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Genetic studies revealed differences between European and North American populations of *Calypogeia azurea*

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Abstract: *Calypogeia azurea*, a widespread, subboreal-montane liverwort species, is one of a few representatives of the *Calypogeia* genus that are characterized by the occurrence of blue oil bodies. The aim of the study was to investigate the genetic variation and population structure of *C. azurea* originating from different parts of its distribution range (Europe and North America). Plants of *C. azurea* were compared with *C. peruviana*, another *Calypogeia* species with blue oil bodies. In general, 339 gametophytes from 15 populations of *C. azurea* were examined. Total gene diversity (H_T) estimated on the basis of nine isozyme loci of *C. azurea* at the species level was 0.201. The mean Nei's genetic distance between European populations was equal to 0.083, whereas the mean genetic distance between populations originating from Europe and North America was 0.413. The analysis of molecular variance (AMOVA) showed that 69% of *C. azurea* genetic variation was distributed among regions (Europe and North America), 15% – among populations within regions, and 16% – within populations. Our study revealed that *C. azurea* showed genetic diversity within its geographic distribution. All examined samples classified as *C. azurea* differed in respect of isozyme patterns from *C. peruviana*.

Key words: Bryophyta, liverworts, *Calypogeia*, genetic variation, population structure, isozymes

1. Introduction

Calypogeia Raddi is a large genus of leafy liverworts with about 90 described species (Schuster 1969). The presence of oil bodies, their color, shape and pattern of distribution in cells of the leaf and underleaf are among the most important taxonomic features of this genus (Buch 1935). *Calypogeia azurea* Stotler & Crotz is one of a few species that are characterized by blue oil bodies (Müller 1957; Schuster 1969; Yang & Lin 2009). In Europe, however, *C. azurea* is the only species of blue oil bodies, which allows unequivocal identification of living plants. *Calypogeia azurea* is monoecious, mainly autoicous, often fertile species. This species has a wide range of geographical distribution throughout the northern hemisphere. It occurs in North America, Europe and Asia and is regarded as subboreal-montane species (Müller 1957; Schuster 1969; Paton 1999; Damsholt 2002). In Central Europe, it is scattered and rare in lowlands, e.g., in Germany (Müller 1957) or Poland, where it is very rare and restricted to the north-eastern part of the country, while in the mountains – it is widespread and grows from low elevations to the alpine zone (Szweykowski 2006). In North America, *C. azurea* is also rare and co-occurs with *C. peruviana* Ness & Mont. – another species with blue oil bodies. These two species, however, differ in their distribution ranges, as well as in some morphological characters (Schuster 1969).

Calypogeia azurea is characterized by a high variability of morphological features (Schuster 1969). It is a relatively large species of the *Calypogeia* genus, and plants can reach up to 5 cm in length and the width of leafy shoots range from 915-3753 μm, on average 2000 μm. Leaves are broadly cordate, usually wider than long (Buczkowska 2004a). High morphological variability of *C. azurea* can be associated with its wide ecological tolerance. It occurs in a variety of habitats, from acidic to mildly alkaline, e.g., on loamy soil, humus, peat, wet stones and rocks or, rarely, on rotten logs

(Müller 1957; Schuster 1969; Paton 1999). Szweykowski & Krzakowa (1990) showed that *C. azurea* is one of the best characterized in terms of peroxidase phenotypes species of the *Calypogeia* genus occurring in Poland. Further isozyme and cytological research revealed that *C. azurea*, species with n = 18 chromosome number, is of allopolyploid origin (Buczkowska *et al.* 2004).

Our knowledge of liverworts genetic variability, in comparison with higher plants, is still limited and concerns, mainly, thallose liverworts (Shaw 2009). Genetic variation and population structure is known only in a few species of leafy liverworts e.g. *Plagiochila asplenioides*, *P. porelloides* (Zieliński & Wachowiak-Zielińska 1994), *Porella baueri* (Boisselier-Dubayle *et al.* 1998), *P. platyphylla* (Wyatt *et al.* 2005), *Ptilidium pulcherrimum*, *P. ciliare* (Adamczak *et al.* 2005). Recently, our knowledge of leafy liverworts genetic variation was enlarged by Bączkiewicz (2012), who examined eight other species and compared genetic diversity of sterile species: *Trichocolea tomentella*, *Bazzania trilobata*, *Lophozia hatcheri*, *Mylia anomala* and fertile species: *Lepidozia reptans*, *Calypogeia integristipula*, *Mylia taylorii*, *Tritomaria quinquedentata*). Although *C. azurea* is a well-known liverwort species, there is no data on its genetic variation. The purpose of the present study was to evaluate the level of genetic variation of *C. azurea* populations originating from Europe and North America based on isozyme markers. Plants of *C. azurea* were compared with *C. peruviana*, another *Calypogeia* species with blue oil bodies.

2. Material and methods

2.1. Plant material

Specimens of *C. azurea* used in the present study originated from of Europe and North America. In total, 339 gametophytes from 13 populations from different regions of Poland and 2 from North America were examined. In each population, 2-10 patches (samples)

Table 1. Collection sites of the studied populations of *Calypogeia azurea* and *C. peruviana* (the latter used as a reference species)

Population No.	Population symbol	Locality	Collector
		C. azurea	
1	LG	NE Poland, Warmińsko-Mazurskie Province, Lake Godle near Ełk	KB, AB
2	TLC	S Poland, Tatra Mts, Rów Zakopiański at N base of Tatra Mts, Las Capowski forest, 971 m a.s.l.	AB, KB
3	TSU	S Poland, Tatra Mts, NE slope of Skupinów Upłaz Mt, 1200 m a.s.l.	KB, AB
4	TSW	S Poland, Tatra Mts, Sucha Woda Valley, Psia Trawka meadow, 1183 m a.s.l.	KB, AB
5	TŻT	S Poland. Tatry Mts., E slope of Żółta Turnia Mt, 1470 m a.s.l.	KB, AB
6	TCS	S Poland, Tatra Mts, Dolina Pięciu Stawów Polskich Valley, N side of Czarny Staw Polski, 1722 m a.s.l.	KB, AB
7	BT	SE Poland, Bieszczady Mts, W slope of Tarnica Mt, 1317 m a.s.l.	KB
8	BR	SE Poland, Bieszczady Mts, W slope of Rozsypaniec Wołosacki Mt, 1215 m a.s.l.	KB
9	BGS	SE Poland, Bieszczady Mts, Wetlina, Valley of Górna Solinka stream, 714 m a.s.l.	KB
10	BP	SE Poland. Bieszczady Mts,Wołowate, SW slope of Pacałowa Mt, 784 m a.s.l.	KB
11	GDK	SE Poland, Gorce Mts, Ochotnica Dolna, Kudowy, 655 m a.s.l.	KB
12	BSP	SE Poland, Beskid Sądecki Mts, Potok Podlipowiec stream, 520 m a.s.l.	KB
13	BSB	SE Poland, Beskid Sądecki Mts, Potok Biały stream, 723 m a.s.l.	KB
14	USA	North America, North Carolina, Southern Appalachian Mts, BS 1133b/7	BS
15	USA	North America, North Carolina, Southern Appalachian Mts, BS 1139a	BS
		C. peruviana	
16	USA	North America, North Carolina, Southern Appalachian Mts, BS 1132c/4	BS
17	USA	North America, North Carolina, Southern Appalachian Mts, BS	BS

Explanations: collectors, AB – Alina Bączkiewicz, BS – Blanka Shaw, KB – Katarzyna Buczkowska; herbarium, POZW – Liverwort Herbarium of the Adam Mickiewicz University

were examined, with the exception of the US populations represented by one patch (Table 1). From each patch (approximately of 100 cm^2), 4-5 gametophytes (shoots) were analyzed, in general 339 gametophytes of *C. azurea*. Plants were initially determined on the basis of morphological traits and oil body characters (Müller 1957; Schuster 1969). Two samples (20 gametophytes) of *C. peruviana*, another *Calypogeia* species with blue oil bodies, were used for comparison as a reference species.

2.2. Isozyme electrophoresis

Individual gametophytes were examined in respect of the following enzyme systems: GOT (E.C. 2.6.1.1), GDH (E.C. 1.4.1.2), EST (E.C. 3.1.1.-), PGI (E.C. 1.1.1.94), MDH (E.C. 1.1.1.37), PGD (E.C. 1.1.1.44) and PGM (E.C. 5.4.2.2). Electrophoretic separation of isozymes was conducted according to the procedure described by Wendel & Weeden (1989). Details of cell extract preparation and buffer systems used are included in Buczkowska & Bączkiewicz (2011). Detected alleles were labeled in accordance with previous studies of *Calypogeia* (Buczkowska *et al.* 2004; Buczkowska 2004b; Buczkowska & Bączkiewicz 2011).

2.3. Data analysis

Data for isozyme loci were analyzed using GenAlEx 6.5 (Peakall & Smouse 2012) to calculate allele frequencies, percentage of polymorphic loci (P%), mean numbers of alleles (A) and effective alleles (Ae) per locus, gene diversity (H_S and H_T). To investigate the genetic structure of populations, an analysis of molecular variance (AMOVA) was done. The level of genetic differentiation between populations was estimated using Φ statistic (an analogue to F_{ST}) and Nei's (1978) genetic distance (D) and genetic identity (I) between populations. Mega 6.06 (Tamura *et al.* 2013) was used to construct an UPGMA dendrogram. Because *C. azurea* is an allopolyploid species, genetic distances were computed in the same way as for *C. sphagnicola* (Buczkowska *et al.* 2012), i.e. genotypes of the haploid form were treated as homozygotes of the diploid data format, and codominant options of GenAlEx were used.

Genetic variability parameters were calculated only for the *C. azurea* population as *C. peruviana* was represented only by 2 samples and, therefore, it was used merely as a reference species to show that plants of *C. azurea* from North America with different isozyme patterns from the European plants were not confused with *C. peruviana*.

3. Results

In seven enzyme systems, nine loci were resolved in the studied *C. azurea* populations. Of the 9 analyzed loci, only one (GDH) was monomorphic in all studied populations. Three populations (two from Bieszczady Mts., and one from the USA) were monomorphic (Table 2). Altogether, 19 different genotypes (G) were detected in the total studied sample of 339 gametophytes, giving the proportion of distinguishable genotypes (G/N) as

Table 2. Number of patches (N_p), number of gametophytes (N), mean number of alleles (A) and effective alleles (A_e) per locus, percentage of polymorphic loci (P), gene diversity (H_S), number of multilocus genotypes (G), and number of private alleles in populations of *Calypogeia azurea*

Population No.	N_p	N	A	A_e	P (%)	H_S	G	Private alleles
1	4	20	1.11	1.10	11.1	0.056	2	–
2	10	54	1.56	1.16	33.3	0.096	8	1
3	6	36	1.56	1.24	44.4	0.155	6	–
4	4	24	1.22	1.20	22.2	0.109	5	–
5	4	36	1.22	1.19	22.2	0.106	4	–
6	3	12	1.44	1.34	44.4	0.195	3	1
7	2	8	1.00	1.00	0.0	0.000	1	–
8	4	30	1.22	1.09	22.2	0.063	3	–
9	3	12	1.00	1.00	0.0	0.000	1	–
10	2	10	1.11	1.02	11.1	0.022	2	–
11	5	28	1.11	1.05	11.1	0.034	2	–
12	5	25	1.11	1.02	11.1	0.017	2	–
13	4	24	1.11	1.07	11.1	0.043	2	–
14	1	10	1.11	1.02	11.1	0.022	2	2
15	1	10	1.00	1.00	0.0	0.000	1	2
Mean	3.9	22.6	1.19	1.10	17.0	0.061	2.9	

Table 3. Hierarchical analysis of molecular variance (AMOVA) of *Calypogeia azurea* populations without (A) and with (B) division of populations into two geographic regions (Europe and North America)

Source of variation	*df*	Sum of squares	Variance component	Variance %	Fixation index[1]
A					
Among populations	13	100,580	0,322	60	Φ_{PT}= 0.598***
Within populations	324	110,411	0,341	40	
B					
Among regions	1	60,868	1,512	69	Φ_{RT}= 0.695***
Among populations within regions	13	100,580	0,322	15	Φ_{PR}= 0.486***
Within populations	324	110,411	0,341	16	Φ_{PT}= 0.843***
Total	338	271,858	2,175	100	

Explanations: A – Φ_{PT} (analogous to F_{ST}) = variation among populations divided by total variation, B – Φ_{RT} = variation among regions divided by total variation; Φ_{PR} = variation among populations within regions divided by the sum of variation among populations within regions and variation within populations; Φ_{PT} = the sum of variation among regions and variation among populations divided by total variation; *** – P≤ 0.001

0.056. The number of genotypes in populations ranged from 1 to 8, with a mean of 2.93. Two genotypes were the most frequent, representing 38.6% and 24.2% of all plants. Five genotypes were very rare and occurred only once, in populations no. 1, 2, 3 and 15. Percentage of polymorphic loci ranged from 0.00 to 44.4% (mean 17.04%). Six private alleles were found in populations number 2, 6, 14 and 15 (Table 2).

Gene diversity (H_S) within the *C. azurea* populations ranged from 0.00 to 0.195, with a mean of 0.061. Genetic variation of populations from the Tatra Mts. was higher than in the lowland population and those originating from the Bieszczady and Beskid Sądecki Mts. (Table 2). The total gene diversity (H_T) based on allelic frequencies based on polymorphic loci over all populations exhibited the value of 0.201. The molecular variance analysis (AMOVA) conducted for the *C. azurea* revealed that most of the genetic variation (69%) was present among regions (Europe and North America), only 15% of molecular variation was distributed among studied populations within regions, whereas variation within populations accounted for 16% of the total variation. The coefficients of genetic differentiation among regions (Φ_{RT}=0.695), among populations within regions (Φ_{PR}=0.486), and within populations (Φ_{PT}=0.843) were statistically significant (P<0.001) (Table 3). Both populations from North America were genetically different from plants occurring in Poland. At three loci, genotypes detected in plants originating from the USA were not observed in populations from Poland: *Got* – genotype 11, *Est*1 – genotypes 44, and 13, *Pgm* – genotype 15, thus they can be regarded as markers, which distinguish the European and American populations (Fig. 1). On the other hand, European and American plants did not

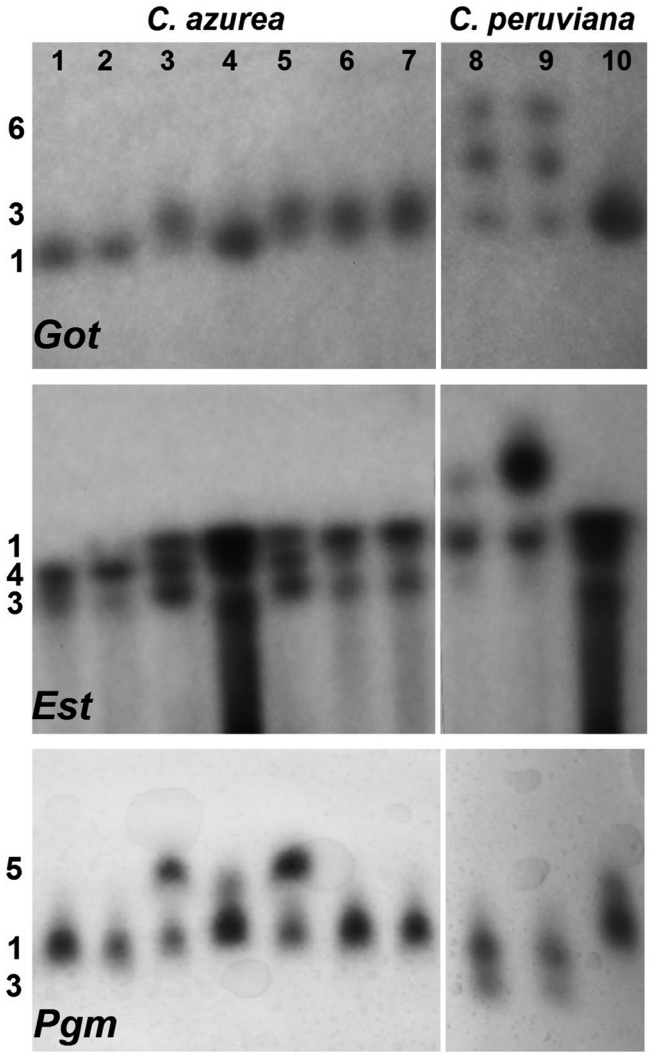

Fig. 1. Isozyme phenotypes of three marker loci: *Got*, *Est* and *Pgm* of *Calypogeia azurea* and *C. peruviana*. *C. azurea*: lines 1, 2 and 3, 5 – plants from North America (population 15 and 14, respectively), lines 6, 7 – plants from Poland (population 5 and 8); 4, 10 – standard; *C. peruviana*: lines 8, 9. Numbers on the left side of the diagram are the numbers of alleles

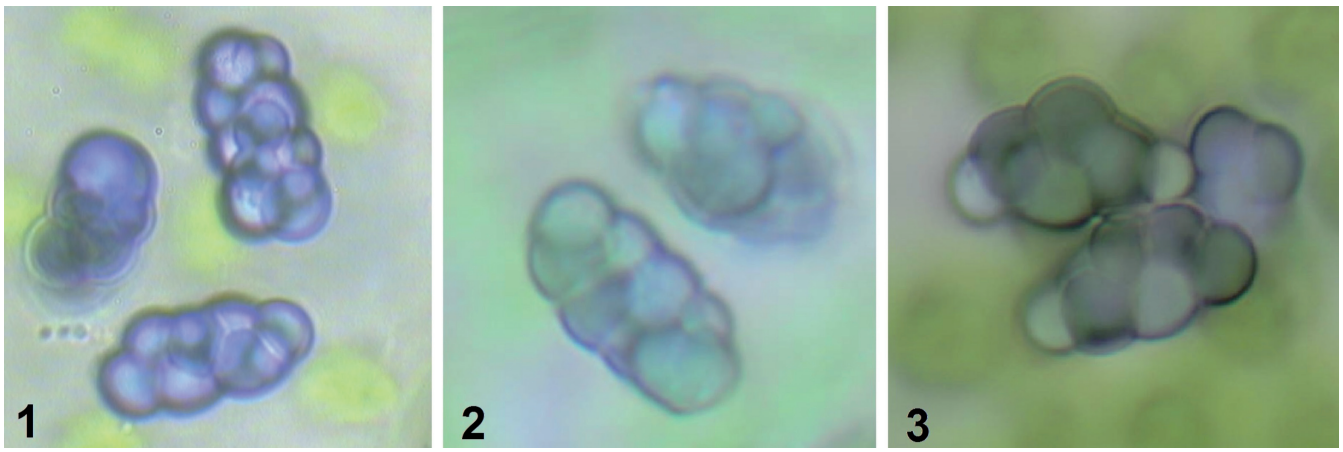

Fig. 2. Sample pictures of oil bodies in the studied plants
Explanations: 1 – *Calypogeia azurea* from Poland (population 5), 2 – *C. azurea* from North America (population 15), 3 – *C. peruviana* (population 17)

differ in terms of color and structure of oil bodies. All the studied plants had blue and composed of several large and distinct globules oil bodies, which were characteristic for *C. azurea* (Fig. 2). All samples of *C. azurea* differed in isozyme pattern from the examined samples of *C. peruviana* used in this study as a reference species.

Genetic distances (D) between Polish populations ranged from 0.0 to 0.279 and genetic identity (I) from 0.756 to 1.00 (Table 4). The mean genetic distance and identity between these populations were equal to 0.083 and 0.924, respectively. Among the Polish population, the most genetically distinct was the 6-TCS population from the Tatra Mts. (Czarny Staw Polski) and populations from Gorce (11-GDK) and Beskid Sądecki (12-BSP and 13-BSB). These populations formed distinct clusters on the dendrogram constructed based on genetic distances (Fig. 3). The values of genetic distances among European and American populations were higher and ranged from 0.242 to 0.655, with a mean of 0.413. The populations 14 and 15 from the USA were genetically distinct from the other studied populations (Table 4, Fig. 3).

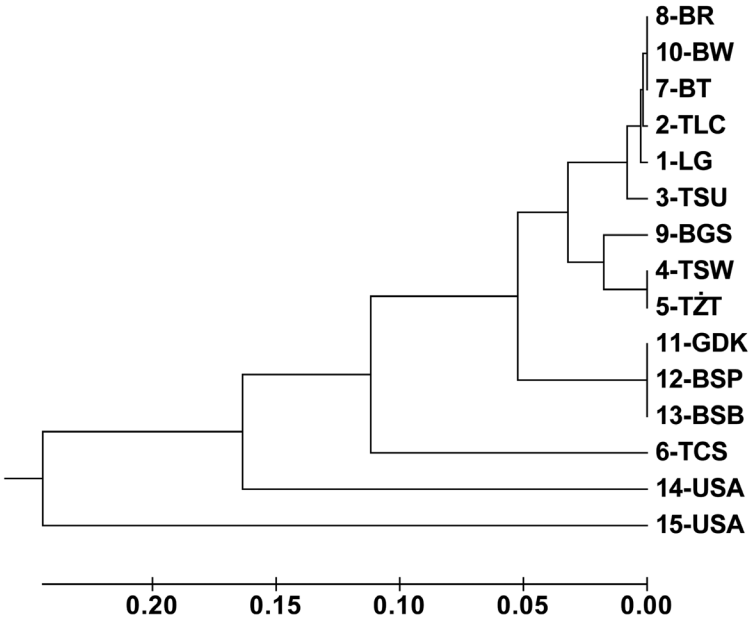

Fig. 3. UPGMA dendrogram of the studied populations of *Calypogeia azurea* based on Nei's genetic distances from isozyme data

Table 4. Nei's (1978) genetic identity I (above diagonal) and genetic distance D (below diagonal) among populations of *Calypogeia azurea* based on isozyme loci

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	****	0.992	0.980	0.946	0.938	0.794	1.000	0.992	0.868	1.000	0.908	0.886	0.923	0.662	0.635
2	0.008	****	0.982	0.976	0.971	0.800	0.997	0.999	0.930	0.994	0.896	0.873	0.912	0.704	0.661
3	0.020	0.019	****	0.966	0.960	0.829	0.985	0.988	0.894	0.987	0.923	0.905	0.936	0.708	0.710
4	0.056	0.024	0.034	****	1.000	0.823	0.955	0.984	0.964	0.961	0.930	0.916	0.939	0.775	0.636
5	0.064	0.029	0.041	0.000	****	0.820	0.947	0.978	0.968	0.953	0.921	0.908	0.931	0.773	0.634
6	0.231	0.224	0.187	0.195	0.198	****	0.785	0.807	0.759	0.794	0.797	0.789	0.802	0.603	0.530
7	0.000	0.003	0.015	0.046	0.055	0.242	****	1.000	0.876	1.000	0.917	0.894	0.932	0.694	0.641
8	0.008	0.001	0.012	0.017	0.023	0.215	0.000	****	0.916	1.000	0.932	0.913	0.945	0.731	0.645
9	0.142	0.073	0.112	0.036	0.032	0.276	0.132	0.088	****	0.873	0.778	0.756	0.792	0.692	0.639
10	0.000	0.006	0.013	0.040	0.048	0.231	0.000	0.000	0.136	****	0.936	0.916	0.950	0.712	0.635
11	0.097	0.110	0.080	0.073	0.082	0.227	0.087	0.070	0.252	0.066	****	1.000	1.000	0.787	0.538
12	0.122	0.136	0.100	0.088	0.097	0.237	0.112	0.091	0.279	0.088	0.000	****	1.000	1.000	0.787
13	0.080	0.092	0.067	0.063	0.072	0.221	0.070	0.056	0.233	0.051	0.000	0.000	****	0.785	0.552
14	0.412	0.351	0.346	0.255	0.257	0.506	0.366	0.313	0.368	0.340	0.239	0.237	0.242	****	0.631
15	0.454	0.413	0.343	0.453	0.455	0.634	0.445	0.439	0.447	0.455	0.619	0.655	0.595	0.461	****

4. Discussion

The results of the present isozyme study revealed that *C. azurea* shows genetic diversity within its geographic distribution. The *C. azurea* genetic variation at the population level estimated on the basis of nine isozyme loci (H_S=0.061) was low and close to the value found in other studied species of the *Calypogeia* genus, e.g., *C. integristipulla* (Bączkiewicz 2012), *C. fissa* (Buczkowska 2004b) and lower than in *Ptilidium pulcherrimum* or *P. ciliare* (Adamczak *et al.* 2005). The genetic variation of *C. azurea* was close to the mean value of genetic variation for liverworts reported by Shaw (2000) (H_S=0.044) and Itouga *et al.* (2002) (H_S=0.059) estimated on the basis of isozyme studies. However, the populations from the Tatra Mts. showed higher genetic variation; the highest H_S value occurred in population 6 from Czarny Staw Polski and reached 0.195. The total gene diversity of *C. azurea* at the species level was not high (H_T=0.201); it was almost the same as in *C. integristipulla* (H_T=0.205; Baczkiewicz 2012). Thus, the level of total gene diversity estimated for monoecious and sexually reproducing species of the *Calypogeia* genus was lower than in other species of leafy liverworts, which reproduce vegetatively such as *Bazzania trilobata* and *Trichocolea tomentella*, H_T=0.299 and H_T=0.263, respectively (Bączkiewicz 2012), or *Ptilidium ciliare* (H_T=0.299; Adamczak *et al.* 2005). Our results confirm the research of Bączkiewicz (2012), who concluded that there were no significant differences in the level of total gene diversity between sexually and asexually reproducing species.

The high value of genetic differentiation coefficient (Φ_{PT}=0.598) indicates a limited gene flow between populations of *C. azurea*, especially, between the North American and European populations. This results in the presence of unique alleles and genotypes in the plants from North America, which were not detected in any European population. Genetic distances between populations originating from North America and Europe (D=0.413) estimated on the basis of nine isozyme loci were close to the values of D=0.472 found between two forms of *C. sphagnicola* (*C. sphagnicola* f. *sphagnicola* and *C. sphagnicola* f. *paludosa*), recognized as reproductively isolated species (Buczkowska *et al.* 2012) and higher than between allopolyploid *Porella baueri* and its two parental species: *P. platyphylla* (I=0.802; D=0.221) and *P. cordeana* (I=0.738; D=0.304; Boisselier-Dubayle *et al*. 1998) or haploid and diploid cytotypes of *Corsinia coriandrina* (I=0.667; D=405; Boisselier-Dubayle & Bischler 1998).

Our results seem to support the hypothesis of Szweykowski & Krzakowa (1990), which assumes genetic differences between *C. azurea* occurring in Europe and North America, based on differences in distribution pattern of oil bodies in leaf cells. The American plants had characteristic blue oil bodies and also did not differ from typical plants of *C. azurea* in terms of morphological

features. Thus, the observed genetic diversity may be another case of cryptic speciation within this widespread liverwort species. We realize that our results were obtained from a small sample size of *C. azurea* from the North American part of distribution and should be considered as preliminary results. Nevertheless, this finding indicates an important phenomenon in the evolution of *Calypogeia* species. Further studies need be undertaken in order to recognize the range of genetic variability of this widely distributed in Holarctis species.

Schuster (1969) stressed that, in some regions in North America, *C. azurea* can be difficult to distinguish from *C. peruviana* – another species also having blue oil bodies and co-occurring with *C. azurea* in North America. Therefore, for comparison, the isozyme analyses were also done for two samples of *C. peruviana*. Our studies showed that all examined plants of *C. azurea* differed in terms of isozyme pattern from *C. peruviana*.

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