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Direct amplification and NaOH extraction: two rapid and simple methods for preparing bryophyte DNA for polymerase chain reaction (PCR)

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SUMMARY

PCR (polymerase chain reaction) has become one of the most important techniques used in molecular systematics. Generally, the methods applied to isolate DNA for PCR amplification depend on multiple steps to isolate and clean the final product. This involves considerable effort and time when many samples have to be processed and poisonous organic solvents are often needed (phenol, chloroform etc.). In this contribution, two very rapid and simple techniques intended for use in higher plants are shown to be useful in bryophyte molecular biology: direct amplification from plant tissues and the NaOH extraction method. In 15 of the 17 investigated bryophytes (two hepatics and 15 mosses) the *trn*L_{UAA} intron of the chloroplast DNA was successfully amplified by the direct approach, while the NaOH extraction method gave amplifiable DNA in all 17 species. DNA amplified by both methods was successfully used in cycle sequencing.

KEYWORDS: direct PCR, NaOH DNA isolation, *trn*L_{UAA} intron, bryophyte DNA extraction.

INTRODUCTION

PCR (polymerase chain reaction)-based techniques are becoming increasingly popular for studying relatedness of bryophyte taxa. The sequencing of PCR-amplified DNA fragments and RAPD (random amplified polymorphic DNA) are two frequently used techniques. The methods that are generally used for DNA extraction from bryophytes are based on those described by Doyle & Doyle (1987, 1990) using CTAB as solvent (cf. Ashton *et al.*, 1994; Boisselier-Dubayle *et al.*, 1995; Capesius & Bopp, 1997; Meißner *et al.*, 1998; Patterson *et al.*, 1998; De Luna *et al.*, 1999; Stenøien, 1999) although other methods, such as the SDS extraction method of Edwards, Johnstone & Thompson (1991) are sometimes used (cf. Cox & Hedderson, 1999). All these methods require a considerable investment in both time and the chemicals needed. Two methods have been published for the very rapid preparation of plant material for PCR, although so far no studies have been published that have applied these techniques to bryophytes: the direct amplification of DNA from plant tissues (Berthomieu & Meyer, 1991; Rogers & Parkes, 1999) and the alkaline isolation of total DNA (Wang, Qi & Cutler, 1993). Such techniques could be very useful when large numbers of samples have to be processed, for example in population genetic investigations or when extremely small bryophytes are studied. Therefore, we compared both methods with several small moss and hepatic species.

MATERIALS AND METHODS

Plant material

Plants of *Metzgeria furcata* (L.) Dum., *Lophocolea bidentata* (L.) Dum. and *Fissidens taxifolius* Hedw. were collected from S.W. Germany in January 2000. *Didymodon sicculus* M.J.Cano, Ros, García-Zamora & J.Guerra, *Pterygoneurum lamellatum* (Lindb.) Jur., *Bryum argenteum* Hedw., and one sample of *Aloina aloides* (Schultz) Kindb. were collected during February 2000 from S.E. Spain. Other samples of *Aloina aloides* were herbarium specimens of Spanish origin sampled in the years 1999, 1997, 1993 and 1990. *Grimmia trichophylla* Grev., *Brachythecium velutinum* (Hedw.) Schimp., *Pterogonium gracile* (Hedw.) Sm, *Homalothecium lutescens* (Hedw.) Robins., *Hypnum cupressiforme* Hedw., *Rhynchostegium megapolitanum* (H.F.Weber & D.Mohr) Bruch, Schimp. *Eurhynchium*

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meridionale (Bruch, Schimp. & W.Gümbel) De Not. and *Homalothecium aureum* (Spruce) Robins. were collected during September 2000 from Sierra Espuña, S.E. Spain. Additional samples of *Homalothecium aureum* were herbarium specimens collected in the years 1999, 1997, 1994, 1991, 1986 and 1981. *Tortula muralis* Hedw. and *Tortula revolvens* (Schimp.) G.Roth. were collected during March 2001 from Murcia, S.E. Spain. All the plants were stored dry until direct PCR or DNA-extraction was carried out. Herbarium vouchers are deposited at MUB.

Preparation of plant material for direct PCR

In the case of terricolous mosses, the lower part of the plants was cut off to remove the soil particles adhering to the rhizoids. All plants were rinsed with double-distilled water (DDW) to remove dirt and biological contaminants as far as possible. The bryophytes were then transferred to 1.5 ml Eppendorf tubes with sterile DDW and treated in an ultrasonic cleaner to remove any remaining foreign material. Such ultrasonic cleaning of small bryophyte samples in plastic tubes works well and is routinely applied in our laboratory for the preparation of material for SEM studies. Clean leaves or thallus fragments of *ca* 0.2 mm^2 were removed with sterile forceps and transferred to thin-walled 0.2 ml Eppendorf tubes, to which PCR components were added. In order to investigate the dependance of the PCR process on the amount of plant material, varying amounts of *Bryum argenteum* plant material were used.

Basically, NaOH extraction followed the protocol of Wang *et al.* (1993) with minor modifications. One whole plant was put in an Eppendorf tube and 5 µl of 0.5 M NaOH were added. The plant was then ground with a stainless steel needle, which had been cleaned with 0.5 M HCl, until no larger fragments were visible. An additional 15 µl 0.5 M NaOH were then added and grinding was continued for 1 or 2 min. The samples were centrifuged at 13,000 rpm for 2 min and the supernatant was diluted 1:10 in 100 mM Tris–HCl, pH 8.3. When necessary, this dilution was used for the preparation of further dilutions with 100 mM Tris–HCl, pH 8.3.

For comparison purposes, DNA was extracted with a commercially available kit, which had previously given good results in our laboratory with higher plants (DNeasy Plant Mini Kit, Qiagen GmbH, Hilden, Germany). This kit uses silica-gel membranes to bind DNA selectively, which can then be washed with an appropriate buffer and later eluted. In a modification of the manufacturer's protocol, one plant was ground in 5 µl buffer AP1 in an Eppendorf tube at room temperature. When no large fragments were visible, 195 µl of AP1 were added and the Qiagen protocol was followed but with all the buffer volumes, with the exception of the washing buffer, reduced by half. The final elution of the DNA from the silica-gel membrane was made in two steps using 50 µl of the elution buffer supplied by the manufacturer. The eluates were then combined to give a final volume of 100 µl. NaOH-extracted or Qiagen-extracted DNA (1 µl) that was then used in the PCR reactions. At least three independent sets of replicate experiments were run for all the extraction methods.

The PCR reactions were performed in 0.2 ml reaction tubes using 1 U Taq DNA polymerase (Appligene Oncor), 0.2 mM dNTPs (Appligene Oncor), 10 mM Tris–HCl, pH 8.3 (RT), 50 mM KCl and 1.5 mM $MgCl_2$ in 25 µl final volume. The primers were designed to amplify the $trnL_{UAA}$ intron of cp DNA. 10 pmol of primers C and D (Taberlet *et al.*, 1991) were used with the modifications introduced by Meißner *et al.* (1998) to improve annealing in bryophytes. An Eppendorf Mastercycler gradient was programmed (2 min 94°C, 35 cycles with 15 sec 94°C, 30 sec 50°C, 1 min 72°C and a final extension step of 2 min at 72°) to perform the amplification reactions. The amplification products were separated on 9% PAA gels and DNA bands were visualized using the silver staining method of Dean & Milligan (1998). Gels were scanned using a TWAIN compatible scanner (Agfa SnapScan 600) with a transillumination module and stored in digital format.

PCR products were purified using the QIAquick Purification Kit (Qiagen). Sequencing reactions were carried out using the Biocycle Sequencing Kit of GATC (Konstanz, Germany) in an Eppendorf Mastercycler (2 min 95°C, 35 cycles 30 s 95°C, 30 s 55°C, 1 min 72°C) and primers C/D of Taberlet. Sequencing reactions were separated in a GATC-1500-system and transferred to Nylon membranes. The biotin-modified ddNTPs of the sequencing kit allowed the visualization of the DNA-bands by the application of a standard protocol with StreptavidinAlkaline Phosphatase (GATC) and BCIP/NBT (Aldrich).

Results and Discussion

Fig. 1 shows an example of the direct amplification of the $trnL_{UAA}$ intron of chloroplast DNA. Amplification products were obtained for all species studied with the exception of *Lophocolea bidentata* and *Rhynchostegium megapolitanum*. In some species (*Grimmia trichophylla*, *Brachythecium velutinum*, *Homalothecium lutescens*, and *Eurhynchium meridionale*) not all replicates resulted in detectable amplification products. Rogers & Parkes (1999) observed that an excess of tissue rather than too little is more likely to cause problems in direct PCR of higher plants, attributing these problems to leaf matrix components such as proteins, carbohydrates, chlorophyll, etc., that may act as Taq polymerase inhibitors. Therefore an attempt was made to reduce the amount of plant material of *Lophocolea bidentata* to the minimum that could be easily handled (*ca* 25 × 25 µm) but no amplification was observed for any *L. bidentata* direct PCR reaction.

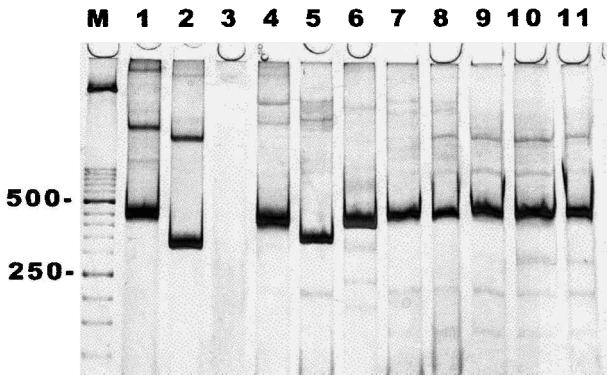

Figure 1. Direct amplification of the *trn*L_{UAA} intron using bryophyte leaves or thallus fragments. Lane M = 50 bp ladder; 1 = *Fissidens taxifolius*; 2 = *Metzgeria furcata*; 3 = *Lophocolea bidentata*; 4 = *Didymodon sicculus*; 5 = *Pterygoneurum lamellatum*; 6 = *Bryum argenteum*; 7 = *Aloina aloides*, collected 2000; 8 = *Aloina aloides*, 1999; 9 = *Aloina aloides*, 1997; 10 = *Aloina aloides*, 1993; 11 = *Aloina aloides*, 1990. One leaf or a thallus fragment of corresponding size was used in a 25 µl reaction volume.

However, the fact that the DNA fragment was successfully amplified starting with template DNA extracted from *L. bidentata* using the Qiagen DNeasy Plant Mini Kit, indicates that the primers used were suitable for amplifying the intron in this taxon (Fig. 2).

Tests with other species showed that an excess of plant material does indeed have an inhibitory effect on direct PCR amplification (Fig. 3). In the case of *Bryum argenteum*, half a stem or more of plant material completely inhibited the reaction, although the strength of the inhibitory effect depended on the species. In *Metzgeria furcata*, for example, thallus fragments more than 1 mm in length

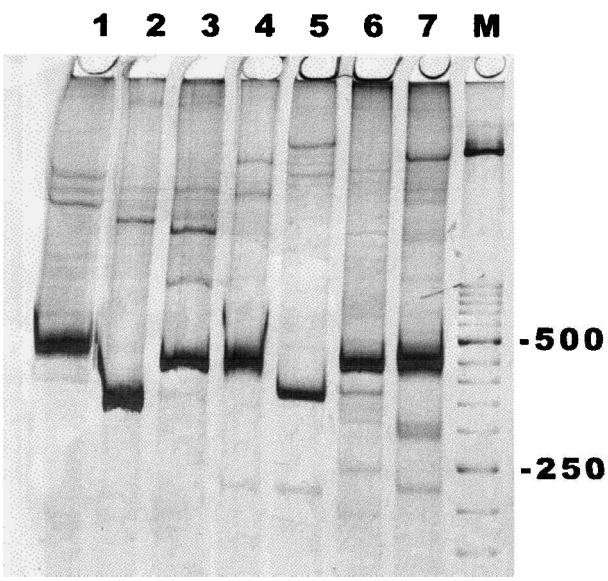

Figure 2. Amplification of the *trn*L_{UAA} intron using DNA extracted with the DNeasy Plant Mini Kit (Qiagen). The DNA of all investigated bryophytes was amplified. Lane 1 = *Fissidens taxifolius*; 2 = *Metzgeria furcata*; 3 = *Lophocolea bidentata*; 4 = *Didymodon sicculus*; 5 = *Pterygoneurum lamellatum*; 6 = *Bryum argenteum*; 7 = *Aloina aloides*, collected 2000; M = 50 bp ladder.

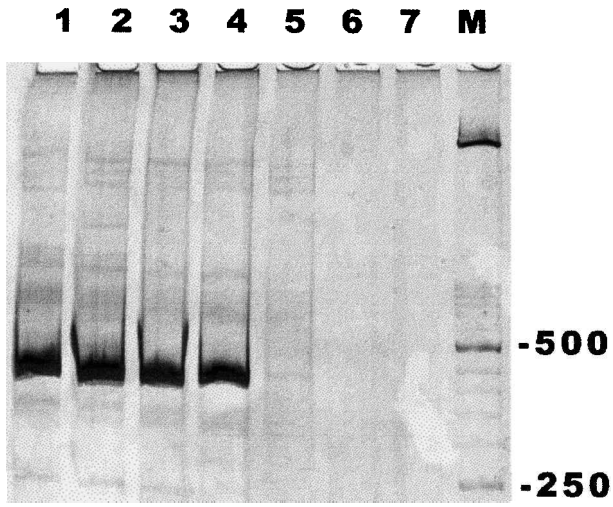

Figure 3. Inhibitory effect of large amounts of plant material on direct PCR amplification. Increasing quantities of *Bryum argenteum* were added to the PCR assays. Lane 1 = ½ leaf; lane 2 = 1 leaf; lane 3 = 2 leaves; lane 4 = 4 leaves; lane 5 = ½ gametophore; lane 6 = 1 gametophore; lane 7 = 2 gametophores; M = 50 bp ladder. The reaction volume in all cases was 25 µl.

clearly inhibited the reaction and the optimal length of fragments was found to be below 0.5 mm. Although these results seem to suggest that hepatics are more resistant to direct PCR than most mosses, perhaps due to the high number of secondary substances that are produced and stored in the oil bodies of these plants, more species must be tested before this can be confirmed.

When the NaOH extraction method was tried in an attempt to overcome the problems that we found in the amplification of *Lophocolea* and *Rhynchostegium*, a 1:10 dilution of the extract gave positive results in all bryophyte species tested, with the exception of *Lophocolea bidentata*. However, higher dilutions worked with *Lophocolea* (Fig. 4), which suggests that a lack of DNA is not the problem, but rather the presence of inhibitory substances in the plants.

In molecular taxonomic studies, investigators frequently depend on herbarium material as a source of DNA. For this reason, we tried to amplify DNA from herbarium specimens of *Aloina aloides* up to ten years old. Fig. 1 shows that the direct PCR amplification of the *trn*L_{UAA} intron was possible with all tested samples. Also DNA extracted using the NaOH method was capable of amplification in the case of herbarium samples of *Aloina* stored for up to 10 years (Fig. 5). The same is true for specimens of *Homalothecium aureum* stored under normal herbarium conditions for up to 19 years (data not shown). However, when interpreting this result, one should keep in mind that both *Aloina aloides* and *Homalothecium aureum* are well adapted to tolerating desiccation and that DNA might be stabilized by special cell components under herbarium conditions. Some bryophytes are known to survive up to 23 years in an ahydrobiotic state (Breuil-Sée, 1993, 1994). Herbarium specimens of species that are less tolerant to

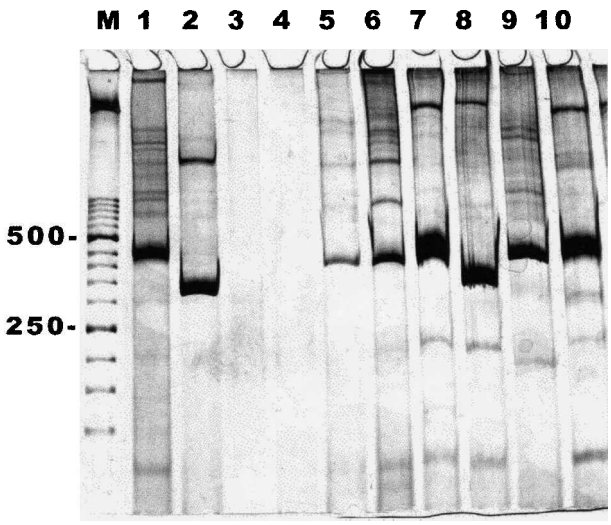

Figure 4. Amplification of the *trn*L_{UAA} intron using NaOH-extracted DNA as template. All dilutions are 1:10 unless otherwise indicated. Lane 1 = *Fissidens taxifolius*; lane 2 = *Metzgeria furcata*; lane 3 = *Lophocolea bidentata*; lane 4 = *L. bidentata,* diluted 1:20; lane 5 = *L. bidentata*, 1:40; lane 6 = *L. bidentata*, 1:80; lane 7 = *Didymodon sicculus*; lane 8 = *Pterygoneurum lamellatum*; lane 9 = *Bryum argenteum*; lane 10 = *Aloina aloides*, collected 2000; M = 50 bp ladder. Note that no amplification was observed with *Lophocolea bidentata* at dilutions of 1:10 and 1:20, but at dilutions of 1:40 and 1:80 the amplification was successful.

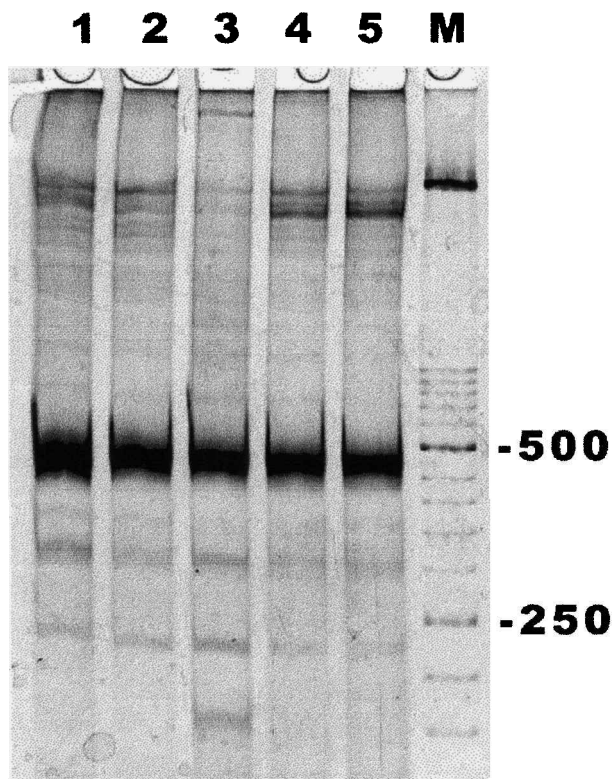

Figure 5. Amplification of the *trn*L_{UAA} intron in *Aloina aloides* stored dry for up to 10 years in the herbarium. The DNA was NaOH-extracted from single plants, diluted 1:10 and then 1 µl of the diluted extract was added to the PCR reaction (25 µl final volume). Lane 1 = collection year 2000; lane 2 = year 1999; lane 3 = year 1997; lane 4 = year 1993; lane 5 = year 1990; M = 50 bp ladder.

desiccation are generally more resistant to PCR amplification (M. Stech, personal communication). Therefore, when collecting plant material for DNA studies when the DNA-extraction is not possible immediately, a rapid drying (using silica gel or $CaSO_4$) is recommended (Sytsma *et al.*, 1993). Nevertheless, all samples collected during 2000 were capable of amplification using the direct PCR approach or the NaOH extraction method, when the experiments were repeated in April 2001. NaOH extracted DNA can be stored at 20ºC in the dilution buffer for up to 1 year and successfully used in amplification reactions.

Both extraction methods were used to amplify DNA with short random primers (RAPD) (Welsh & McClelland, 1990; Williams *et al.*, 1990). A high proportion of the amplified bands were not reproducible (data not shown). This may be due to the low concentration of DNA in the reaction mix. Williams *et al.* (1993) reported, that in the case of soybean, *Glycine max*, a concentration of 1.5 ng/25 µl reaction was necessary to guarantee the reproducibility of the bands. This corresponds to the DNA content of 850 haploid genomes. It is probable, that the amount of available DNA in the reaction mix is well below this level. In the case of direct DNA amplification the access to the DNA might be partially restricted by the chromosomal DNA structure and the proteins associated with the DNA. DNA extracted with NaOH is denatured and although such denatured DNA is suitable for PCR-based amplification it may not be for some other downstream applications such as restriction enzyme digestion. In some of our gels more than one band could be seen. In such a case gel extraction methods are necessary to clean the amplified DNA prior to its use in downstream applications (sequencing reaction etc.). However, this is more time-consuming than the application of spin columns in the case of single bands.

The most important downstream-reaction of amplified DNA is DNA-sequencing. In order to ascertain that the products of NaOH-extraction and direct amplification are usable, we sequenced the *trn*L_{UAA} intron of *Tortula muralis* obtained by direct amplification and of *Tortula revolvens* using NaOH extracted DNA template. In both cases the DNA sequencing reactions were successful.

Conclusions

Our conclusion is that direct PCR amplification from bryophyte fragments or NaOH extraction of DNA could be an interesting alternative to the extraction of DNA by other methods, which, although more sophisticated and possibly yielding cleaner DNA, are more complicated. No organic solvents or any other treatment (heating, proteinase K etc.) are necessary and the time needed is drastically reduced, since manipulation is minimized, which is of special importance when large numbers of specimens are being studied. The reduced number of steps may also decrease the risk of cross contamination. This is especially

true in the case of direct PCR, since it is not necessary to handle DNA-containing liquids. These may form aerosols and often contaminate pipettes, if no filter tips are used.

The techniques of direct amplification and NaOH extraction can be applied to very small samples, which may prove useful when sequencing families such as the Pottiaceae. In some lanes additional bands can be found. Therefore, in these cases it might be necessary to use gel extraction methods for the cleaning of the PCR products prior to their use in sequencing reactions. One problem that is frequently found in the family Pottiaceae is the existence of putative hybrids (Guerra, Ros & Cano, 1994; Ros, Guerra & Cano, 1994; Ros *et al.*, 1996) that cannot be analysed by isozymes since often only a single specimen of very small size is available. This makes PCR-based techniques the best choice for confirming the hybrid origin, and the availability of simple methods, which allow easy handling of such samples, should be of great interest.

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