroplast regions are proposed as a potential candidate for plastid barcoding loci in mosses and liverworts (Liu et al. 2010). According to Hollingsworth et al. (2009), *rpo*C1 and *trnH-psbA* were the most universal loci and amplified well across all studied species of the liverwort genus *Asterella* s.l. However, the locus *rpo*C1 has relatively low levels of species discrimination. Our results showed that both regions also worked well in the *Calypogeia* genus.

Two clades of *C. sphagnicola* identified in our study correspond to morphologically described forms of the species (Schuster 1969). The clade identified as *C. sphagnicola* f. *sphagnicola* comprises small plants with abundant gemmiparous shoots, spherical and undivided, or, very rarely, is composed of 2–4 segment oil bodies (5–6 μm in diameter). Plants from a clade of *C. sphagnicola* f. *paludosa* are more robust and always without gemmae. They also are bigger and usually divided into several (5–9) segment oil bodies (4–6 × 4–9 μm). Biometrical analysis based on genetically determined material confirmed morphological differences, and the forms differ statistically significantly in respect to 34 quantitative morphological traits, e.g., the width and length of leaves, width of underleaves, length of stem internodes (between two adjacent underleaves) as well as cell size of underleaf and stem (Buczkowska et al. 2009). However, the quantitative traits are less reliable; therefore, species delimitation on the morphological disparities only may be difficult. The best diagnostic features are oil body characters and the presence or absence of gemmiparous shoots. Plants in the present study regarded as *C. sphagnicola* f. *sphagnicola* morphologically correspond to the syntype specimen of *C. sphagnicola* from the UPS Herbarium (B-511887, Sweden, in prov. Skåne, ad Hörby, in sphagneto repens, J. Persson, June 1901) cited by Arnell (1902) in the protolog of the species. Plants of the syntype were small (width of shoots ranges from 990 to 1,170 μm, on average 1,070 μm) with gemmiparous shoots; broadly ellipsoidal gemmae were 2-celled (16 × 23 μm). We are not yet able to understand the morphological variation of *C. paludosa*; the plants investigated so far do not exactly match the morphology of Warnstorf (1906) type material (S Herbarium: B20850—Berlin, Moor sűdlich an der Chaussee Erkner-Gottesbrűck zwischen Sphagnum, E. Schultz, 23.01.1906 and B20841—Kl. Pankow am Cressinsee zwischen Sphagnum, Jaap, 04.08.1898). Plants of the type specimens differ from the studied diploid form in underleaf shape,

which had a broadly rounded sinus and often angulation on the lobes' external margins. Extension of sampling and inclusion of plants from other parts of the range are necessary to arrive at a final conclusion on the identity of the diploid entity. Therefore, the name of the diploid form is only provisional, based on Schuster's (1969) determination key.

Conclusion

The two present-day forms of *C. sphagnicola* are reproductively isolated because of differences in ploidy level and allopatric distribution: *C. sphagnicola* f. *sphagnicola* is haploid, whereas *C. sphagnicola* f. *paludosa* is a diploid form. The haploid form corresponds to the syntype specimen of *C. sphagnicola*, but the name of the diploid form was used only temporarily. A fixed heterozygous pattern of four isozyme loci indicated an allopolyploidal origin of the diploid form with the haploid form *C. sphagnicola* f. *sphagnicola* as one of the putative progenitors. The phylogenetic analysis based on chloroplast *rpo*C1 and *trn*H-*psb*A regions supports species rank for the present-day forms and suggests that the chloroplasts of *C. sphagnicola* f. *paludosa* were not inherited from *C. sphagnicola* f. *sphagnicola* but rather from the other unknown parental taxon.

Acknowledgments This work was financially supported by grant no. N303 344235 from the Polish Ministry of Science and Higher Education. We thank the Directors of the Tatra National Park and the Bieszczady National Park for their support provided during the fieldwork, and Patrycja Gonera for help in the laboratory. The authors thank Jochen Heinrichs and two anonymous reviewers for helpful comments on an earlier draft. We also wish to thank the curators of the herbaria: UPS, LD and S for the loan of specimens.

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