Biodiv. Res. Conserv. 5-8: 25-30, 2007

BRC
www.brc.amu.edu.pl

Semi-specific intron-exon splice junction markers in bryophyte studies

Jakub Sawicki* & Monika Szczecińska

Department of Botany and Nature Protection, University of Warmia and Mazury in Olsztyn, Plac Łódzki 1, 10-727 Olsztyn, Poland, *e-mail: jakub.sawicki@uwm.edu.pl

Abstract. We present a protocol for use of intron-exon splice junction (ISJ) markers in studies on bryophytes. The advantages and disadvantages of the method are discussed and compared with other PCR-based markers like AFLP, ISSR and RAPD.

Key words: intron-exon splice junction markers, bryophytes

1. Introduction

Isoenzymatic markers have been used in studies on the variation and taxonomy of bryophytes since the 1970s (Krzakowa & Szweykowski 1977; Zieliński 1987; Wyatt *et al*. 1989; Odrzykoski & Szweykowski 1991; Cronberg 1996). Apart from their numerous advantages, including low costs and the possibility to perform a rapid and simple analysis, isoenzymatic markers have also some limitations. In order to obtain stable enzymatic patterns, the material under analysis must retain vitality under culture conditions, to avoid phenological and habitat-related changes in expression. In the case of peat mosses the analysis of some enzymatic systems was possible only after several months of culturing, because the material collected shows no isoenzymatic activity (Cronberg 1995). Moreover, the level of genetic variation determined with the use of isoenzymatic markers is often low. For example, species-specific enzymatic markers were not found in the Scots pine, *Pinus sylvestris* and in the dwarf pine, *P. mugo*. However, these pine species have different DNA markers of both unique and RAPD-type nonspecific sequences (Zieliński & Polok 2005). Similarly, the third sibling species, enzymatically undistinguishable from the typical form of the liverwort *Pellia endiviifolia*, was identified (Polok *et al*. 2005c).

PCR-based DNA analysis offers new options that can be applied in studies on bryophytes. It makes it possible to analyze a practically unlimited number of loci as well as to use herbarium materials. Genome-scanning AFLP, ISSR and RAPD markers are highly advantageous since no prior sequence information is required, which considerably reduces the cost and time of analysis. Among various classes of markers, RAPD markers have gained popularity in 90s. They are widely used in both taxonomic studies and population genetics (Ashton *et al*. 1994; Boisselier-Dubayle & Bishler 1994; Boisselier-Dubayle *et al*. 1995; Stenoien 1999; Stenoien & Sastad 1999; Stenoien & Flatberg 2000; Scott & Crandall-Stotler 2002; Polok *et al*. 2005a). However, they are often criticized for low reproducibility of results (Vekemans & Jacquemart 1997; Perez *et al*. 1998). AFLP markers (Vanderporten & Tignon 2000; Snall *et al*. 2004) and ISSR markers (Hassel & Gunnarsson 2003; Gunnarsson *et al*. 2005) are used less frequently in studies on bryophytes.

An interesting alternative to the DNA markers discussed above are semi-specific ISJ markers, based on sequences commonly found in plants and indispensable for post-transcription DNA processing (Weining & Langridge 1991). ISJ primers are partly complementary to the sequences on the exon-intron boundary (Fig. 1). These primers are not available commercially, and so they are

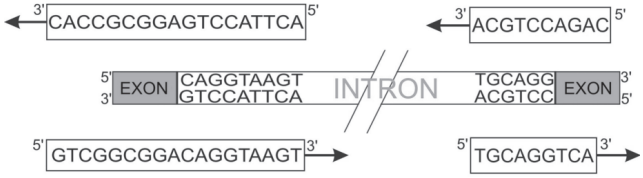

Fig. 1. The scheme of ISJ-PCR reaction

less commonly used than RAPD. The amplified bands are treated as single dominant loci and scored either present or absent. At first ISJ markers were used to assess genetic variation in cereal species (Weining & Langridge 1991; Weining & Henry 1995). Semi-specific intron-exon splice junction markers have been used both in intra- and interspecific studies. Lewandowska (2001) applied ISJ markers to estimate the genetic diversity of *Lolium multiflorum* and *L. perenne*. These primers were also used to identify the sibling species of the liverwort *Pellia endiviifolia* (Zieliński & Polok 2005) and turned out to be effective species-specific markers in studies on *Polygonatum* (Polok *et al*. 2005b; Szczecińska *et al*. 2006) and *Conocephalum* (Sawicki *et al*. 2005). Polok *et al*. (2005a) applied ISJ markers to evaluate the genetic similarity of three species of the genus *Sphagnum*. In addition, ISJ markers were used to analyze the genetic diversity of the fungus *Uncinula necator* (Stummer *et al*. 2000).

The present paper describes a method for applying ISJ markers to studies on bryophytes. These markers are compared with other markers, and their advantages and disadvantages are discussed.

2. Material and methods

2.1. Plant material

In our studies we used ISJ primers on several moss (*S. capillifolium*, *S. fimbriatum*, *S. girgensohnii*, *S. rubellum*, *S. russowii*, *Orthotrichum speciosum*) and liverwort species (*Aneura maxima*, *A. pinguis*, *Bazzania trilobata*, *Pellia borealis*, *P. endiviifolia*, *P. epiphylla*). In the case of *Sphagnum* species from the section *Acutifolia* primers were tested on fresh, frozen and dried herbarium specimens. We also used a plant material from in vitro cultures of *S. fimbriatum* and *S. girgensohnii*. The highest yield and DNA quality were obtained from fresh and frozen samples, however, we didn't notice any differences in banding patterns. For the analysis of *Orthotrichum speciosum* we used dried, two years old samples from herbarium specimen, which despite very low amount of isolated DNA worked well with ISJ primers. During studies on thallose liverworts (*Aneura maxima*, *A. pinguis*, *Conocephalum conicum*, *C. salebrosum*, *Pellia borealis*, *P. endiviifolia*, *P. epiphylla* and *P. nessiana*) we used fresh and frozen material. DNA of the leafy liverwort, *Bazzania trilobata*, was isolated from fresh, frozen and dried material. In this case, results were similar to those obtained for *Sphagnum* species.

2.2. DNA Isolation

DNA isolation for the purposes of ISJ-PCR may be performed by any method enabling to obtain DNA of relatively high purity. In our study, DNA purity was assessed spectrophotometrically and ranged between 83% and 96% (the ratio of absorbance at 260 nm to 280 nm should be higher than 1.600). It was found impossible to amplify DNA in the material isolated by rapid methods proposed by Pedersen *et al*. (2006) and Werner *et al*. (2002). In our studies we used the modified CTAB procedure (Doyle & Doyle 1990) as well as the DNeasy® Plant Mini Kit (Qiagen), which let us obtain sufficient amount of DNA from a single gametophyte.

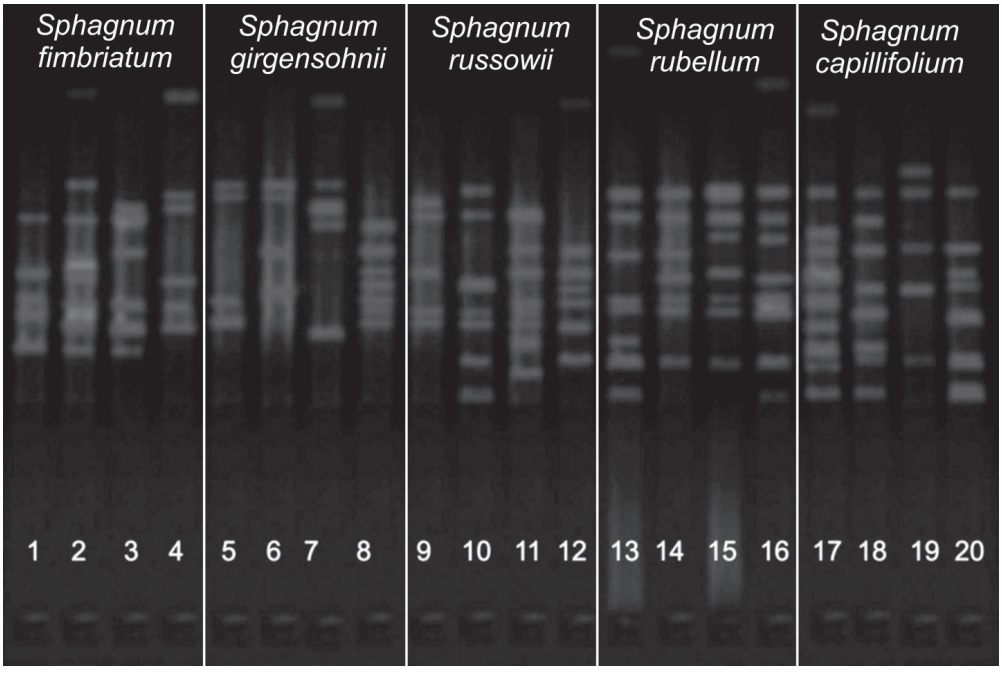

Fig. 2. DNA amplification patterns for five *Sphagnum* species (1-4 *S. fimbriatum*, 5-8 *S. girgensohnii*, 9-12 *S. russowii*, 13-16 *S. rubellum*, 17-20 *S. capillifolium*) with ISJ-2 primers

2.3. ISJ-PCR protocol

Every primer listed in Table 1 works very well with the examined species. However, it's important to use high annealing temperature to get clear, reproducible bands. ISJ-PCR amplifications were performed in a volume of 20 ml containing 20 mM (NH4)SO4, 50 mM Tris-HCl (pH 9.0 at 25˚C), 1.5 mM MgCl2, 1 ml BSA (0.25 mg/ml), 200 mM each dATP, dGTP, dCTP, dTTP, 1.0 mM of each primer, one unit of Taq polymerase and 4 ml of DNA (20ng/ml). The reaction was processed at 94˚C for 1 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.

Table 1. ISJ primers used in studies on bryophytes

Primer	Primer sequence	bp
ISJ-1	5' CAGACCTGC 3'	9
ISJ-2	5' ACTTACCTGAGGCGCCAC 3'	18
ISJ-3	5' TGCAGGTCA 3'	9
ISJ-4	5' GTCGGCGGACAGGTAAGT 3'	18
ISJ-5	5' CAGGGTCCCACCTGCA 3'	16
ISJ-6	5' ACTTACCTGAGCCAGCGA 3'	18
ISJ-7	5' TGCAGGTCAGGACCCT 3'	16
ISJ-8	5' GACCGCTTGCAGGTAAGT 3'	18
ISJ-9	5' AGGTGACCGACCTGCA 3'	16
ISJ-10	5' ACTTACCTGCATCCCCCT 3'	18
ISJ-11	5' TGCAGGTCAAACGTCG 3'	16
ISJ-12	5' GGACTGGAGCAGGTAAGT 3'	18

2.4. Electrophoresis and gel visualisation

Electrophoresis of ISJ-PCR products was performed in 1.5% agarose gels, containing 0.5 mg/ml ethidium bromide. As gel and separation buffer 1x TBE was used. Electrophoresis of samples was carried out at run at 100 V for 2 hours. After electrophoresis the gel was placed on a UV-transilluminator and photographed (Fig. 2).

3. Results

The number and length of amplified bands depend on primer sequence, annealing temperature and reaction mixture composition. The optimizations presented below, originally adapted for *Sphagnum girgensohnii*, were successfully applied also to the other taxa analyzed in the study.

3.1. The annealing temperature and reagent concentrations

The annealing temperatures of ISJ primers considered optimum for vascular plants, i.e. 36˚C and 48˚C for short and long primers, respectively (Polok *et al*. 2005b; Chmiel & Polok 2005; Szczecińska *et al*. 2006), could not be applied to species of the genus *Sphagnum*. The obtained band patterns were often unrepeatable and difficult to interpret due to a high number of amplification products. A reasonable compromise between the number of bands revealed by the primer and pattern reproducibility was a temperature of 56˚C for 16- to 18-nucleotide primers and 37˚C for 10-nucleotide primers. Higher temperatures caused too high decrease in primer efficiency, or even a complete lack of amplification products in some species. However, it seems that annealing temperatures ranging between 48 and 58˚C should be tested in studies on other species, not analyzed here, especially when high-purity DNA is used. This would enable to obtain repeatable patterns at lower temperatures, thus resulting in higher primer efficiency. Annealing temperature optimization is also necessary when new, random primers are to be used. Annealing time had no influence on the generated band patterns. Neither the number nor the length of amplification products were affected by the time ranges tested in this experiment (60, 90 and 120 sec).

Two types of Taq polymerases were used, namely RedTaq Genomic (Sigma) with PCR buffer containing no Mg ions, and HifiTaq (Novazym) with PCR buffer containing $MgCl_2$. In the case of RedTaq polymerase the best compromise between the distinctness and repeatability of band patterns was 1.5 ml of a 5M $MgCl_2$ solution. The concentration of Mg ions in HifiTaq polymerase buffer was sufficient to achieve reproducible results and well-visible patterns. The concentration of nucleotides (dNTP) was not modified relative to vascular plants (Polok *et al*. 2005a; Szczecińska *et al*. 2006). The normal course of ISJ-PCR is related to the amount of DNA in the reaction mixture. We tried four DNA concentrations ranging from 40 to 100 ng per 20 ml of a PCR solution with *S. girgensohnii* and the ISJ-2 primer. The polymerase chain reaction with 40 ng of DNA generated much less intense bands, compared to 60 and 80 ng of DNA.

3.2. Testing the reproducibility

To test the reproducibility of ISJ-PCR amplification products we perform three independent DNA extractions from samples of *S. girgensohnii* growing in greenhouse, dried herbarium specimen (8 month old), in vitro culture and frozen material (stored in -20˚C) collected in field.

PCR reactions were performed in two different thermalcyclers (Eppendorf and Bio-Rad) using polymerases from Novazym (Hifi Polymerase) and Sigma (RedTaq Genomic DNA Polymerase). Each time we obtained identical band patterns for tested samples and primers. Moreover, the ISJ-PCR amplification of *Orthotrichum speciosum* sample in two labs located at the Department of Botany and Nature Protection in Olsztyn and Pfizer Plant Research Laboratory (New York Botanical Garden) gave identical results, despite using different reagents and laboratory equipment.

3.3. Primer efficiency

The tested ISJ primers amplified from 4 to 18 loci (Table 2), depending on the species. Scored bands ranged between 300-1800 bp. Among *Sphagnum*

Table 2. Efficiency of applied ISJ primers

Taxons	Number of bands revealed by ISJ primers											
	ISJ-1	ISJ-2	ISJ-3	ISJ-4	ISJ-5	ISJ-6	ISJ-7	ISJ-8	ISJ-9	ISJ-10	ISJ-11	ISJ-12
Aneura sp.	11	11	9	7	16	12	9	10	10	11	10	9
Bazzania trilobata	7	15	7	16	15	5	6	14	8	10	9	7
Conocephalum sp.	8	10	8	11	12	6	8	17	12	15	13	8
Orthotrichum speciosum	4	11	7	9	10	12	6	15	13	11	12	9
Pellia sp.	13	9	9	11	10	9	11	8	9	11	10	8
Sphagnum sp.	9	8	13	13	8	7	12	16	9	18	10	9

species the primers ISJ-10 and ISJ-8 were found to be most efficient, since they allowed to distinguish 18 and 16 bands, respectively. Primers ISJ-3, ISJ-4 (13 1oci) and ISJ-7 (12 loci) were almost equally efficient during the analysis of peat mosses. ISJ-8 was found to be the most efficient primer in the case of *Orthotrichum speciosum,* as it amplified 15 loci. The high efficiency of ISJ primers was confirmed in studies on thallose liverworts of the genera *Aneura*, *Conocephalum* and *Pellia*. During the analysis of members of the genus *Aneura* the most loci were revealed by ISJ-5 (16) and ISJ-6 (12), whereas ISJ-4 was found to be the least efficient primer (7). The number of bands amplified by other primers ranged from 9 to 12. Similarly as with *Sphagnum* sp., primers ISJ-8 and ISJ-10 were also the most efficient in the case of species of the genus *Conocephalum* – they amplified 17 and 15 loci, respectively. ISJ primers showed comparable efficiency in studies on the genus *Pellia*, where they amplified from 8 (ISJ-8 and ISJ-12) to 13 (ISJ-1) loci. As for *Bazzania trilobata,* ISJ-4 was found to be the most effective primer, since it amplified as many as 16 bands. Primers ISJ-2, ISJ-5 and ISJ-8 were only slightly less efficient: they revealed 15 and 14 loci, respectively.

3.4. Intraspecific variation

Intraspecific polymorphism of ISJ markers was analysed among populations of *S. fimbriatum* and *S. girgensohnii*. Analysis of 48 individuals from twelve populations of *S. fimbriatum* from north-eastern Poland revealed 70 ISJ loci and 81% of them were polymorphic. The highest polymorphism revealed primer ISJ-4, which all 13 loci were polymorphic. Two primers, ISJ-7 and ISJ-10 amplified only monomorphic loci. The number of polymorphic loci amplified by rest of ISJ starters ranged from 2 (ISJ-5) to 7 (ISJ-6).

Analysis of 96 individuals from sixteen populations of *S. girgensohnii* with ISJ markers revealed polymorphism at a level of 71%. Again, the highest number of polymorphic bands was amplified by ISJ-4 primer – 10 out of 12 loci were polymorphic. Three primers didn't reveal any variation among populations: ISJ-2, ISJ-7 and ISJ-11. The number of polymorphic loci amplified by rest of ISJ starters ranged from 1 (ISJ-3) to 8 (ISJ-6).

To test application of ISJ markers on a population level, one population of *S. girgensohnii* from Bialskie Mts (Poland) was analyzed. The 25 individuals analyzed represented as many as 20 different genotypes. The frequency of particular genotypes ranged from 0.240 to 0.040. The average gene diversity (H_e) determined using ISJ markers was 0.132. The gene diversity at a locus within the population varied for polymorphic loci from 0.080 to 0.516. The average effective number of alleles calculated based on ISJ markers applied in the experiment was 1.214. The effective number of alleles at a locus ranged from 1 for monomorphic loci to 1.985 for locus amplified by ISJ-4 primer. The mean Shannon's information index (I) of phenotypic diversity was 0.178.

4. Discussion

ISJ primers are not available commercially, and so they are used less commonly than AFLP, RAPD or ISSR. However, due to their numerous advantages, they may be a viable alternative to the above classes of markers. Just like those markers, the PCR-ISJ method requires no prior information on the DNA sequence of the taxon analyzed.

Due to higher annealing temperatures as compared to RAPD markers, the band patterns obtained using ISJ primers are more repeatable. This concerns particularly 16- and 18-nucleotide primers, although in our study also 10-nucleotide ones permitted high reproducibility of results. Higher annealing temperatures do not cause a considerable decrease in primer efficiency. The analysis of 20 peat moss populations of the section *Acutifolia* showed that the efficiency of ISJ primers was only slightly lower than the efficiency of RAPD primers: the average number of bands amplified per primer was 10.1 and 11.2, respectively (Sawicki 2006). Identical efficiency of ISJ and RAPD primers (on average 11.2 bands per primer) was observed during the analysis of the sibling species of the *Aneura pinguis* complex (Bączkiewicz *et al*. 2008).

In studies on vascular plants the average number of loci amplified by ISJ primers was almost twofold higher, compared to RAPD primers (Polok *et al*. 2005c; Szczecińska 2006). However, it should be noted that in the

case of vascular plants, ISJ-PCR was carried out at a lower annealing temperature. In the experiment with three *Sphagnum* species of the section *Acutifolia* a decrease in annealing temperature from 56 to 48˚C caused an increase in the number of bands amplified by ISJ-4 from 13 to 21 (Polok *et al*. 2005a). Nevertheless, our attempts to perform amplification at 48˚C were unsuccessful, since no reproducible band patterns were obtained for most primers.

ISJ markers have lower requirements with regard to DNA purity than RAPD markers, which in the case of vascular plants permits isolation by the NaOH method (Polok, personal communication). In *S. girgensohnii* stable band patterns were generated already for DNA purity of 79%, while RAPD markers did not reveal amplification. Also in the liverwort *Bazzania trilobata* ISJ markers, in contrast to RAPD, provided reproducible results for DNA purity of 82%.

The level of genetic variation revealed by ISJ primers makes it possible to use them both in intra-population and inter-specific studies. The percentage of polymorphic ISJ loci for the analyzed peat mosses varied from 0.36 for *S. rubellum* to 0.68 for *S. girgensohnii* (Sawicki 2006). Semi-specific ISJ markers were found to be more polymorphic than RAPD markers in green species of the section *Acutifolia* (*S. fimbriatum* and *S. girgensohnii*), whereas an opposite tendency was recorded in red species (*S. capillifolium*, *S. rubellum* and *S. russowii*). Differences in the polymorphism of ISJ loci were also observed between the sibling species of the *Aneura pinguis* complex, for which the values of P ranged from 0.00 to 0.48 (Bączkiewicz *et al*. 2008). The cryptic species of the *Aneura pinguis* complex were characterized by higher polymorphism at ISJ loci than at RAPD loci, except for *A. pinguis*-C. In this species none of the tested marker classes showed intraspecific diversity.

ISJ and RAPD markers reveal polymorphism at a comparable level, but this is dependent on the taxon under analysis. Higher polymorphism of ISJ markers, compared with RAPD, was observed in the species of the genus *Polygonatum* (Polok *et al*. 2005c; Szczecińska 2006; Szczecińska *et al*. 2006), while in the species of the genus *Lolium* RAPD markers were more polymorphic (Lewandowska 2001; Polok 2005).

Semi-specific ISJ markers are not used commonly for taxonomic analysis, but they turned out to be effective species-specific markers in studies on *Pellia* (Polok *et al*. 2005c), *Pinus* (Chmiel & Polok 2005) and *Polygonatum* (Polok *et al*. 2005b; Szczecińska *et al*. 2006). Species-specific bands were also found for peat mosses of the section *Acutifolia* (Sawicki 2006) and liverworts of the genus *Aneura* (Bączkiewicz *et al*. 2008).

ISJ markers are also an interesting alternative to AFLP, mostly due to a shorter and simpler procedure as well as lower equipment requirements. In addition, they do not require separation on polyacrylamide gel, which reduces the risk of contact with its toxic ingredients. An unquestionable advantage of AFLP is the number of loci obtained as a result of separation. One of the disadvantages of ISJ markers is their dominant character, which in the case of diploid organisms makes it impossible to distinguish between a dominant homozygote and a heterozygote. However, this is not a problem in the analysis of haploid bryophytes. The PCR-ISJ method is a viable alternative to AFLP, ISSR and RAPD markers used in bryological studies. Due to its low cost and simplicity, this procedure can be applied in most laboratories with minimum equipment requirements.

Acknowledgements. We thank Kornelia Polok for introducing us to ISJ-PCR methodology and Nils Cronberg for helpful comments on an earlier version of the manuscript.

References

Ashton N. W., Antonishyn N. A. & Baker K. E. 1994. Molecular phylogenetic aspects of genetical and physiological studies of moss development. J. Hattori Bot. Lab. 76: 41-57.

Bączkiewicz A., Sawicki J., Buczkowska K., Polok K. & Zieliński R. 2008. Application of different DNA markers in studies on cryptic species of *Aneura pinguis* (Hepaticae, Metzgeriales). Cryptogamie briologie 29(1): 3-21.

Boisselier-Dubayle M. C. & Bishler H. 1994. A combination of molecular and morphological characters for delimitation of taxa in European *Porella*. J. Bryol. 18: 1-11.

Boisselier-Dubayle M. C., Jubiler M. F., Lejeune B. & Bischler H. 1995. Genetic variability in the three subspecies of *Marchantia polymorpha* (Hepaticae): izozymes, RFLP and RAPD markers. Taxon 44: 363-376.

Chmiel J. & Polok K. 2005. Use of DNA markers for assessing the genetic identity between native and introduced stone pine (*Pinus cembra*) in the Tatra Mountains. In: W. Prus-Głowacki W. (ed.). Variability and Evolution – New Perspectives, seria Biologia 72: 137-147. UAM Poznań.

Cronberg N. 1995. Isozyme electrophoresis of *Sphagnum*: an analysis and methodology. Lindbergia 20: 40-48.

Cronberg N. 1996. Isozyme evidence of relationships within *Sphagnum* sect. *Acutifolia* (*Sphagnaceae, Bryophyta*). Pl. Syst. Evol. 203: 41-64.

Doyle J. J & Doyle J. L. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Gunnarsson U., Hassel K. & Soderstrom L. 2005. Genetic structure of endangered peat moss *Sphagnum angermanicum* in Sweden: a results of historic and contemporary processes? Bryologist 108: 194-203.

Hassel K. & Gunnarsson U. 2003. The use of inter simple sequence repeats (ISSR) in bryophyte population studies. Lindbergia 28: 152-157.

Krzakowa M. & Szweykowski J. 1977. Peroxidases as taxonomic markers in two critical *Pellia* taxa (Hepaticae, Pelliaceae). Bull. Acad. Polon. Sci., Ser. Sci. Biol. 25: 203-204.

Lewandowska K. 2001. Estimation genetic similarity of *Lolium perenne* L. and *L. multiflorum* Lam. on the basis of molecular markers. Ph. D. Thesis, University of Warmia and Mazury in Olsztyn.

Odrzykoski I. J. & Szweykowski J. 1991. Genetic differentiation without concordant morphological difference in the thallose liverwort *Conocephalum conicum*. Pl. Syst. Evol. 178: 135-151.

Pedersen N., Russell S. J. & Newton A. E. 2006. A novel molecular protocol for rapid extraction of DNA from bryophytes and the utility of direct amplification of DNA from a single dwarf male. Bryologist 109(2): 257-264.

Perez T. J., Albornoz J. & Dominguez A. 1998. An evaluation of RAPD fragment reproducibility and nature. Mol. Ecol. 7: 1347-1357.

Polok K. 2005. Evolutionary status of close related *Lolium* L. taxa. In: W. Prus-Głowacki (ed.). Variability and Evolution – New Perspectives, seria Biologia 72: 195-207. UAM Poznań.

Polok K., Hołdyński Cz., Sawicki J., Szczecińska M. & Zieliński R. 2005a. Genetic similarity of Polish *Sphagnum* species on the base of RAPD and ISJ makers. In: W. Prus-Głowacki W. (ed.). Variability and Evolution – New Perspectives, seria Biologia 72: 209-216. UAM Poznań.

Polok K., Hołdyński Cz., Szczecińska M., Sawicki J. & Zieliński R. 2005. Genetic similarity of Polish *Polygonatum* species on the base of RAPD and ISJ markers. In: W. Prus-Głowacki (ed.). Variability and Evolution – New Perspectives, seria Biologia 72: 217-223. UAM Poznań.

Polok K., Sawicki J., Kubiak K., Szczecińska M., Korzekwa K., Szandar K. & Zieliński R. 2005c. Genetic divergence within *Pellia endiviifolia* (Dicks.) Dum. from Poland. In: W. Prus-Głowacki W. (ed.). Variability and Evolution – New Perspectives, seria Biologia 72: 241-252. UAM Poznań.

Sawicki J. 2006. Assesment of taxonomical position of selected *Sphagnum* species (section *Acutifolia*) on the base of molecular markers. Ph. D. Thesis, University of Warmia and Mazury in Olsztyn.

Sawicki J., Nadolski Ł. & Zieliński R. 2005. Identification of *Conocephalum conicum* and *C. salebrosum* on the basis of DNA markers. In: B. Jackowiak & Z. Celka (eds.). Taksonomia, chorologia i ekologia roślin w dobie zagrożenia różnorodności biologicznej. Materiały konferencji naukowej dedykowanej Profesorowi dr hab. Waldemarowi Żukowskiemu z okazji 70-lecia urodzin, p. 197. UAM Poznań.

Scott K. M. & Crandall-Stotler B. 2002. RAPD polymorphism as an indicator of population structure, breeding system and speciation in *Fossombronia*. Bryologist 105(2): 225-232.

Snall T., Fogelqvist J. & Riberio P. J. 2004. Spartial genetic structure in two congeneric epiphytes with different dispersal strategies analysed with three different method. Mol. Ecol. 13: 2109-2119.

Stenoien H. K. 1999. Protocols for DNA isolation and random amplified polymorphic DNA (RAPD) analysis on *Sphagnum*. Lindbergia 24: 43-37.

Stenoien H. K. & Flatberg K. I. 2000. Genetic variability in the rare Norwegian peat moss *Sphagnum troendelagicum*. Bryologist 103: 794-801.

Stenoien H. K. & Sastad S. M. 1999. Genetic structure in three haploid peat mosses (*Sphagnum*). Heredity 82: 391-400.

Stummer B. E., Zanker T. & Scott E. S. 2000. Genetic diversity in population *Uncinula necator*: comparison of RFLP- and PCR-based approaches. Mycological Research 104: 44-52.

Szczecińska M. 2006. Genetic diversity of lowland and montane populations of *Polygonatum verticillatum* (L.). All. Ph. D. Thesis, University of Warmia and Mazury in Olsztyn.

Szczecińska M., Sawicki J., Polok K., Hołdyński Cz. & Zieliński R. 2006. Comparison of three *Polygonatum* species from Poland based on DNA markers. Ann. Bot. Fenn. 43(5): 379-388.

Vanderporten A. & Tignon M. 2000. Amplified fragments length polymorphism between populations *Amblystegium tenax* exposed to contrasting water chemistries. J. Bryol. 22: 257-262.

Vekemans X. & Jacquemart A. L. 1997. Perspectives on the use of molecular markers in plant population biology. Belgian J. Bot. 129: 91-100.

Weining S. & Langridge P. 1991. Identification and mapping of polymorphisms in cereals based on polymerase chain reaction. Theor. Appl. Genet. 82: 209-216.

Weining S. & Henry R. J. 1995. Molecular analysis of the DNA polymorphism of wild barley (*Hordeum spontaneum*) germplam using the polymerase chain reaction. Genetic Resource and Crop Evolution 42(3): 273-280.

Werner O., Ros R. M. & Guerra J. 2002. Direct amplification and NaOH extraction: two rapid and simple methods for preparing bryophyte DNA for polymerasae chain reaction (PCR). J. Bryol. 24: 127-131.

Wyatt R., Odrzykoski I. J. & Stoneburner A. 1989. Bryophyte isozymes: systematic and evolutionary implications. In: D. E. Soltis & P. S. Soltis (eds.). Isozymes in plant biology, pp. 221-240. Dioscorides Press.

Zieliński R. 1987. Genetic variation of the liverwort genus *Pellia* with special reference to central European territory. Rozpr. Stud. Uniw. Szczec. 108(24): 1-297.

Zieliński R. & Polok K. 2005. Molecular evolution and plant taxonomy. In: W. Prus-Głowacki (ed.). Variability and Evolution – New Perspectives, seria Biologia 72: 37-55. UAM Poznań.