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Application of different DNA markers in studies on cryptic species of *Aneura pinguis* (Jungermanniopsida, Metzgeriales)

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Abstract – The liverwort *Aneura pinguis* is represented in Europe by 4 cryptic species (A, B, C and D). They differ in morphology and ecological preferences. Three molecular marker systems (RAPD, ISJ and katG) were used to identify particular cryptic species. Ten samples of 4 cryptic species of *A. pinguis* and 2 samples of *A. maxima* (as an outgroup) were analysed. The 18 primers representing 3 different DNA marker categories revealed 147 bands, and 56 of them were species-specific. Among the cryptic species of *A. pinguis*, the greatest number of 21 specific bands was observed in species D, compared to 8 in species C, 7 in species B, 5 in species A, and 15 in *A. maxima*. The value of genetic similarity between analysed populations at the intraspecific level was I=0.90. In contrast, the I value between particular studied cryptic species ranged from 0.385 to 0.690. The similarities showed that species C and D are the most distinct, whereas species A and B are the most similar. The results of DNA analysis are consistent with earlier enzymatic data and support the division of *A. pinguis* into 4 cryptic species.

DNA markers / RAPD / ISJ / katG / cryptic species / *Aneura pinguis* / Metzgeriales / Hepaticae

INTRODUCTION

Cryptic species have separate gene pools due to reproductive isolation but they do not differ morphologically, or show only slight differences (Mayr, 1948, 1996). They are identified in routine allozyme or DNA analyses (Avise, 2004) and have proved to be common in animals (Sonneborn, 1975; Macdonald & Goldstein, 1999; Smith & Fonseca, 2004; Chang *et al*., 2006), plants (Lewis & Flechtner, 2004; Whittall *et al*., 2004; Fernandez *et al*., 2006; Grundt *et al*., 2006), fungi (Fell *et al*., 1992; Girandt *et al*., 1999; Naumova *et al*., 2004; Balajee *et al*., 2005) and bacteria (Wimpee *et al*., 1991; Laguerre *et al*., 1994).

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Cryptic species were first identified in certain bryophyte groups, e.g. liverworts and mosses with the help of allozymes in the late 1970s and in the early 1980s. The first case of cryptic speciation in liverworts was reported from Poland, in *Conocephalum conicum* (L.) Dumort. (Szweykowski & Krzakowa, 1979), and *Pellia epiphylla* (L.) Corda (Zielinski, 1987a). They were later identified also in other liverworts, such as *Pellia endiviifolia* (Dick.) Dumort. (Zielinski, 1987a), *Marchantia polymorpha* (Nees) Burgeff (Boisselier-Dubayle & Bischler, 1989; Boisselier-Dubayle *et al*., 1995), *Riccia dictyospora* (Howe) (Dewey, 1989), *Aneura pinguis* (L.) Dumort. (Szweykowski & Odrzykoski, 1990), *Corsinia coriandrina* (Spreng.) Lindb. (Boisselier-Dubayle & Bischler, 1998), *Reboulia hemispherica* (L.) Raddi (Boisselier-Dubayle *et al.*, 1998), *Targonia hypophylla* L. (Boisselier-Dubayle & Bischler, 1999) and *Conocephalum japonicum* (Thumb.) Grolle (Miwa *et al*., 2003; Miwa *et al.*, 2004), as well as in moss species, including *Leucobryum glaucum* (Hedw.) Ångstr. and *L. albidum* (P. Beauv.) Lindb. (Patterson *et al*., 1998), *Plagiomnium cuspidatum* Sieb. & Zucc. (Wyatt & Odrzykoski, 1998), *Neckera pennata* Hedw. (Appelgren & Cronberg, 1999), *Mielichhoferia elongata* (Hoppe & Hornsch.) Nees & Hornsch. and *M. mielichhoferiana* (Funck) Loeske (Shaw, 2000), *Rhytidiadelphus subpinnatus* (Lindb.) T. J. Kop. and *R. squarrosus* (Hedw.) Warnst. (Vanderpoorten *et al*., 2003).

Similarly as the cryptic species discovered in other groups of living organisms, they are characterized by the presence of distinct gene pools (different multilocus genotypes) as well as by the absence of morphological differences in the vast majority of cases (Nei, 1987; Avise, 2004). If present, such differences are slight only. Genetic similarity (*I*) between populations within a cryptic species often exceeds 0.90, while between cryptic species is often lower than 0.60 (Odrzykoski, 1987; Zielinski 1987a; Dewey, 1989). For the above-mentioned cryptic species of liverworts, it ranged from 0.155 to 0.667, with a mean value of 0.354 (Bączkiewicz *et al*., 2005). In comparison, morphologically well-distinguished species have parameters of genetic similarity at the intraspecific level, $I>0.90$ (Gottlieb, 1981; Crawford, 1983) and at the interspecific level, $I=0.67$ (Gottlieb, 1981) or 0.87 (Nei, 1987).

The origin of cryptic species is related to the process of speciation, involving natural selection that favours various genotypes in contrasting environmental conditions. The analysis of allele frequency in populations enables to determine whether these populations have a common gene pool or not, i.e. whether they represent the same or different biological species. Genetic variation patterns at the intraspecific and interspecific level differ considerably; therefore cryptic species can be detected relatively easily, already during routine population-based studies (Zielinski, 1987a). Populations representing separate gene pools, i.e. separate biological species, differ with regard to the presence of alternative alleles at some loci, being their diagnostic alleles.

Populations belonging to different biological species have separate multilocus genotypes, which are not subject to recombination due to the existence of a reproductive barrier (Szweykowski *et al*., 1981; Odrzykoski, 1987). The reproductive barrier is probably a by-product of genetic differences between species (Avise, 2004). The lack of experimental data on existence a barrier is not a factor excluding the possibility to distinguish cryptic species, since their presence may be confirmed directly by the differences in their gene pools, as mentioned above (Szweykowski *et al.*, 1981; Zielinski, 1987b; Szweykowski & Odrzykoski, 1990).

The cryptic species within bryophytes are sympatric or allopatric, which indicates that their speciation must have been affected by ecological or

geographical conditions (Odrzykoski & Szweykowski, 1991; Szweykowski *et al*., 1995; Shaw, 2001; Bączkiewicz *et al*., 2005).

The identification of cryptic species within various groups of living organisms is of great significance to population genetics, taxonomy and biogeography, so they are subject to extensive research (Wyatt *et al*., 1989a,b; Wachowiak-Zielinska & Zielinski, 1995; Mayr, 1996; Avise, 2004; Zielinski & Polok, 2005).

Aneura pinguis (L.) Dumort. is a dioecious thallose liverwort, whose chromosome number is n=9. It can be found in Europe, America, Asia, Africa, Australia, New Zealand and Madagascar (Schuster, 1992; Paton, 1999; Damsholt, 2002). Due to its wide ecological tolerance, *A. pinguis* is common in Poland both in lowlands and in upper parts of mountains (Bączkiewicz *et al*., 2005). *A. pinguis* is taxonomically homogenous, although it shows considerable morphological variation (Furuki, 1991; Schuster, 1992; Paton, 1999). The first two cryptic species of *A. pinguis,* known as A and B, were detected in Poland by Szweykowski & Odrzykoski (1990). They had separate multilocus genotypes with reference to 4 of the 7 analysed enzymatic loci, coding for AAT, PGD, PGM and SDH (57.1%), and displayed different ecological preferences. Cryptic species A grew on wet limestone and its detritus, whereas cryptic species B colonized the humus layer overlying the rocks. In the meantime, the third cryptic species (C) of *A. pinguis* was identified (Bączkiewicz *et al.,* unpublished data). These species differed in respect of four characteristics of oil-bodies, particularly their number per cell and size (Buczkowska *et al*., 2005), as well as with regard to 13 of 18 morphological and anatomical characters of the gametophyte (Buczkowska *et al*., 2005). Isoenzymatic studies have revealed the presence of cryptic species A also in the territories of Germany and Slovakia, of cryptic species B in Lithuania and Ireland, and of cryptic species C in Ireland. The fourth cryptic species, D, was reported from Great Britain and Ireland. These cryptic species had distinct multilocus genotypes, in 5 to 7 of the 12 analysed loci (Bączkiewicz & Buczkowska, 2005). Further enzymatic studies showed that the cryptic species of *A. pinguis* in Poland differ significantly in terms of location and distribution. The populations found in the Tatra Mts are represented by species A (90.9%), C (7.9%) and B (1.2%), the populations from the Pieniny Mts by species A (96.0%) and B (4.6%), while the populations from the Bieszczady Mts by species B (96.0%) and C (4%). The lowland populations are represented primarily by species B (about 68%), but also C (about 28%) and A (about 4%). Genetic similarity within particular cryptic species is very high, i.e. 0.956 in A, 0.772 in B and 0.964 in C, while genetic similarity between them varies from 0.219 to 0.361 (Bączkiewicz *et al*., unpublished data).

New, highly variable DNA markers have been introduced into studies on bryophytes recently (Fiedorow *et al*., 2001; Shaw, 2001; Pacak *et al*., 2002; Vanderpoorten *et al*., 2003; Feldberg *et al*., 2004). This provided the basis for confirming the presence of cryptic species distinguished by using isoenzymes, and for proposing new categories of PCR-based diagnostic markers (Zielinski & Polok, 2005). For example, ISJ (Intron Splice Junction) markers have confirmed the existence of 2 cryptic species in *C. conicum* species S and L (Zielinski, unpublished data), and of a third cryptic species in *P. endiviifolia* (Zielinski & Polok, 2005).

No DNA marker system has been developed for the cryptic species of *A. pinguis* to date. Therefore, the aims of the present study were: (1) to find DNA markers specific to its cryptic species, (2) to compare the effectiveness of 3 DNA marker systems for distinguishing the cryptic species, (3) to compare the results of DNA and isoenzymatic analysis of cryptic species of *A. pinguis*.

MATERIAL AND METHODS

Plant material

From various localities, 10 samples of the *A. pinguis* complex (3 samples of species A, B, C, and 1 of D) and 2 samples of *Aneura maxima* (Schiffn.) Steph. as an outgroup were studied (Table 1). Each cryptic species was identified by the isozyme method (Bączkiewicz & Buczkowska, 2005). *A. maxima* was identified morphologically according to Furuki (1991), Schuster (1992), Buczkowska & Bączkiewicz (2006). From each sample, 10-15 thalli were randomly chosen for the molecular study. They were all stored at – 80ºC until used. Vouchers were deposited in the POZW herbarium.

DNA extraction

In total, 0.3-1.5 g of thalli were taken from each sample. DNA was isolated by the modified CTAB procedure (Murray & Thompson, 1980). Briefly, the liquid-nitrogen-groundthalli were thoroughly mixed with 3 ml of preheated CTAB isolation buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl and 2% ß-mercaptoethanol) and incubated at 56°C for 1 h. After 3 chloroform/isoamyl alcohol extractions, the DNA was precipitated and dissolved in sterile, deionized H_2O. The purity of DNA samples was assessed spectrophotometrically and reached 88-94%. The DNA content of the samples was 11.2 µg to 46.4 µg.

Table 1. Localities and herbarium number (POZW) of studied samples of *A. pinguis* cryptic species A, B, C and D, and *A. maxima*.

Species	*No of sample*	*Localities*	*POZW No*
A. pinguis-A	A 1	Pieniny Mts, Skalskie stream, Skalskie reserve	P 63-4
	A 2	Tatra Mts, Jaworzynka Valley	T 73-2
	A 3	Tatra Mts, Chochołowska Valley	T 86-3
A. pinguis-B	B 1	Tatra Mts, Wielka Sucha Valley	T 79-1
	B 2	Wielkopolska region, Diabli Skok reserve	DS 8-1
	B 3	Wielkopolska region, in Poznań city	PO 1-3
A. pinguis-C	C 1	Tatra Mts, Sucha Woda Valley	T 74-4
	C 2	Tatra Mts, Skupinów Upłaz Mt., NE slope	T 132-1
	C 3	Tatra Mts, Pańszczyca Valley	T 157-1
A. pinguis-D	D	Great Britain, Scotland, North Ebudes	ANG 3-1
A. maxima	M 1	Wielkopolska region, Diabli Skok reserve	DS 5-2
	M 2	Tatra Mts, Capowski Forest	T 161-1

RAPD markers

The sequences of 6 RAPD primers used for DNA amplification in this study are given in Table 2. The PCR reaction was conducted in a volume of 20 µl containing PCR buffer [20 mM $(NH_4)_2SO_4$ and 50 mM Tris-HCl, pH 9, at 25°C], 2 mM $MgCl_2$, 1 × concentrated Enhancer containing betaine, 200 µM each of dATP, dGTP, dCTP, and dTTP, 0.3 µM primer, 1 unit of Tfl polymerase (Epicentre Technology) and 60 ng of template DNA. The reaction proceeded at 94°C for 3 min., followed by 45 cycles at 94°C for 1 min., 37°C for 1 min., and 72°C for 2.5 min., with a final extension step of 72°C for 5 min.

ISJ markers

The sequences of 4 ISJ primers used for DNA amplification in this study are given in Table 2. The PCR reaction was conducted in a volume of 20 µl containing PCR buffer [20 mM $(NH_4)_2SO_4$ and 50 mM Tris-HCl, pH 9 at 25°C], 2 mM $MgCl_2$, 1 × concentrated Enhancer containing betaine, 200 µM each dATP, dGTP, dCTP, dTTP, 0.3 µM primer, 1 unit of Tfl polymerase (Epicentre

Table 2. The sequences of RAPD, ISJ and katG primers used in the study.

Type of marker	*Abbreviation*	*No. of nucleotides*	*Primer sequence*
RAPD	OPA-01	10	5'CAGGCCCTTC3'
	OPB-14	10	5'TCCGCTCTGG3'
	OPB-19	10	5' ACCCCCGAAG 3'
	OPD-02	10	5'GGACCCAACC3'
	OPD-03	10	5'GTCGCCGTCA3'
	OPD-07	10	5' TTGGCACGGG 3'
ISJ	ISJ1	10	5'CAGACCTGCA 3'
	ISJ2	18	5'ACTTACCTGAGGCGCCAC3'
	ISJ4	18	5'GTCGGCGGACAGGTAAGT3'
	ISJ5	16	5' CAGGGTCCCACCTGCA 3'
katG	katG 4-1	19	5' TCGACTTGACGCCCTGACG3'
	katG 4-2	18	5'CAGGTCCGCCCATGAGAG3'
	katG 5-1	19	5' CGACAACGCCAGCTTGGAC 3'
	katG 5-2	20	5' GGTTCACGTAGATCAGCCCC 3'
	katG 6-1	20	5' GCAGATGGGGCTGATCTACG 3'
	katG 6-2	18	5' ACCTCGATGCCGCTGGTG 3'
	katG 11-1	20	5' TGCTCGACAAGGAGAACCTG 3'
	katG 11-2	20	5' TCCGAGTTGGACCCGAAGAC 3'

Technology) and 60 ng of template DNA. The reaction proceeded at 94°C for 3 min., followed by 45 cycles at 94°C for 1 min., 56°C for 1 min., and 72°C for 2.5 min., with a final extension step of 72°C for 5 min.

katG markers

The 4 pairs of primers complementary to *Mycobacterium tuberculosis* (Zopf) *katG* gene (Table 2) encoding catalase-peroxidase were used according to a method developed by Zielinski & Polok (2005). The amplification mixture of a 20-µl volume contained 20 mM $(NH_4)SO_4$, 50 mM Tris-HCl (pH 9.0 at 25°C), 1.5 mM $MgCl_2$, 1 × concentrated Enhancer with betaine, 200 µM each of dATP, dGTP, dCTP, dTTP, 1.0 µM of each primer, 0.75 unit of Tfl polymerase (Epicentre Technology) and 100 ng of template DNA. The reaction proceeded at 94°C for 1 min., followed by 30 cycles at 94°C for 1 min., 58°C for 1 min., and 72°C for 1.5 min., with a final extension step of 72°C for 5 min.

Electrophoresis

PCR samples were loaded on a 1.2% (for RAPD and ISJ markers) or 1.5% (katG) agarose gel containing 0.5 µg/ml of ethidium bromide and separated in 1 × TBE buffer at 120 V constant power. Gels were observed in UV light (312 nm) and photographed.

Data analysis

All bands that could be reliably read were treated as single dominant loci and scored as either present (1) or absent (0) across all genotypes. The degree of genetic similarity was determined with Nei's (1978) formula and calculated by using POPGENE-1.32 software (Yeh & Boule, 1999). The dendrogram was created on the basis of UPGMA. Statistica 6.0 was used for principal component analysis.

RESULTS

Primer efficiency

The analysis of 10 samples of the four cryptic species of *A. pinguis* and 2 samples of *A. maxima* by 18 primers representing 3 DNA marker categories enabled to distinguish 147 different bands. The highest number of 57 different bands was revealed by 6 RAPD primers – on average 9.5 bands per primer. The 4 ISJ primers used in the study showed a similar efficiency, revealing a total of 45 different bands (11.3 bands per primer). Four pairs of primers complementary to the bacterial sequences of the *katG* gene enabled to distinguish also 45 different bands (11.3 bands per pair of primers).

From 147 distinguished bands, 112 were present in *A. pinguis* (cryptic species A, B, C and D) and 49 in *A. maxima* (Table 3). Numbers of bands detected by RAPD, ISJ and katG markers were, respectively: 45, 37 and 30 in cryptic species of *A. pinguis* and 23, 15 and 11 in *A. maxima*.

Table 3. Efficiency of RAPD, ISJ and katG markers in identification of polymorphic and specific bands in *A. pinguis* cryptic species and *A. maxima*.

Species	*RAPD*		*ISJ*		*katG*		*Total*
	N	%	*N*	%	*N*	%	
	Bands detected in *A. pinguis* and *A. maxima*						
Aneura pinguis	45	100.0	37	100.0	30	93.3	112
Aneura maxima	23	13.0	15	13.3	11	18.2	49
	Bands specific for *A. pinguis* cryptic species and *A. maxima*						
A. pinguis-species A	4	20.0	1	12.5	0	0	5
A. pinguis-species B	1	3.0	4	14.8	2	11.8	7
A. pinguis-species C	0	0	1	50.0	7	41.2	8
A. pinguis-species D	5	26.3	4	33.3	12	54.6	21
Aneura maxima	9	40.9	4	26.7	2	18.2	15
Total	19	33.3	14	31.1	23	51.1	56

DNA polymorphism in cryptic species of A. pinguis *and in* A. maxima

In the analysed material, 144 of 147 identified bands were polymorphic (98%). In *A. pinguis*, the RAPD and ISJ markers detected 100% of polymorphic bands and katG detected 93.3%, while in *A. maxima* the corresponding values were: 13.0%, 13.3.% and 18.2% (Table 3).

The highest polymorphism was observed in *A. pinguis* species B (36.7% of polymorphic bands), followed by *A. pinguis* species A (11.6%) and *A. pinguis* species C (2.0%). In *A. maxima*, 4.8% of bands were polymorphic. The ISJ markers turned out to be the most polymorphic within the species examined. 46.7% of polymorphic ISJ bands were found in *A. pinguis* species B, 15.6% in *A. pinguis* species A, and 4.4% in *A. maxima*. There were no polymorphic ISJ bands in *A. pinguis* species C. Lower polymorphism was revealed by RAPD markers. The value of polymorphic RAPD bands varied from 38.6% in *A. pinguis* species B to 12.3% in *A. pinguis* species A, and reached 5.3% *A. maxima*. The 4 pairs of katG primers revealed the lowest level of polymorphism. However, as in the case of ISJ and RAPD markers, the highest percentage of polymorphic katG bands was recorded in *A. pinguis species* B (24.4%). Interestingly, the percentage of polymorphic katG bands in the other cryptic species of *A. pinguis* (A and C) was 4-fold lower (6.7%). Moreover, a low level of polymorphism was also found in *A. maxima* samples (4.4%).

Genetic similarities at the intra- and interspecific levels

Values of genetic similarities between analysed populations at the intraspecific level were very high and often exceeded I=0.90 (Table 4). The

Table 4. Nei's genetic similarities (above the diagonal) and genetic distances (below the diagonal) among *A. pinguis* cryptic species and *A. maxima*.

Samples	*A1*	*A2*	*A3*	*B1*	*B2*	*B3*	*C1*	*C2*	*C3*	*D*	*Am-1*	*Am-2*
A1		0.918	0.932	0.544	0.660	0.599	0.539	0.548	0.522	0.599	0.680	0.646
A2	0.085		0.918	0.585	0.660	0.612	0.5303	0.539	0.530	0.585	0.653	0.646
A3	0.071	0.085		0.585	0.674	0.612	0.5303	0.539	0.530	0.585	0.680	0.646
B1	0.608	0.536	0.536		0.735	0.728	0.417	0.426	0.400	0.442	0.497	0.490
B2	0.416	0.416	0.395	0.308		0.803	0.530	0.539	0.530	0.517	0.571	0.551
B3	0.513	0.491	0.491	0.318	0.220		0.548	0.556	0.548	0.551	0.619	0.585
C1	0.618	0.634	0.634	0.874	0.634	0.602		0.989	0.978	0.374	0.530	0.496
C2	0.602	0.618	0.618	0.853	0.618	0.586	0.011		0.967	0.383	0.539	0.504
C3	0.651	0.634	0.634	0.917	0.634	0.602	0.023	0.034		0.391	0.513	0.478
D	0.513	0.536	0.536	0.816	0.660	0.596	0.984	0.961	0.939		0.497	0.476
Am-1	0.385	0.426	0.385	0.700	0.560	0.480	0.634	0.618	0.668	0.700		0.952
Am-2	0.437	0.437	0.437	0.714	0.596	0.536	0.702	0.685	0.738	0.742	0.049	

highest similarity was found between 3 samples of *A. pinguis* species C, which were almost identical with the *I* value ranged from 0.967 to 0.989. A little higher differentiation was observed in samples of *A. pinguis* species A, where the genetic similarity coefficient ranged from 0.918 to 0.932. In contrast to species A and C, 3 samples of *A. pinguis* species B were the most differentiated (from 0.728 to 0.803). The differentiation between samples of *A. maxima* was similar to *A. pinguis* species A and C (*I*=0.952).

The genetic similarities were much lower at the interspecific level than at the intraspecific level. The *I* value between species A, B, C and D of *A. pinguis* ranged from 0.385 to 0.690, confirming their distinction. The *I* value was the highest between species A and B (*I*=0.690) and the lowest between C and D (*I*=0.385). The DNA markers applied in the study indicated a similar degree of genetic identity between cryptic species of *A. pinguis* as between them and the two *A. maxima* populations. The *I* value ranged from 0.492 to 0.685 and was the highest between *A. pinguis* species A and *A. maxima* and the lowest between *A. pinguis* species D and *A. maxima* (0.492). It is worthy of note that the genetic similarity between *A. pinguis* species C and D (0.385) was even lower than between individual cryptic species and *A. maxima* (Table 5).

The cluster analysis, presented in the form of an UPGMA dendrogram, divided the 12 studied populations into 5 groups, corresponding to 4 cryptic species of *A. pinguis* and A. *maxima* (Fig. 1). The outermost position is occupied by *A. maxima* and *A. pinguis* species C. The scatter diagram based on PCA analysis also grouped the 12 studied populations into 5 entities, according to their biological status (Fig. 2).

There were only slight differences between applied DNA markers in the level of genetic similarity assessment among taxa examined (Table 5). For the RAPD markers the similarities between *A. pinguis* cryptic species ranged from 0.555 to 0.746 (mean 0.654), and for ISJ from 0.541 to 0.737 (mean 0.657). Greater

Table 5. Nei's genetic similarities among *A. pinguis* cryptic species A, B, C and D, and *A. maxima* based on three DNA marker categories.

Species pair	*RAPD*	*ISJ*	*katG*	*Total*
A. pinguis A - B	0.637	0.621	0.818	0.690
A. pinguis A - C	0.746	0.737	0.674	0.552
A. pinguis A - D	0.559	0.660	0.609	0.605
A. pinguis A - *A. maxima*	0.600	0.613	0.859	0.685
A. pinguis B - C	0.681	0.691	0.712	0.550
A. pinguis B - D	0.636	0.541	0.455	0.550
A. pinguis B - *A. maxima*	0.550	0.564	0.730	0.611
A. pinguis C - D	0.667	0.689	0.293	0.385
A. pinguis C - *A. maxima*	0.649	0.697	0.631	0.520
A. pinguis D - *A. maxima*	0.436	0.539	0.517	0.492

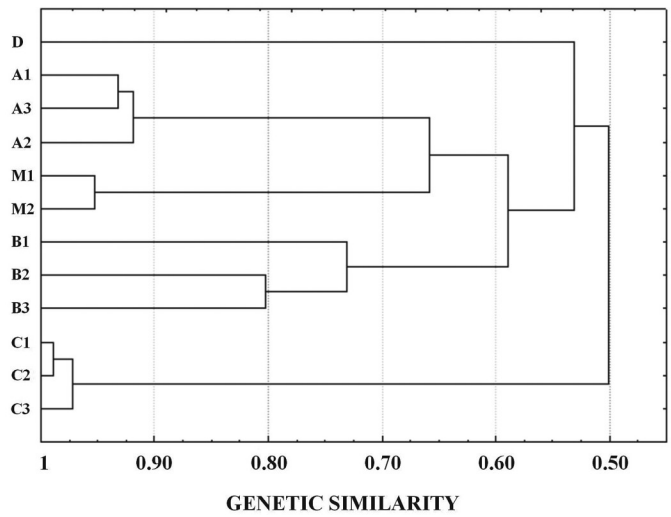

Fig. 1. UPGMA grouping of *A. pinguis* cryptic species and *A. maxima* based on Nei's genetic similarity.

differences were revealed by katG markers, where the *I* value ranged from 0.293 to 0.818 (mean 0.594). Furthermore, the *I* value between particular cryptic species of *A. pinguis* and *A. maxima* for RAPD markers ranged from 0.436 to 0.649 (mean 0.559), for ISJ from 0.539 to 0.697 (mean 0.603), and for katG from 0.517 to 0.859 (mean 0.684).

The differences in *I* value were observed between *A. pinguis* spe-cies A and B for katG markers, (0.818), RAPD (0.637), and ISJ (0.621). In the case of *A. pinguis* species D and C, the lowest *I* value was found in the katG markers,

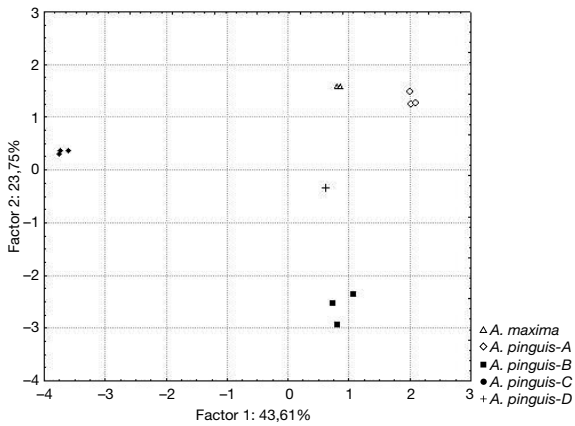

Fig. 2. Principal component analysis of *A. pinguis cryptic* species and *A. maxima.*

I=0.293. Also in this case, the RAPD and ISJ markers revealed a similar degree of genetic similarity: *I*=0.667 and *I*=0.689, respectively.

Species-specific DNA markers

The 3 categories of DNA markers applied in the study revealed a total of 56 species-specific bands (38.1%), including 41 (36.7%) in cryptic species of *A. pinguis* and 15 (30.6%) in *A. maxima* (Table 3). Among cryptic species of *A. pinguis*, the greatest number of 21 specific bands were observed in species D, 8 in C, 7 in B, and 5 in A.

The greatest number of 23 species-specific bands was revealed by katG markers. Most of them were observed in *A. pinguis* species D (12) and C (7). Two other species-specific bands of this category were found in *A. pinguis* species B and *A. maxima*. The RAPD markers revealed 19 species-specific bands: 9 in *A. maxima*, 5 in *A. pinguis* species D, 4 in A, and 1 in B. The lowest number of 14 species-specific bands was identified with ISJ markers. Among them, 4 were found in *A. pinguis* species B, D and in *A. maxima*. In *A. pinguis* species A and C the ISJ markers revealed only 1 species-specific band.

For molecular identification of cryptic species in the *A. pinguis* complex, katG markers appeared the most useful. Except the katG-6 primers, which make it possible to distinguish only *A. pinguis* species D from the other cryptic species, the other katG primers permit to identify up to 4 cryptic species at once. The katG-4 primers enable identification of all *A. pinguis* cryptic species (Fig. 3). *A. pinguis* species D has bands 1, 5 and 9, but *A. pinguis* species A has only band 5, while bands 3 and 4 are characteristic for *A. pinguis* species B, and bands 2, 6,

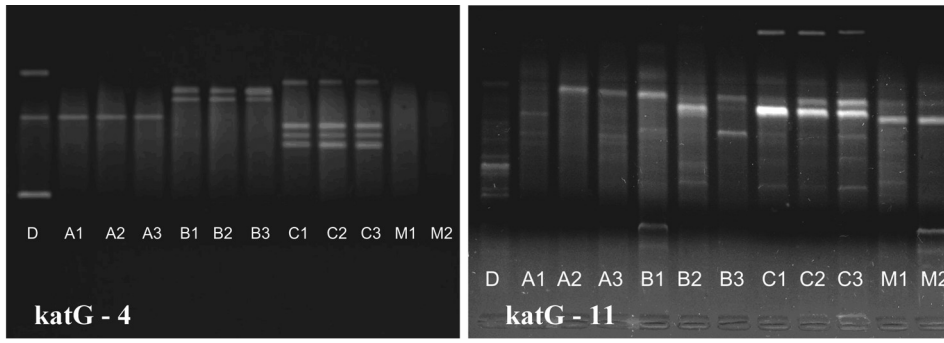

Fig. 3. Amplification products generated by katG-4 and katG-11 primers.

7 and 8 are characteristics for *A. pinguis* species C. No amplifications are found in *A. maxima* populations. The katG-5 primers enable to distinguish *A. pinguis* species C, D and *A. maxima*. Bands 3 and 4 are present only in *A. pinguis* species C, whereas bands 1 and 9 are characteristics for *A. pinguis* species D and *A. maxima*, respectively. The katG-11 primers enable molecular identification of the same species as katG-5 (Fig. 3). Five bands (2, 3, 8, 13 and 16) are characteristics for *A. pinguis* species D. The first band was present only in *A. pinguis* species C and band 7 was found only in *A. maxima* samples.

DISCUSSION

The present study, conducted by using DNA markers, confirmed the presence of 4 cryptic species of *A. pinguis*, previously detected with isoenzymes (Szweykowski & Odrzykoski, 1990; Bączkiewicz *et al*., 2005; Andrzejewska, unpublished). The tested cryptic species have distinct gene pools, as confirmed by the presence of specific DNA markers and different multilocus genotypes, as well as by low values of genetic similarity between populations at the interspecific level, in comparison with the intraspecific level.

The DNA markers used in this study were found to be more effective with regard to the number of revealed diagnostic loci, as compared to isoenzymes. The analysis of 12 enzymatic loci (Bączkiewicz *et al*., 2005) enabled to identify a total of 9 diagnostic loci. Particular cryptic species had from 1 (species D) to 4 (species B) of such loci, absent in the other species. The compared pairs of cryptic species had different multilocus genotypes, in which 5 to 7 loci (approx. 50%) were represented by alternative alleles.

A comparable analysis performed with the use of 6 RAPD primers, 4 ISJ primers and 4 pairs of primers complementary to the bacterial sequence of the *katG* gene, revealed a total of 56 specific loci, from 5 to 21 for each of the examined cryptic species. It must be stressed that species D has a higher number of specific loci (21) than the outgroup species *A. maxima* (15). Cryptic species D has been identified most recently in this group. It has been reported only from Great Britain and Ireland (Bączkiewicz *et al*., 2005). Species D differs considerably from the other 3 cryptic species in terms of the structure of oil-bodies, which are coarsely granular and big (5-12 μm) in species D, whereas homogenous to finely granular and smaller (1-10 μm) in species A, B and C (Buczkowska *et al*., 2005). Paton (1999) demonstrated 2 types of oil-bodies, smaller (1-5 μm) and bigger (5-12 μm), in *A. pinguis* from Great Britain, which may correspond to cryptic species B and D (Bączkiewicz *et al*., 2005).

The high number of diagnostic markers observed at the DNA level allowed us to verify the degree of differences between the cryptic species of *A. pinguis,* as well as to shift species D from the position with the lowest number of specific markers (1) to the position with the highest number of these markers (21). Despite the restrictions of the isoenzymatic analysis, related primarily to the limited number of enzymatic loci analysed, which in most cases ranges from 10 to 20, this method may be successfully used for screening tests. It should be emphasized that almost all cryptic species within bryophytes were detected by this method. Isoenzymes are highly effective in identifying cryptic species, since they permit the analysis of enzyme-coding genes, which are most probably subject to selection (Nevo, 1983). Two cryptic species of *P. epiphylla*, which had alternative

alleles at 6 loci of the 17 analysed (35.3%), were detected in this way (Zielinski, 1987a). They also differed in thallus size under natural conditions, and showed statistically significant differences in the majority of morphological and anatomical characters of the thallus determined in culture. Their distribution in Poland is allopatric. *P. epiphylla* N, n=9, with small-sized thalli, is rare and can be found only in northern Poland (and in Scandinavia), while *P. epiphylla* S, n=9, with larger thalli resembling those of *P. borealis* Lorbeer, is more common in southern Poland, especially in the mountains (and in the south of Europe) (Zielinski, 1987a; Szweykowski *et al*., 1995).

Comparative studies on the cryptic species of *P. endiviifolia*, conducted by using isoenzymes and DNA markers, revealed further limitations of the isoenzymatic analysis. The first two cryptic species were detected within this taxon by using isoenzymes. They are referred to as a typical form and an aquatic form. They differed in 10 of the 13 enzymatic loci analysed (77%) (Zielinski, 1987a). The typical form grows on a solid substratum, usually limestone, and forms characteristic curled thalli (f. *fabroniana*), whereas the aquatic form occurs in stagnant waters, often under the surface. An analysis of *P. endiviifolia* populations with DNA markers confirmed the presence of the first 2 forms, later called A and B, and revealed the existence of the third, well-headed cryptic species C (Polok *et al.*, 2005c). These three cryptic species of *P. endiviifolia* differed in thallus morphology examined in culture (Polok *et al*., 2005c). Similarly as cryptic species A, B, C of *A. pinguis*, also cryptic species of *P. endiviifolia* are located in a small area in Poland and display ecological tolerance to varying environmental conditions, constituting a genetic mechanism of their differentiation.

The analysed enzymatic loci make a very limited and specific part of the genome, so they do not always enable to detect the existing differences, as illustrated by the example of cryptic species C of *P. endiviifolia*. Another well-known example of conservatism of enzymatic loci in the complex of closely related pine species, i.e. *Pinus sylvestris* L.*, P. mugo* Turra*, P. uliginosa* Neumann and *P. uncinata* Raymond ex A.DC., making it impossible to detect diagnostic allozymes as well as to identify these taxa at the molecular level and to determine relationships between them (Neet-Sarqueda, 1994; Prus-Głowacki *et al*., 1998; Lewandowski *et al*., 2000). However, diagnostic DNA markers may be easily generated in these species (Zielinski & Polok, 2005).

The markers compared in this study (RAPD, ISJ and katG), represent 3 distinct categories, amplifying random, semirandom and unique genome sequences. All of these categories were found to be suitable for generating species-specific bands. They revealed 19, 14 and 23 diagnostic loci, respectively.

Some authors discuss the application of RAPDs in taxonomy, because several factors can influence their reproducibility, e.g. DNA purity, concentration of the template, primers and magnesium chloride, type of primers and *Taq* polymerase, thermocycler and reaction conditions (Edwards, 1998; Harris, 1999; Rabouam *et al*., 1999). However, diagnostic RAPD markers have made it possible to distinguish taxonomically difficult species in bryophyte genera, e.g. *Porella* (Boisselier-Dubayle & Bischler, 1994), *Polytrichum* (Zouhair *et al*., 2000) or *Sphagnum* (Såstad *et al*., 1999; Polok *et al*., 2005a), as well as in genera of vascular plants, e.g. *Picea* (Nkongolo *et al*., 2003), *Astragalus* (Mehrnia *et al*., 2005), *Polygonatum* (Polok *et al*., 2005b; Szczecińska *et al*., 2006) and members of Poaceae (Polok, 2005). Other works on the application of RAPDs in taxonomy of liverworts also showed that this method can be used to study the relationships between cryptic species, e.g. *Pellia* (Pacak *et al*., 1998;

Polok *et al*., 2005c) and *Marchantia* (Boisselier-Dubayle *et al*., 1995). The results of our study indicate a high reproducibility of RAPDs when DNA of high purity was used.

The semirandom, ISJ markers were used for the first time in studies on *Zea mays* (L.) by Rafalski (1997). The markers were also used with success for identification 3 *P. endiviifolia* cryptic species of (Polok *et al*., 2005c) as well as *Rosa* sp. (Nowak & Polok, 2005) and *Polygonatum* sp. (Polok *et al*., 2005b).

The katG markers, characterized by high conservativeness and a low level of intraspecific polymorphism, are very useful for taxonomy. Their effectiveness increases with an increase in the genetic distance between the examined taxa (Zielinski & Polok, 2005). For instance, in three grass species, *Calamagrostis arundinacea* (L.) Roth., *Lolium perenne* L. and *Phragmites australis* (Cav.) Trin. *ex* Steud., the proportions of katG specific bands were: 65.2%, 47.5% and 36.7%, respectively (Krzakowa *et al*., 2007). The katG markers were the only system that allowed researchers to distinguish the majority of 7 species of the genus *Lolium*, which was impossible to accomplish with AFLP, ITS and STS markers (Polok *et al*., 2006). The katG markers were effective in identification of species-specific bands in many angiosperms, including various grasses as well as species of the genera *Rosa* and *Polygonatum* (Zielinski & Polok, 2005; Szczecińska *et al*., 2006). In addition, the katG system was successfully applied for population and phylogenetic studies of *Pinus cembra* L. and *P. pumila* (Pall.) Regel (Chmiel & Polok, 2005), *P. sylvestris* and *P. mugo* (Zielinski & Polok, 2005) as well as liverwort species of the genus *Pellia* (Polok, unpublished data). Thus, RAPDs, ISJs and katGs offer an efficient tool for species identification, including cryptic species.

Apart from species-specific bands, also more variable bands were detected, which may have a potential application while examining genetic variation within cryptic species of the *A. pinguis* complex. Preliminary research showed the highest degree of polymorphism in species B (36.7%), followed by A (11.6%) and C (2%). The katG markers proved to be as effective as RAPD and ISJ markers with regard to polymorphism detection within cryptic species, as well as to the identification of diagnostic loci in these species. This may be due to a considerable degree of similarity between cryptic species, which – as most closely related – constitute the last segment of the genealogical tree, but also to the selection of primers for the amplification of sequences complementary to the bacterial *katG* gene, applied in the study. Tests performed on a variety of plant species demonstrated that particular pairs of primers reveal both monomorphic and polymorphic sequences (Polok, unpublished data). This may indicate a different degree of conservatism of individual fragments of the bacterial *katG* gene, whose sequences can be found in the analysed plants (Zamocky, 1994). An example can be the presence of monomorphic sequences in *C. arundinacea*, amplified with primers designed to the part of the *katG* gene coding for N-termini of catalase-peroxidase (katG9, katG10, katG12) and polymorphic sequences on C-termini of this protein (katG2, katG3, katG4) (Krzakowa *et al*., 2007). In studies on *A. pinguis*, the primers used for katG4, katG5, katG6 and katG11 sequences amplified the sequences encoding both N-termini and C-termini of proteins.

The analysis of 12 putative enzyme loci in 1652 individuals of *A. pinguis* species A, B and C from Poland (representing 14, 12 and 9 localities, respectively) revealed a similar degree of polymorphism. In species A, the *P* value (percent of polymorphic loci) was 28.6%, compared to 13.2% in B and 5.6% in C (Bączkiewicz *et al*., unpublished data). The analysis of genetic similarity between

the tested populations of *A. pinguis* clearly indicates that it exists at two levels: intra- and interspecific. The populations within species A and C, as well as within *A. maxima*, display genetic similarity exceeding *I*=0.900, while in the case of the populations of species B this value is much lower and ranges from 0.728 to 0.803. Genetic similarity between 4 cryptic species of *A. pinguis* is as low as 0.385 to 0.690. It is comparable to the values of genetic similarity between each of these species and *A. maxima*. The results correspond to the grouping of these populations carried out lately, on the basis of enzymatic data (Bączkiewicz *et al*., unpublished data), when genetic similarity within particular cryptic species was very high, *i.e.* 0.96, 0.77, 0.96, respectively, while genetic similarity between them varied from 0.219 to 0.361. Three categories of markers, RAPD, ISJ and katG, revealed a comparable degree of genetic similarity between the cryptic species analyzed in the study.

On the basis of DNA markers, the analysed species C and D are the most distinct, whereas species A, B and *A. maxima* are more similar. This result is different from isozyme results, where species A and D were the most distinct (Bączkiewicz & Buczkowska, 2005). Results of the molecular study confirmed our previous hypothesis (Bączkiewicz & Buczkowska, 2005) that *A. pinguis* species D, occurring on the British Isles, is another cryptic species within the *A. pinguis* complex. Genetic similarities between cryptic species of *A. pinguis* are comparable or even lower than between other species. For example, genetic similarities based on RAPD markers between other cryptic liverwort species were 0.713-0.717 in the *P. epiphylla* (L.) Corda complex (Pacak *et al*., 1998) and 0.562-0.649 in the *M. polymorpha* complex (Boisselier-Dubayle *et al*., 1995). Comparable values of genetic similarity were also reported between morphologically recognized species in mosses, e.g. in the genera *Polytrichum* (Zouhair *et al*., 2000), *Sphagnum* (Såstad *et al*., 1999), and in vascular plants, e.g. *Picea* (Nkongolo *et al.* 2003), *Pinus* (Chmiel & Polok, 2005), *Carica* (Jobin-Decor *et al*., 1997), *Brassica* (Lázaro & Aguinagalde, 1998), *Lippia* (Viccini *et al*., 2004), and *Polygonatum* species (Polok *et al*., 2005b; Szczecińska *et al*., 2006).

It is much more difficult to confirm the presence of a reproductive barrier between species (including cryptic ones) in bryophytes than in higher plants. Gametophytes growing under controlled conditions, e.g. in a greenhouse, often do not produce generative organs of one or both sexes. Field investigations on hybridization are also difficult or impossible to conduct in the case of allopatric distribution of parental forms. Such a situation was observed in *P. epiphylla* species S and N, which show allopatric distribution and produce no female generative organs under culture conditions (Zielinski, unpublished data). However, in 2 cryptic species of *C. conicum* species S and L, the analysis of gametophytes from mixed populations revealed no recombinants, indicating a complete reproductive isolation between them (Szweykowski *et al*., 1981; Odrzykoski, 1987). No recombinants were detected between 2 cryptic species of *P. endiviifolia*, the typical form A and the aquatic form B, in a mixed population from the Tatra Mountains, being the only known locality of their co-occurrence (Zielinski, 1987a). No recombinants between cryptic species in mixed populations were recorded in *A. pinguis*, either (Bączkiewicz *et al*., unpublished data). It should be stressed that this species produces no generative organs under culture conditions (Buczkowska *et al*., unpublished data).

The identification of cryptic species in bryophytes significantly extends our knowledge in the field of population genetics, taxonomy and biogeography of this group of plants (Stoneburner *et al*., 1991). When 2 alternative multilocus

genotypes were detected for the first time in *C. conicum* with the use of allozymes, it was assumed that they possibly constituted 2 excluding recombination systems within this species (Szweykowski *et al*., 1981). A consequence of such an interpretation of the reasons for the detected genetic variation in *C. conicum* could be a considerable overestimation of variation parameters (*P*, *A* and *H*) in this species. However, this hypothesis was excluded during further research, which showed that these 2 alternative genetic systems belong to separate gene pools. On the basis of numerous morphological and anatomical diagnostic features, species S of *C. conicum* has acquired the status of species – *C. salebrosum* Szweykowski, Buczkowska & Odrzykoski (Szweykowski *et al*., 2005). Morphological differences have also been detected between *P. epiphylla* species S and N (Zielinski, 1987a), *P. endiviifolia* species A, B, C (Polok *et al*. 2005c), as well as in the cryptic species of *A. pinguis* (Buczkowska *et al*., 2005; Buczkowska *et al*., 2006). It can be expected that as in the case of *C. conicum*, detailed biometric tests in other cryptic species within bryophytes will be followed by concrete taxonomic decisions.

For example in *C. conicum, P. endiviifolia* and *A. pinguis*, thorough population-based studies (including an analysis of the distribution range of bryophyte species) showed that cryptic speciation takes place on both ecological and geographical planes. As for the ecological perspective, it has been found that the part of variation considered plasticity (Schuster, 1966) has a genetic basis and results from the adaptation of particular cryptic species to a given habitat. *C. conicum* is a good illustration of cryptic speciation of geographical character. A total of 5 cryptic species of *C. conicum* have been identified (*I*=0.178–0.593), of which L is limited to Europe only, A and C are endemic to North America, while J occurs exclusively in Japan (Odrzykoski & Szweykowski, 1991). Species S is common both in Europe and in the United States, where it forms sympatric populations with species A. The cryptic species of *P. endiviifolia* reported from Poland have different gene pools than *P. endiviifolia* in Japan (*I*=0.231–0.461), which additionally can be differentiated into at least 2 cryptic species (*I*=0.692) (Zielinski, 1987a). Species C of *P. endiviifolia* (Polok *et al*., 2005c) has been recently recognized not only in Poland, but also in the Czech Republic (Sawicki unpublished data). *A. pinguis* shows a similar mode of cryptic speciation. Thus, it seems that broad geographical studies of the species at the DNA and morphological level, including herbarium materials, may be of great taxonomic value.

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