Biodiv. Res. Conserv. 32: 1-8, 2013

BRC
www.brc.amu.edu.pl

DOI 10.2478/biorc-2013-0016

Submitted 17.08.2013, Accepted 18.11.2013

Chloroplast DNA sequences confirmed genetic divergence within *Calypogeia muelleriana* (Calypogeiaceae, Marchantiophyta)

Katarzyna Buczkowska[1*], Patrycja Gonera[1], Alina Bączkiewicz[1], Stanisław Rosadziński[2] & Mariola Rabska[1]

[1]Department of Genetics, Faculty of Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland
[2]Department of Plant Ecology and Environmental Protection, Faculty of Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland
* corresponding author (e-mail: androsac@amu.edu.pl)

Abstract: Nine species of the genus *Calypogeia* Raddi are currently known from Europe: *C. azurea*, *C. integristipula*, *C. neesiana*, *C. suecica*, *C. muelleriana*, *C. sphagnicola*, *C. fissa*, *C. arguta*, and *C. azorica*. Recently, another species, morphologically resembling *C. muelleriana* but genetically distinct from it, was detected using isozyme markers. In the present study, relationships between the newly detected species (*C. sp. nov.*) and typical *C. muelleriana* were analyzed using the DNA sequences data of three regions from the chloroplast genome: introns of *trn*G and *trn*L genes and intergenic spacer *trn*H-*psb*A. *Calypogeia sp. nov.* differs from *C. muelleriana* s. str. (typical form) in all examined chloroplast regions. It differs as well from *C. azurea*, which was used as a reference species. The number of fixed nucleotide differences between *C. muelleriana* s. str. and *C. sp. nov.* is almost the same as between *C. muelleriana* s. str. and *C. azurea*. The results of the present study suggest a closer affinity of *C. sp. nov.* to *C. azurea* than to *C. muelleriana* s. str. in Europe, *C. muelleriana* s. str. was noted in Poland, Germany, Holland, United Kingdom and Azores. Samples determined as *C. sp. nov.*, besides Poland, were so far detected also in North America.

Key words: Liverworts, *Calypogeia*, chloroplast genome, *trn*G, *trn*L, *trn*H-*psb*A

1. Introduction

The genus *Calypogeia* Raddi belonging to the family Calypogeiaceae is distributed worldwide, primarily in subtropical to tropical climates (Bischler 1963; Schuster 1969). Nine species of this genus are known from Europe and Macaronesia: *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. – endemic for of the Azores (Söderström *et al.* 2002, 2007).

Simple morphological structure of gametophyte with the limited number of diagnostic characters is the main reason of taxonomic problems in liverworts (Szweykowski 1984). Nowadays, many bryologists are of an opinion that some species, described in old works, remain to be rediscovered and characterized again (Bischler & Boisselier-Dubayle 2000; Schumacker & Váňa 2005). This opinion concerns also the genus *Calypogeia*, which is considered by many taxonomists to be one of the most difficult groups of liverworts (Schuster 1969; Szweykowski 2006). Within the genus, new species are still described in different parts of distribution, e.g., *C. khasiana* in India (Singh & Nath 2007). Likewise, the number of *Calypogeia* species in Europe is probably higher than presently known. The isozyme studies of European species of the genus revealed the presence of plants genetically distinct from the well-known and accepted species, which probably represent unrecognized taxa. Three genetically distinct taxa were detected within the complexes of *C. sphagnicola* (Buczkowska *et al.* 2012a, 2012b) and *Calypogeia fissa* (Buczkowska 2004a; Buczkowska *et al.* 2012c). Differences in oil body characters and some morphological traits were also found between some of these groups (Buczkowska *et al.* 2011).

C. muelleriana exhibits a marked morphological variation within its geographic range. According to Schuster (1969), this species shows a different pattern of variability in North America and Europe. In North America two subspecies: widespread *C. muelleriana* subsp. *muelleriana* and rare, locally distributed *C. muelleriana* subsp. *blomquistii,* were distinguished (Schuster 1969). Moreover, extraordinary polymorphism of the first subspecies was pointed out by Schuster (1969). However, the author emphasized that this polymorphism is mainly environmentally induced. *C. muelleriana* also causes taxonomic problems in the eastern part of its geographic range, since difficulties with the delimitation of *C. muelleriana* from *C. azurea* and *C. sphagnicola* were emphasized by Schlyakov (1979). Damsholt (2002) recognized two varieties: *C. muelleriana* var. *muelleriana* and *C. muelleriana* var. *erecta*. Similarly, morphological variation of *C. muelleriana* was reported from Poland by Szweykowski (2006). The author detected two forms: typical and atypical. The typical form is widespread in south-western part of Poland, whereas the atypical form occurs in the northern part of lowlands. Isozyme and molecular markers have proved that these two forms are genetically different (Buczkowska & Bączkiewicz 2011; Buczkowska & Dabert 2011). Plants classified as the typical form exactly correspond to the type specimen of *C. muelleriana* (Buczkowska 2004b). The atypical form represents a new taxon tentatively named as *C. sp. nov.* (Buczkowska & Dabert 2011). The new taxon morphologically resembles *C. muelleriana* s. str., but it differs in oil body characteristics and underleaf shape (Buczkowska 2010). However, the final taxonomic treatment of the newly distinguished taxon demands future studies.

Unfortunately, isozyme markers and oil body features are useless in the case of herbarium material. Therefore, other diagnostic markers for the taxa, suitable also for dried specimens, would be helpful. Recently, commonly applied strategy in taxonomy is used of different DNA markers e.g. ISSR, ISJ, RAPD, AFLP or SCAR (Hassel & Gunnarsson 2003; Sawicki & Szczecińska 2007; Celka *et al.* 2010, 2012; Grądzielewska 2011) and DNA sequencing (e.g. Szweykowska-Kulińska *et al.* 2002; Pacak & Szweykowska-Kulińska 2003; Shaw *et al.* 2003; Taberlet *et al.* 2006; Górniak *et al.* 2006; Wickett & Goffinet 2008; Fuselier *et al.* 2009; Heinrichs *et al.* 2010; Kreier *et al.* 2010; Plášek *et al.* 2011; Krawczyk *et al.* 2013).

In the present study, we use DNA sequences of three regions from chloroplast genome (introns of *trn*G and *trn*L genes and intergenic spacer *trn*H-*psb*A) to assess the level of genetic diversity between *C. muelleriana* s. str. and the recently detected taxon *C. sp. nov.* in order to provide additional arguments for distinguishing the new taxon as a separate species.

2. Material and methods

2.1. Plant material

In general, 26 samples of the *C. muelleriana* complex were examined: 16 of *C. muelleriana* s. str. (the typical form) and 10 of the new taxon – *C. sp. nov.* (Table 1). Plants were initially determined on the basis of morphological traits and oil body characters according to Müller (1951-1958), Schuster (1969) and own observations (Buczkowska 2010). Each sample was divided into 2 parts: one was deposited as a voucher in the POZW Herbarium, while the other was used for isozyme analyses and a greenhouse culture. Plants classified as the typical form of *C. muelleriana* exactly correspond to its type specimen (Buczkowska 2004b).

Samples identified on the basis of isozyme pattern (Buczkowska & Bączkiewicz 2011) were used for DNA extraction in the first part of the study: 10 samples of typical *C. muelleriana* and 8 samples of *C. sp. nov.* (Table 1). 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). In the second stage of research, DNA was extracted from herbarium samples. Moreover, 3 samples of *C. azurea* and two samples of *Tritomaria quinquedentata* (Huds.) H. Buch were used as an outgroup in DNA analysis.

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 and a standard CTAB procedure (Murray & Thompson 1980) downscaled to fit 1.5 ml eppendorf tubes was used for DNA extraction. 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.

Explanations: [1] – sequences new in the present study are marked in bold, [2] – the new species detected within the *C. muelleriana* complex (Buczkowska & Dabert (2011). Collectors, AB – Alina Bączkiewicz, AS-V – Alfons Schäfer-Verwimp, BKA – Barbara K. Andreas, DAC – Des A. Callaghan, DGL – David G. Long, HG – H. Greven, HB – Hanna Barczak, JS – Jerzy Szweykowski, KB – Katarzyna Buczkowska, RG – R. Gradstein, SR – Stanisław Rosadziński. Herbaria, S-V – Herb. Schäfer-Verwimp, POZW – Herbarium of Adam Mickiewicz University, DC – Herb D. A. Callaghan, DGL – Herb. D. G. Long

Table 1. Localities of the samples of the *C. muelleriana* complex used for DNA studies and GenBank accession numbers

Locality	Collector	Herbarium No.	Accession number[1]		
			*trn*L	*trn*G	*trn*H-*psb*A
C. muelleriana s. str. (typical form)					
NE Poland, Warmińsko-Mazurskie Province, Lake Godle near Ełk	KB, AB	POZW 41708	**KF371552**	**KF371604**	**KF371578**
NW Poland, Pomorskie Province, Lake Orle near Miastko	KA, AB	POZW 41346	**KF371550**	**KF371602**	**KF371576**
		POZW 41182	**KF371551**	**KF371603**	**KF371577**
NW Poland, Pomorskie Province, Lake Lubygość near Kartuzy	KB, AB	POZW 42214	**KF371553**	**KF371605**	**KF371579**
		POZW 42218	**KF371555**	**KF371607**	**KF371581**
NW Poland, Pomorskie Province, Lake Głęboczko near Bytów	KB, AB	POZW 42222	**KF371554**	**KF371606**	**KF371580**
W Poland, Lubuskie Province, Nabłoto foresty	SR, KB	POZW 42316	**KF371559**	**KF371611**	**KF371585**
W Poland, Lubuskie Province, Nowogród Bobrzański	SR, KB	POZW 42314	**KF371557**	**KF371609**	**KF371583**
W Poland, Lubuskie Province, Starosiedle foresty	SR, KB	POZW 42323	**KF371556**	**KF371608**	**KF371582**
W Poland, Lubuskie Province, Biecz forestry	SR, KB	POZW 42318	**KF371558**	**KF371610**	**KF371584**
Holland, Maarssen, 2 km North of Utrecht, Molenpolder, on wet peat	JS, RG, HG	POZW 34160	**KF371563**	**KF371615**	**KF371589**
Germany, Baden-Württemberg, western Bodenseegebiet, Kreis Konstanz, Schiener-Berg-Nordabhang above Bankholzen, 630 m a.s.l.	AS-V	S-V 28577	**KF371560**	**KF371612**	**KF371586**
Germany, Baden-Württemberg, Bodenseekreis, Südliches Oberschwaben: Heiligenberg, 650 m a.s.l.	AS-V	S-V 31465	**KF371561**	**KF371613**	**KF371587**
Germany, Baden-Württemberg, western Bodenseegebiet, Kreis Konstanz, Schiener-Berg-Nordabhang above Bankholzen, 630 m a.s.l.	AS-V	S-V 28576	**KF371562**	**KF371614**	**KF371588**
Azores Archipelago, São Miguel Island, east part of island, Planalto dos Graminhais west of Pico do Vara, 940 m a.s.l.	AS-V	S-V 29472	**KF371564**	**KF371616**	**KF371590**
United Kingom, South Lancashire, in damp sandstone crevice of disused quarry wall	DAC	DC 1421	**KF371565**	**KF371617**	**KF371591**
United Kingdom: Scotland, Lanarkshire, Low Moss	DGL	DGL 29844	AY453769	-	-
C. muelleriana – atypical form = *C. sp. nov.*[2]					
NE Poland, Warmińsko-Mazurskie Province, Lake Godle near Ełk	KB, AB	POZW 41706	**KF371571**	**KF371623**	**KF371597**
		POZW 41707	**KF371570**	**KF371622**	**KF371596**
NW Poland, Pomorskie Province, Lake Orle near Miastko	KB, AB	POZW 42211	**KF371566**	**KF371618**	**KF371592**
		POZW 41151	**KF371567**	**KF371619**	**KF371593**
NW Poland, Pomorskie Province, Lake Lubygość near Kartuzy	KB, AB	POZW 42220	**KF371568**	**KF371620**	**KF371594**
		POZW 42216	**KF371569**	**KF371621**	**KF371595**
NW Poland, Pomorskie Province, Lake Smołowe near Miastko	KB, AB	POZW 42285a	**KF371573**	**KF371625**	**KF371599**
		POZW 42285b	**KF371574**	**KF371626**	**KF371600**
NE Poland, Podlaskie Province, Wigierski National Park, Lake Sucharek near Suwałki	JS, KB, HB	POZW 35596	**KF371572**	**KF371624**	**KF371598**
North America, Tunk Lake, west of Cherryfield, woods on S side of road, 60 1183 m a.s.l.	BKA	dhl825	**KF371575**	**KF371627**	**KF371601**
North America, USA, Davis 130 (DUKE)	EC Davis	DUKE	AY608121	AY608169	-
C. azurea					
S Poland, Tatra Mts, Sucha Woda Valley, Psia Trawka meadow, 1183 m a.s.l.	KB, AB	POZW 41746	JQ658804	JQ658787	JF776843
SE Poland, Bieszczady Mts, W slope of Mt Rozsypaniec Wołosacki, 1215 m a.s.l.	KB	POZW 41949	JQ658803	JQ658786	JF776841
NE Poland, Jez. Godle lake near Ełk	KB, AB	POZW 41748	JQ658802	JQ658785	JF776842
Tritomaria quinquedentata					
S Poland, Tatra Mts, Jaworzynka Valley, 1338 m a.s.l. (TQ705)	KB, AB	POZW 41479	JQ658805	JQ658788	JF776850
S Poland, Tatra Mts, Miętusia Valley, 1037 m a.s.l. (TQ1007)	KB, AB	POZW 41204	JQ658806	JQ658789	JF776851

Primers used for the amplification and sequencing of *trn*H-*psb*A spacer (psbAF and trnHR) were according to Sang *et al.* (1997) and for the introns of *trn*G and *trn*L genes (primers A and B) from Pacak & Szweykowska-Kulińska (2003). PCR amplification was carried out as described in the previous publication (Buczkowska *et al.* 2012a). Purified PCR products of the studied chloroplast regions 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).

2.3. Data analysis

Chromatograms of DNA sequences were edited and assembled using Sequencher 4.5 (Genecodes Inc.). Contigs were aligned manually with MEGA 5.2 (Tamura *et al.* 2011). Regions of ambiguous alignment and incomplete data (i.e., at the beginning and end of sequences) were excluded from the analyses and lacking sequences were coded as missing. Minimum Evolution (ME) analysis was conducted using MEGA version 5.2. The pairwise distances were estimated with the Kimura 2-parameter method. Initial trees were generated using the neighbor-joining (NJ) method. The ME tree was searched using the Close Neighbor Interchange (CNI) algorithm at a search level of 2. Statistical significance of clades within inferred trees was evaluated using the bootstrap method (Felsenstein 1985) with 2000 replicates. Incongruence between the *trn*H-*psb*A, *trn*G and *trn*L data was assessed by comparing clade support on the consensus ME tree. 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).

3. Results

Seventy-eight new sequences were generated for this study (Table 1). Eighteen sequences used in this study were obtained from the previous examination (GenBank), as indicated in Table 1. The lengths of analyzed sequences are given in Table 2. The new taxon detected within the *C. muelleriana* complex – *C. sp. nov.*, differs from *C. muelleriana* s. str. (typical form) in all examined chloroplast regions. It differs as well from *C. azurea*. The number of fixed nucleotide differences between *C. muelleriana* s. str. and *C. sp. nov.* is almost the same as between *C. muelleriana* s. str. and *C. azurea*. The two pairs of taxa differ in respect of 34 and 33 substitutions and 3 insertions/deletion, respectively (Table 3). However, sequences of all analyzed regions were very similar in pair of *C. sp. nov.* and *C. azurea* (Table 3). For all pairs of taxa, the higher number of substitutions was found in the introns of *trn*G and *trn*L than in the intergenic spacer *trn*H-*pab*A.

The tree based on ME analysis revealed two well-supported clades (Fig. 1). According to the molecular data, *C. muelleriana* s. str. and *C. sp. nov.* form two different well-supported groups (Fig. 1). The results showed that *C. sp. nov.* is closer related to *C. azurea* than to *C. muelleriana* s. str. Two sequences from GenBank determined as *C. muelleriana* were compared with the sequences of the *C. muelleriana* complex obtained in this study. The sequence of the *trn*L gene intron from United Kingdom (AY453769) is the same as in *C.*

Table 2. The length (in bp) of *trn*G and *trn*L gene introns and *trn*H-*psb*A intergenic spacer of the *C. muelleriana* complex, *C. azurea* and *T. quinquedentata*

Taxon	*trn*G	*trn*L	*trn*H-*psb*A
C. muelleriana s. str.	657	296	233
C. sp. nov.[1]	658	296	234
C. azurea	658	296	225
T. quinquedentata	625	290	222

Explanation: [1] – the new species detected within the *C. muelleriana* complex (Buczkowska & Dabert (2011)

Table 3. Fixed nucleotide differences in chloroplast regions among the studied taxa of *C. muelleriana* complex and *C. azurea*

Taxa pair	*trn*G	*trn*L	*trn*H-*psb*A	Total[2]
C. muelleriana s. str. – *C. sp. nov.*[1]	17s; 2i	10s	7s; 1i	34s; 3i
C. muelleriana s. str. – *C. azurea*	17s; 2i	11s	5s; 1i	33s; 3i
C. sp. nov.[1] – *C. azurea*	3s	1s	2s	6s

Explanations: [1] – the new species detected within the *C. muelleriana* complex (Buczkowska & Dabert (2011), [2] – s – substitutions, i – insertions/deletions

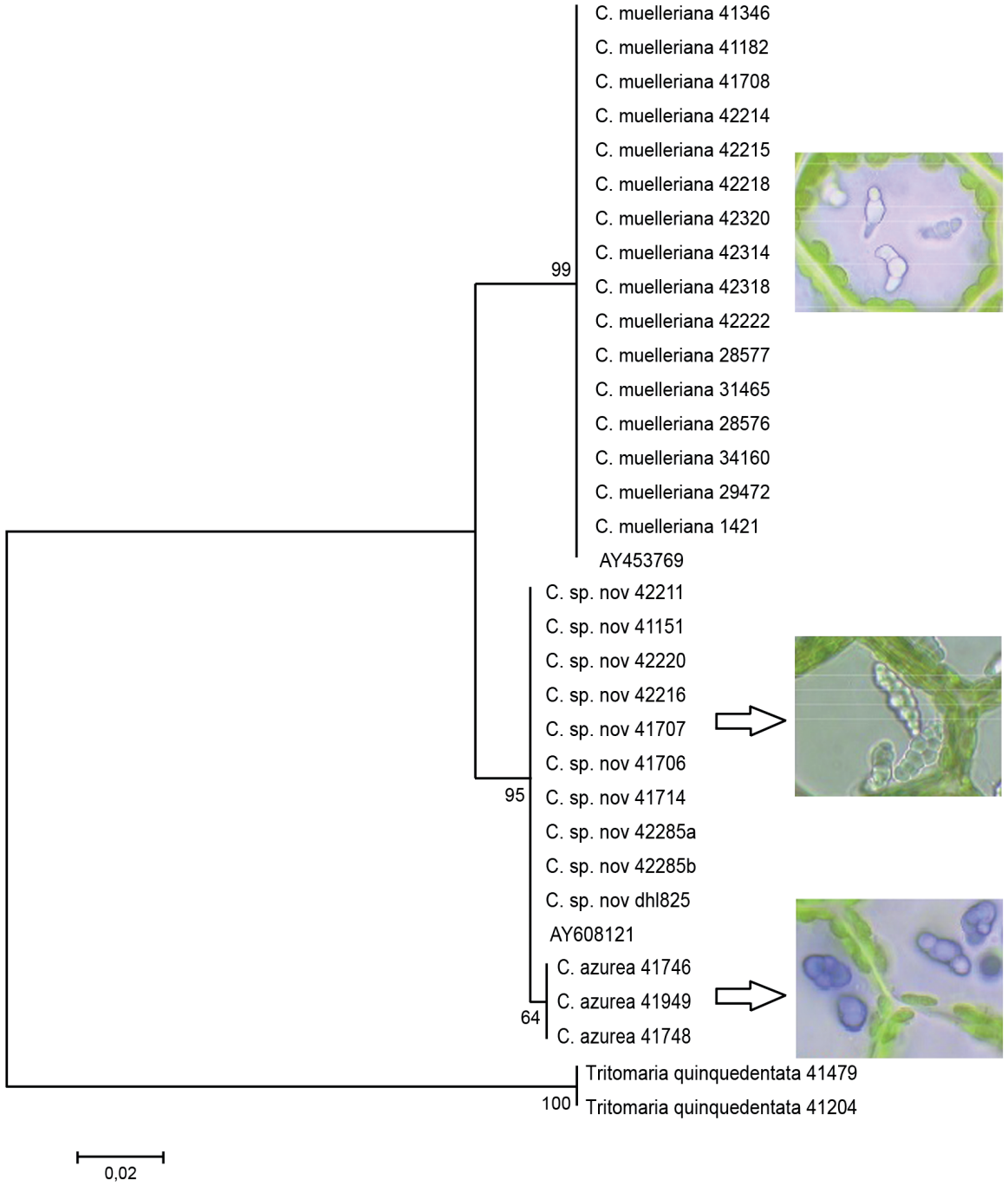

Fig. 1. Phylogram resulting from Minimum Evolution (ME) analysis based on combined data of all studied chloroplast sequences (*trn*G, *trn*L and *trn*H-*psb*A). Bootstrap values are given above branches

muelleriana s. str., whereas the sequences of introns of *trn*L and *trn*G genes from USA (AY608121 and AY608169) belong to the new taxon – *C. sp. nov.* (Fig. 1).

4. Discussion

Isozyme and molecular markers revealed that two morphological forms of *C. muelleriana* (typical and atypical) noted by Szweykowski (2006) are in fact two genetically different taxa. The typical form corresponds to the type specimen of *C. muelleriana* (Buczkowska 2004b; Buczkowska & Bączkiewicz 2011), whereas the atypical form is a new taxon and was tentatively named as *C. sp. nov.* (Buczkowska & Dabert 2011). The two taxa differ also in oil body characters and some morphological traits (Buczkowska 2010). Despite the fact that in Poland both species occur sympatrically and sometimes grow in mixed patches, recombinant gametophytes were not observed. This indicates that

the two species are genetically isolated (Buczkowska & Bączkiewicz 2011).

The distinctness of these two taxa was further supported by a sequence analysis in the present study. The number of fixed nucleotide differences between *C. muelleriana* s. str. and *C. sp. nov.* as well as between *C. muelleriana* s. str. and *C. azurea* was over 5 times higher than between *C. sp. nov.* and *C. azurea*. The highest number of the fixed nucleotide differences between the examined taxa occurred in the intron of *trn*G gene, and was comparable to the number of differences detected for the pair of species within *C. sphagnicola* complex (Buczkowska *et al.* 2012b). The studied chloroplast regions (*trn*G, *trn*L and *trn*H-*psb*A) are commonly applied in molecular taxonomy of bryophytes (Szweykowska-Kulińska *et al.* 2002; Taberlet *et al.* 2006; Wilson *et al.* 2007; Wickett & Goffinet 2008; Fuselier *et al.* 2009; Heinrichs *et al.* 2010; Kreier *et al.* 2010; Sawicki *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 the sequences of these chloroplast regions three forms of the *C. sphagnicola* complex were earlier proved to be distinct species (Buczkowska *et al.* 2012a, 2012b). Differences detected in the introns of *trn*G and *trn*L genes were comparable with differences reported for other liverwort species (Szweykowska-Kulińska *et al.* 2002, Pacak & Szweykowska-Kulińska 2003; Jankowiak & Szweykowska-Kulińska 2004). In the *trn*H-*psb*A spacer, the same number of mutations was noted between the pairs of unequivocally different species: *C. suecica* and *C. neesiana* or *C. neesiana* and *C. azurea* (Buczkowska *et al.* 2012a) and for the species of the genus *Orthophyllum* (Sawicki *et al.* 2010).

The results of the present study suggest a closer affinity of *C. sp. nov.* to *C. azurea* than to *C. muelleriana* s. str. The sequences derived from *C. azurea* and *C. sp. nov.* differ only at 6 of the 1188 analyzed nucleotide sites. However, the taxa differ morphologically, especially in terms of oil bodies - a very important taxonomic feature in *Calypogeia* (Buczkowska 2004b, 2010). In *C. sp. nov.* oil bodies are colorless, while in *C. azurea* are blue (Fig. 1). Both species of the *C. muelleriana* complex as well as *C. azurea* have the allopoliploid origin (Buczkowska *et al.* 2004; Buczkowska & Bączkiewicz 2011). It suggests that *C. sp. nov.* could inherited their chloroplast genome from the same parental species as *C. azurea*, whereas *C. muelleriana* s. str. inherited the chloroplasts from the other unknown parent.

Isozyme markers and other molecular and morphological data indicate that *C. muelleriana* s. str. and *C. sp. nov.* represent two different taxa. Morphological differences give possibility to describe the new detected taxon as a separate species. However, a large herbarium material needs to be examined in detail to establish finally the taxonomic status of the analyzed taxon. Many species were so far described in the *Calypogeia* genus, some of them were reduced to synonyms of particular species (Müller 1951-1958; Schuster 1969), therefore it has to be checked if any of the several published names matches the discussed above taxon. Both the DNA sequences received from the material previously determined by isozyme markers and oil body characters can help this search. They can be used as reference sequences for identification of those herbarium samples in which oil bodies were not observed, and also help in verification of the type specimens.

Based on the results of the present study, some preliminary conclusions regarding geographic distribution of these two species can be drawn. In Poland, *C. muelleriana* s. str. occurs mainly in the south-western part of the country, where it is widespread. To date, DNA sequences confirmed the presence of this species in Germany, Holland, United Kingdom and Azores. Samples determined as *C. sp. nov.*, besides Poland, where it is rare, were so far detected also in North America. According to Schuster (1969), *C. muelleriana* in North America shows a wide range of phenotypes that in majority are probably environmentally induced. However, the genetic studies of the genus *Calypogeia* suggest that one of the reasons of the high morphological variation of *C. muelleriana* reported by Schuster (1969) may be the presence of an unrecognized so far species – *C. sp. nov.*, which morphologically resembles *C. muelleriana*. The DNA sequences revealed that some specimens from USA determined as *C. muelleriana* in fact belong to *C. sp. nov.* e.g. GenBank accesion number AY608121. 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 species distinguished within the *C. muelleriana* complex.

Acknowledgements. This work was financially supported by the grant no. N303 344235 from the Polish Ministry of Science and Higher Education. We would like to thank D. A. Callaghan, A. Schäfer-Verwimp and Barbara K. Andreas for providing plant material.

References

Bischler H. 1963. The genus *Calypogeia* Raddi in Central and South America. I-III. Candollea 18: 19-128.

Bischler H. & Boisselier-Dubayle M. C. 2000. New approaches to the systematics of liverworts. Nova Hedwigia 70: 37-44.

Buczkowska K. 2004a. Genetic differentiation of *Calypogeia fissa* Raddi (Hepaticae Jungermanniales) in Poland. Pl. Syst. Evol. 247: 187-201.

Buczkowska K. 2004b. The genus *Calypogeia* Raddi (Hepaticae Jungermnniales) in Poland biometrical analysis of morphological and anatomical variation. Nova Hedwigia 78: 121-146.

Buczkowska K. 2010. Morphological differentiation of *Calypogeia muelleriana* (Jungermanniales, Hepaticae) in Poland. Biodiv. Res. Conserv. 17: 23-32.

Buczkowska K., Adamczak M., Bączkiewicz A., Chudzińska E. & Wachowiak W. 2006. *In vitro* propagation of cryptic species of *Aneura pinguis* (Metzgeriales, Hepaticae). Cryptogamie Bryol. 27: 241-251.

Buczkowska K. & Bączkiewicz A. 2011. New taxon of the genus *Calypogeia* (Jungermanniales, Hepaticae) in Poland. Acta Soc. Bot. Pol. 80: 327-333.

Buczkowska K. & Dabert M. 2011. The development of species-specific SCAR markers for delimitation of *Calypogeia* species. J. Bryol. 33: 291-299.

Buczkowska K., Sawicki J., Szczecińska M., Rosadziński S., Rabska M., Bączkiewicz A. 2011. Two morphologically distinct groups of the *Calypogeia fissa* complex were found in Europe. Biodiv. Res. Conserv. 23: 7-19.

Buczkowska K., Odrzykoski I. J. & Chudzińska E. 2004. Delimitation of some European species of *Calypogeia* Raddi (Jungermnniales, Hepaticae) based on cytological characters and multienzyme phenotype. Nova Hedwigia 78: 147-163.

Buczkowska K., Sawicki J., Szczecińska M., Klama H. & Bączkiewicz A. 2012a. Allopoliploid speciation of *Calypogeia sphagnicola* (Jungermanniopsiada, Calypogeiaceae) based on isozyme and DNA markers. Pl. Syst. Evol. 298: 549-560.

Buczkowska K., Sawicki J., Szczecińska M., Klama H. & Bączkiewicz A. 2012b. Isozyme and DNA markers reveal a new genetically distinct taxon of *Calypogeia sphagnicola* (Jungermanniopsida, Calypogeiaceae). Pol. Bot. J. 57: 95-107.

Buczkowska K., Gonera P. & Hornik B. 2012c. PCR-based molecular markers for identification of taxa from the *Calypogeia fissa* complex (Jungermanniopsida, Calypogeiaceae). Biodiv. Res. Conserv. 28: 9-18.

Celka Z., Szczecińska M. & Sawicki J. 2010. Genetic relationship between some of *Malva* species as determined with ISSR and ISJ markers. Biodiv. Res. Conserv. 19: 23-32.

Celka Z., Szczecińska M., Sawicki J. & Shevera M. V. 2012. Molecular studies did not support the distinctiveness of *Malva alcea* and *M. excisa* (Malvaceae) in Central and Eastern Europe. Biologia 67: 1088-1098. DOI: 10.2478/s11756-012-0107-9

Damsholt K. 2002. Illustrated Flora of Nordic Liverworts and Hornworts. 456-476 pp. Nordic Bryological Society. Lund.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.

Fuselier L., Davison P. G., Clements M., Shaw B., Devos N., Heinrichs J., Hentschel J., Sabovljevic M., Szovenyi P., Schuette S., Hofbauer W & Shaw A. J. 2009. Phylogeographic analysis reveal distinct lineages of the liverworts *Metzgeria furcata* (L.) Dumort. and *Metzgeria conjugata* Lindb. (Metzgeriaceae) in Europe and North America. Biol. J. Linn. Soc. 98: 745-756.

Górniak M., Mytnik-Ejsmont J., Rutkowski P., Tukałło P., Minasiewicz J & Szlachetko D. L. 2006. Phylogenetic relationships within the subtribe *Spiranthinae* s.l. (Orchidaceae) inferred from the nuclear ITS region. Biodiv. Res. Conserv. 1-2: 18-24.

Grądzielewska A. 2011. Application of the ISSR metod to estimate the genetic similarity of *Dasypyrum villosum* (L.) P. Candargy Greek populations to *Triticum* and *Secale* species. Biodiv. Res. Conserv. 21: 7-12. DOI 10.2478/v10119-011-0002-1

Hassel K. & Gunnarsson U. 2003. The use of inter simple sequence repeats (ISSR) in bryophyte population studies. Lindbergia 28: 152-157.

Hey J. & Wakeley J. 1997. A coalescent estimator of the population recombination rate. Genetics 145: 833-846.

Heinrichs J., Hentschel J., Bombosch A., Fiebig A., Reise J., Edelmann M., Kreier H. P., Schäfer-Verwimp A., Caspari S., Schmidt A. R., Zhu R. L., Konrat M., Shaw B. & Shaw A. J. 2010. One species or at least eight? Delimitation and distribution of *Frullania tamarisci* (L) Dumort s l (Jungermanniopsida Porellales) inferred from nuclear and chloroplast DNA markers. Molec. Phylog. Evol. 56: 1105-1114.

Jankowiak K. & Szwekowska-Kulińska Z. 2004. Organellar inheritance in the allopolyploid liverwort species *Porella baueri* (Porellaceae): reconstructing historical events using DNA sequence analysis. Monographs in Systematic Botany from the Missouri Botanical Garden 98: 404-414.

Krawczyk K., Szczecińska M. & Sawicki J. 2013. Evaluation of 11 single-locus and seven multilocus DNA barcodes in *Lamium* L. (Lamiaceae). Molec. Ecol. Res. DOI: 10.1111/1755-0998.12175

Kreier H. P., Feldberg K., Mahr F., Bombosch A., Schmidt A. R., Zhu R. L., Konrat M., Shaw B., Shaw A. J. & Heinrichs J. 2010. Phylogeny of the leafy liverwort *Ptilidium*: Cryptic speciation and shared haplotypes between Northern and Southern Hemispheres. Molec. Phylog. Evol. 57: 1260-1267.

Murray M. & Thompson W. F. 1980. Rapid isolation of molecular weight plant DNA. Nucleic Acids Res. 8: 4321-4325.

Müller K. 1951-1958. Die Lebermoose Europas. In: Dr. L. Rabenhorst's Kryptogamen Flora von Deutschland, Österreich und der Schweiz. Akademische Verlagsgesellschaft Geest & Portig K-G, Leipzig.

Pacak A. & Szweykowska-Kulińska Z. 2003. Organellar inheritance in liverworts. An example of *Pellia borealis*. J. Mol. Evol. 56: 11-17.

Plášek V., Sawicki J., Marková I. & Wierzcholska S. 2011. *Orthotrichum affine* var. *bohemicum* (Orthotrichaceae), a new variety of epiphytic moss from the Czech Republic. Acta Soc. Bot. Pol. 80(4): 335-340.

Sang T., Crawford D. J. & Stuessy T. F. 1997. Chloroplast DNA phylogeny reticulate evolution and biogeography of *Paeonia* (Paeoniaceae). Am. J. Bot. 84: 1120-1136.

Sawicki J., Plášek V. & Szczecińska M. 2010. Molecular studies resolve *Nyholmiella* (Orthotrichaceae) as a separate genus. J. Syst. Evol. 48: 183-194.

Sawicki J. & Szczecińska M. 2007. Semi-specific intron-exon splice junction markers in bryophyte studies. Biodiv. Res. Conserv. 5-8: 25-30.

Schumacker R. & Váňa J. 2005. Identification keys to the liverworts and hornworts of Europe and Macaronesia (Distribution and status). 209 pp. Sorus, Poznań.

Schuster R. M. 1969. The Hepaticae and Anthocerotae of North America east of the hundredth meridian, vol. 2. Columbia University Press, New York-London.

Shaw A. J., Cox C. J. & Boles S. B. 2003. Polarity of peatmoss (*Sphagnum*) evolution: who says bryophytes have no roots? Am. J. Bot. 90: 1777-1787.

Shlyakov R. N. 1979. Pieczenocznyje mchi sjeviera S.S.S.R. t. 2 Lofoziewyje, (Hepatics of the northern territories of the USSR, vol. 2, Lophoziaceae). Nauka, Leningrad.

Singh A. P. & Nath V. 2007. A New *Calypogeia* Raddi from India. Taiwania 52: 320-323.

Söderström L., Urmi E. & Váňa J. 2002. Distribution of Hepaticae and Anthocerotae in Europe and Macaronesia. Lindbergia 27: 3-47.

Söderström L., Urmi E. & Váňa J. 2007. The distribution of Hepaticae and Anthocerotae in Europe and Macaronesia – Update 1-427. Cryptogamie Bryol. 28: 299-350.

Szweykowska-Kulińska Z., Pacak A. & Jankowiak K. 2002. New DNA markers for discrimination between closely-related species and for the reconstruction of historical events; an example using liverworts. Cell. Mol. Biol. Lett. 7: 403-416.

Szweykowski J. 1984. Species problems and taxomonic methods in Bryophytes in: R. M. Schuster The New Manual of Bryology. The Hattori Bot. Lab. Nichinan, Miyazaki, vol. 2, pp. 1130-1171.

Szweykowski J. 2006. An annotated checklist of Polish liverworts. In: Z. Mirek (ed.) Biodiversity of Poland, 4, pp. 18-20. W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M. & Kumar S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. 28: 2731-2739.

Taberlet P., Coissac E., Pompanon F., Gielly L., Miquel C., Valentini A., Vermat T., Corthier G., Brochmann C. & Willerslev E. 2006. Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. Nucleic Acids Res. 35: e14.

Wickett N. J. & Goffinet B. 2008. Origin and relationships of the myco-heterotrophic liverwort Crypthotallus mirabilis Malmb. (Metzgeriales, Marchantiophyta). Bot. J. Linn. Soc. 156: 1-12.

Wilson R., Gradstein S.R., Schneider H. & Heinrichs J. 2007. Unravelling the phylogeny of Lejeuneaceae (Jungermanniopsida): evidence for four main lineages. Molec. Phylog. Evol. 43: 270-282.