Cryptogamie, Bryologie, 2005, 26 (2): 151-165

Comparison of allozyme variability in Polish populations of two species of *Ptilidium* Nees (Hepaticae) with contrasting degrees of sexual reproduction

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(Received 15 March 2004, accepted 29 August 2004)

Abstract – The genetic structure of 19 populations of two species of the genus *Ptilidium* – *P. pulcherrimum* and *P. ciliare* from Poland and Slovakia was studied. Nine putative loci in nine enzyme systems were resolved. Two loci (*Got* and *Me*) were monomorphic at the genus level. Both species were fixed for alternative alleles at three loci (*Acp*, *Idh* and *Prx*-A) that can be used as diagnostic markers for these two species. Both species proved to be polymorphic: *P. ciliare* for three (*Gdh*, *Pgm* and *Sdh*), and *P. pulcherrimum* for four loci (*Gdh*, *Pgm*, *Sdh* and *Pgd*). Gene diversity statistics show a lower total gene diversity (H_T) of asexually reproducing *P. ciliare* (0.299 ± 0.089) as compared to sexually reproducing *P. pulcherrimum* (0.379 ± 0.044). The G_{ST} in *P. ciliare* was 0.263, in *P. pulcherrimum* 0.374. In both species a relation between the allele numbers and allele frequencies with geographical regions was observed. Montane populations of *P. ciliare* displayed a lower number of alleles than the populations derived from the lowlands, whereas in *P. pulcherrimum* no genetic differentiation between both geographical regions was observed.

***Ptilidium ciliare* / *Ptilidium pulcherrimum* / isozymes / genetic variation / sexual reproduction / vegetative spread**

INTRODUCTION

Considering that the haplophase dominates the life cycle of bryophytes, that their gametophytes are directly subjected to natural selection and that self-fertilization and vegetative reproduction is frequent bryophytes were considered

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to exhibit little genetic variation and therefore to be more slowly evolving than flowering plants (Anderson, 1963; Schuster, 1966; Crum, 1972). However, numerous isozyme investigations of natural populations indicate that the level of genetic variation of many bryophyte species is comparable to the one typical for outcrossing vascular plants, although within bryophytes the level of genetic variation is lower for liverworts than for mosses (Szweykowski, 1984a; Wyatt, 1985, Wyatt *et al.*, 1989; Stoneburner *et al.*, 1991; Shaw, 2000). Numerous observations also suggest a correlation between the level of genetic variation and the type of reproduction system, higher for outbreeding than for inbreeding vascular plants (Hamrick & Godt, 1990; Wyatt, 1994, Wyatt *et al.*, 1989; Stoneburner *et al.*, 1991). In bryophytes, self-fertilization leads to the production of completely homozygous sporophytes with all identical spores. Propagation through such genetically homogeneous spores is therefore equivalent to asexual reproduction and results in low levels of genetic variability (Longton, 1976; Wyatt *et al*., 1989; Shaw, 2000). Furthermore, the level of genetic differentiation among populations is correlated to the dispersal distance of sperm and diaspores, and the rate of self-fertilization (Wyatt, 1985; Wyatt, 1994; Miles & Longton, 1990; Itouga *et al.*, 2002a). Nevertheless, the data describing genetic variability of haploid species representing different reproduction systems are still insufficient (Hofman, 1991; Boisselier-Dubayle *et al.*, 1995; Boisselier-Dubayle & Bischler, 1997; Cronberg, 1998; Cronberg *et al.*, 1997; Stenøien & Såstad, 2001; Itouga *et al.*, 2002a).

Within liverworts, about 68% of species are unisexual (Wyatt & Anderson, 1984), including the genus *Ptilidium* Nees displaying an isolated systematic position (Schuster, 1966). The lack of gemmae, or other specialized modes of asexual reproduction is characteristic of this genus (Schuster, 1966). It consists of three species: *P. pulcherrimum* (Web.) Vain., *P. ciliare* (L.) Hampe and *P. californicum* (Austin) Underw. (Schuster, 1966). In Europe only two species are known – *P. ciliare* and *P. pulcherrimum* (Paton, 1999; Grolle & Long, 2000), significantly differing in ecological requirements. *Ptilidium pulcherrimum* has a wider scale of ecological tolerance than *P. ciliare*. It is a pioneer species, mostly an epiphyte, but it also occurs on decaying logs, rarely on acid soil and on rocks. *Ptilidium ciliare* is mostly an epilith growing mainly on rocks covered by a thin layer of soil as well as on acid, sandy soils (Schuster, 1966; Müller, 1951-1958; Paton, 1999). Both species are characterized by a similar circumboreal type of distribution. However, *P. ciliare* extends further North than *P. pulcherrimum*. *Ptilidium pulcherrimum* occurs mainly in lowlands, but also in subalpine regions, whereas *P. ciliare* occurs indifferently, from lowlands to the alpine belt (Schuster, 1966; Müller, 1951-1958; Paton, 1999). In Poland, they are common plants occurring from the lowlands to the alpine zone and they occupy sites characteristic of them, similarly to their entire range (Szweykowski, 1958; Szweykowski, 2004). They are very rare gathered together in one tuft (Szweykowski & Koźlicka, 1969).

Both studied species differ in frequency of sexual reproduction. *Ptilidium pulcherrimum* is frequently found with gametangia and sporophytes (Müller, 1951-1958; Schuster, 1966; Paton, 1999; Szweykowski, 2004), which suggests a predominant occurrence of sexual reproduction. *Ptilidium ciliare* differs from *P. pulcherrimum* in its being preponderantly sterile, rarely producing perianths and sporophytes (Müller, 1951-1958; Schuster, 1966; Paton, 1999; Szweykowski, 2004). Thus, this species appears to rely mainly on vegetative propagation. In asexual populations of *P. ciliare*, the absence of segregation and recombination may lead to the reduction of the level of genetic variation. Furthermore, the absence of gemmae limits the gene flow between populations. The next important difference

between the species is that *P. pulcherrimum* often grows on substrates with a limited temporal persistence and therefore needs to disperse at a higher rate than *P. ciliare*, which grows in potentially more stable substrates. Thus, it may be assumed that genetic variation in *P. ciliare* will be lower than in *P. pulcherrimum*. On the other hand, however, Schuster (1966) highlights a significant morphological polymorphism of *P. ciliare*, confirmed by biometrical studies (Szweykowski & Koźlicka, 1969; Adamczak unpubl.). Isozyme investigations limited only to peroxidases in *P. pulcherrimum* have revealed the existence of five isozyme phenotypes (Krzakowa & Koźlica, 1988).The lack of isozyme data for *P. ciliare* makes it impossible to compare both species.

The aim of the work was to study the genetic variation of these two closely related species – *P. ciliare* and *P. pulcherrimum* that differ in their mode of reproduction. We have attempted to provide the answer to the following questions: (1) what is the variation within populations, (2) between populations, and (3) what are the differences between the studied species?

MATERIALS AND METHODS

Plant material. Plants used in this study consisted of 19 populations collected from different regions in Poland and Slovakia (Table 1, Fig. 1a, 1b). Number of colonies collected in population depended on populations size, 1-5 colonies were usually collected from each population, except for one (population no. 19), where 20 colonies were found. The number of stems analyzed was proportional to size of the colony, with a minimum of 6 stems analyzed per colony. Each colony was divided into two parts: one was deposited as a voucher in the POZW herbarium and the second was used for isozyme analyses, except for three very small collections, which were used only for isozyme studies. Plants were stored at 4°C until the beginning of studies. Prior to analysis each stem was identified according to their morphological characters (Müller, 1951-1958; Schuster, 1966).

Electrophoretic analysis. Electrophoretic analyses were carried out on 8-61 plants from each of 19 populations. In total 213 plants (stems) of *P. ciliare* and 229 of *P. pulcherrimum* were examined. Electrophoretic isozyme separation was conducted according to the procedure described by Odrzykoski (1995). Crude cell extract was prepared by homogenization of a single stem in 30 µl distilled water for peroxidases and in 60 µl of extraction buffer for the remaining enzymes, and soaked on paper wicks (Whatman 3MM). The saturated wicks were placed into a slot of a 10% starch gel (Starch Art W 621-2 starch for electrophoresis). Isozymes were separated in three buffer systems: A – Tris-citrate (pH 8.2), lithium-borate (pH 8.3), B – tris-histidine (pH 7.0) and C – morpholine-citrate (pH 6.1) in dilution of electrode buffer 1:9 (Wendel &Weeden, 1989). The enzymes stained and buffer systems used for their resolution were listed in Table 2. Lithium-borate 10 mm high gels were separated at a constant voltage, at 230V/6.5 h. Tris-histidine and morpholine-citrate (10 mm) gels were separated at a constant current of 40mA/7.5 h. After separation the isozymes were detected on the gel slabs using standard staining methods (Wendel & Weeden, 1989). GOT, EST, ACP and PRX were stained in liquid assay, the remaining enzymes were stained for 2-3 hours in 37°C using the agar-overlay method. The most anodal alleles were labeled for each locus as 1, then 2 for the next one and so on.

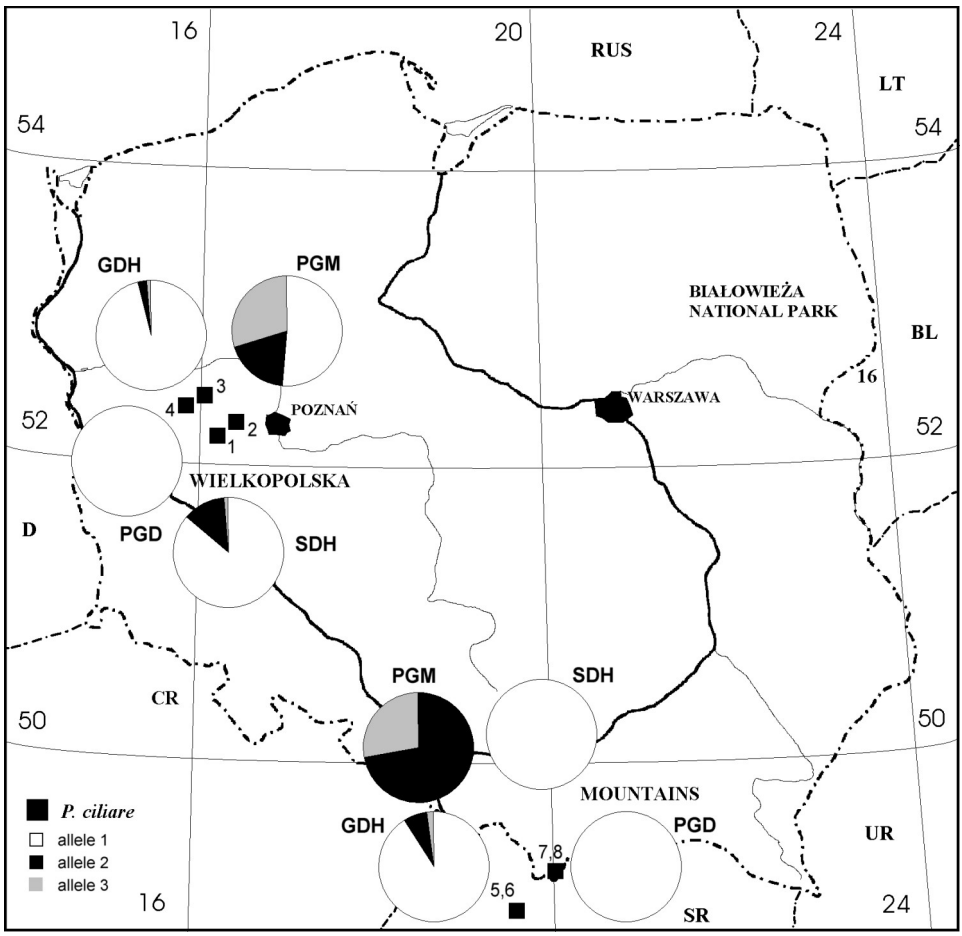

Fig. 1a. Map of Poland showing locations of studied populations and geographical patterns of variation in allele frequencies at polymorphic loci in *P. ciliare*. Numbers of populations correspond to those in Table 1.

Data analysis. Allele frequencies, percentage of polymorphic loci (P), mean numbers of alleles per locus (A) were calculated for each population and as the mean of each species and the Mann-Whitney U-test was used to test their statistical significance between the studied species. Gene diversity statistics (H_T, H_S, D_{ST}, G_{ST}) for each species were calculated (Nei, 1973; Nei, 1978). Nei's (1972) genetic distance were estimated between the populations of each species and between the species. Analyses were performed using POPGENE-1.32 (Yeh *et al.*, 2000). Three measures of clonal diversity, i.e. the number of genotypes per population (G), the proportion of distinguishable genotypes (G/N) (Ellstrand & Roose, 1987) and multilocus genotype diversity (D_G) as a modification of the

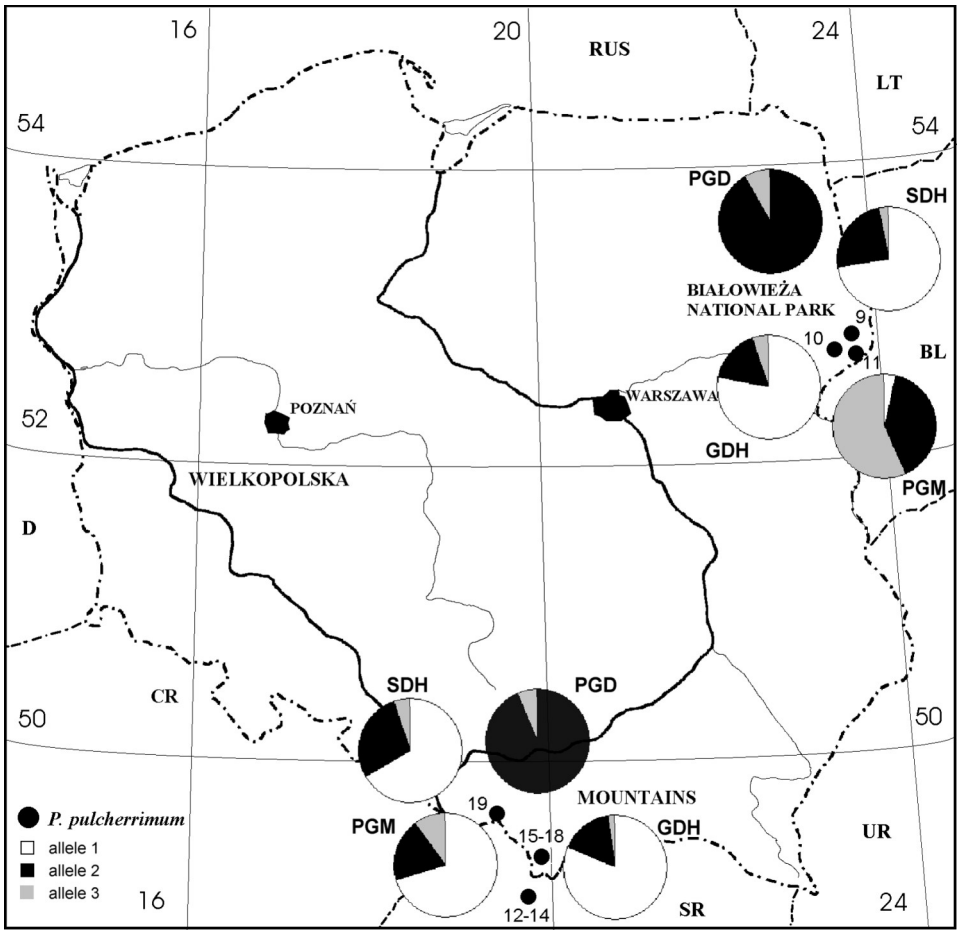

Fig. 1b. Map of Poland showing locations of studied populations and geographical patterns of variation in allele frequencies at polymorphic loci in *P. pulcherrimum*. Numbers of populations correspond to those in Table 1.

Simpson index (Pielou, 1969, Ellstrand & Roose, 1987) were also estimated. Multilocus genotype diversity (D_G) was calculated according to the formula: $D_G = 1 - \Sigma\{[n_i (n_i - 1)] / N (N - 1)]\}$, (i = 1 to C); where *n* is the number of individuals in clone *i*, *C* is the number of clones, and *N* is the sample size. Values of D_G range from 0 to 1, and higher values correspond to greater clonal diversity. Proportion of distinguishable genotypes (G/N) was calculated as the number of distinct genotypes (G) relative to the number of stems sampled (N). This measure ranged from 0 to 1, and higher values correspond to greater clonal diversity. In both cases, the potential level of diversity increases with the number of loci assayed.

Table 1. Collection sites of studied populations of *P. ciliare* (1-8) and *P. pulcherrimum* (9-19). The POZW No. denotes voucher number in the herbarium.

Population No.	*Locality*	*Samples POZW No.*	*Collector*	*Date*
Ptilidium ciliare				
	LOWLANDS OF WESTERN POLAND			
1	Poland, Wielkopolska, Porażyn village, sterile	39557	MA, AA	01.10.13
2	Poland, Wielkopolska, Piotrowo village, sterile	39851, 39852, 39517	AB	01.05.10
3	Poland, Wielkopolska, Grubsko village, sterile	39518, 39529, 39530, 39531, 39533, 39534	KB	01.12.03
4	Poland, Wielkopolska, Sowia Góra hill, sterile	39794, 39795, 39796, 39797, 39798, 39799, 39800, 39801, 39802, 39803, 39804, 39805, 39806, 39807, 39808, 39809, 39810, 39811	MA	02.10.05
	MOUNTAINS			
5	Slovakia, Tatry Mts., Račkova Valley, sterile	39860	MA, AA	01.08.11
6	Slovakia, Tatry Mts., Maly Baranec Mt., sterile	39853	MA, AA	01.08.08
7	Poland, Tatry Mts., Jaworzynka Valley, sterile	39841, 39842, 39843, 39844	AB, KB	02.08.06
8	Poland, Tatry Mts., Goryczkowa Valley, sterile	39845, 39846, 39847	KB, AB	02.08.11
Ptilidium pulcherrimum				
	LOWLANDS OF EASTERN POLAND			
9	Poland, Białowieża National Park, dept. 476A, ♀	39835, 39836, 39837	MA, AA	02.09.24
10	Poland, Białowieża National Park, dept. 254Dc, sterile	39838, 39839, 39840	MA, AA	02.09.27
11	Poland, Białowieża National Park, dept. 500, ♀, ♂ and spor.	39641, 39642, 39643	MA, AA	02.03.11
	MOUNTAINS			
12	Slovakia, Tatry Mts., Račkova Valley, sterile	39855, 39859	MA, AA	01.08.11
13	Slovakia, Tatry Mts., Maly Baranec Mt., ♀	39854, 39856, 39858	MA, AA	01.08.08
14	Slovakia, Tatry Mts., Jamnicka Valley, sterile	39857	MA, AA	01.08.09
15	Poland, Tatry Mts., Goryczkowa Valley, sterile	39848	AB, KB	02.08.11
16	Poland, Tatry Mts., Pańszczyca Valley, sterile	39849	AB, KB	02.08.12
17	Poland, Tatry Mts., Kościeliska Valley, sterile	39647, 39648, 39649	KB, AB	02.04.26
18	Poland, Tatry Mts., Sucha Woda Valley, ♀, ♂ and spor.	39659, 39664, 39665, 39673	AB, KB	02.04.27
19	Poland, Beskidy Mts., Babia Góra Mt., ♀, ♂	39812, 39813, 39814, 39815, 39816, 39817, 39818, 39819, 39820, 39821, 39822, 39823, 39824, 39825, 39826 39827, 39828, 39829 39831, 39830	TK	02.09.19

Collectors: AA – Artur Adamczak; MA – Małgorzata Adamczak; AB – Alina Bączkiewicz; KB – Katarzyna Buczkowska; TK – Tomasz Karpiński.

RESULTS

Electrophoretic phenotypes. A consistent satisfactory resolution was achieved for 9 out of 16 screened enzyme systems (Table 2). All analyzed enzymes displayed single-banded patterns, which probably represented a single gene product, except PRX, where two isozymes were detected, but the slowly migrated *Prx*-B was eliminated due to its inconsistent staining.

Genetic diversity. Totally 20 alleles were detected, 15 in *P. ciliare* and 16 in *P. pulcherrimum*, 11 alleles were in common for both species, but differed in frequency (Table 3). Two loci (*Got* and *Me*) were monomorphic at the genus level and were excluded from statistical analysis. Both species were fixed for alternative alleles at three loci (*Acp*, *Idh* and *Prx*-A), which can act as diagnostic markers for these two species. Moreover, *P. ciliare* was fixed for allele 1 in *Pgd*, whereas in *P. pulcherrimum* two alleles (allele 2 and 3) were observed in this locus. The remaining loci (*Gdh*, *Pgm* and *Sdh*) appeared to be polymorphic in both species and expressed three alleles.

In both species rare alleles (with frequencies below 0.05) were detected, three in *P. ciliare* (*Gdh* allele 2 and 3, *Sdh* allele 3), two (*Gdh* allele 3 and *Sdh* allele 3) in *P. pulcherrimum*. In spite of a similar number of alleles detected, there is a difference in the level of intrapopulation polymorphism between these two species. In *P. ciliare*, only three of seven loci (*Gdh*, *Pgm* and *Sdh*) were polymorphic. The percentage of polymorphic loci (P) within populations ranged from 11.11 to 33.33% (with a mean of 20.831%). The mean number of alleles per locus (A) ranged from 1.111 to 1.556, with a mean of 1.2776. Gene diversity (H_S) within the populations for all loci ranged from 0.024 to 0.124, with a mean of 0.0736 (Table 4). In *P. pulcherrimum*, four loci (*Gdh*, *Pgm*, *Sdh* and *Pgd*) were polymorphic. The percentage of polymorphic loci (P) within populations ranged from 22.22 to 44.44% (mean = 32.320%). The mean number of alleles per locus (A) ranged from 1.222 to 1.667 (mean = 1.4440). Gene diversity (H_S) within the populations for all loci ranged from 0.048 to 0.183, with the mean of 0.1055 (Table 4).

In total 15 and 25 different genotypes (G) were detected in *P. ciliare* and *P. pulcherrimum*, respectively. In *P. ciliare*, three genotypes representing 24.78, 27.48 and 35.59% of all plants, were widespread and found in almost all populations. Nine genotypes were rare and occurred only in one population, and six genotypes were observed only once. Two widespread (representing 15.13, and

Table 2. List of enzymes studied, their abbreviations (Abbr.), enzyme commision number (E.C.) and buffer systems.

Enzyme	*Abbr.*	*E.C.*	*Buffer systems*
Acid phosphatase	ACP	3.1.3.2	A
Phosphogluconate dehydrogenase	PGD	1.1.1.44	C
Glutamate dehydrogenase	GDH	1.4.1.2	A
Glutamate oxaloacetate transaminase	GOT	2.6.1.1	A
Isocitrate dehydrogenase	IDH	1.1.1.41	C
Malic enzyme	ME	1.1.1.40	C
Phosphoglucomutase	PGM	5.4.2.2	B
Peroxidase	PRX	1.11.1.7	A
Shikimate dehydrogenase	SDH	1.1.1.25	B

Table 3. Allele frequencies for 9 putative gene loci in studied populations of *P. ciliare* (1-8) and of *P. pulcherrimum* (9-19).

		Populations																		
		P. ciliare								*P. pulcherrimum*										
Locus	Allele	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Got	1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Me	1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Acp	1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	–	–	–	–	–	–	–	–	–	–	–
	2	–	–	–	–	–	–	–	–	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Idh	1	–	–	–	–	–	–	–	–	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	2	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	–	–	–	–	–	–	–	–	–	–	–
Per	1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	–	–	–	–	–	–	–	–	–	–	–
	2	–	–	–	–	–	–	–	–	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Pgd	1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	–	–	–	–	–	–	–	–	–	–	–
	2	–	–	–	–	–	–	–	–	0.937	0.889	0.933	1.000	0.929	0.900	1.000	1.000	0.917	0.833	0.967
	3	–	–	–	–	–	–	–	–	0.063	0.111	0.067	–	0.071	0.100	–	–	0.083	0.167	0.033
Gdh	1	0.914	1.000	0.924	1.000	1.000	1.000	0.750	0.875	0.875	0.778	0.700	0.357	0.452	0.900	0.867	0.875	1.000	1.000	1.000
	2	0.029	–	0.076	–	–	–	0.250	0.042	0.125	0.111	0.267	0.643	0.548	0.100	0.067	–	–	–	–
	3	0.057	–	–	–	–	–	–	0.083	–	0.111	0.033	–	–	–	0.066	0.125	–	–	–
Pgm	1	0.600	0.500	0.547	0.393	–	–	–	–	0.125	–	–	0.571	0.571	0.700	0.800	0.875	0.833	0.833	0.443
	2	0.371	–	0.208	0.197	0.875	0.750	0.417	0.833	0.250	0.556	0.367	0.143	0.262	0.200	0.200	0.125	0.167	0.167	0.361
	3	0.029	0.500	0.245	0.410	0.125	0.250	0.583	0.167	0.625	0.444	0.633	0.286	0.167	0.100	–	–	–	–	0.196
Sdh	1	0.657	0.833	0.962	1.000	1.000	1.000	1.000	1.000	0.937	1.000	0.233	–	0.214	0.200	1.000	1.000	1.000	1.000	0.902
	2	0.343	0.167	–	–	–	–	–	–	0.063	–	0.700	1.000	0.714	0.600	–	–	–	–	0.033
	3	–	–	0.038	–	–	–	–	–	–	–	0.067	–	0.071	0.200	–	–	–	–	0.065
N		35	12	53	61	8	8	12	24	16	9	30	14	42	10	15	8	12	12	61

Table 4. Sample size (N), mean number of alleles per locus (A), percentage of polymorphic loci (P), allelic diversity (H_S), number of multilocus genotypes (G), proportion of distinguishable genotypes (G/N), Simpson diversity index (D_G).

Population No.	*N*	*A*	*P*	$H_S \pm SD$	*G*	*G/N*	D_G
P. ciliare							
1	35	1.556	33.33	0.124 ± 0.207	8	0.229	0.696
2	12	1.222	22.22	0.086 ± 0.180	3	0.250	0.667
3	53	1.444	33.33	0.090 ± 0.197	6	0.113	0.707
4	61	1.222	11.11	0.071 ± 0.213	3	0.049	0.649
5	8	1.111	11.11	0.024 ± 0.073	2	0.250	0.250
6	8	1.111	11.11	0.042 ± 0.125	2	0.250	0.429
7	12	1.222	22.22	0.096 ± 0.192	4	0.333	0.712
8	24	1.333	22.22	0.056 ± 0.112	5	0.208	0.377
Total	213						
Mean		1.2776	20.831	0.0736	4.1	0.2102	0.5608
P. pulcherrimum							
9	16	1.556	44.44	0.109 ± 0.177	6	0.375	0.675
10	9	1.444	33.33	0.118 ± 0.192	5	0.556	0.806
11	30	1.667	44.44	0.164 ± 0.219	8	0.267	0.770
12	14	1.333	22.22	0.115 ± 0.229	4	0.286	0.615
13	42	1.667	44.44	0.183 ± 0.247	8	0.190	0.848
14	10	1.667	44.44	0.153 ± 0.218	6	0.600	0.845
15	15	1.333	22.22	0.062 ± 0.125	4	0.267	0.371
16	8	1.222	22.22	0.049 ± 0.097	2	0.250	0.250
17	12	1.222	22.22	0.048 ± 0.100	3	0.250	0.439
18	12	1.222	22.22	0.062 ± 0.123	3	0.250	0.545
19	61	1.556	33.33	0.098 ± 0.211	8	0.131	0.716
Total	229						
Mean		1.4440	32.320	0.1055	5.2	0.3110	0.6255

28.99% of all plants) and nine local genotypes occurring only in one population (seven only once) were found in *P. pulcherrimum*. The number of genotypes in populations of *P. ciliare* ranged from two to eight, with the mean of 4.1, whereas in *P. pulcherrimum* from two to eight, with the mean of 5.2. The proportion of distinguishable genotypes (G/N), on the average, was equal to 0.2102 and 0.3110 in *P. ciliare* and *P. pulcherrimum*, respectively. The Simpson index (D_G) was lower in *P. ciliare* (0.5608) than in *P. pulcherrimum* (0.6255) (Table 4). The Mann-Whitney U-test showed that the species do not differ statistically significant in respect of these statistics. Gene diversity statistics based on polymorphic as well as on all studied loci have shown a lower genetic variation of *P. ciliare* than that of *P. pulcherrimum* (Table 5).

Genetic distances between populations of each species as well as between the species were computed. The values of genetic distances D (Nei, 1972) showed a lower differentiation between populations of *P. ciliare*, with the D value ranged from 0.015 to 0.073, than those of *P. pulcherrimum* that ranged from 0.001 to 0.210. Genetic distance between the species was equal to 0.739. The UPGMA phenogram based on Nei's (1972) genetic distance was constructed for both species. Populations of *P. ciliare* were divided into two distinct clades, the first including 4 lowland populations and the second including 4 populations from the

Table 5. Means of total genetic diversity (H_T), genetic diversity within (H_S) and between (D_{ST}) and value of (G_{ST}) for populations of *P. ciliare* and *P. pulcherrimum* computed for all and for polymorphic loci separately.

	H_T	H_S	D_{ST}	G_{ST}
P. ciliare				
Polymorphic loci	0.299 ± 0.089	0.221 ± 0.039	0.078	0.263
All loci	0.099 ± 0.045	0.074 ± 0.022	0.025	0.263
P. pulcherrimum				
Polymorphic loci	0.379 ± 0.044	0.237 ± 0.021	0.142	0.374
All loci	0.168 ± 0.056	0.106 ± 0.023	0.062	0.374

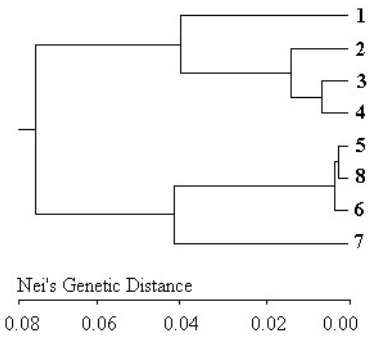

Fig. 2. UPGMA phenogram based on Nei's (1972) genetic distances among 8 populations of the *P. ciliare*.

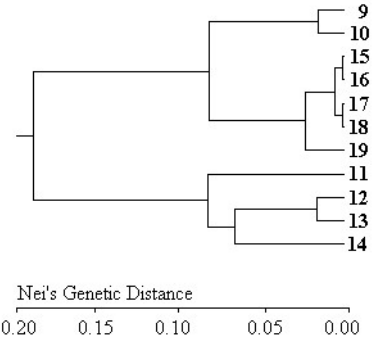

Fig. 3. UPGMA phenogram based on Nei's (1972) genetic distances among 11 populations of the *P. pulcherrimum*.

mountains (Fig. 2). Populations of *P. pulcherrimum* are also divided into two distinct clades, though their relation with geographic region was not clear (Fig. 3).

Relationship between genetic differentiation and geographic regions. Our results show that both species differ in numbers as well as in the frequencies of alleles in different geographical regions (Fig. 1a, 1b). *Ptilidium ciliare* populations from the mountains had a lower number of alleles than those coming from the lowlands, whereas in *P. pulcherrimum* the same number of alleles was found in both regions. *Ptilidium ciliare*, two loci had alleles typical for only particular geographic regions (Fig. 1a, Table 3).

DISCUSSION

The two species of the genus *Ptilidium* differ in the level of genetic variation as estimated on the basis of seven loci. The mean values of the measures of genetic variability for *P. pulcherrimum* (A = 1.4440, P = 32.320%, H_S = 0.1055)

are higher than those for *P. ciliare* (A = 1.2776, P = 20.8312, H_S = 0.0736). The level of variation in *P. pulcherrimum* is so high that is comparable with variation of mosses and even with that of vascular plants (A = 1.53, P = 34.2%, H_S = 0.113 – Hamrick & Godt, 1990). However, the variation of *P. ciliare* is lower than *P. pulcherrimum* and typical of liverworts. The mean value of genetic variation (H_S) for liverworts estimated on the basis of isozyme studies ranges from 0.044 (Wyatt *et al.*, 1989; Shaw, 2000) to 0.059 (Itouga, 2002b). Frequency of sexual reproduction of the studied species is probably the main reason of the difference observable at the level of allelic and genotypic diversity between those species. It is often assumed that outbreeding and recombination may generate novel genetic variation on haplotypic level (Stenøien & Såstad, 2001), and a shift from sexual to vegetative reproduction decreases genetic diversity because of reduced genetic recombination (Mischler, 1988). In *P. pulcherrimum* populations, the sexual reproduction appears to be the most common one since about 50% of the studied populations were fertile or with sporophytes. It can increase the genetic variation because of the possibility of recombination. The relatively high value of proportion of distinguishable genotypes (G/N = 0.3110), which is higher than the mean (0.25) and median (0.13) values reported by Diggle *et al.* (1998) for asexual species, indicate the domination of sexual reproduction in *P. pulcherrimum*. In contrast to *P. pulcherrimum* all analyzed plants of *P. ciliare* were sterile, which indicates the preponderance of vegetative reproduction. Hence it is not surprising that the G/N value is low, and even lower than the mean value reported for asexual species (Diggle *et al.*, 1998). Our observation is congruent with the extreme rarity of sexual reproduction of *P. ciliare* reported by many authors (Müller, 1951-1958; Schuster, 1966; Paton, 1999; Szweykowski, 2004). The level of genetic diversity can be also related to the persistence of the habitat (Cronberg *et al.*, 1997; Stenøien & Såstad, 1999). *Ptilidium ciliare* is a species growing in stable habitats, and may thus not need to reproduce as often as a *P. pulcherrimum*, which grows in more transient habitats, in order to disperse and maintain genetic diversity. Longevity of the substratum is one of the most important habitat properties shaping the evolution of bryophytes (During, 1979). Stenøien & Såstad (2001) pointed out that haplotypic diversity is a function of not only recombination among loci but also mutations within loci, and that a high mean number of mutants per locus per generation may generate high levels of haplotypic diversity, without significant recombination. This can be a reason of a relatively high level of genetic diversity observed in asexual *P. ciliare* in comparison with other liverwort species.

The mode of reproduction has also a considerable influence on the level of differentiation among populations. There is a relationship between G_{ST} value and life history characteristics of vascular plants. G_{ST} values were generally higher for annual plants than for perennial plants, similarly to autogamous plants where these values were also higher than G_{ST} values for outcrossed plants (Hamrick, 1989). This relationship is also confirmed in bryophytes, dioecious species reproducing only by spores usually have lower G_{ST} values, e.g., *C. conicum* (L.) Dumort. G_{ST} = 0.230 (Odrzykoski, 1986) than monoecious e.g., *Riccia dictyaspora* Howe G_{ST} = 0.692 (Dewey, 1989), or *Preissia quadrata* (Scop.) Nees G_{ST} = 0.928 (Boisselier-Dubayle & Bischler, 1997). High G_{ST} values are also expected for many clonal species, where the majority of genetic variation is often found among populations (Ellstrand & Roose, 1987). Taking into consideration the factors affecting the level of interpopulation variation and mode of reproduction of the studied species, a higher G_{ST} value should be expected for *P. ciliare* rather than for *P. pulcherrimum*. Contrary to expectations a lower G_{ST} value was observed in

P. ciliare. The value of G_{ST} in *P. pulcherrimum* is slightly higher than that expected in dioecious species, reproducing via spores, but it is lower than in other liverworts with the same kind of reproduction, like *Asterella liukiuensis* (Horik.) Horik. for which the G_{ST} value is 0.681 (Itouga *et al.*, 2002b), or *Porella platyphylla* (L.) Pfeiff. (G_{ST} = 0.893; Boisselier-Dubayle *et al.*, 1998). The G_{ST} value in *P. ciliare*, where sporophytes are sporadically noticeable and, which, moreover, lacks gemmae (Schuster, 1966), is lower than expected. There may be several reasons to explain our observations for *P. ciliare*. First of all, sexual reproduction may occur more often than it is commonly believed, an inconsistency that may result from the insufficiently known biology of this species, and consequently there may exist the gene flow. The second hypothesis suggests that the present genetic differentiation of *P. ciliare* is a relict of the glacial period. This species could have reproduced sexually before the period of glaciers, but lost this ability later in the course of post-glacial migration. Such shift in the mode of reproduction has been proposed for example for *Selaginella rupestris* (L.) Spring (Tryon, 1971). Based on the G_{ST} value it is impossible to separate the effect of current and past gene flows (Shaw, 2000). A high level of protein polymorphism in liverwort colonies reproducing mainly in a vegetative way is also frequently explained in the light of the hypothesis about establishing colonies by more than one meiospore (Krzakowa & Szweykowski, 1979; Szweykowski, 1984b; Shaw, 2000). As many authors point out very rare production of sporophytes by *P. ciliare* (Schuster, 1966; Müller, 1951-1958; Paton, 1999), which was confirmed also in our studies, the second hypothesis seems to be more likely and convincing one. Similarly, the high level of genetic variation in asexually reproduced *Sphagnum pulchrum* (Braithw.) Warnst. was attributed to gene flow in the past by Daniels (1982).

Our study reveals that populations of *P. ciliare* can be segregated in two distinct groups – one consisting of montane populations, and another – of lowland populations from western Poland (Fig. 2). These lowland populations are characterized by a higher number of alleles. The presence of a unique allele (*Pgm* 1) with a high frequency in these lowland populations, which has not been found in the montane ones (Fig. 1a, Table 3), indicates that present-day gene flow between lowland and montane populations is highly restricted, and that these groups can constitute separate evolutionary lineages. The occurrence of this private allele only in lowland populations can be explained as a relict of an old gene pool (montane populations could have lost that allele) or there may have been a fixed acquired mutation of adaptative value for specific habitat conditions. Some authors (Krzakowa & Szweykowski, 1979; Cummins & Wyatt, 1981) suggested, for example, that genetic variability is maintained by close adaptation to local microhabitats in which selective advantages vary over short distances. In *P. pulcherrimum* the same number (14) of identical alleles were found in the lowlands – the primary forest of Białowieża National Park (in eastern Poland) and in the Tatry Mts. (the World Biosphere Reserve) where the old gene pool has been preserved. However, out of 25 genotypes occurring in *P. pulcherrimum*, 10 of them were found only in mountainous populations, 5 were characteristic only of Białowieża National Park, and the remaining 10 are common in the lowlands and in mountains, 2 of which are more frequent (Fig. 1b).

Acknowledgements. Grateful thanks are extended to the late Prof. Jerzy Szweykowski for his ideas and interest. We are grateful to Artur Adamczak for his help in collecting the plant material used in this study. Thanks are due to reviewers for valuable comments on manuscript and linguistic advice. We would like to express our thanks to the Management of the Tatra National Park for many facilities provided during the field work.

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