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Differentiation and genetic variability of three cryptic species within the *Aneura pinguis* complex (Jungermanniidae, Marchantiophyta)

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ABSTRACT – 1652 individuals of *Aneura pinguis* from Poland were surveyed for variation in 12 putative gene loci. Based on isozyme data, we distinguished three cryptic species. No evidence for gene flow between these species was found. To date, no qualitative morphological characters are available, which would allow delimitation of the cryptic species of *A. pinguis*. Hence, these species are not formally described, but assigned as cryptic species A, B, and C. The mean genetic distance (*D*) between them is 1.3393. The highest genetic variation within populations (H_S) was found in species A, and the lowest in species B. Individual species of *A. pinguis* differ in their habitat preferences. Species A is the most common, it occurs mostly in the Western Carpathians, grows mainly on calcareous rocks and humus. Species B is the most frequent in the Eastern Carpathians on clay soil. Species C is the rarest, it can be found both in lowlands and mountains, but mainly in lowlands and on various substrata. All studied cryptic species occur partly sympatrically.

Liverworts / *Aneura pinguis* / cryptic species / allozyme / cpDNA-*trn*L / genetic variation / geographic distribution / ecological preferences.

RÉSUMÉ – Différentiation and variabilité génétique de trios espèces cryptiques dans le complexe *Aneura pinguis* (Jungermanniidae, Marchantiophyta). Des investigations portant sur 12 loci enzymatiques ont été menées sur une population de 1652 individus d'*Aneura pinguis* collectés en Pologne. Trois espèces cryptiques ont été identifiées sur la base des données des isoenzymes. Des flux de gènes n'ont pas été remarqués entre ces espèces. Du fait d'un manque de caractères qualitatifs et morphologiques différenciant ces espèces, ces dernières on été décrites comme espèces cryptiques A, B et C. La distance moyenne génétique (D) entre elles équivalait á 1,33. La plus grande variation génétique dans la population (Hs) a été observée chez l'espèce A, contrairement à l'espèce B avec la plus faible variation. Les autres espèces *A. pinguis* se différentient par leurs préférences écologiques. L'espèce A est la plus fréquente et se localise principalement dans les Carpates Occidentales sur des terrains calcaires ou humiques. L'espèce B est plus répandue dans les Carpates Orientales sur des sites argileux. L'espèce C est la plus rare et pousse plus fréquemment dans les plaines qu'en montagnes, mais aussi dans différentes zones. Toutes les espèces étudiées se manifestent partiellement de manière sympatrique.

Hépatiques / *Aneura pinguis* / espèces cryptiques / allozyme / cpDNA-*trn*L / variations génétiques / distribution géographique / préférences écologiques

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INTRODUCTION

Most species of bryophytes have been distinguished on the basis of morphological differences (Szweykowski, 1982; Bischler & Boisselier-Dubayle, 2000). The introduction of isozyme electrophoresis and molecular studies to taxonomy of bryophytes has resulted in the discovery of cryptic species within recognized particular species. Cryptic species are characterized by a complete or almost complete absence of morphological differences, as well as by the presence of distinct gene pools (different multilocus genotypes) (Mayr, 1970, 1996; Nei, 1978; Avise, 2004). Because they are morphologically indistinguishable, they cannot be classified as classical taxonomic species, because the commonly accepted morphological species concept (MSC) (Mayden, 1997; Agapow, 2004) does not apply in their case. On the other hand, however, they conform to the concept of species based on the lack of recombination (Mayr, 1942).

Genetic investigations on the taxonomy of liverworts and mosses and analyses of genetic diversity of populations have recently revealed that widespread species often consist of complexes of cryptic species. At present, the cryptic species are treated as species complexes, which can usually be identified only on the basis of DNA or isozyme markers. The first cryptic species in liverworts were identified in the 1970s, while investigating isozyme variability in *Conocephalum conicum* (L.) Dumort. (Szweykowski & Krzakowa, 1979). Later, the cryptic species were discovered in other thalloid liverworts, e.g., in: *Pellia epiphylla* (L.) Corda (Zieliński, 1987), *Marchantia polymorpha* (Nees) Burgeff (Boisselier-Dubayle *et al.,* 1989), *Riccia dictyospora* (Howe.) (Dewey, 1989), *Aneura pinguis* (L.) Dumort. (Szweykowski & Odrzykoski, 1990), and *Metzgeria furcata* (L.) Dumort. (Fuselier *et al.,* 2009). Recently, molecular studies showed cryptic speciation in leafy liverworts, for example, in: the genus *Herbertus* (Feldberg *et al.,* 2004), *Frulania tamarisci* (L.) Dumort. (Heinrichs *et al.,* 2010), *Ptilidium ciliare* (L.) Hampe (Kreier *et al*. 2010), *Porella platyphylla* (L.) Pfeiff. (Heinrichs *et al.,* 2011) and in mosses, such as: *Mielichhoferia elongata* (Hoppe & Hornsch.) Nees & Hornsch. (Shaw, 2000), *Hamatocaulis vernicosus* (Mitt.) Hedenäs (Hedenäs & Eldenäs, 2007), *Platyhypnidum riparioides* (Hedw.) Dixon (Huttunen & Ignatov, 2010), and in the genera *Orthotrichum* (Hedw.) (Sawicki *et al.,* 2012) and *Scleropodium* (Bruch & Schimp.) (Carter, 2012). Molecular studies revealed that cryptic speciation in bryophytes is more common, than it was previously thought. Therefore, some authors propose that the concept of DNA taxonomy should be applied for lineages of organisms with low morphological complexity (Heinrichs *et al.*, 2009, 2011).

Aneura pinguis (L.) Dumort. is a thalloid liverwort species with a broad holarctic distribution. It is common from lowlands up to the high montane zone and grows in various habitats: on calcareous rocks, basic humus, peat bogs, wet sand on lake shores, and fallen decorticated logs (Szweykowski, 2006). It is found all over Europe, North America, Mexico, India, Japan, Australia and New Zeland (Schuster, 1966; Paton, 1999; Damsholt, 2002). Until the 1990s, the species was universally regarded as morphologically homogeneous within its entire distribution range. So far, genetic studies of *A. pinguis* (Szweykowski & Odrzykoski, 1990; Bączkiewicz & Buczkowska, 2005; Wachowiak *et al.,* 2007; Bączkiewicz *et al.,* 2008) revealed four groups, where genetic distances between them were much greater than average interspecies distances in vascular plants species. Thus, in the meantime, an informal taxonomy was used and they were named as cryptic species A, B, C and D. Three of them (A, B, C) occur abundantly in Central Europe and D in Great Britain (Bączkiewicz & Buczkowska, 2005).

Biometric studies of A, B and C cryptic species of the *A. pinguis* complex demonstrated slight differences between them in quantitative features, e.g., in: the size of the thallus and cells, and thickness and number of cells in the cross-section of the thallus (Buczkowska *et al.*, 2006). These species also subtly differ in the structure and size of oil bodies (Buczkowska *et al.*, 2005). However, these differences are not sufficiently distinct and cannot be diagnostic features, still, they may help with their identification. Moreover, these cryptic species differ in the composition of volatile compounds present in the cells of these species (Wawrzyniak *et al.,* 2014).

Nevertheless, *A. pinguis* is still treated as a taxonomically single species, because these cryptic taxa were not formally described to date. Efforts have been made to separate the species, however, we feel that current evidence is insufficient to describe the species using Linnaean nomenclature. Thus, our present objective is to determine as many differences between these species as possible. A comparison of genetic variation of the three cryptic species of *A. pinguis* (A, B and C) in connection with geographic and ecological distribution will complement our knowledge about the differences between them.

Aims of this study were to: (1) analyze allozyme variability of the three cryptic species of *A. pinguis* (A, B and C) in order to estimate their genetic diversity, (2) describe their current geographic distribution in Poland and (3) study the correlations between their habitats and allozyme patterns.

MATERIALS AND METHODS

Sampling of plant material

From 24 localities from six regions in Poland: four mountain ranges in the south (Tatra, Pieniny, Beskid Sądecki, and Bieszczady Mts.) and two lowlands in the north (north-eastern and north-western Poland) a big fragments of 256 patches were sampled. From each locality, 1-40 fragments of patches were sampled, depending on the frequency of occurrence of *A. pinguis* in the particular locality. Distances between patches which samples were collected were more than 5 m. The cryptic species of *A. pinguis* could not be identified according to their morphological characters, so their identification was based on an initial genotypic analysis of isozyme markers and by cpDNA tRNA ^{Leu} region marker (Wachowiak *et al*., 2007). From each sampled patches, 5-20 thalli were analyzed, depending on the their size. Totally 1652 thalli were analyzed. One locality was treated as single population for each species, because sometimes in one locality we were identified two or three species of *A. pinguis*, in total we receive 35 populations (Table 1). After isozyme analysis, the rest of sampled patches were deposited as a voucher in the POZW herbarium in Poznań. Thallies from some patches were used for biometric analyses (Buczkowska *et al.* 2006).

Electrophoretic analysis

Plants were stored at 4ºC until the beginning of studies. Crude cell extract was prepared by homogenization of a single thallus in 80 µl of extraction buffer pH 7.5: (100 mM tris, 1 mM EDTA $Na_2 2H_2O$, 10mM KCL, 10mM $MgCl_2 \times 6H_2O$, 10 ml

Table 1. Regions, localities, studied populations and the number of individuals in each population of three cryptic species of the *A. pinguis* complex

No.	*Locality*	*Populations*						*Mixed colonies*
		Species A		*Species B*		*Species C*		
	Mountains:	*1*	*2*	*1*	*2*	*1*	*2*	
1	Tatra Mts., Biały Potok Valley	A1	126	B1	6	–	–	–
2	Tatra Mts., Kościeliska Valley	A2	144	–	–	–	–	–
3	Tatra Mts., Mt. Skupniów Upłaz, NE slope	A3	249	–	–	C1	22	–
4	Tatra Mts., Kozinicki Żleb gully	A4	108	–	–	–	–	–
5	Tatra Mts., Wielka Sucha Valley	A5	112	B2	20	–	–	–
6	Tatra Mts., Sucha Woda Valley	A6	15	–	–	C2	42	2
7	Tatra Mts., Pańszczyca Valley	A7	65	–	–	C3	11	–
8	Tatra Mts., Jaworzynka Valley	A8	105	–	–	C4	7	–
9	Pieniny Mts., stream on N slope of Mt. Repowa	A9	59	–	–	–	–	–
10	Pieniny Mts., Skalski stream, Skalski reserve	A10	81	B3	31	–	–	1
11	Pieniny Mts., small stream, near Skalski reserve	A11	78	–	–	–	–	–
12	Pieniny Mts., near the Skalski stream source	A12	9	–	–	–	–	–
13	Beskid Sadecki Mts., slope of Mt. Lipowiec	A13	9	–	–	–	–	–
14	Bieszczady Mts., Górna Solinka Valley	–	–	B4	60	–	–	–
15	Bieszczady Mts., Sianki, Niedźwiedzi stream	–	–	B5	51	C5	10	–
16	Bieszczady Mts., old quarry near Brzegi Górne	–	–	B6	31	–	–	–
17	Bieszczady Mts., Mt. Ryczywół, S slope	–	–	B7	18	–	–	–
	Lowlands:							
18	Białowieża Primary Forest aurochs' reserve	A14	9	B8	40	C6	9	–
19	Białowieża Primary Forest, 254Dc region	–	–	B9	5	–	–	–
20	Redykajny reserve, near Olsztyn	–	–	B10	18	–	–	–
21	Ruskie lake, near Olsztyn	–	–	–	–	C7	17	–
22	Pomerania, Garczyn lake, near Kościerzyna	–	–	–	–	C8	11	–
23	Wielkopolska region, Diabli Skok reserve	–	–	B11	45	C9	11	–
24	Wielkopolska region, Poznań	–	–	B12	15	–	–	–
	Total	14	1169	12	340	9	140	3

10% triton X-100 and 50 ml ME). The saturated paper wicks (Watmann 31 ETCHR) were placed into of 10% starch gel (Starch Art W 641-2 starch for electrophoresis). Two gel/electrode buffer systems were used: (A) lithium-borate (pH 8.3) – electrode buffer, tris-citrate (pH 8.2) – gel buffer; and (B) morpholine-citrate (pH 6.1), gel buffer was prepared by dilution of electrode buffer 1:15 (Wendel & Weeden, 1989). Buffer system A was used for GOT (E.C. 2.6.1.1), GDH (E.C. 1.4.1.2), PGI (E.C. 5.3.1.9), and ACP (E.C. 3.1.3.2). Buffer system B was used for SDH (E.C. 1.1.1.25), IDH (E.C. 1.1.1.41), MDH (E.C. 1.1.1.37), PGD (E.C. 1.1.1.44), and PGM (E.C. 5.4.2.2). Gels were stained with standard methods (Wendel & Weeden, 1989). Tris-citrate gels were separated at a constant voltage of 260 V for 4 h. Morpholine-citrate gels were separated at a constant current of 50 mA for 4.5 h. After separation, enzymes were stained for 2-3 hours at 37°C.

Data analysis

To compare genetic diversity of the studied *A. pinguis* species (A,B,C), the same statistical analysis was done separately for each species. In each species, for each population the following features were estimated: allele frequencies, the mean number of alleles per locus (A), the number of rare alleles, percentage of polymorphic loci (P) and the mean genetic diversity over loci (H_S) within populations (Nei, 1973). To detect the possible effects of selection, the Ewens-Watterson test for neutrality (1000 permutations) (Manly, 1985) was performed for each locus. For each studied cryptic species, Nei's gene-diversity statistics (Nei, 1978) was used to partition the total allelic diversity (H_T) into the components of diversity within the studied populations (H_S) and to calculate the genetic differentiation (G_{ST}). Gene flow (Nm) was estimated indirectly from G_{ST}, using the formula $Nm = 0.5(1-G_{ST})/G_{ST}$, adapted for haploid organisms (McDermott & McDonald, 1993). In bryophytes, this formula is used for both nuclear and chloroplast markers, as the migrating diaspores always comprise a haploid genome, and it is assumed in all cases that the distance of sperm migration is very short, normally not exceeding 10 cm (McLetchie, 1996; Korpelainen *et al*., 2005). These analyses were performed using POGENE 1.32 (Yeh *et al*., 2000). The pairwise genetic distances (D) and identity (I) among populations were evaluated on the basis of Nei's genetic distance matrix (Nei, 1978), an UPGMA phenogram was constructed and a principal coordinate analysis (PCoA) was performed. Analyses were calculated using the program GenALEx ver 6.3. (Peakall & Smouse, 2006) and STATISTICA program version 8.0 for Windows (StatSoft, 2008). To investigate genetic structure of populations, a hierarchical analysis of molecular variance (AMOVA) was done by GenALEx ver 6.3 statistic program. AMOVA was used to describe the percentage share of genetic diversity within populations and among populations. The level of genetic differentiation between populations was estimated using Φ statistic (an analogue to *F*). Significance levels for populations were determined using a permutation test (1000 permutations).

The parameters of genotypic diversity within populations were also estimated. All collected gametophytes were sorted by a unique multilocus genotype (MLG). Each of the detected distinct G (= MLG) was assumed to be a distinct genet. The proportion of distinguishable genotypes (G/N) was calculated as the number of unique multilocus genotypes (MLG) divided by the sample size (Ellstrand & Roose, 1987). The proportion of distinguishable genotypes was calculated for each population and as a total mean for all three species.

RESULTS

Geographic distribution and ecology

Three cryptic species were identified in the *A. pinguis* complex: A, B and C. Individual species of *A. pinguis* differ in their geographic distribution and their habitat preferences. 1169 out of 1652 studied thalli belong to species A. It was recognized in 14 of 24 investigated localities, mostly in mountains, including the Tatra, Pieniny and Beskid Sądecki Mts., where over 90% of the examined thalli belonged to this species. Species A, occurring in mountains, grows mainly on calcareous rocks, rock detritus, soil, sometimes on humus. However, species A was

absent in the Bieszczady Mts. Only one patch of species A was found in lowlands, in the Białowieża Primary Forest – in population no. 14A, here it grows on humus. Species B was found at 12 localities, mostly in lowlands (Wielkopolska, Warmia – near the city of Olsztyn, and Białowieża Primary Forest), where about 70% of individuals of the *A. pinguis* complex belong to this species, it was found mainly on humus or rotten wood. In the mountains, this species is characteristic for the Bieszczady Mts. – 96% of examined thalli in this region belonged to cryptic species B. In the Pieniny and Tatra Mts., species B is rather rare. In mountains species B was found on clay soil. Species C is the rarest species of the complex in Poland, it was recognized at 9 localities (only in 21 patches). Thirteen patches were found in the Tatra, one in the Bieszczady Mts. and 7 in lowlands (Wielkopolska, Warmia – near the city of Olsztyn, Pomerania and Białowieża Primary Forest). Species C occurs on various substrata – in mountains mainly on rock detritus, sometimes on clay soil while in lowlands on humus.

Only in the Białowieża Primary Forest, all three cryptic species (A, B and C) can occur sympatrically. Apart from them, we found species A with B at 3 localities, A with C at 4 localities and B with C at 2 localities. Except for three mixed patches: two of them in the Sucha Woda Valley population in the Tatra Mts., where species A co-occurred with C, and one patch in the Skalski stream population in the Pieniny Mts., where species A co-occurred with B, species grew in separate patches (Table 1, Fig.1).

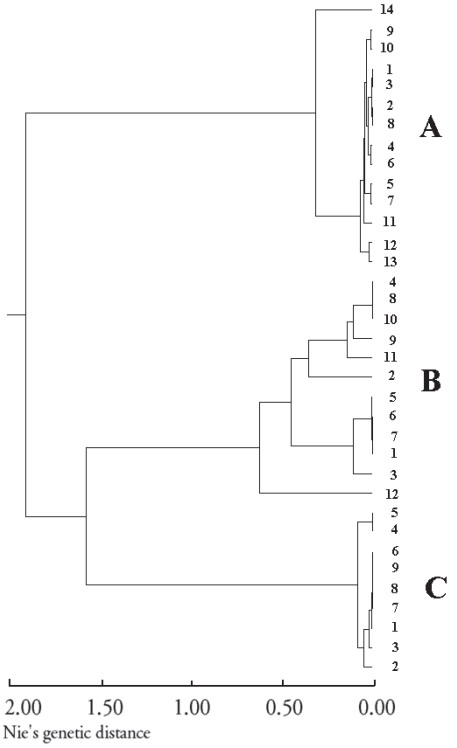

Fig. 1. UPGMA phenogram based on Nei's (1978) genetic distances among populations of three cryptic species of the *A. pinguis* complex.

Electrophoretic patterns

The 12 putative gene loci of 9 enzyme systems were studied in the species of *A. pinguis* complex from different regions of Poland (Table 1). The Ewens-Watterson test for neutrality showed that allele frequencies at all loci were selectively neutral in the studied populations. In total, all studied loci of the *A. pinguis* complex were polymorphic and had haploid banding patterns. In total, 40 alleles were detected in all studied species. The mean allele frequencies for the studied loci in all examined species are shown in Table 2. Individual cryptic species of the A. *pinguis* complex did not share alleles at *Got*, *Idh* and *Mdh*-B loci, which can be used as markers for species identification. Alleles: 1 at *Got*, 3 and 4 at *Idh*, and 3 at *Mdh*-B were specific for species A, alleles: 2 and 4 at *Got*, 2 at *Idh*, and 1 and 2 at *Mdh*-B for species B and additionally, allele 3 at *Sdh*. For species C were specific alleles: 3 at *Got*, 1 at *Idh*, 4 at *Mdh*-B, and 2 at *Mdh*-A (Table 2). The percentage of polymorphic loci (*P*) within populations of the species complex ranged

Table 2. The mean allele frequencies for 12 putative loci in the studied (A, B, C) cryptic species of the *A. pinguis* complex. Diagnostic alleles are boldfaced.

Locus	*Allele*	*A*	*B*	*C*
Got	**1**	1.000	–	–
	2	–	0.962	–
	3	–	–	1.000
	4	–	0.038	–
Sdh	1	0.981	–	–
	2	0.009	–	1.000
	3	–	1.000	–
	4	0.007	–	–
Idh	**1**	–	–	1.000
	2	–	1.000	–
	3	0.774	–	–
	4	0.226	–	–
Gdh	1	0.974	0.515	0.989
	2	–	0.485	–
	3	0.026	–	0.011
Pgi-A	1	1.000	0.377	0.040
	2	–	0.540	0.960
	3	–	0.083	–
Pgi-B	null	–	0.460	0.960
	1	0.828	0.507	–
	2	0.171	–	–
	3	–	0.033	0.040
Mdh-A	1	1.000	1.000	–
	2	–	–	1.000
***Mdh*-B**	**1**	–	0.006	–
	2	–	0.994	–
	3	1.000	–	–
	4	–	–	1.000
Mdh-C	1	–	0.006	–
	2	1.000	0.994	1.000
Pgd	1	–	0.474	–
	2	0.080	0.526	1.000
	3	0.841	–	–
	4	0.080	–	–
Pgm	1	0.763	–	–
	2	0.237	0.167	–
	3	–	0.833	1.000
Acp	1	0.930	–	0.349
	2	0.070	0.913	0.651
	3	–	0.087	–

from 0.0 to 50.0% (the mean – 15.77%). The mean number of alleles per locus (*A*) within studied populations of *A. pinguis* ranged from 1.0 to 1.58 with a mean of 1.16. The most rare alleles were in species A, whereas in species B and C, except population 11B, they were not found (Table 3). Allelic data revealed 40 multilocus genotypes (MLGs) among 1652 analyzed thalli, divided into three groups corresponding to the three cryptic species: A, B and C. Twenty-two genotypes belonged to species A, 13 to species B, and 5 to species C (Table 3).

Genetic variation within the cryptic species

***Aneura pinguis* – species A.** Of the 12 studied loci, five (*Got*, *Pgi*-A, *Mdh*-A, *Mdh*-B and *Mdh*-C) were monomorphic in all populations. Two alleles were characteristic only for the Tatra Mts.: allele 4 at *Sdh* and 2 at *Pgd*. Moreover, allele 4 at *Pgd* was found only in the populations from the Białowieża Primary Forest and the Pieniny Mts., but in the Pieniny Mts. it occurred with a low frequency (Table 2). The percentage of polymorphic loci in populations (*P*) ranged from 0.0 to 50.0%, with a mean of 28.57% . The mean number of alleles per locus (*A*) ranged from 1.00 to 1.58, with a mean of 1.302 (Table 3). Mean allelic diversity within populations (*Hs*) for all loci ranged from 0.0 to 0.148, with a mean of 0.0694. The total allelic

Table 3. Sample size (*N*), percentage of polymorphic loci (*P*), mean number of alleles per locus (*A*), number of rare alleles, allelic diversity (H_S), number of multilocus genotypes (ML*G*), and proportion of distinguishable genotypes (*G/N*) for three cryptic species of the *A. pinguis* complex (MLG = G)

Population No.	*N*	*P*	*A*	*Rare alleles*	$H_S \pm SD$	*No. of MLG*	*G/N*
A. pinguis – species A							
1 A	126	33.33	1.33	1	0.045 ± 0.070	5	0.040
2 A	144	16.67	1.17	1	0.014 ± 0.034	3	0.021
3 A	249	41.67	1.50	4	0.062 ± 0.085	16	0.064
4 A	108	33.33	1.33	4	0.048 ± 0.118	6	0.055
5 A	112	50.00	1.58	1	0.124 ± 0.161	9	0.080
6 A	15	25.00	1.25	0	0.093 ± 0.178	4	0.267
7 A	65	33.33	1.33	0	0.108 ± 0.176	5	0.080
8 A	105	41.67	1.50	4	0.047 ± 0.072	6	0.057
9 A	59	33.33	1.33	0	0.132 ± 0.202	4	0.067
10 A	81	50.00	1.50	1	0.148 ± 0.190	7	0.086
11 A	78	33.33	1.33	0	0.109 ± 0.172	8	0.103
12 A	9	8.33	1.08	0	0.041 ± 0.143	2	0.222
13 A	9	0.00	1.00	0	0.00	1	0.111
14 A	9	0.00	1.00	0	0.00	1	0.111
Mean		28.571	1.302	1.1	0.0694	5.5	0.0974
A. pinguis – species B							
1 B	6	0.00	1.00	0	0.00	1	0.167
2 B	20	0.00	1.00	0	0.00	1	0.050
3 B	31	41.67	1.50	0	0.185 ± 0.245	6	0.193
4 B	60	0.00	1.00	0	0.00	1	0.017
5 B	51	33.33	1.33	0	0.069 ± 0.102	2	0.039
6 B	31	0.00	1.00	0	0.00	1	0.032
7 B	18	0.00	1.00	0	0.00	1	0.056
8 B	40	0.00	1.00	0	0.00	1	0.025
9 B	5	0.00	1.00	0	0.00	1	0.200
10 B	18	0.00	1.00	0	0.00	1	0.056
11 B	45	33.33	1.33	3	0.041 ± 0.092	4	0.089
12 B	15	50.00	1.50	0	0.071 ± 0.080	3	0.200
Mean		13.194	1.138	0.25	0.0305	1.9	0.0937
A. pinguis – species C							
1 C	22	8.33	1.08	0	0.025 ± 0.086	2	0.091
2 C	42	33.33	1.33	0	0.133 ± 0.211	5	0.119
3 C	11	8.33	1.08	0	0.041 ± 0.143	2	0.182
4 C	7	0.00	1.00	0	0.00	1	0.143
5 C	10	0.00	1.00	0	0.00	1	0.100
6 C	9	0.00	1.00	0	0.00	1	0.111
7 C	17	0.00	1.00	0	0.00	1	0.059
8 C	11	0.00	1.00	0	0.00	1	0.091
9 C	11	0.00	1.00	0	0.00	1	0.091
Mean		5.554	1.054	0	0.0221	1.7	0.1097

diversity (H_T) based on allele frequencies of 7 polymorphic loci over all populations was 0.2130, genetic diversity between populations was slightly lower than within populations: H_S = 0.1192, D_{ST} = 0.0938. The G_{ST} value was 0.4405 and N_m value was 0.635.

From 22 different MLGs detected in this species, only one was widespread (present in 53% of the studied plants) and occurred in 12 out of 14 populations (106 colonies). Fourteen MLGs had a local distribution (present in 0.1-5.0% of the studied plants) and occurred only in one population. Some MLGs were restricted to a given region, for example, 10 MLGs were characteristic for the Tatra region, while for the Pieniny Mts. – 3. Only in 30 of 192 patches more than 2 MLGs occurred, but the mean number of genotypes per population (*G*) was 5.5.The proportion of distinguishable genotypes (*G/N*) was on average 0.0974 (Table 3).

***Aneura pinguis* – species B.** Three loci (*Sdh*, *Idh* and *Mdh*-A) were monomorphic in all populations. Populations from N-W Poland had specific alleles at four loci: at *Mdh*-B, *Mdh*-C and *Acp* – private alleles were found in the population from Poznań, and at *Pgi*-2 – in the population from the Diabli Skok reserve (Table 3). Only 4 of 12 studied populations were polymorphic. The percentage of polymorphic loci in the populations (*P*) ranged from 0.0 to 50.0%, with a mean of 13.19%. The mean number of alleles per locus (*A*) ranged from 1.0 to 1.5, with a mean of 1.138. Mean allelic diversity within populations (*Hs*) for all loci ranged from 0.0 to 0.185, with a mean of 0.0305 (Table 3).

The total allelic diversity (H_T) based on allele frequencies of 9 polymorphic loci over all populations was 0.3262 and most of the genetic variation in this species – resulted from the differences between populations: H_S = 0.0407, D_{ST} = 0.2855. The G_{ST} value was 0.8753 and N_m value was 0.071. Two out of 12 detected in this species MLGs were broadly widespread. They were present in 35% and 28% of the studied thalli and were found in 5 and 6 populations, respectively. Eight MLGs had a local distribution (present in 0.1-5.0% of the studied plants) and occurred only in one population. Only 8 of 43 patches had more than 2 MLGs, but the mean number of genotypes per population was 1.9. The proportion of distinguishable genotypes (*G/N*) was on average 0.0937 (Table 3).

***Aneura pinguis* – species C.** Eight loci (*Got*, *Sdh*, *Idh*, *Mdh*-A, *Mdh*-B, *Mdh*-C and *Pgd*, *Pgm*) were monomorphic in all populations. *Gdh*, *Pgi*-A, and *Pgi*-B were characteristic for the Tatra Mts. (Table 2). The percentage of polymorphic loci in populations (*P*) ranged from 0.0 to 33.3%, with a mean of 5.554%. Only 3 of 9 examined populations were polymorphic. The mean number of alleles per locus (*A*) ranged from 1.0 to 1.33, with a mean of 1.05. Mean allelic diversity within populations (*Hs*) for all loci ranged from 0.0 to 0.133, with a mean of 0.0221 (Table 3). The total allelic diversity (H_T) based on allele frequencies of 4 polymorphic loci over all populations was 0.1569, genetic diversity between population was slightly higher than within populations: H_S = 0.0662, D_{ST} = 0.0907. The G_{ST} value was 0.5778 and N_m value was 0.365.

From 5 MLGs detected in this species, 2 were the most widespread (present in 58% and 27% of the studied thalli and, occurred in 7 and 5 populations, respectively). Three MLGs were local (present in 0.1-5.0% of the studied thalli), each found only in one population. In 5 of 21 patches two or three MLGs were detected, but the mean number of genotypes per population (*G*) was 1.7. The proportion of distinguishable genotypes (*G/N*) was on average 0.1097, so it was the highest among the studied species (Table 3).

Genetic differentiation between the cryptic species

The highest genetic variation within populations (H_S) was in species A, while the lowest in species B. Species A had the highest number of alleles per locus (*A*), the polymorphic loci (*P*) and number of genotypes (*G*) (Table 3). The Mann-Whitney U-tests show that species A and C differ significantly in respect of *A*, *P*, H_S and *G*, while species A and B in respect of *P* and H_S, while species B and C do not differ significantly in respect of these statistics (Table 4). The hierarchical AMOVA analysis of genetic structure showed that in the studied A and C species of *A. pinguis*, genetic diversity within populations is higher than between populations, whereas in species B, genetic diversity between populations is higher than within populations. The fixation index (Φ_{PT}) in this species was 0.828, however, in all studied species, the genetic differentiation among populations (Φ_{PT}) was statistically significant. Consequently, the lowest gene flow between populations was observed in species B (Table 5). The UPGMA phenogram based on Nei's genetic distance values (Nei, 1978) among 35 studied populations of the *A. pinguis* complex illustrates the magnitude of genetic differences among the three cryptic species A, B, C (Fig. 1). Species A is the most genetically distinct from species B and C. Species C is the

Table 4. The Mann-Whitney U-test statistics used for testing statistical significance of differences between the means of genetic variability measures of three cryptic species of *A. pinguis*; significance levels; ns – p > 0.05, * – p < 0.05, ** – p < 0.01 (with the Bonferroni correction)

	species A – species B				*species A – species C*				*species B – species C*			
Genetic variability measures	*U statistic values*	*Z corrected for ties*	*p-level*		*U statistic values*	*Z corrected for ties*	*p-level*		*U statistic values*	*Z corrected for ties*	*p-level*	
A	46.00	2.04	0.0413	ns	18.50	2.90	0.0038	**	49.00	0.42	0.6711	ns
P	47.00	1.98	0.0474	ns	18.50	2.89	0.0038	**	49.00	0.42	0.6713	ns
HS	41.00	2.25	0.0244	*	25.50	2.41	0.0158	*	51.50	0.21	0.8322	ns
G	28.00	2.97	0.0029	*	18.00	2.90	0.0036	**	52.00	0.17	0.8653	ns
G/N	68.00	0.82	0.4102	ns	39.00	–1.51	0.1297	ns	36.00	–1.28	0.1999	ns

Table 5. Analysis of molecular variance (AMOVA) for three cryptic species of *A. pinguis* among and within populations

Source of variation	*df*	*Sum of squares*	*Variance component*	*variance (%)*	*Fixation index*
A					ΦPT = 0.228***
Among populations	13	140.29	0.130	23	
Within populations	1155	206.94	0.439	77	
B					ΦPT = 0.828***
Among populations	11	325.66	1.072	83	
Within populations	328	0.223	0.223	17	
C					ΦPT = 0.362***
Among populations	8	22.49	0.171	36	
Within populations	131	39.41	0.301	64	

ΦPT (analogous to FST) = variation among populations divided by total variation; *P ≤ 0.05; **P £ 0.01, *** P ≤ 0.001.

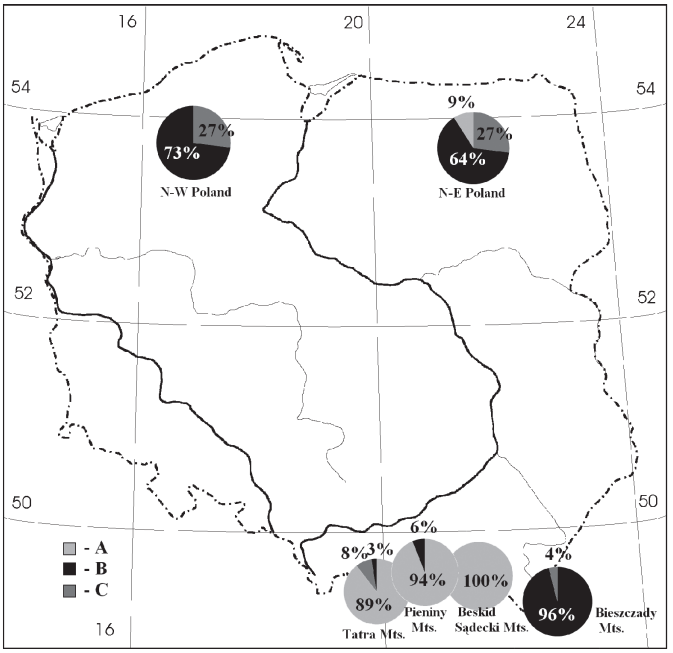

Fig. 2. Frequency of the cryptic species of *A. pinguis* in the studied regions of Poland.

most homogenous, while species B is the most heterogeneous. In species A, the most distinct is population A14 from the Białowieża Forest, which is the only lowland population of this species. The most distinct in species B was population B12 from Poznań (Fig. 2). The phenogram is consistent with the PCoA diagram (Fig. 3). The

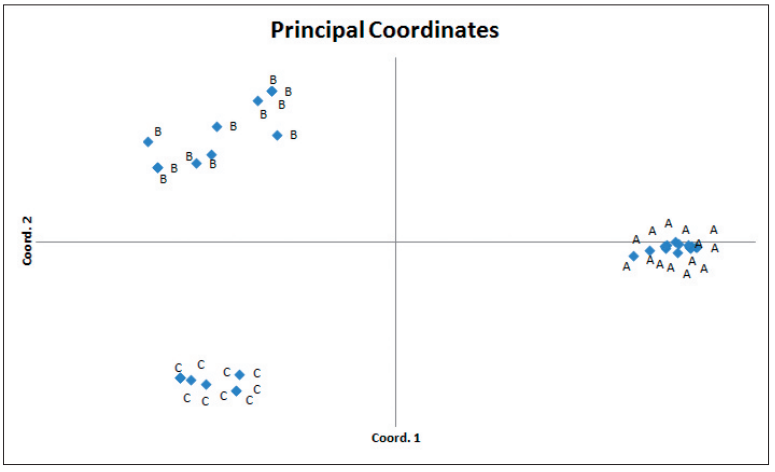

Fig. 3. Two-dimensional scatter plot of PCoA based on Nei's (1978) genetic distances among population of three (A, B, C) cryptic species of the *A. pinguis* complex. The percentage explained variability: PC 1 = 51.64%; PC 2 = 34.93%.

Table 6. Nei's (1978) genetic identities (above the diagonal) and genetic distances (below the diagonal) between three cryptic species of the *A. pinguis* complex

	A	*B*	*C*
A	****	0.219	0.228
B	1.518	****	0.361
C	1.481	1.019	****

mean Nei's genetic identity (I) values between populations within individual cryptic species of *A. pinguis* are high, on average 0.9555, 0.7719 and 0.9638 in species A, B and C, respectively. Consequently, the mean genetic distance (D) between populations within each of the cryptic species of *A. pinguis* (A, B, C) was low: 0.0499, 0.2984, 0.0275, respectively, whereas genetic identity (I) between the cryptic species is very low, ranging from 0.219 to 0.361. The highest genetic distance was between species A and B (1.518), and the lowest between B and C (1.019). The mean genetic distance between the cryptic species was 1.3393 (Table 6).

DISCUSSION

This study supports a previous discovery that *Aneura pinguis* shows taxonomic differentiation and is a complex of cryptic species (Bączkiewicz & Buczkowska, 2005). In Poland, it consists of at least three cryptic species: A, B, and C. An important evidence for this finding is the existence of three groups of MLGs corresponding to three cryptic species. These groups do not share the same alleles in three loci, which were used as markers. In liverworts, relative species and cryptic species often differ in alleles in 1-3 loci (Akiyama & Hiraoka, 1994; Boisselier-Dubayle & Bischler, 1999; Bączkiewicz *et al*., 2003; Buczkowska, 2004). Most patches of the different species of the *A. pinguis* complex occur separately but sometimes they grew sympatrically in one locality and even on the same substrata; only 3 of the 256 studied patches were mixed, but even there, we did not find any recombinant gametophytes (Table 1). This observation can be a proof of genetic isolation between particular cryptic species.

Cryptic species of *Aneura pinguis* differ in their geographic distributions. Species A, with the exception of one patches in the Białowieża Primary Forest (lowland), has been recorded only in the Western Carpathians, while species B grows mainly in the Eastern Carpathians and in lowlands. Differences between the Western and Eastern Carpathians were noted earlier in the hepatic flora. Some hepatic species that are widespread and common in the Western Carpathians and in the Sudetes are either completely absent in the Eastern Carpathians or very rare (Szweykowski & Buczkowska, 1996). Similarly, Pawłowski (1972) already noted differences between the Western and Eastern Carpathians in the phanerogam flora. Species C is rare, both in lowlands and in the Western and Eastern Carpathians. Our data indicate partial connection between the species and substrata. All species may grow on humus or wet soil, but species A dominates on calcareous rocks, rock detritus or soil, B on clay soil and C on humus and wet sandy soil. Species C was

formerly can be found on wet sand of oligotrophic lakes in northern of Poland (Andrzejewska, 2000), but recently is rare here it is probably due to anthropogenic transformations of these areas.

The most patches of all species of *Aneura pinguis* complex are usually genetically monomorphic and consist of a single genotype. However, genetic exchange is probably present, since 50-80% of plants are fertile (Buczkowska *et al*., 2006), and 15.6-38.8% of patches in each species are polymorphic. Low intracolony variability is usually observed in liverworts (e.g. Boisselier-Dubayle & Bischler, 1997; Boisselier-Dubayle *et al*., 1998a, 1998b; Buczkowska, 2004; Adamczak *et al*., 2005; Bączkiewicz, 2012).

An evidence confirming independent evolutionary lineages in the studied cryptic species of *Aneura pinguis* is also provided by their genetic distances (*D*). Genetic distances between species A, B and C (Table 6) were close to the values found between the cryptic species of other liverwort complexes (e.g. Akiyma & Hiraoka, 1994; Boisselier-Dubayle *et al*., 1995; Boisselier-Dubayle & Bischler, 1999; Miwa *et al*., 2003, 2004) and even higher than between the genera of vascular plants (e.g. Rieseberg & Soltis, 1987; Gottlieb, 1981). Genetic distance (*D*) and genetic identity (*I*) play a significant role in identifying cryptic species within complexes. Gottlieb (1981) calculated that the mean genetic identity for 21 pairs of congeneric plant species was $I = 0.67 \pm 0.04$ (SE). Similarly, Crawford (1983) reported that genetic similarity between populations of one species is always $I > 0.90$. According to Stoneburner *et al*. (1991), genetic identities (*I*) in bryophytes between populations within cryptic species ranged from 0.856 to 0.939, and between populations of different cryptic bryophyte species ranged from 0.211 to 0.454.

The genetic diversity (H_S) within the populations of *Aneura pinguis* is the highest in species A (0.0694), nevertheless, it is typical for most thalloid liverworts that are mostly less variable than leafy liverworts (Bączkiewicz, 2012). The total allelic diversity (H_T) based on allele frequencies of polymorphic loci is the highest in species B. It is the consequence of the fact that in species B, the majority of genetic variation (83%) results from variation between populations (Table 5). On the other hand, in species A and C, the value H_T is lower than in species B and the majority of genetic variation (77% and 64% respectively) results from variation within populations. This result confirms the rule that in liverworts the percentage share of differentiation between populations in the total genetic diversity of species is largely correlated with the level of genetic diversity of the species (H_T). In the species with high total genetic diversity (H_T), differences between populations tend to be rather small. In turn, in the species with lower total genetic diversity, the percentage share of differentiation among populations in the total diversity of species was much higher (Bączkiewicz, 2012).

The genetic distance between the studied representatives of the *Aneura pinguis* complex, characteristic alleles, lack of recombinant gametophytes, differences in pattern of genetic diversity and different ecological and geographic preferences supported the hypothesis of evolutionarily isolated species and contributes to the formal description of these species in the nearer future.

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