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Fast identification method for an allopolyploid liverwort *Pellia borealis* Lorbeer (Hepaticae, Metzgeriales)

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Abstract – A simple method was described for the identification of an allopolyploid liverwort *Pellia borealis* based on isozyme analysis. A perfect correlation was observed between the polyploid chromosome number (n=18) and the fixed heterozygous isoenzyme pattern of two diaphorase loci (DiaA and DiaC) in a large collection of *Pellia epiphylla* sensu lato from Polish populations. Therefore, their isozyme pattern may be used as a reliable diagnostic marker, which makes it possible to distinguish between a polyploid and two parental cryptic species from the *P. epiphylla* complex. Due to the alloploid origin of *P. borealis* we postulate that the species rank of the taxon should be maintained and geographical distribution revised.

Isozyme markers / chromosome number / allopolyploidy / *Pellia borealis* / *Pellia epiphylla* complex

INTRODUCTION

Liverworts from genus *Pellia* the *Epiphyllae* section (Schust.) occurs in North America, Europe and Asia (de Sloover & Messe, 1982; Newton, 1986a; Szweykowski *et al*., 1995; Odrzykoski *et al*., 1996). Recent studies on genetic variability of this section have provided new evidence supporting a hypothesis of allopolyploid origins of *Pellia borealis* Lorbeer as a species originated by hybridization between two cryptic species from the *Pellia epiphylla* (L.) Corda complex and subsequent polyploidization (Odrzykoski *et al.*, 1996; Chudzińska *et al.,* 2005; Fiedorow *et al.*, 2001; Pacak & Szweykowska-Kulińska, 2000, 2003). This hypothesis was formulated by Jachimsky (1935), based on comparative karyotype studies. He argued that *P. borealis* is an allopolyploid of *P. epiphylla* and an unknown "race" of *Pellia,* with a chromosome number n=9 as the parental species.

The polyploid *Pellia borealis* is not recognized as a separate species in the most recent European floras (e.g. Grolle, 1983; Grolle & Long, 2000), mostly due to the lack of well-established diagnostic characters. Only Schuster (1992) treated *P. borealis* as a separate species based on the opinion that good diagnostic traits include the shape of epidermal cells and the size of spores, as it was suggested earlier by Müller (1954). Because chromosome numbers alone cannot be used as a diagnostic indicator in a traditional taxonomical procedure, a polyploid is

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considered as a chromosomal race of *P. epiphylla*, and its hybrid origin and genetic individuality are neglected.

All European species of *Pellia,* including taxa from the *Pellia epiphylla* complex, may be distinguished based on their peroxidase electrophoretic pattern (Szweykowski *et al.,* 1981). However, as it was found later, the expression of some peroxidase isozymes may be subjected to developmental fluctuations, thus identification of freshly collected samples is often problematic (Zieliński, 1986). The differences in phenotype between *P. epiphylla*-species S and *P. borealis* are relatively insignificant, particularly when the material is analysed with no preceding culture under comparable conditions. For this reason, the peroxidase phenotype is applicable mainly for separation of sterile plants of the *P. epiphylla* complex from *P. neesiana* and from two cryptic species of the *P. endiviifolia* complex (Szweykowski *et al*., 1995). At present alternative, more reliable isoenzymatic markers are preferred for identification of plants immediately after their collection in the field (cf. Odrzykoski *et al*., 1996), but detection of all of these enzymes on electrophoretic gels is expensive and cannot be recommended as a routine method for large-scale identification purposes.

In this paper, a simple method is described for the identification of the polyploid *P. borealis* and two cryptic species from the *P. epiphylla* complex based on the electrophoretic pattern of only two enzymes. Plants used in this study were analysed simultaneously with respect to their chromosomal numbers and electrophoretic phenotypes of enzymes, of which one (DiaC) represents a new diagnostic marker. We have studied a large collection of samples from over one hundred Polish populations and found that the diaphorase isozyme pattern may be reliably used to distinguish between the polyploid and both parental species. In view of the difficulties in the identification of the polyploid based on morphometric differences and sometimes on the peroxidase isozyme pattern, these new markers may facilitate studies on the geographical distribution and ecological preferences of species from this section.

MATERIAL AND METHODS

Plants originating from 157 populations were examined (see the Appendix). Separate thalli from each colony were analyzed, and the tip of each thallus was subjected to cytological analyses, while the remaining part was used for enzyme extraction. Most samples were prepared for analysis directly after their collection from the field; the others were cultivated in a greenhouse or a culture chamber with a regulated day length (12 h) and temperature (16-18˚C). A fragment of each colony was also deposited as a voucher in the Bryophyte Herbarium in Pozna (POZW).

Chromosome counting

Tips of freshly growing thalli were immersed in 0.02 M8-hydroxyquinoline for 5 h at RT and fixed in a mixture of absolute alcohol and glacial acetic acid (3:1), at least one week at a temperature 5-10˚C. Squash preparations were made, after fixative was replaced with alcohol, in acetic acid and stained with DAPI (2-5 minutes) or acetic haematoxylin (48 h) and embedded in Hoyer's medium. For C-banding, the 2% Giemsa technique was applied, following the procedure of

Schwarzacher *et al*. (1980) with modifications (Chudzińska, 1998). Chromosome counting was performed in 5 to 10 metaphase plates. Microphotographs were taken using an Olympus BX40 microscope equipped with fluorescence optics, where DAPI fluorescence was visualized with the use of excitation and emission filters of 380 and 460 nm, respectively.

Isozyme electrophoresis

Two enzymes were studied: NADH diaphorase (DIA, E.C. 1.8.1.4 or E.C. 1.6.99.2) and peroxidase (PER, E.C. 1.11.1.7) as a control. The peroxidase phenotype made it possible to distinguish between sterile gametophytes of species from the *P. epiphylla* complex and *P. neesiana* (Szweykowski *et al*., 1981). Isoenzymes were separated using standard starch gel electrophoresis, coupled with histochemical localization of the enzymes. The extraction and separation methods were identical to those used for distinguishing two formerly cryptic species from the *Conocephalum conicum* (L.) Dum. complex and have been described in detail by Odrzykoski (1995). Following electrophoresis, the starch block was sliced into three layers and one was stained to detect peroxidase activities, while the other ones were analyzed for the activity of diaphorases employing two staining techniques (see Manchenko, 1994, for discussion on diaphorase zymograms). The staining mixture contained 10 mg NADH in 10 ml 0.2 M Tris-HCl buffer supplemented with 3 mg MTT, 0.5 mg 2,6-dichlorophenol- indophenol (DCIP) mixed with 10 ml liquid 2% agar in an aqueous solution (the agar-overlay technique). The staining mixture poured on the gel covers its surface with a transparent film (overlay), through which diaphorase isoenzymes may be observed and photographed. The other slice was stained with 10 mg menadione instead of DCIP as the electron acceptor (Wendel & Weeden, 1989; Manchenko, 1994).

RESULTS

The diaphorase electrophoretic phenotype for haploid gametophytes consists of four isoenzymes (DiaA, DiaB, DiaC and DiaD). They may be visualized by a combination of two staining techniques on separate gels. Staining intensity of the DiaA isozyme is higher with DCIP, while that of DiaC when menadione is used as an electron acceptor. Different electrophoretic variants of the two diaphorase isoenzymes (DiaA and DiaC) were observed in two cryptic species. The other two (DiaB and DiaD) have no different variants. Among plants with a haploid chromosome number (n=9) an individual karyotype pattern may be distinguished for 36 phenotypes typical for the cryptic species *P. epiphylla*-species *S* (DiaA1, DiaC2) and 35 showed the karyotype and phenotype characteristics for *P. epiphylla*-species *N* (DiaA2, DiaC1). Chromosomes with big heterochromatin fragments analyzed and compared using the Giemsa C banding method allows us to distinguish chromosomes between cryptic species from the *Pellia epiphylla* complex and to confirm that they are parental species of *Pellia borealis*. Plants with a polyploid chromosomal number (n=18) were detected in 86 samples. All polyploid samples expressed a putative heterozygous phenotype for both diaphorase loci, composed with variants present in the parental species (DiaA12 and DiaC12). An example of chromosome metaphase plates and the electrophoretic pattern of diaphorase isoenzymes is presented on Fig.1 and the list of studied samples can be found in Appendix 1.

DISCUSSION

Traditional taxonomic procedures frequently fail to detect significant genetic differentiation of some liverwort species with a broad geographical range (Shaw, 2009). One of the intriguing examples has been found within genus *Pellia*; section *Epiphyllae,* with three (or four) known species. Two species, i.e. *P. ephiphylla* (L.) Corda and *P. neesiana* (Gottsche) Limpr., had a wide amphi-Atlantic or circumboreal distribution (Schuster, 1992). The third species (*P. x appalachiana* Schust.), of a postulated but unconfirmed hybrid origin, is restricted to the Southern Appalachians (Schuster, 1992; Self & Crandall-Stotler, 2001). The fourth taxon, a polyploid *P. borealis*, is most commonly regarded as a synonym of *P. epiphylla* and only exceptionally as a separate species, as long as it is described exclusively from Europe (Schuster, 1992; Szweykowski, 2006).

The first description of *Pellia borealis* species as a separate species was presented by Lorbeer (1934) shortly after the discovery of polyploidy within *P. epiphylla* by Heitz (1927). Lorbeer pointed out that the polyploid could be distinguished from *P. epiphylla* by larger dimensions of gametophyte cells and a higher average number of oil bodies. Müller (1954) also suggested that the polyploid could be distinguished by the shape of marginal and epidermal cells of the gametophyte (marginal thallus cells, epidermal capsule cells), which was later confirmed by Schuster (1992) based on plants originating from Poland. However, these findings have not been confirmed in other studies (e.g. Paton & Newton, 1967; Mendelak 1972; de Slover & Messe 1982). Recently, some differences were detected in the length of hairs, which cover the apex of the thallus, in the height of a rib and in the length of marginal cells studied using scanning electron microscopy (Orzechowska *et al.,* 2006). These results need to be confirmed on a more extensive material. Therefore a possibility of distinguishing *P. borealis* from *P. epiphylla* based on morphometric characters alone is still an open question.

One year after the description of *P. borealis* as separate species Jachimsky (1935) discovered that the karyotype of the polyploid is composed with a unique set of chromosomes and it is hard to find homologous pairs expected from an autopolyploid of recent origins. He suggested that a polyploid may be a result of hybridization between *P. epiphylla* and an unknown species of *Pellia.* Newton (1986a), taking advantage of chromosomal staining techniques, confirmed the results of Jachimsky, as to chromosomal differences between *P. epiphylla* and *P. borealis*, but she was not able to discriminate between auto- and allopolyploidy, as the origin of *P. borealis.* However, she could exclude *P. neesiana* as a possible parental species of the postulated allopolyploid. No other species from this section was known and may be considered as the second parental species (Newton, 1986b).

New species were discovered during studies on peroxidase polymorphism in Polish populations of *Pellia* (Szweykowski *et al*., 1981). Originally, all samples of *P. epiphylla* with the PX3 peroxidase phenotype found in four populations from North Poland were regarded as representing infra-specific polymorphism. However, after detection of additional differences in four other isozyme loci between those samples and *P. epiphylla* possessing the PX1 and PX2 phenotypes, two "genetic forms" were distinguished, i.e."*P. epiphylla S"* and "*P. epiphylla N*" (Zieliński, 1984). This author also suggested that these "forms" may represent "distinct genetic pools", because the level of differentiation of isozyme loci is much higher than usually found within a single species of other liverworts. Subsequently, he proposed that *P. epiphylla* is a complex of two sibling (cryptic) species (Zieliński, 1987). These species were informally named as *P. epiphylla-*

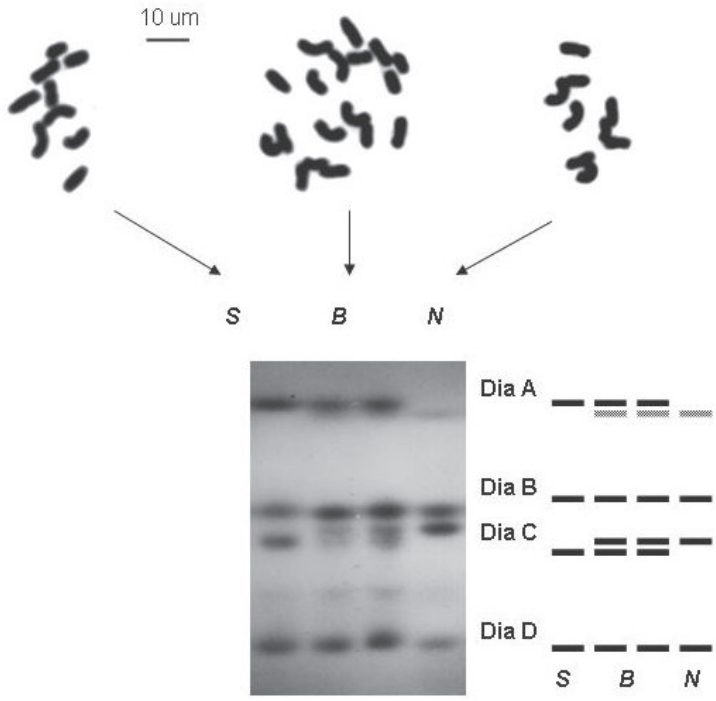

Fig. 1. Electrophoretic phenotypes of Diaphorase izozymes in three species from *Pellia epiphylla* complex; S- *P. epiphylla S,* B- *P. borealis,* N- *P. epiphylla N*.

species *S* and *P. epiphylla*-species *N*, according to the nomenclature proposed for cryptic species by Odrzykoski & Szweykowski (1991). The geographic distribution pattern and ecological preferences of the cryptic species and *P. borealis* is poorly known except for Poland. Intensive studies showed the common presence of an allopolyploid all over the country and an almost allopatric distribution of the two parental species (Szweykowski *et al.,* 1995).

Discovery of a new species facilitated a repeated testing of the hypothesis presented by Jachimsky on the allopolyploid origin of *P. borealis.* Comparisons of electrophoretic phenotypes of three species-specific enzyme loci demonstrate that all polyploid samples from several geographically distinct populations express a heterozygous phenotype composed with variants characteristic of cryptic species, thus supporting alloploidy (Odrzykoski *et al*., 1996).

Recently, numerous cytological and molecular techniques have been exploited to investigate polyploid genome organisation. One of them involves differential staining of chromosomes (Chudzińska *et al.,* 2005), while another the application of the RAPD technique (e.g. Boisselier-Dubayle *et al*., 1995; Pacak *et al*., 1998; Wachowiak *et al*., 2007). The allopolyploid origin of *P. borealis* has been confirmed recently using intron sequence differences of the tRNAGly (UAA) gene. The cryptic species are differentiated by e.g. indel of three nucleotides (GCT), which may also be detected as differences in the size of amplification products on acrylamide gels. In the diploid genotype of *P. borealis* one copy of the

sequence is identical to the sequence in *P. epiphylla*-species *S* and the other to the sequence in *P. epiphylla*-species *N* (Fiedorow *et al.,* 2001). Other studies on sequence differentiation of additional regions of chloroplasts and mitochondrial DNA resulted in the designation of *P. epiphylla*-species *N* as a donor of organellar DNA into a polyploid genome of *P. borealis* (Pacak & Szweykowska-Kulińska, 2000, 2003).

Electrophoretic analysis of isozyme variants remains the simplest and easiest routine method, for identification of all species, which can be used to differentiate between cryptic species of the *P. epiphylla* complex and the alloploid *P. borealis*. Two enzymes, including DiaA, described earlier by Odrzykoski *et al*. (1997) and the new marker DiaC, detected upon alternate staining of diaphorase isoenzymes, are sufficient. Its monomeric structure is easy to interpret and heterozygotes are represented by a single band from each parent species. The proposed identification method can be used for the identification of living gametophytes only, but the development of a set of DNA-based molecular markers useful for the identification of haploid taxa from freshly dried or herbarium specimens using already known sequence differences or other sequences proposed as DNA "barcodes" is only a question of time.

We hope that the identification method based on isozyme analysis may facilitate further studies on the distribution, habitat preferences and evolutionary history of the polyploid *Pellia borealis* and may help in the discovery of diagnostic morphometric differences between cryptic species from these groups.

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Appendix

Numbers from Hepatic Herbarium (POZW) housed in the Department of Genetics of the A. Mickiewicz University in Poznań, Poland.

***Pellia epiphylla*- species S** (gametophytic chromosome number n=9)

TATRY RANGE (Carpatians, S Poland): **POZW 29472;** Chowańcówka stream, **POZW 32465;** Boczań, **POZW 32477**; Tatry Wysokie Range, **POZW 32481**; Zawoja-Markowe, **POZW 32482**; Dolina Białego, Tatry Zachodnie, **POZW 33903** Las pod Gubałowskie Forest, Podhale, **POZW 33950**; Ostry Wierch, **POZW 33973**; Las Hurkotne, **POZW 33976;** Przyporniak, **POZW 34456**; Wyżnia Kira Miętusia, Tatry Zach. **POZW 35893;** Magura Witowska, GÓRY IZERSKIE RANGE (Western Sudety Mountains): **POZW 35239;** Mała Kamienna Stream, **POZW 35244;** Mała Kamienna, **POZW 35248;** Mała Kamienna, **POZW 32466**; Góry Izerskie Range, **POZW 32478**; Polana Izerska, BIESZCZADY (Eastern Carpatians, S Poland): **POZW 34736**; Hnatowe Berdo, **POZW 34747;** Wołosatka Stream, **POZW 34751**; Pszczeliny, **POZW 34760**; Pszczeliny, **POZW 34905;** Wetlina, **POZW 34906**; Przełęcz. Orowicza, **POZW 34907;** Przełęcz Orłowicza, **POZW 34991;** Jawornik, **POZW 34998**; Dolina Górnej Solinki Valley, **POZW 32769**; Smerek, KARKONOSZE RANGE (Western Sudety Range, S Poland): **POZW 31256;** Malina Valley, **POZW 31262**; Wilczy Potok Stream, **POZW 34391**; Kamieńczyk Waterfall, GÓRY STOŁOWE RANGE (Sudety Range, S Poland): **POZW 32483**; Karłów, **POZW 32484;** Karłów, BESKIDY RANGE (S Poland): **POZW 32474**; Barania Góra, Wisła, **POZW 32475;** Barania Góra, Wisła-Czarne, **POZW 32480**; Wisła-Nowa Osada, Barania Góra Mountain, **POZW 32476;** Zawoja Policzne, Beskid Żywiecki Range, **POZW 32479**; Rajcza, Beskid Żywiecki Range.

***Pellia epiphylla*- species** *N* (gametophytic chromosome number n=9)

POJEZIERZE MAZURSKIE (woj. Podlaskie, NE Poland): **POZW 27197;** Frącki, gm. Głęboki Bród, Suwałki, **POZW 32464;** Jez. Długie, Augustów, **POZW 37571;** Suwałki, (woj. Warmińsko-Mazurskie): **POZW 35399;** Bagno Radykajny, Olsztyn, **POZW 203,** Miłomłyn, Ostróda, **POZW 203;** J.Ruskie, Ostróda, POJEZIERZE POMORSKIE (woj. Pomorskie, N Poland): **POZW 35451;** Jezioro Kulkówko, **POZW 35458;** Jezioro Małe Płocice, Kartuzy, **POZW 35459;** Jezioro Małe Płocice, Kartuzy, **POZW 35460;** *Ibidem*, **POZW 35462;** Gdańsk, **POZW 35463;** Gdańsk, **POZW 35464;** Gdańsk, **POZW 35465;** Gdańsk, **POZW 35466;** Gdańsk, **POZW 35469;** Gdańsk, **POZW 35470;** Gdańsk, **POZW 35762;** Supsk **POZW 35765;** Słupsk, **POZW 35778;** Słupsk, **POZW 35780;** Słupsk, **POZW 35772;** Jelenie Małe Lake, WOJ. WIELKOPOLSKIE: **POZW 32471;** Peskownica, Kuźnica Zelichowska, Piła, **POZW 205;** *Ibidem*, Pestkownica, WOJ. MAZOWIECKIE: **POZW 32472;** Klembów, Warszawa, **POZW 34387;** Klembów B+N, **POZW 165;** Kampinos, Warszawa, **POZW 33953;** Klembów, WOJ. ZACHODNIOPOMORSKIE**: POZW 32473;** Warnowo, Linówek peatbog, Wolin.

Pellia borealis (gametophytic chromosome number n=18)

BIESZCZADY (Eastern Carpatians, S Poland): **POZW 37252;** Ustrzyki, KOTLINA TORUŃSKA (Bydgoszcz, NW Poland): **POZW 30854;** Jez. Dębno, near Barwice, **POZW 32190;** Jez. Ostrowite Lake, Chojnice, **POZW 32192;** *ibidem,* GÓRY BYSTRZYCKIE (Sudety Range, S Poland): **197 POZW;** Piekielna Dolina Valley, **POZW 32452;** Duszniki Zdrój, **POZW 169;** Śnieżnik, GÓRY IZERSKIE RANGE (Western Sudety Mountains): **341 POZW; 343 POZW; POZW 35031;** Wrześnica, **POZW 35232;** Mała Kamienna, **POZW 35270;** Wkrześnica, GÓRY KACZAWSKIE RANGE (Western Sudety Mountains): **POZW 35046;** Dol. Kamienicy Valley, *ibidem* **POZW 35054;** GÓRY STOŁOWE RANGE (Sudety Range, S Poland): 131 **POZW;** Karłów, **POZW 32468;** Karów, **POZW 32467;** *ibidem*, **POZW 28558;** Czerwony Potok Stream, KARKONOSZE RANGE (Western Sudety Range, S Poland): **POZW 35227;** Kowary, 190 **POZW;** Szklarka waterfall, **POZW 35267;** Jakuszyce, **POZW 36831;** Jelenia Góra, **POZW 36833;** Jelenia Góra, **POZW 31264;** Góra Chojnik, POBRZEŻE SŁOWIŃSKIE (near Koszalin, N Poland): **POZW 28561;** Jez. Chlewe Małe, **POZW 28605;** Radew River, **POZW 28606;**

Sarnowo, **POZW 32470;** Jez. Lubygość Lake, Mirachowo, **POZW 35530;** Słupsk, **POZW 35537;** *ibidem*, **POZW 35567,** Slupia River, Kartkowo, **POZW 35772**, Jelenie Duze Lake, Parchowo, **POZW 35780,** Kamionka River, Jutrzenka, Borzytuchom, **POZW 34393;** Czołpino, Słowiński PN, **POZW 32457;** Koczała, Słupsk, **POZW 32458;** Lipnica, Wieczywno Małe Lake, Słupsk, **POZW 32460;** Osowo Lęborskie, Słupsk, **POZW 36928** Słupsk, **POZW 36949** Słupsk, **POZW 36958,** Słupsk, **POZW 34393;** Czołpino, Słowiński PN, **POZW 32457;** Koczała, Słupsk, **POZW 32458;** Lipnica, Jez. Wieczywno Małe Lake, Słupsk, **POZW 32460;** Osowo Lęborskie, Słupsk, POJEZIERZE MAZURSKIE (N Poland): **POZW 32464;** Augustów, **POZW 32459;** Ostróda, near Olsztyn, **POZW 32463;** Jez. Ruskie Lake, Ostróda, WIELKOPOLSKA (W Poland): **124 POZW;** Diabli Skok, near Piła, **POZW 32455;** Diabli Skok, PODHALE (Karpaty Range, S Poland): **POZW 32461;** Ludźmierz, **POZW 32469;** Szaflary, **POZW 32462;** Ojców near Kraków, ŻUŁAWY WIŚLANE (N Poland): **POZW 36888;** Gdańsk, **POZW 36894;** Gdańsk, **POZW 32198;** Leśny Młynek, near Brusy, **POZW 32204;** Gdańsk, **POZW 32220;** *ibidem*, **POZW 34179,** Jez. Osuszyno Lake, **POZW 34185;** Jez. Księże Lake, **POZW 34204;** Jez. Małe Płocice Lake, **POZW 34243;** Krugliniec, Wda River, **POZW 34337;** Łobez, **POZW 34340;** *ibidem*, **POZW 34376** Piaskowa River, Radowo Małe, **POZW 34216;** Jez. Przymusińskie Lake, near Gdańsk, ROZTOCZE (SE Poland, near Zamość): **POZW 27135;** Susiec, Puszcza Solska, **POZW 27140;** Susiec, KOTLINA KŁODZKA (SW Poland): **POZW 36465;** Lądek Zdrój, **POZW 36483;** Kłodzko, **POZW 36837;** *ibidem*, **POZW 36838** *ibidem*, **POZW 28559;** Topielisko Peatbog, **POZW 28560;** Orlica River, Czerwone Bagno, **POZW 35323;** Kłodzko, NIZINA SZCZECIŃSKA (NW Poland): **POZW 32467;** Puszcza Bukowa, near Szczecin, **POZW 34346;** Mściecino, **POZW 34348;** Głowienica, Puszcza Goleniowska, **POZW 34370;** Piaskowa River, Radowo Małe, **POZW 34380;** *ibidem*, TATRY RANGE (Carpatians, S Poland): **POZW 35839;** Zakopane, **POZW 35862;** *ibidem* **POZW 37158;** *ibidem*, **POZW 28477;** Molkówka, **POZW 28481;** Pol. Molkówka, **POZW 29273;** Wołoszyn, **POZW 31310;** Las Pod Gubałowskie, **POZW 32465;** Boczań Nad Kuźnicami, **POZW 32715;** Capówka, Podtatrze, **POZW 32733;** Dol. Pańszczycy Valley, **POZW 32736;** Dol. Skalnite Valley, **POZW 32850;** Tatry, **POZW 32858;** Polana Cicha, **POZW 34486;** Kuźnice, **POZW 34487;** *ibidem*, **POZW 34552;** Małe Ciche, **POZW 34695;** Tatry, **POZW 34699;** Dol. Suchej Wody Valley, NIZINA ŚLĄSKA (SW Poland): **POZW 32453;** Masyw Ślęży, **POZW 32454;** Rościsławice near Wrocław, **POZW 32456;** Sulistrowiczki near Wrocław.