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Two ploidy levels of genetically delimited groups of the *Calypogeia fissa* complex (Jungermanniopsida, Calypogeiaceae)

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Abstract: *Calypogeia fissa* is a suboceanic-mediterrean and amphiatlantic species, which comprises two subspecies: *C. fissa* subsp. *fissa* occurring in Europe and *C. fissa* subsp. *neogea* Schust. known from North America. Recently, within the European part of distribution, three groups (P_S, P_B and G) were distinguished with the aid of genetic and molecular markers. The flow cytometry results revealed that two of the detected groups of the European *C. fissa*, which are frequent in Poland (P_S and P_B), differ in ploidy level: the P_S group is haploid, whereas the P_B group is diploid. Isozyme pattern at two loci may suggest an allopolyploid origin of the diploid P_B group.

Key words: *Calypogeia*, bryophytes, liverworts, ploidy level, flow cytometry, isozyme markers

1. Introduction

Molecular and cytogenetic studies of liverworts have revealed that some species are genetically heterogeneous and, in fact, consist of morphologically cryptic or nearly cryptic taxa (Shaw 2001). Cytological mechanisms, such as polyploidization, was one of the speciation ways within such complexes e.g. *Pellia borealis* (Odrzykoski *et al.* 1996), *Corsinia coriandrina* (Boisselier-Dubayle & Bischler 1998), *Porella bauerii* (Boisselier-Dubayle *et al.* 1998a), or the *Reboulia hemisphaerica* complex (Boisselier-Dubayle *et al.* 1998b) consisted of haploid and polyploid taxa. In some of the above examples, the species with a duplicated chromosome number are allopolyploids which arose as a result of hybridization and its parental species were identified e.g. two haploid cryptic species of the *P. epiphylla* complex (S and N) are proved to be parental species of the polyploid *P. borealis* (Odrzykoski *et al.* 1996; Fiedorow *et al.* 2001). In turn, *P. bauerii* is a hybrid between two different species, namely *P. platyphylla* and *P. cordeana* (Boisselier-Dubayle *et al.* 1998a).

Hybridization and genome duplication are also important processes of speciation in the *Calypogeia* genus. The genus includes species with haploid (n=9) and diploid (n=18) set of chromosome number (Newton 1973; Fritch 1991). Moreover, polymorphism in chromosome numbers was observed in some species e.g. *C. neesiana*, *C. suecica*, *C. azurea*, in which plants with n=9 or n=18 were noted depending on geographic origin (Newton 1973; Fritch 1991; Paton 1999; Chudzińska *et al.* 2001). Evidence from cytological and isozyme studies revealed that three species with diploid set of chromosomes: *C. azurea*, *C. muelleriana* and *C. sphagnicola* f. *paludosa* have an allopolyploid origin (Buczkowska *et al.* 2004; Buczkowska *et al.* 2012a). For *C. fissa*, only one chromosome number (n=18) was reported (Lorbeer 1934; Müller 1951-1958; Damsholt 2002).

Calypogeia fissa – a suboceanic-mediterrean and amphiatlantic species (Mller 1951-1958; Schuster 1969) comprises two subspecies: *C. fissa* subsp. *fissa* occurring in Europe and *C. fissa* subsp. *neogea* Schust. reported from North America (Schuster 1969; Damsholt 2002). Recently, within the European part of distribution, three groups (P_S, P_B and G) were distinguished with the aid of genetic and molecular markers (Buczkowska 2004; Buczkowska *et al*. 2012b). The P_S group, which comprises much smaller plants than the two remaining groups, is the most distinct both in terms of genetic and morphological features (Buczkowska 2004;

Buczkowska *et al.* 2011). A clear difference in the size of plants suggests differences in ploidy level between these groups. In the present study, we used flow cytometry to estimate the ploidy level of two genetically distinct groups (P_S and P_B) of the *C. fissa* complex occurring in Poland. Isozyme markers were also applied in order to check whether the studied groups may have arisen as a result of allopoliploidization.

2. Material and methods

2.1. Plant material

Plants used in the present studies were initially determined as belonging to the *C. fissa* complex on the basis of morphological traits and oil body characters according to Schuster (1969) and Szweykowski (2006). Next, genetic groups (P_S and P_B) of *C. fissa* subsp. *fissa* were identified on the basis of isozyme markers according to Buczkowska (2004). In isozyme analysis, *C. fissa* subsp. *neogea* was used for comparison (Table 1). Electrophoretic separation of the following enzyme systems: EST, GOT, GDH and PGD was conducted according to the procedure described by Wendel & Weeden (1989). Details of cell extract preparation and buffer systems used are included in Buczkowska & Bączkiewicz (2011). Detected alleles were labeled in accordance with previous studies of *Calypogeia* (Buczkowska 2004; Buczkowska *et al.* 2004; Buczkowska & Bączkiewicz 2011).

2.2. Flow cytometry

About 10-15 shoots from each accession carefully cleaned were used for analyses. A modified

Table 1. Collection sites of the studied *Calypogeia* samples

Sample No.	Locality	Collector	Herbarium No.
	***Calypogeia fissa* subsp. *fissa* – group P_S**		
1	W Poland, Lubuskie Province, Bogumiłów near Żary	SR	42628*
2	Central Poland, Wielkopolskie Province, Antonin near Ostrów Wlkp.	KB	42225*
3	Central Poland, Wielkopolskie Province, Antonin near Ostrów Wlkp.	KB	42227
4	Central Poland, Wielkopolskie Province, Antonin near Ostrów Wlkp.	KB	42629*
	***Calypogeia fissa* subsp. *fissa* – group P_B**		
5	NW Poland, Pomorskie Province, Lake Małe Sitno near Czarna Dąbrówka	KB, AB	42345*
6	NW Poland, Pomorskie Province, Lake Kamień near Miastko	KB, AB	42205*
7	W Poland, Lubuskie Province, Starosiedle forest division	SR, KB	42275*
8	W Poland, Lubuskie Province, Rzeczyca river, humus in *Carici elongate-Alnetum*	SR	42630
	Calypogeia fissa* subsp. *neogea		
9	North America, North Carolina, the Southern Appalachian, Dry Falls	BS	42626
10	North America, North Carolina, the Southern Appalachian, Dry Falls	BS	42622
11	North America, North Carolina, the Southern Appalachian	BS	42620
12	North America, North Carolina, the Southern Appalachian	BS	42617
	***Calypogeia muelleriana s. str.* (typical form = group A[1])**		
13	NE Poland, Pomorskie Province, Lake Orle near Miastko	KB, AB	41182*
14	NW Poland, Pomorskie Province, Lake Lubygość near Kartuzy	KB, AB	42214*
15	W Poland, Lubuskie Province, Starosiedle forest division	KB, AB	42323*
	***Calypogeia muelleriana* (atypical form = group B[1])**		
16	NE Poland, Warmińsko-Mazurskie Province, Lake Godle near Ełk	KB, AB	41706*
17	NE Poland, Warmińsko-Mazurskie Province, Lake Godle near Ełk	KB, AB	41707*
18	NW Poland, Pomorskie Province, Lake Lubygość near Kartuzy	KB, AB	42220*
	Calypogeia azurea		
19	S Poland, Tatra Mts., NE slope of Skupinów Upłaz Mt.	KB, AB	41776*
20	S Poland, Gorce Mts., Ochotnica Dolna Kudowy	KB	42390*
21	SE Poland, Bieszczady Mts., Wetlina, Górna Solinka Valley	KB	41948*
	Calypogeia suecica		
22	S Poland, Tatra Mts., stream near Lake Toporowy Staw Wyżni	KB, AB	41727*
23	S Poland, Beskid Sądecki Mts., stream Czarny	KB	42366*
24	SE Poland, Bieszczady Mts., Górna Solinka Valley	KB	41936*

Explanations: [1] – according to Buczkowska & Bączkiewicz (2011); collectors, AB – Alina Bączkiewicz, BS – Blanka Shaw, KB – Katarzyna Buczkowska, SR – Stanisław Rosadziński; * – samples used for flow cytometry

procedure described by Šmarda (2006) was used to prepare the samples. Plant material was ground in a mortar with Otto I buffer (0.1M citric acid, 0.5% Tween 20). The crude nuclei suspension was filtered through a 30 µm nylon filter. 1 mL of Otto II buffer (0.4M $Na_2HPO4.12H_2O$) supplemented with 2 µg/mL 4,6-diamidino-2-phenylindole (DAPI) was then added to the nuclei suspension. *C. suecica* (Arnell & J. Press.) Müll. Frib., the haploid (n=9) species (Buczkowska *et al.* 2004) was used as external standard. For verification of the results, ploidy level was also tested by flow cytometry for samples of other species with known (n=18) chromosome numbers: *C. azurea* Stotler & Crotz, *C. muelleriana* (Chudzińska *et al.* 2001; Buczkowska *et al.* 2004). *C. muelleriana* is genetically heterogenous and consists of two taxa: *C. muelleriana* s.str. (typical form, group A) and the new taxon – group B (Buczkowska & Bączkiewicz 2011). Chromosome number (n=18) was determined only for the A group. Therefore, in the present studies the DNA content was measured for both *C. muelleriana* groups. Three different samples of each *C. fissa* group and remaining species were measured (Table 1). The measurements were repeated 3 times for each sample.

3. Results

The DNA content of groups P_S and P_B of the *C. fissa* complex was determined by flow cytometric measurements and compared with reference species (Fig. 1). Nuclei DNA content in specimens determined by genetic markers as the P_S group of *C. fissa* subsp. *fissa*

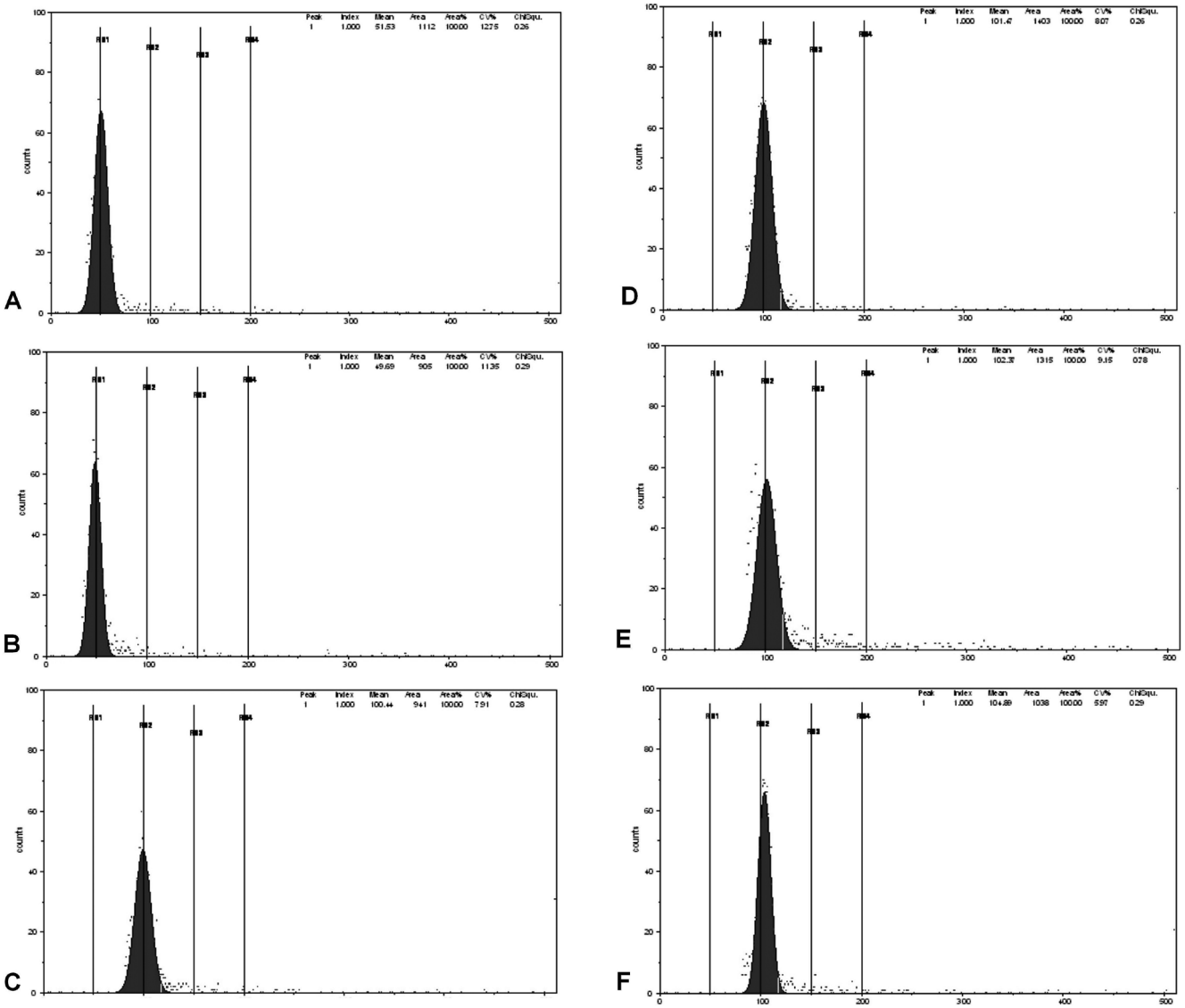

Fig. 1. Histograms of flow cytometry analysis of DNA content in the studied groups of the *Calypogeia fissa* complex and references species Explanations: A – *Calypogeia suecica* (n=9), B – *Calypogeia fissa* subsp. *fissa* group P_S, C – *Calypogeia fissa* subsp. *fissa* group P_B, D – *Calypogeia azurea* (n=18), E – *Calypogeia muelleriana* s. str. – group A (n=18), F – *Calypogeia muelleriana* – group B; RN (range-gate), RN1 = (2C), RN2 = (4C), RN3 = (8C)

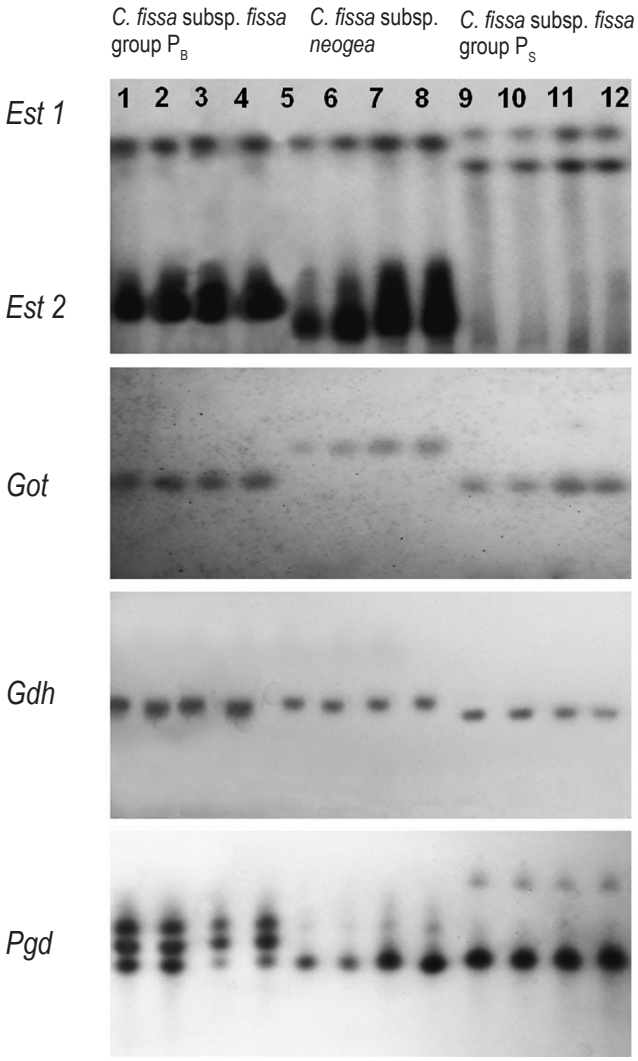

Fig. 2. Isozyme phenotypes of studied groups of the *Calypogeia fissa* complex
Explanations: 1-4 *Calypogeia fissa* subsp. *fissa* group P_B, 5-8 *C. fissa* subsp. *neogea,* 9-12 *C. fissa* subsp. *fissa* group P_S

was equal to the DNA content found in *C. suecica*, with known (n=9) chromosome number and half as high as in specimens from the P_B group of *C. fissa* subsp. *fissa*. DNA content of the P_B group was comparable to the values obtained for *C. azurea* and *C. muelleriana* s.str. (n=18). DNA ploidy level of *C. muelleriana* – group B assessed by flow cytometry was the same as *C. muelleriana* s.str. with (n=18) chromosome number (Fig. 1).

Isozyme phenotypes obtained for the studied groups of the *C. fissa* complex are given in Fig. 2. For the P_S and P_B groups, two different phenotypes in the EST enzyme system and only one, the same for both groups in *Got* locus, were found. Two different phenotypes were observed in the *Pgd* locus: single-banded in all samples of the P_S group and triple-banded pattern in samples of the P_B group. The triple-banded isozyme phenotype possibly represented heterozygous genotypes. Heterozygous genotype in the P_B group was also observed in *Gdh*, which, in heterozygote, appeared always as a slightly diffused broad band because of its multiple banded phenotype.

4. Discussion

In previous experiments, three distinct groups (P_S, P_B and G) within European subspecies of *C. fissa* were detected by isozyme and molecular studies (Buczkowska 2004; Buczkowska *et al.* 2012b). Results of the present study support the hypothesis that *C. fissa* is a complex species consisting of genetically isolated taxa. Two of the examined groups of European *C. fissa*, which are frequent in Poland (P_S and P_B), differ in their ploidy level. The P_S group has half as high DNA content as in specimens from the P_B group. Nuclei DNA content in the P_S group is equal to the DNA amount found in *C. suecica*, with a known (n=9) chromosome number, whereas in the P_B group is comparable with *C. azurea* and *C. muelleriana* – species with n=18 (Buczkowska *et al.* 2004). In view of the obtained results, the studied groups of the *C. fissa* complex can be regarded as a separate species in accordance with the biological species concept, which assume reproductive isolation. The P_S and P_B groups of *C. fissa* subsp. *fissa* are haploid and diploid species, respectively. Unfortunately, due to the lack of a sufficient amount of living material we failed to identify the ploidy level in the G group of European *C. fissa* and *C. fissa* subsp. *neogea*. Data concerning the ploidy level in bryophytes are still incomplete as chromosome counting in some species is very difficult. Flow cytometry is a very helpful method in determining the ploidy level in bryophyte species (Śliwińska *et al.* 2000; Ricca *et al.* 2008; Temsch *et al.* 2010).

Isozyme phenotypes at two loci (*Pgd* and *Gdh*) observed in the P_B group express fixed heterozygous pattern which can suggest their allopolyploid origin. Similarly, fixed heterozygotes in the same loci were also found in diploid (n=18) *C. sphagnicola* f. *paludosa* regarded as allopolyploids (Buczkowska *et al.* 2012a). Previous studies and known chromosome number indicate that polyploidization played an important role as the speciation process in the *Calypogeia* genus (Newton 1973; Inoue 1976; Fritch 1991; Buczkowska *et al.* 2004). Two cytotypes were detected in the *C. sphagnicola* complex: haploid *C. sphagnicola* f. *sphagnicola* and diploid *C. sphagnicola* f. *paludosa* which were recognized as species of allopolyploid origin based on fixed heterozygosity in isozyme loci (Buczkowska *et al.* 2004; Buczkowska *et al.* 2012a). The isozyme loci also provided evidence for an allopolyploid origin of *C. azurea* and both taxa (group A and B) of the *C. muelleriana* complex (Buczkowska *et al.* 2004; Buczkowska & Bączkiewicz 2011). Results of isozyme

and molecular studies give evidence that in bryophytes, allopolyploids are more common than it was previously assumed (Såstad 2005). An allopolyploid origin was detected in several liverwort species, e.g. *P. borealis* (Fiedorow *et al.* 2001), *P. bauerii* (Boisselier-Dubayle *et al.* 1998) and mosses e.g. *Rhizomnium pseudopunctatum* (Jankowiak *et al.* 2005)*, Plagiomnium curvatulum* (Jankowiak-Siuda *et al.* 2008).

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References

Boisselier-Dubayle M. C. & Bischler H. 1998. Allopolyploidy in the thalloid liverwort *Corsinia* (Marchantiales). Bot. Acta 111: 490-496.

Boisselier-Dubayle M. C., Lambourdiere J. & Bischler H. 1998a. The leafy liverwort *Porella baueri* (Porellaceae) is an allopolyploid. Pl. Syst. Evol. 210: 175-197.

Boisselier-Dubayle M. C., Lambourdiere J. & Bischler H. 1998b. Taxa delimitation in *Reboulia* investigated with morphological, cytological, and isozyme markers. Bryologist 101: 61-69.

Buczkowska K. 2004. Genetic differentiation of *Calypogeia fissa* (L.) Raddi (Hepaticae, Jungermanniales) in Poland. Pl. Syst. Evol. 247: 187-201.

Buczkowska K., Odrzykoski I. J. & Chudzińska E. 2004. Delimitation of some European species of *Calypogeia* Raddi (Hepaticae Jungermnniales) based on cytological characters of oil bodies and multienzyme phenotype. Nova Hedwigia 78: 147-163.

Buczkowska K. & Bączkiewicz A. 2011. New taxon of the genus *Calypogeia* (Jungermanniales, Hepaticae) in Poland. Acta Soc. Bot. Pol. 80: 327-333.

Buczkowska K., Sawicki J., Szczecińska M., Klama H. & Bączkiewicz A. 2012a. Allopoliploid speciation of *Calypogeia sphagnicola* (Jungermanniopsiada, Calypogeiaceae) based on isozyme and DNA markers. Pl. Syst. Evol. 298: 549-560.

Buczkowska K., Gonera P. & Hornik B. 2012b. PCR-based molecular markers for identyfication of taxa from the *Calypogeia fissa* complex (Jungermanniopsida, Calypogeiaceae). Biodiv. Res. Conserv. 28: 9-18.

Buczkowska K., Sawicki J., Szczecińska M., Rosadziński S., Rabska M. & Bączkiewicz A. 2011. Two morphologically distinct groups of the *Calypogeia fissa* complex were found in Europe. Biodiv. Res. Conserv. 23: 7-19.

Chudzińska E., Barczak H. & Szweykowski J. 2001. Chromosome numbers in some Polish critical or rare Liverworts (*Hepaticae*). Cryptogamie, Bryol. 22(2): 85-93.

Damsholt K. 2002. Illustrated Flora of Nordic Liverworts and Hornworts. 840 pp. Nordic Bryological Society, Lund.

Fiedorow P., Odrzykoski I. J., Szweykowski J. & Szweykowska-Kulińska Z. 2001. Phylogeny of the European species of the genus *Pellia* (Hepaticae, Metzgeriales) based on the molecular data from nuclear *tRNA*^{Leu} (CAA) intergenic sequences. Gene 262: 309-315.

Fritch R. 1991. Index to bryophyte chromosome counts. Bryoph. Bibl. 40: 19-20.

Inoue H. 1976. Chromosome studies in some Arctic hepatics. Bull. Nat. Sci. Mus. Tokyo Ser. B 2: 39-46.

Jankowiak-Siuda K., Pacak A., Odrzykoski I., Wyatt R. & Szweykowska-Kulińska Z. 2008. Organellar inheritance in the allopolyploid moss *Plagiomnium curvatulum*. *Taxon* 57: 137-143.

Jankowiak K., Rybarczyk A., Wyatt R., Odrzykoski I., Pacak A. & Szweykowska-Kulinska Z. 2005. Organellar inheritance in the allopolyploid moss *Rhizomnium pseudopunctatum*, Taxon 54: 383-388.

Lorbeer G. 1934. Zytologie der Lebermoose mit besonderer Berücksichtigung allgemeiner Chromosomenfragen. I. Teil. Jahrb. Wiss. Bot. 80: 567-818.

Müller K. 1951-1958. Die Lebermoose Europas In: Dr L. Rabenhorst's Kryptogamen Flora von Deutschland Österreich und der Schweiz. 3rd ed. Akademische Verlagsgesellaschaft Geest & Portig K.-G., Leipzig.

Newton M. 1973. Chromosome studies in some British and Irish bryophytes, II. J. Bryol 7:379-398.

Odrzykoski I. J., Chudzińska E. & Szweykowski J. 1996. The hybrid origin of the polyploid liverwort *Pellia borealis*. Genetica 98: 75-86

Paton J. 1999. The Livervort Flora of the British Isles. Harley Books, Colchester.

Ricca M., Beecher F. W., Boles S. B., Temsch E., Greilhuber J., Karlin E. F. & Shaw A. J. 2008. Cytotype variation and allopolyploidy in North American species of the *Sphagnum subsecundum* complex (Sphagneceae). Am. J. Bot. 95: 1606-1620.

Såstad S. M. 2005. Patterns and mechanisms of polyploid formation in bryophytes. Regnum Vegetabile 143: 317-334.

Schuster R. M. 1969. The Hepaticae and Anthocerotae of North America east of the hundredth meridian vol 2, Columbia University Press, New York-London.

Shaw A. J. 2001. Biogeographic patterns and cryptic speciation in bryophytes. J. Biogeog. 28: 253-261.

Šmarda P. 2006. DNA ploidy levels and intraspecific DNA content variability in Romanian fescues (*Festuca*, *Poaceae*) measured in fresh and herbarium material. Folia Geobot. 41: 417-432.

Szweykowski J. 2006. An annotated checklist of Polish liverworts. In: Z. Mirek (ed.) Biodiversity of Poland, 4,

114 pp. W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków.

Śliwińska E., Krzakowa M. & Melosik I. 2000. Estimation of ploidy level in four Sphagnum species (Subsecunda section) by flow cytometry. In: M. Krzakowa & I. Melosik (eds.). The variability in Polish populations of sphagnum taxa (Subsecunda section) according to morphological anatomical and biochemical traits. Bogucki Press SC, Poznań, pp. 137-150.

Temsch E., Greilhuber J. & Krisai R. 2010. Genome size in liverworts. Preslia 82: 63-80.

Wendel J. E. & Weeden N. F. 1989. Visualisation and interpretation of plant isozymes. In: D. E. Soltis & P. S. Soltis (eds.). Isozymes in Plant Biology, pp. 4-45. Dioscorides Press, Portland, Oregon.