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The effect of different DNA isolation protocols and AFLP fingerprinting optimizations on error rate estimates in the bryophyte *Campylopus introflexus*

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Amplified fragment length polymorphism (AFLP) has become a standard method for investigating genetic variation in plants. Nevertheless, only a few applications in bryophytes have been published and there is still a need to optimize the method. We optimized DNA isolation and AFLP protocols for *Campylopus introflexus* (Hedw.) Brid. As DNA quality is crucial for successful AFLP analysis, three different DNA extraction protocols were compared and the Invisorb Plant Mini Kit produced the highest DNA amount (15, 38 and 47 ng μl^{-1} for dry, fresh and new cultivated tissues, respectively) and purity. Newly grown stems gave the purest DNA (absorbance at the wavelengths of 260 nm and 280 nm was 1.86). However, banding patterns obtained from dry herbarium specimens (up to two years old) corresponded with those from fresh material at a similarity level of 94.71%. The replicability of AFLP profiles was not dependent on the way plants were stored. We compared commercial kits and the influence of modifications of the protocols for obtaining reliable results. We tested the reproducibility of AFLP fingerprints produced by the final optimized protocol. An increased amount of restriction enzymes and prolonged restriction and ligation of up to 10 h were the most important modifications for improving results. The modified protocol was applied to 30 samples and four selective primer combinations, and gave an average genotyping error rate of 0.0451.

Amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995), which are polymerase chain reaction (PCR)-based markers for the rapid screening of genetic diversity, have become a standard method for population genetics in plants. AFLPs allow rapid generation of hundreds of highly reproducible DNA markers in almost any target organism. Endonuclease digestion of total genomic DNA followed by selective PCR amplifications and electrophoresis of a subset of the fragments results in a unique fingerprint for each genetic individual (Meudt and Clarke 2007, Mueller and Wolfenbarger 1999).

AFLP consists of restriction, ligation and two rounds of PCR. First, DNA is cut with two restriction enzymes, a rare cutter (e.g. *Eco*RI) and a frequent cutter (e.g. *Mse*I). Second, adaptors with known sequences are ligated to the ends of DNA fragments to generate a DNA template for PCR amplification. The number of fragments is reduced by using adaptor-specific primers that amplify only fragments with both *Eco*RI and *Mse*I ends. Adding one to three selective nucleotide(s) to these primers is an accurate and efficient way of selecting a specific set of restriction fragments for amplification. Specific selection is mostly achieved by two consecutive PCRs. In the first step (pre-amplification) the restricted/ligated DNA is amplified with primers, both of which have a single selective nucleotide. The second PCR (selective amplification) uses both primers with two or three selective nucleotides (Karp et al. 1996, Blears et al. 1998, Weising et al. 2005, Meudt and Clarke 2007).

AFLP is especially suitable when studying intraspecific polymorphisms or relationships among closely related organisms (Karp et al. 1996, Arens et al. 1998). It requires no prior molecular knowledge of the target organism (Meudt and Clarke 2007). AFLP is mostly insensitive to the starting DNA concentration (Vos et al. 1995, Rosendahl and Taylor 1997) but requires relatively pure DNA without contamination from proteins and secondary compounds (Jones et al. 1997, Meudt and Clarke 2007). When optimized it is generally highly replicable with a genotyping error rate of less than 2% (Vos et al. 1995, Jones et

al. 1997, Arens et al. 1998, Mueller and Wolfenbarger 1999, Bonin et al. 2004). However, AFLP is a dominant marker that does not allow homo- and heterozygotes to be distinguished. Fingerprints are scored as a binary matrix (presence/absence of bands). Problems associated with possibly subjective data scoring are largely overcome with a large number of loci (Bonin et al. 2004). Error rates were estimated in few of the studies using AFLP. But, for the numbers to be useful, it is necessary to have reference ranges for error rates, especially when publishing surprising or controversial results (Bonin et al. 2004). Publishing of error rates in bryophyte studies has been very rare (Fernandez et al. 2006); therefore estimates of other reference ranges are useful.

Whereas many studies dealing with genetic variation in higher plants have been published (Chauhan et al. 2004, Mahmud et al. 2007, Arens et al. 1998, Pelser et al. 2003, Schönswetter et al. 2004), the use of AFLP in bryophytes has been limited (Vanderpoorten and Tignon 2000, Pfeiffer et al. 2006). However, AFLPs have been proved sufficiently polymorphic at the intra-specific level in bryophytes for studies of population genetic processes (Snäll et al. 2004, Zartman et al. 2006), cryptic speciation (Fernandez et al. 2006), clone identification (Pfeiffer et al. 2006), species differentiation (Rowntree et al. 2010) or linkage mapping (McDaniel et al. 2007). Several studies have also reported that bryophytes require special modification of DNA isolation protocols (Schlink and Reski 2002, Fernandez et al. 2006) because the cells of mosses contain a lot of secondary metabolites (Mittmann et al. 2007, Xie and Lou 2009). Although the extraction process influences the quality of isolated DNA (which is one of the most important factors necessary for successful and reliable AFLP fingerprinting), no study on bryophytes has dealt with this aspect. AFLP is still not a standard method in bryophytes and no essential modifications of the standard protocol or suggestions for special treatment have been published to date. Therefore it is still necessary to optimize the original protocol (Vos et al. 1995) for each newly investigated species.

In this study we optimized the DNA isolation and AFLP protocols for *Campylopus introflexus* (Hedw.) Brid. In particular, we aimed to (a) compare different DNA extraction protocols with respect to obtaining a sufficient quantity and quality of DNA for AFLP, and (b) test the reproducibility of AFLP fingerprints after different modifications to the protocol.

Methods

Sample collection

Campylopus introflexus is one of the most aggressively invasive mosses in Europe, originating from the Southern Hemisphere (Frahm 1984). During the last few decades it spread from western Europe to the north and east, and in some habitats it has became a dominant (Hassel and Söderström 2005, Daisie 2009). For the optimization work within this study we used samples from the Czech Republic (16 populations), Poland (1 population) and South Africa (1 population) (Table 1). From five populations we sampled more than one cushion. Samples were obtained from all the usual habitats of *C. introflexus* (peatbogs, spruce and pine forests, sand dunes). We confirmed that in four populations individual cushions (to 3 × 3 cm) contain only one AFLP genotype. We analysed the upper parts (1–3 cm) of 3–10 leafy stems per cushion from a total of 30 *C. introflexus* cushions. Preliminary analyses showed that all stems from one cushion are genetically identical. As the starting material for DNA extraction, gametophyte tissue in three different conditions was used: (a) dry stems from herbarium specimens, max. 2 years old, (b) fresh stems collected from the field, (c) new stems from *ex situ* cultivation on sterile soil (1–3 months at 22°C).

DNA extraction

Brown tissue and inorganic matter were manually removed from all samples. Samples were washed several times in distilled water. The moss tissue (gametophytes) was used dried, wet or re-hydrated in distilled water. About 100–800 mg of clean tissue was homogenized 1) in a mixer mill MM-200 (Retsch) using microcentrifuge tubes with 3-mm glass beads, then ground to a fine powder (in the case of dry gametophytes), or 2) using a grinding mortar with a pestle that was placed in liquid nitrogen and then ground to a fine powder manually (in the case of wet or rehydrated gametophytes). Three extraction protocols were tested: a) CTAB extraction protocol (Doyle and Doyle 1990), b) DNeasy Plant Mini Kit (Qiagen), and c) Invisorb Spin Plant Mini Kit (Invitek) with modifications described below.

In the CTAB extraction protocol (Doyle and Doyle 1990), samples were incubated in 700 µl of extraction buffer with 0.2% beta-mercaptoethanol for 30 min at 60°C with constant shaking, with the addition of 0.02 g PVP (MW 40000) in the first few minutes of shaking. After that 500 µl of chloroform:isoamylalcohol (24:1) was added to the liquid phase of each sample than several times inverted and incubated for 5 min at room temperature. This step was repeated twice. Samples were centrifuged for 6 min at 13 500 rpm and the supernatant was precipitated by 500 µl isopropanol for 30 min at –20°C. Pellets were washed in 96% and then 70% ethanol, dried down, and resuspended in 50 µl 1×TE buffer.

For extraction using the Invisorb Kit (Invitek) the manufacturer's protocol was followed. Ten µl proteinase K (10 mg ml^{-1}) was added to the mixture in the lysis step. The final DNA product was eluted in 50 µl pre-heated

Table 1. Collection sites for experimental material of *C. introflexus*.

Code	Locality	GPS coordinates [WGS-84]
Afrika	South Africa, Cape Town: NP Table Mountain	16°65′51.2S″, 14°030′26″E
Bork	Czech Republic, Soběslav: Nature reserve Borkovická blata	49°23′72.2″N, 14°62′58.3″E
Bor	Czech Republic, Skuteč: Nature reserve Maštale	49°81′68.6″N, 16°14′19.2″E
Gerl	Czech Republic, Pancíř Mt.: Gerlův potok I. zone of NP Šumava	49°16′72″N, 13°29′91″E
Hrebec	Czech Republic, Svitavy: Hřebeč village	49°72′77.2″N, 16°57′57.8″E
Jilov	Czech Republic, Jílové u Prahy: Petrov village	49°89′30.4″N, 14°46′03.0″E
Kasper	Czech Republic, Kašperské hory	49°15′70.8″N, 13°55′60.0″E
Kralov	Czech Republic, Prachatice: Nature reserve Kralovické louky	49°00′49.4″N, 14°08′36.4″E
Kyjov	Czech Republic, Kyjov	50°91′91.1″N, 14°44′44.7″E
NBrunst	Czech Republic, Pancíř Mt.: Nature reserve Nový Brunst	49°17′8″N, 13°27′13″E
Osika	Czech Republic, Albeř	49°02′86.7″N, 15°15′49.7″E
Osli	Czech Republic, Proseč: Nature reserve Maštale	49°81′68.6″N, 16°14′19.2″E
PL dun	Poland, Smołdzino: Słowiński Park Narodowy	54°74′81″N, 17°40′34″E
Popice	Czech Republic, Jihlava: Popice village	49°34′09.5″N, 15°53′80.0″E
Raje	Czech Republic, Třeboň: Nature reserve Vrájích	48°98′60.8″N, 14°70′91.8″E
Tisina	Czech Republic, Tachovská Huť: Tišina Mt.	49°93′18.3′N, 12°55′05.8″E
Valmez	Czech Republic, Hostašovice	49°50′53.3″N, 17°99′12.2E
Zvonk	Czech Republic, Přední Zvonková: Racín village	48°70′86.7″N, 14°04′46.4″E

Elution Buffer D and incubated for 45 min. Extraction using the DNeasy Plant Mini Kit (Qiagen) followed the manufacturer's protocol.

Extracted DNA was visualized on a 0.8% TAE agarose gel and the DNA concentration was measured photometrically using a BioPhotometer (Eppendorf) (as absorbance of UV light of wavelength of 260 and 280 nm [= A260, A280]). DNA concentration was adjusted to 20 ng μl^{-1}. Purity was expressed as the ratio of A260/A280. Clean DNA should have a ratio between 1.8 and 2.

AFLP fingerprinting

The AFLP methods largely followed the procedures described by Vos et al. (1995). Commercial AFLP kits (Invitrogen) were used for the restriction, ligation and pre-amplification steps in order to ensure standard concentrations and conditions across samples. These kits have successfully been used repeatedly to generate AFLP fingerprints in vascular plants and fungi (Bless et al. 2006, Prohens et al. 2006, Mahmud et al. 2007, Bory et al. 2008).

The AFLP Core Plant Reagent Kit I (Invitrogen) was used for restriction and ligation. The original protocol (<http://tools.invitrogen.com/content/sfs/manuals/10482016.pdf>) was followed with a few modifications which are routinely used in our laboratory: incubation of the restriction mixture for 15 min at 70°C after restriction was eliminated, ligation was incubated at 37°C, and the ligation mixture was not diluted with TE buffer. As the plant samples were very small all steps were scaled down to a final volume of restriction mix before incubation of 10 µl. Complete restriction of DNA was tested on 1.8% TBE agarose gels.

Pre-amplification was carried out using the AFLP Pre-Amp Primer Mix I (Invitrogen) following the manufacturer's protocol (<http://tools.invitrogen.com/content/sfs/manuals/aflpi_man.pdf>). Whole reaction was made in ten times reduced volume (final volume of preamplification mix before PCR was 5.1 µl), in order to save material. Pre-selective PCR started with an initial step of 72°C for 2 min, followed by 20 cycles of 10 s at 94°C, 30 s at 56°C and 120 s at 72°C. Final elongation was carried out at 60°C for 30 min. The product was visualized on 1% TBE agarose gel after being diluted ten times with ddH_2O.

A total of 2.3 µl of pre-amplified DNA was added to the selective amplification premix (5.1 µl ddH_2O, 1 µl 10× polymerase buffer, 0.2 mM dNTP, 0.5 pmol *Eco*RI primer, 0.5 pmol *Mse*I primer, 0.2 U DNA polymerase). Selective PCR amplification started at 92°C for 2 min, 65°C for 30 s and 72°C for 2 min. A touchdown protocol was applied in the following eight cycles of 1 s at 94°C, 30 s at 64°C (1°C decrease each cycle), and 60 s at 72°C. This was followed by 23 cycles of 1 s at 94°C, 30 s at 56°C

and 120 s at 72°C. Final elongation took place at 60°C for 30 min. For all PCR amplifications a Touchgene gradient (Techne) thermocycler was used and the ramping time was lowered to 0.9°C s^{-1}. Two precipitations produced the final purification. First, PCR products with 1 µl of sodium acetate and 25 µl of 96% ethanol were chilled for 20 min at –20°C. Precipitated products were spun at 4°C for 30 min at 12 500 rpm and the supernatant was discarded. Second, 100 µl of 70% ethanol was added and samples were spun at 4°C for 5 min at 12 500 rpm. Purified products were desiccated at 65°C for 10 min. Just before the products were run on the sequencer, 10 µl of the mixture HiDi formamide: GeneScan-500 Rox (20:1, Applied Biosystems) was added to each sample. Fragment analysis was performed on an ABI 3100 Avant automated sequencer (Applied Biosystems). Raw data were analysed and scored using GeneMarker ver. 1.8 (SoftGenetics).

The protocol described above gave very inconsistent results that were not readily scoreable. In the next step we therefore tested a few modifications of the standard protocol to improve resolution of banding patterns. Some modifications were succesfully used in previous AFLP studies (Snäll et al. 2004, Pfeiffer et al. 2005, Rowntree et al. 2010), but explicit tests of how they influenced the results were not described. The following modifications to the AFLP protocol were subsequently used during optimization and only the best results (assessed by PCR success and after that by the most distinct AFLP banding patterns) from a particular modification step were included in the subsequent testing:

1. Denaturation time increased to 10 seconds in all pre-amplification and selective amplification steps.
2. Different DNA polymerases (REDTaq DNA Polymerase (Sigma), JumpStart REDTaq DNA Polymerase (Sigma), Immolase DNA Polymerase (Bioline).
3. Amount of template DNA for restriction (50, 100, 200, 600 and 1000 ng)
4. Amount of mixture (AFLP Core Plant Reagent Kit I) of restriction enzymes *EcoR*I/*Mse*I (1.0 U of each – amount in original protocol, 1.25 U of each – increased amount, both in a 10 µl restriction mixture).
5. Duration of restriction and ligation (4, 6 and 10 h)
6. Dilution of the pre-amplification product (1:9.7, 1:6.8, 1:4)

After optimization, 36 different primer combinations were investigated using the optimized protocol. Finally, four primer combinations that produced polymorphic and evenly distributed fragments in the range of 50–500 bp were selected for the final analysis of 30 samples and for error rate assessment.

Assessment of AFLP error rate

Distinct polymorphic peaks in the 50- to 500-bp range were scored as present or absent, and the only characters included in the analysis were those for which unambiguous determinations could be made in all electropherograms. We followed all the suggestions made by Bonin et al. (2004) to minimize error rate. We assessed the reproducibility of the AFLP reactions by repeating the entire AFLP procedure on 30 individuals following the optimized protocol. We estimated the genotyping error rate (Bonin et al. 2004) after scoring by comparing the 1/0 matrices obtained for the duplicated samples. Differences detected at this point could be due either to the technical work, and/or to the subjectivity introduced during the scoring process. Samples with large intensity differences for some peaks are often erroneously scored when using automated peak detection. In weak samples it is necessary to correct automatic detection manually, otherwise the genotyping error rate increases considerably. The error rate was estimated by dividing the number of differences by the total number of comparisons among all pairs. This was done separately for each primer combination.

Results

DNA extraction

DNA extraction protocols gave different results for the amount of DNA obtained and its purity (Table 2). The cheapest CTAB method yielded, on average, 74, 31 and 40 ng $µl^{-1}$ for dry, fresh and new stems, respectively. However, the amount of DNA obtained varied widely (from 7 to 180 ng $µl^{-1}$) and the purity was inappropriate for subse-

Table 2. Comparison among extraction approaches in terms of the average concentration of DNA obtained, (± SE [standard error of the mean]), minimum and maximum value (ng $µl^{-1}$) and average purity measured as the ratio of absorbance (A260/A280).

Extraction protocol	Source of DNA								
	Dry stems			Fresh stems			New stems		
	average (±SE)	min/max	purity	average	min/max	purity	average	min/max	purity
CTAB	74.04±50.86	7/180	1.58±0.4	31±36.77	5/57	1.65±0.45	40.29±34.72	10/80	1.85±0.39
DNeasy Kit	3.67±1.53	2/5	1.3±0.38	0.33±1.53	0/2	–	–	–	–
Invisorb Kit	15.3±8.96	3/70	1.79±0.25	38.76±46.73	5/224	1.77±0.18	47.64±51.51	7/280	1.86±0.04

quent AFLP analysis in most of the samples. The DNeasy Kit gave very low DNA concentrations (about 3.6 ng μl^{-1} on average), with variable purity. Such low amounts of DNA were insufficient for AFLP. The Invisorb Kit gave most stable results of all the protocols tested and yielded, on average, 15, 38 and 47 ng μl^{-1} for dry, fresh and new stems, respectively. The amount of obtained DNA varied among samples but was sufficient for subsequent AFLP analysis in most cases. The highest DNA purity (A_{260}/A_{280} ratio 1.86) was achieved when new stems were used as the starting material for extraction. Dry herbarium specimens were also suitable for use (after soaking in distilled water for 8 hours) but the amount of DNA was considerably lower (15 ng μl^{-1} on average).

AFLP fingerprinting

Good quality starting DNA is one of the most important prerequisites for successful AFLP analysis. For that reason only DNA extracted using the standard CTAB protocol and Invisorb Kit was used. For the testing alternative protocols the source DNA was extracted from dry stems (in the case of CTAB extraction) and from fresh and new stems (Invisorb Kit). There were no differences between fresh and new stems from Invisorb Kit extractions during monitoring the impact of modifications in AFLP protocol.

During successive optimization steps, CTAB-extracted DNA always gave worse (less clear) banding patterns (or non-amplification during PCR) than Invisorb Kit-extracted DNA. Thus for the final optimized AFLP protocol we used the Invisorb Kit-extracted DNA. The standard (unoptimized) protocol (Methods) produced unpredictable results for about 50% of the samples with an error rate of about 0.5. Consistent results (clear scorable banding patterns) were only achieved by incorporation of all six additional optimization steps (Table 3).

First, extension of the denaturation step (94°C) in the pre-amplification PCR to 10 s had no effect on success-

Table 3. Modifications to the AFLP protocol (tested in the same order as in the table, with successful steps added to subsequent analyses; order of tested modifications correspond with descriptions in Methods). Effects were assessed by PCR success and after that, by distinctness of the AFLP banding pattern: no effect – the results were not different from unmodified protocol, + modification produced more distinct patterns than unmodified protocol, ++ the patterns after modification were the most distinct, – the results were ambiguous.

Extraction protocol + included modifications + →	→ testing modified step		CTAB (dry stems)	Invisorb (fresh stems)
Source protocol from Methods (SP)				
1. SP	increasing time of all denaturation steps during PCR to 10 s		no effect	no effect
2. SP + denaturation 10 s	polymerases	JumpStart 0.1 µl	+	++
		JumpStart 0.2 µl	+	+
		REDTaq 0.1 µl	++	+
		Immolase 0.1 µl	not tested	no effect
3. SP + denaturation 10 s + JumpStart 0.1µl	more template DNA for restriction	50 ng	+	+
		100 ng	+	++
		200 ng	++	+
		600 ng	–	+
		1000 ng	–	–
4. SP + denaturation 10 s + JumpStart 0.1µl + 100 ng template DNA	increased amount of restriction enzymes *EcoR*I (1.25 U) and *Mse*I (1.25 U)		–	++
5. SP + denaturation 10 s + JumpStart 0.1µl + 100 ng template DNA+ *EcoR*I (0.5 µl) and *Mse*I (0.5 µl)	changes in duration of restriction and ligation	4 hours	–	–
		6 hours	–	+
		10 hours	–	++
6. SP + denaturation 10 s + JumpStart 0.1µl + 100 ng template DNA + *EcoR*I (0.5 µl) and *Mse*I (0.5 µl) + 10 hod to r/l	decreasing dilution of product after pre-amplification	1: 9.7	no effect	no effect
		1: 6.8	no effect	no effect
		1: 4	no effect	+
SP + denaturation 10 s + JumpStart 0.1µl + 100 ng template DNA + *EcoR*I (0.5 µl) and *Mse*I (0.5 µl) + 10 hod to r/l + dilution 1 : 4 = final protocol				

ful PCR amplification. Second, the use of REDTaq and JumpStart Taq DNA polymerases improved the analysis; REDTaq gave slightly sharper bands for CTAB-extracted samples and JumpStart Taq for Invisorb Kit-extracted samples. Third, using template DNA between 100 and 200 ng positively influenced the results. Fourth, increased amounts of restriction enzymes during the restriction step (*Eco*RI and *Mse*I both at 1.25 U in a 10 µl restriction mixture) appeared to be crucial and markedly improved the results. Fifth, prolonging the restriction and ligation to 6 or 10 h (for each step) (the latter being better) again improved the results. Sixth, the use of less diluted pre-amplification products (1:4) gave better results.

The optimized protocol is therefore as follows (Table 3): Invisorb Kit-extracted DNA, extension of denaturation time in pre-amplification PCR to 10 s, use of JumpStart Taq polymerase (0.1 µl) and 100 ng of template DNA, increased amount of restriction enzymes *Eco*RI (0.5 µl) and *Mse*I (0.5 µl), prolonged restriction and ligation steps to 10 h each, and decreased dilution of pre-amplification products to 1:4. This optimized protocol was used to test the utility of 36 primer combinations and for reproducibility tests with error rate assessment.

Nine primer combinations (*Eco*RI-ACA/*Mse*I-CAT, *Eco*RI-ACA/ *Mse*I-CTC, *Eco*RI-AAG/ *Mse*I-CAA, *Eco*RI-AGG/ *Mse*I-CAA, *Eco*RI-AAG/ *Mse*I-CTA, *Eco*RI-AAC/ *Mse*I-CAA, *Eco*RI-AGC/ *Mse*I-CAC, *Eco*RI-AAC/ *Mse*I-CTG, *Eco*RI-AAC/ *Mse*I-CTT) appeared to yield polymorphic bands in our dataset (Table 1). Only *Eco*RI-ACA/*Mse*I-CAT, *Eco*RI-AAG/ *Mse*I-CAA, *Eco*RI-AAC/ *Mse*I-CAA and *Eco*RI-AGG/ *Mse*I-CAA amplified evenly distributed and easily scorable DNA fragments over the range of 50–500 bp. These four primer combinations provided 253 scorable fragments including 189 polymorphic markers, and were thus chosen for analysis of the whole data set.

The error rate assessment showed high reproducibility of AFLP profiles (Fig. 1). A total of 2372 comparisons were made and 107 differences in band patterns were found (Table 4). The genotyping error rate for individual primer combinations varied from 0.038 (*Eco*RI-ACA/ *Mse*I-CAT) to 0.054 (*Eco*RI-AGG/*Mse*I-CAA), and the average for all samples was 0.0451. All comparisons were made on AFLP profiles from plants with same storage history and using the same type of DNA isolation. We standardized storage history in these analyses because repeatability of AFLP profiles is affected by whether DNA was extracted from fresh, rehydrated, or dry plants. This was tested on DNA isolated from same cushion stored as herbarium specimens, as field-grown, and as cultivated tissues (Fig. 2, Table 5). The genotyping error rates when comparing AFLP profiles in these cases varied from 0.033 to 0.097. There seemed to be some consistent differences in banding patterns between plants with different storage histories but sample sizes were insufficient to determine if these were significant. The genotyping error rate for

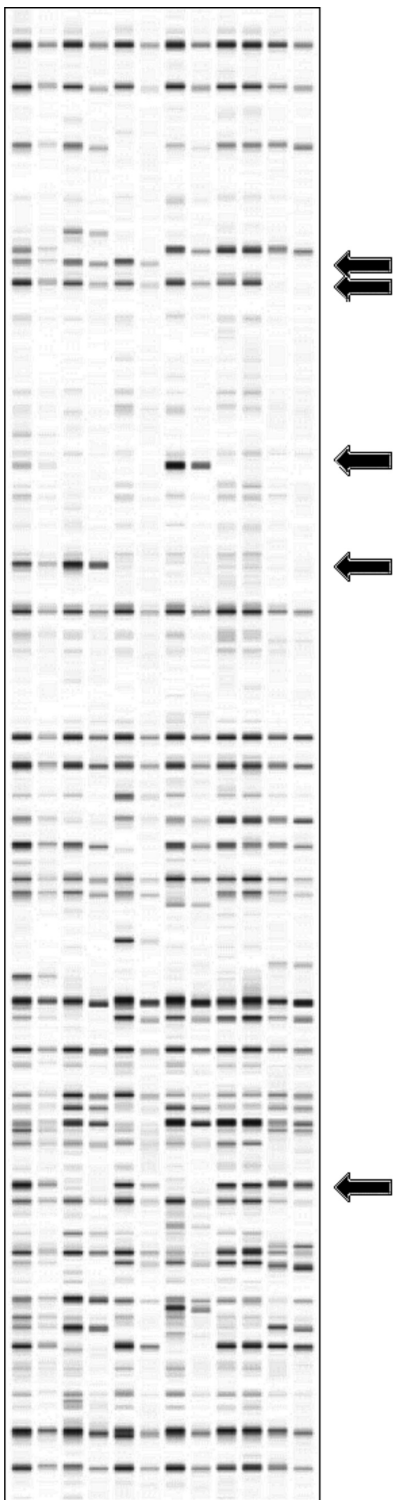

Figure 1. Polymorphic fragments over 70–370-bp range for six different pairs of duplicated samples (Kasper, Valmez_B, Valmez_C, Tisina, PL_dun, Osika), primer *Eco*RI-ACA/ *Mse*I-CAT (Genographer). Major polymorphic fragments are marked.

Table 4. Summary of count of error rate.

Primer combination	No. of tested pairs of replicated samples	No. of assessed bins	No. of non-replicated bins	Error rate
*Eco*RI-ACA/*Mse*I-CAT	19	719	28	0.0389
*Eco*RI-AGG/*Mse*I-CAA	10	442	24	0.0543
*Eco*RI-AGG/*Mse*I-CAA	4	63	3	0.0476
*Eco*RI-AAC/*Mse*I-CAA	25	1148	52	0.0453
Total	58	2372	107	0.0451

herbarium specimens was 0.059, for fresh field-collected plants 0.058, and for cultivated tissue 0.043. These values do not differ from estimates of genotyping error rates for the whole set of populations.

Discussion

Optimization of DNA extraction

Our results demonstrate the importance of selecting the appropriate DNA extraction protocol for bryophyte species. Both the quantity and purity of the DNA differed markedly between the extraction protocols tested in this study. The best scorable AFLP profiles were obtained using the Invisorb Plant Mini Kit. Isolation of bryophyte DNA using different kits is common (Snäll et al. 2004, Pfeiffer et al. 2005, 2006), but extraction yields are rarely reported. Several previous studies have proposed modifications to the standard extraction protocols (Schlink and Reski 2002, Fernandez et al. 2006). Different methods yielded 20–30 ng per sample (*Physcomitriella patens*; Schlink and Reski 2002, Mittmann et al. 2007), 50 ng (*Ceratodon purpureus*, Mittmann et al. 2007), or up to 400 ng (*Grimmia laegivata*; Fernandez et al. 2006) using the CTAB protocol, with modifications that greatly increased the success of the PCR. In our work we obtained more than 1.000 ng of DNA per sample using the CTAB protocol but the purity was lower than for kit-extracted samples. Quality of DNA from CTAB isolation could be increased by purification (Rowntree et al. 2010). As demonstrated here, purity is one of the most important factors influencing the success and reliability of AFLPs. Unfortunately, purity of DNA samples used for other studies has rarely been described so it is difficult to compare our results. Interestingly, only Invisorb-extracted samples were usable in our study, whereas the DNeasy Plant Mini Kit produced very low amounts of DNA in all samples. Thus, although this extraction kit is frequently and successfully used for many vascular plants species (Weising et al. 2005) it might not be appropriate for some bryophyte species. On the other hand, Werner et al. (2002) successfully used this kit for 17 bryophyta species. Other extraction methods have been used for mosses very rarely (NaOH extraction or direct amplification – Werner et al. 2002, Xin et al. 2003, Mittmann et al. 2007).

We also tested the ability to extract DNA from dry herbarium specimens and the quality of the DNA obtained for AFLP fingerprinting. Lower yields might be caused by differences in the amounts of secondary compounds, intra- and extracellular water content, and/or DNA degradation. However, DNA from dry and rehydrated stems is suitable for AFLP analysis. This is consist-

Table 5. Percentage of polymorphic markers among plants from the same cushion depending on the way the specimen was stored (H – herbarium speciment, C – cultivated plants, F – fresh plants from field; primer combination *Eco*RI/*Mse*I).

	ACA/CAT	AGG/CAA	AAC/CAA	AAG/CAA
scorable fragments	74	41	79	59
H – F	4.05%	4.88%	3.80%	5.08%
H – C	5.41%	7.32%	5.06%	3.39%
F – C	4.05%	7.32%	3.80%	5.08%
F – C – H	6.76%	9.76%	6.33%	6.78%
Replicated F	4.05%	7.32%	5.06%	6.78%
Replicated H	5.41%	4.88%	6.33%	6.78%
Replicated C	4.05%	4.88%	5.06%	3.39%

ent with the results of Fernandez et al. (2006) who found that even old herbarium specimens of *Grimmia laevigata* were suitable for high quality DNA extraction. Werner et al. (2002) and Pedersen et al. (2006) successfully used herbarium specimens of several bryophyte species up to even 20 years old to produce DNA sufficient for PCR, but they used another technique to extract DNA (CTAB protocol, NAOH extraction, another commercial kits). This provides an advantage for bryophytes over vascular plants, for which the use of fresh or silica gel-dried leaves is recommended in order to obtain DNA with sufficient quality for AFLP (Weising et al. 2005) and for which herbarium specimens are often unsuitable because of DNA degradation. It is appropriate to use DNA from specimens stored prior to DNA extraction in the same way, because of increasing genotyping error rates from samples stored in different manners. The differences could reflect the occurrence of faint bands, especially from dried material, resulting from partial DNA degradation. However, the differences (genotyping error rate) in AFLP profiles for plants stored in different ways were similar to differences from replicated samples stored in the same way. No manner of storage is much better or worse than another in terms of repeatability.

Figure 2. Banding pattern over 50–500-bp range of primer combination *Eco*RI-AAC/ *Mse*I-CAA for one cushion (population code Bork) – DNA isolation by 1) Invisorb from plants from cultivation, 2) Invisorb from fresh plant from field, 3) Invisorb from herbarium speciments, 4) CTAB from herbarium speciments.

Another source of artifactual variation in bryophytes could be from surface contamination of fresh (not in vitro cultivated) gametophytes by fungi or other organisms. Although all stems were thoroughly washed with distilled water prior to DNA extraction some remnant contamination could theoretically influence the AFLP results. That this level of contamination is not a real problem was demonstrated by the high correspondence between AFLP profiles from washed and unwashed stems (about 95%; Fernandez et al. 2006). Potential endophytic and epiphytic or other surface contaminants (fungi, algae) have 100× smaller genomes than bryophyta and therefore their presence in samples is unlikely to induce significant problems. Several studies reported the need for *in vitro* stem cultivation for obtaining DNA suitable for PCR (*Amblystegium tenax*, Vanderpoorten and Tignon 2000; *Physcomitrella patens*, Schlink and Reski 2002). In our study higher yields were obtained from cultivated plants than from fresh field-collected plants. Moreover, cultivated plants had a number of advantages: clean new parts without old dead leaves, neither soil nor other surface contamination on leaves, easier homogenization of young growing parts (upper 1–2 cm).

AFLP fingerprinting

AFLP have not been used commonly for systematic or population studies in bryophytes. All the studies published to date followed the original AFLP protocol (Vos et al. 1995) with slight modifications, or used commercially available kits. Fernandez et al. (2006) used the Applied Biosystems protocol (AFLP Plant Mapping Kit) without fundamental modifications. Vanderpoorten and Tignon (2000) used the AFLP Analysis system I (Life Technol-

ogies). We used the AFLP Core Plant Reagent Kit and AFLP Pre-Amp Primer Mix I (both Invitrogen) in our study and tested the impact of substantial modifications.

There were no differences in the AFLP analyses using Invisorb-extracted DNA from rehydrated versus new (in vitro cultivated) stems. The first modification (increased denaturation time in pre-amplification and selective amplification PCR cycles) helped to stabilize subsequent steps. Similar protocols were used in several previous studies (Pfeiffer et al. 2005, Rowntree et al. 2010), but without comparisons across methodologies. The selection of Taq polymerase also seemed to be an important factor in our study; the best results were achieved with hot start polymerase (JumpStart, Sigma). This is not surprising since hot start polymerases generally perform better. The amount of template DNA for endonuclease restriction was optimized to 100 ng. This is a low level relative to kit-manufacturer recommendation. DNA quantities in previous moss studies are highly variable, from 10 ng (Snäll et al. 2004) to 500 ng (Pfeiffer et al. 2006). Lower quantities gave inconsistent results, whereas a higher amount of DNA probably led to imperfect restriction, producing results with a background smear. The next improvement was an increase in the amount of restriction endonucleases. Bless et al. (2006) used the same AFLP kit as in our study (Invitrogen) and also had to increase enzyme (*Eco*RI/*Mse*I) amounts to produce good results. Similarly, Pfeiffer et al. (2005) had to increase enzyme amounts to improve results in their study of the bryophytes, *Hypopterygium tamarisci* and *Rhytidium rugosum*. It has repeatedly been reported that lower amounts of restriction enzymes are sufficient when the template DNA is clean, however (Weising et al. 2005).

Complete but specific restriction and ligation are essential for the whole AFLP analysis. Therefore we tested the effect of a prolonged duration of both restriction and ligation (up to 10 hours each) to ensure their completeness. This is a frequently used modification of the general AFLP protocol (Snäll et al. 2004, Pfeiffer et al. 2005, Zartman et al. 2006) that contributes to the stability of reaction. The substantial improvement that we observed might be due to the relative sensitivity of restriction enzymes to impurities (secondary metabolites, etc.) present in the DNA solution (Weising et al. 2005), and the increased amounts of enzymes helped to stabilize the restriction step without the occurrence of non-specific fragments. Although the duration of both the restriction and ligation steps was markedly prolonged in our experiments we found no non-specific restriction products but rather very good reproducibility of the results. Zartman et al. (2006) also reported that prolonging the duration of the restriction step was necessary for reliable results.

The results obtained from the optimized protocol could be used for population studies in a broad range of mosses and liverworts. Both UPGMA (unweighted pair group method with arithmetic mean) and NJ (neighbor-joining) cluster trees were constructed from similarity matrices by using Jaccard's similarity coefficient in FAMD software (Schlüter and Harris 2006). However, clustering of samples from different primer combinations of the scored AFLP fragments was not congruent. This may be because genetic variation of *C. introflexus* was limited and so various primer combinations produced seemingly different banding patterns but the differences were likely not significant.

Reproducibility tests

Every genetic data set includes some erroneous genotypes that can cause problems in the interpretations and conclusions. One of the classical genotyping errors is caused by sensitivity to sample contamination by fungal DNA (Dyer and Leonard 2000). In four of 93 samples we found an increased amount of non-replicable bands (the genotyping error rate for these samples was 16–28%), presumably due to fungal contamination. These samples were easy to recognize and were excluded from further scoring. Although AFLP is more reproducible than e. g. RAPDs, Bonin et al. (2004) pointed out the need to state the error rate in molecular studies. They distinguished between the technical difference rate (due only to laboratory procedures), genotyping error rate (due to laboratory work and differences in scoring between two replicated samples), and scoring differences between two different people evaluating the data. The first two error sources should not exceed 5%. All our data were scored by one person with no attempt to quantify the latter error source.

We calculated the genotyping error rate for our modified protocol and obtained results comparable with error rates in studies devoted to the reproducibility of AFLP profiles (Jones et al. 1997, Bonin et al. 2004) and with those obtained for mosses using the standard AFLP protocol (Fernandez et al. 2006). We repeated the entire AFLP procedure for 30 individuals from 18 populations and achieved a genotyping error rate of 4.51% (for comparing genotyping error rate mentioned for *Betula nana* in Bonin et al. 2004 was 2.6%, for *Grimmia laevigata* in Fernandez et al. 2006 was 5.9%).

Overall, AFLP can be sucessfully and effectively used as molecular markers for genetic studies of bryophytes. Our optimized protocol is expected to work well for species closely related to *C. introflexus*. We did not, however, test how well our optimized protocol applies to species increasingly distant phylogenetically from our study organism. For some species, modification of the protocol might improve results. At the start of the optimization process it is sufficient to try various DNA isolation approaches – CTAB, diverse commercial kits, purification – because secondary metabolites could inhibit subsequent PCR. Optimum amounts of DNA for restriction should be identified because too low or too high concentrations

may cause problems. Low concentrations of restriction enzymes and/or short time of restriction/ligation can also be a source of variation. The restriction and ligation steps are probably the most crucial in the whole AFLP process. Particular care should be given to different peak intensities and difference in the way investigators score presence/absence of bands. The first problem could be solved by manual scoring and the juxtaposition of samples with various intensity of profiles. Ideally, a given study should be scored by a single individual. Repeatability for bryophytes appears to be similar to that found for other organisms (up to to 5% error).

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