Optimization of the genomic DNA extraction in some mosses

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Abstract

The presence of organic compounds and high amount of secondary metabolites (polysaccharides, phenolic component, etc.) in mosses cause difficulties in DNA extraction that are followed by problems in PCR reactions. In lower plants, various methods have been used for DNA extraction including silica gel and different commercial kits. These methods mostly use hazardous (like phenol or liquid nitrogen) or costly (proteinase K) materials. Commercial kits are high cost. In order to develop an appropriate and cost effective procedure for DNA extraction in lower plants, the CTAB protocol was modified. Triton X-100, SDS, activated charcoal and ammonium acetate were used for the elution of the contaminations instead of the hazardous and risky materials. The method was compared with three extraction kits (Vivantus, Biobasic, and Rana), and tested on nine species of mosses including *Neckera complanata*, *Anomodon viticulosus*, *Trichostomum brachydontium*, *Dicranum scoparium*, *Tortula* sp., *Plagiomnium cuspidatum*, *Homalothecium sericeum*, *Eurhynchium* sp., and *Neckera crispa* from Iran. The quality and quantity of the extracted DNA was examined with spectrophotometer and agarose gel electrophoresis. The lack of expensive proteinase K in this procedure had no unfavorable effect on the final results and helped to decrease the costs.

Keyword: Expensive, non-flowering plants, proteinase K, risky materials, Triton X-100

بهینه‌سازی روش استخراج DNA ژنومی در برخی خزه‌ها

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خلاصه

در خزه‌گیان (بریوفیت‌ها)، به علت حضور ترکیبات آلی و متابولیت‌های ثانویه بسیار زیاد از قبیل پلی‌ساکاریدها و ترکیبات فنولی، استخراج DNA و در پی آن واکنش PCR با مشکلات متعددی مواجه است. با این حال، روش‌های متعددی جهت استخراج ژنوم در گیاهان غیرآوندی (بی‌گل) از جمله روش سیلیکاژل و همچنین کیت‌های مختلف تجاری توسط محققان مورد استفاده قرار گرفته است که از یک سو، پرهزینه و از سوی دیگر، ممکن است از لحاظ زیست محیطی دارای مواد خطرناکی باشند. با توجه به استفاده از مواد خطرناک همچون فنول، نیتروژن مایع و همچنین مقرون به صرفه نبودن موادی همچون پروتییناز K و کیت‌های استخراج، یافتن روشی مناسب برای کاهش این گونه ترکیبات و کاهش هزینه‌ها بسیار ضروری به نظر می‌رسد. این مطالعه، با هدف معرفی روش استخراج بهینه شده CTAB در نه گونه خزه در ایران شامل: *Homalothecium sericeum*، *Neckera complanata*، *Anomodon viticulosus*، *Trichostomum brachydontium*، *Dicranum scoparium*، *Tortula* sp.، *Plagiomnium cuspidatum*، *Eurhynchium* sp. و *Neckera crispa* و مقایسه این روش با سه کیت استخراج Vivantus DNA، Biobasic و Rana انجام شد. کمیت DNAهای استخراج شده توسط روش نانومتری مشخص و جهت بررسی کیفیت DNAی استخراج شده علاوه بر الکتروفورز ژل آگارز ۱٪، از دو نشانگر مولکولی SCoT و ISSR نیز استفاده گردید. نتایج نشان داد که DNAی ژنومی استخراج شده با وجود آلودگی نمونه‌ها به متابولیت‌های ثانویه، نسبتا دارای خلوص و کیفیت مطلوب‌تری می‌باشد و حذف مواد پرهزینه مانند نیتروژن مایع و پروتییناز K و نیز استفاده از ترکیباتی همچون SDS، Triton X-100 و آمونیوم استات در جهت بهینه کردن کیفیت DNA ژنومی از کارآیی مناسب‌تری برخوردار می‌باشد.

واژه‌های کلیدی: پروتییناز K، گیاهان بی‌گل، مواد خطرناک، هزینه‌بر، Triton X-100

Introduction

The isolation of high quality DNA is important in any molecular biology work. Molecular markers provide access to the enormous amount of information contained in genetic material. Accessibility to the molecular information opens perspectives for the identification of an organism and statement of the evolutionary relationships within taxa. In this way, various practical data about the taxonomy, phylogeny, biogeography, and also population aspects, could be obtained from the analysis of genome.

As far as molecular study concerns, bryophytes have been understudied comparing with flowering plants (Angeles *et al.* 2005, Crespo Padro *et al.* 2014). Selection of an appropriate DNA extraction protocol is essential in these lower plants (Goffinet *et al.* 2004, Mikulaskova *et al.* 2012). The absence of a cogent DNA extraction protocol with high yield can be a remarkably restricting point for molecular analysis of bryophytes. Extraction of the intact and high-quality DNA is always faced with various problems that influence the quality of the PCR results (Angeles *et al.* 2005, Heidari *et al*. 2011) one of them being the presence of polysaccharides and polyphenols in the DNA sample and which are frequently found in bryophyte tissue in high concentrations. These substances usually indicate themselves by the formation of a highly viscous and brown color solution, making it useless for the molecular studies (Sahu *et al*. 2012). These contaminants inhibit the activity of the key enzymes like the DNA polymerases and decrease the solubility of the extracted DNA (Goffinet & Buck 2004, Mittmann *et al*. 2007). Several studies were browsed for the lower plants DNA extraction including the CTAB-based protocols, NaOH extraction and various commercial kits (Werner *et al*. 2002, Pedersen *et al*. 2006, Xin *et al*. 2003, Mittmann *et al*. 2007, Schlink & Reski 2002, Fernandez *et al*. 2006, Mikulaskova *et al*. 2011). Although, the commercial kits have been to some extent efficacious in eradicating the DNA extraction pitfalls, they have not been cost-effective in high throughput experiments. In addition, their extracted DNA yields have not been reported properly (Mikulaskova *et al*. 2011). In this survey, in order to acquire an appropriate and cost-effective procedure for the DNA isolation by considering Sahu *et al*. (2012), the Cetyl trimethyl ammonium bromide (CTAB) protocol was modified in some mosses. The efficiency of the best protocol was assessed by the application of the resulted DNA in two major DNA molecular markers including ISSR and SCoT marker.

Materials and Methods

- Plant samples for the DNA extraction

Dry leaves of mosses including *Neckera complanata* (Hedw.) Huebener., *Anomodon viticulosus* (Hedw.) Hook. & Taylor., *Trichostomum brachydontium* Bruch., *Dicranum scoparium* Hedw., *Tortula* sp., *Plagiomnium cuspidatum* (Hedw.) T.J. Kop., *Homalothecium sericeum* (Hedw.) Schimp., *Eurhynchium* sp., and *Neckera crispa* (Hedw.) were collected from Mazandaran and Gilan provinces, Iran (Table 1). Samples were stored in −20 ºC until use. The identification of the specimens was done on the basis of Ghahreman *et al.* (2003), Akhani & Kürschner (2004), Smith (2004), and Kürschner & Frey (2011). The voucher specimens are deposited in the "HSBU" Herbarium (Shahid Beheshti University, Tehran, Iran). The reference numbers are shown in table 1.

Table 1. Features of extracted samples along with other relevant data

No.	Taxon	Herbarium No.	DNA yield (µg/ml of sample)	A260/A280
1	*Neckera complanata*	HSBU201901	45.1	1.7
2	*Anamodon viticulosus*	HSBU201902	97.0	1.7
3	*Trichostomum brachydontium* sp.	HSBU201903	4.2	1.1
4	*Dicranum scoparium*	HSBU201904	4.2	1.3
5	*Tortula* sp.	HSBU201905	97.0	1.7
6	*Plagiomnium cuspidatum*	HSBU201906	46.4	1.8
7	*Homalothecium sericeum*	HSBU201907	109.0	1.8
8	*Eurhynchium* sp.	HSBU201908	56.0	1.7
9	*Neckera crispa*	HSBU201909	44.3	1.7

- Extraction methods

The CTAB DNA extraction method of Doyle & Doyle (1990) with some modifications was employed for isolating DNA from nine moss species and three extraction kits were tested for five species: DNA Mini-Preps Biobasic DNA extraction kit (Bio Basic Inc., Canada), GF-1 Plant DNA extraction Vivantis kit (Vivantis Inc., Malaysia), and Rana kit (Rana Inc., Iran) (Fig. 1 and Table 2).

The CTAB protocol was optimized for the DNA extraction including a combination of β-mercaptoethanol, polyvinyl pyrrolidone (PVP), sodium N-lauroyl sarcosine, sodium dodecyl sulfate (SDS) as described below:

- Grind −20 ºC stored leaves (0.05 g) to fine powder with a mortar and pestle and transfer in 1.5 mL microcentrifuge tubes.
- Add 0.3 g activated charcoal (Daejung, Korea) and 400 µL extraction buffer (50 mM EDTA (Duksan, Korea), 120 mM Tris-HCl (Solarbio, China), 1.5 M NaCl (Merck, Germany), 0.5 M sucrose (Carlo Erba, Italy), 1.5% Triton X-100 (Panreac, Spain) 0.1% β-mercaptoethanol (Merck, Germany), 2% CTAB (Carlo Erba, Italy) and 100 µL 1% PVP (polyvinyl pyrrolidone, PVP K10, MW 10.000) (Merck, Germany), 100 µL 1% SDS (w/v) (Sigma, USA) and 50 µL 2% sodium N-lauroyl sarcosine (w/v) (Sigma, USA), incubate at 65 ºC for 45 min (invert four times during incubation).
- Centrifuge at 10,000 rpm (9000 × g) for 15 min at room temperature.
- Transfer the aqueous phase (about 200 µL) into a new tube.
- Add 600 µL of Chloroform (Merck, Germany), Isoamyl alcohol (Carlo Erba, Italy) (24: 1) and shake for 5 min.
- Centrifuge at 12,000 rpm (13,000 × g) for 10 min.
- Repeat the prior step twice.
- Transfer the aqueous phase into a new tube.
- Add 300 µL of chilled isopropanol (Carlo Erba, Italy) and 200 µL NaCL 5 M in the presence of 30 µL ammonium acetate 3 M (Carlo Erba, Italy) and keep at −20 ºC for 1 hr to precipitate the DNA.
- Centrifuge at 12,000 rpm (13,000 × g) for 10 min.
- Discard the supernatant and add 200 µL 70% chilled ethanol (Carlo Erba, Italy) and spool out

the pellet by pipetting and centrifuge again at 10,000 rpm (9000 × g) for 10 min.

- Discard the supernatant and air dry the pellet at room temperature.
- Add 70 µL of high salt TE buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA (pH 8).
- Add 200 µL of chloroform: Isoamyl alcohol (24: 1), invert and centrifuge at 10,000 rpm (9000 × g) for 10 min.
- Transfer upper phase to a new 1.5 µl microtube and add 400 µL 96% ethanol.
- Centrifuge at 10,000 rpm (9000 × g) for 10 min.
- Discard the supernatant and air dry.
- Add 70 µL of diluted ddH2O to dissolve the precipitate.
- Store final solution at −20 ºC/−40 ºC till further use.

For extraction using three extraction kits, the manufacturer's instruction protocols were followed.

- Qualitative and quantitative analysis of the extracted DNA

The DNA quantity and quality were evaluated using a UV-Visible spectrophotometer (Dragon, China) at 260 nm. The DNA purity was determined by calculating the absorbance ratio A260/280 and using a picodrop (Hinxton, UK). For quality and yield assessments, electrophoresis was done for all DNA samples; 4 µl of each DNA extract was loaded and visualized on a 1% agarose gel using an E-Gel96® Pre-cast Agarose Electrophoresis System (Invitrogen) (Figs 2–3).

Table 2. Features of extracted samples with three kits for comparison with our protocol

Method	**Taxon**	**DNA yield of sample (µg/ml)**	**A260/A280**	**DNA yield mean of sample (µg/ml)**
Biobasic kit	*Neckera complanata*	2.0	1.7	3.92
	Homalothecium sericeum	1.5	1.7	
	Eurhynchium sp.	3.0	1.9	
	Neckera crispa	3.4	1.2	
	Anomodon viticulosus	9.7	0.9	
Vivantis kit	*Neckera complanata*	3.2	2.8	4.24
	Homalothecium sericeum	2.1	1.8	
	Eurhynchium sp.	10.9	2.09	
	Neckera crispa	3.5	2.1	
	Anomodon viticulosus	1.5	2.1	
Rana kit	*Neckera complanata*	36.0	2.7	24.82
	Homalothecium sericeum	24.6	1.73	
	Eurhynchium sp.	26.0	2.9	
	Neckera crispa	25.5	1.56	
	Anomodon viticulosus	12.0	1.16	
Our protocol	*Neckera complanata*	45.1	1.72	70.28
	Homalothecium sericeum	109.0	1.8	
	Eurhynchium sp.	56.0	1.7	
	Neckera crispa	44.3	1.73	
	Anomodon viticulosus	97.0	1.7	

- Genomic DNA analysis (ISSR-PCR and electrophoresis)

The PCR amplification reaction was carried out with nine samples and one ISSR primers in a 25 µL reaction volume containing 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$ (Cinna Gen Co, Iran), 1 mM dNTP mix (Cinna Gen Co, Iran), 0.2 µM of primer (Cinna Gen Co, Iran), 1 U of *Taq* DNA polymerase-500 (Cinna Gen Co, Iran), and 15–40 ng of template DNA. ISSR-PCR was performed in the thermocycler (Biorad, USA) for 40 cycles consisting of denaturation at 94 ºC for 60 sec, annealing varying from 52–55 ºC for 60 sec, extension at 72 ºC for 90 sec, and 72 ºC for 6 min for the final extension. The amplified product was checked in 1% agarose gel electrophoresis.

- SCoT-PCR amplification

The PCR amplification were carried out with nine samples in 25 µL reaction containing 1 U of *Taq* DNA polymerase-500, 1 mM dNTPs-Mix, 1X PCR buffer, 2.5 mM $MgCl_2$, 20 mM of amplification primer, and 10–50 ng of the template DNA. Thermal program was carried out in thermocycler (Biorad, USA). The profile used consisted of an initial denaturation for 5 min at 94 ºC, followed by 36 cycles in three segments: 1 min at 94 ºC, 1 min at 53–56 ºC, 90 sec at 72 ºC, and 10 min at 72 ºC for the final extension.

Results

- DNA isolation

In the present study, several protocols of DNA isolation were used each given different results for the amount of DNA obtained and its purity but a high yield and quality of DNA was only obtained with our modified method. The Biobasic kit gave low DNA concentrations (about 3.92 µg/ml on average), with variable purity. Such low amounts of DNA were insufficient for ISSR. Also, the amount of DNA was considerably low (4.24 µg/ml) with Vivantis kit yielded. Our extraction protocol was based on the CTAB method with significant modifications (Fig. 1 and Table 2).

The use of −20 ºC stored leaf samples successfully substituted the need for costly liquid nitrogen. The total DNA isolated from the samples was checked by a Picodrop and U.V visible Spectrophotometer (Dragon, China). The yield of the DNA ranged between 44.3–109 µg/ml for all individual samples (Table 2). Our average procedure yield was 70.28 µg/ml. The ratio of the absorbance at 260–280 nm (A260/A280) was 1.726, fit for PCR applications which indicated insignificant levels of contaminating proteins and polysaccharides but high RNA content since no RNAse treatment was used. The integrity of DNA extracted by each method was assessed by gel electrophoresis individually. Four µl of each extracted DNA was analyzed on a 1% agarose gel and visualized by U.V illumination. Figures 2 and 3 demonstrate a typical sample of the DNA extracted by our modified protocol compared to extracted samples following three kits.

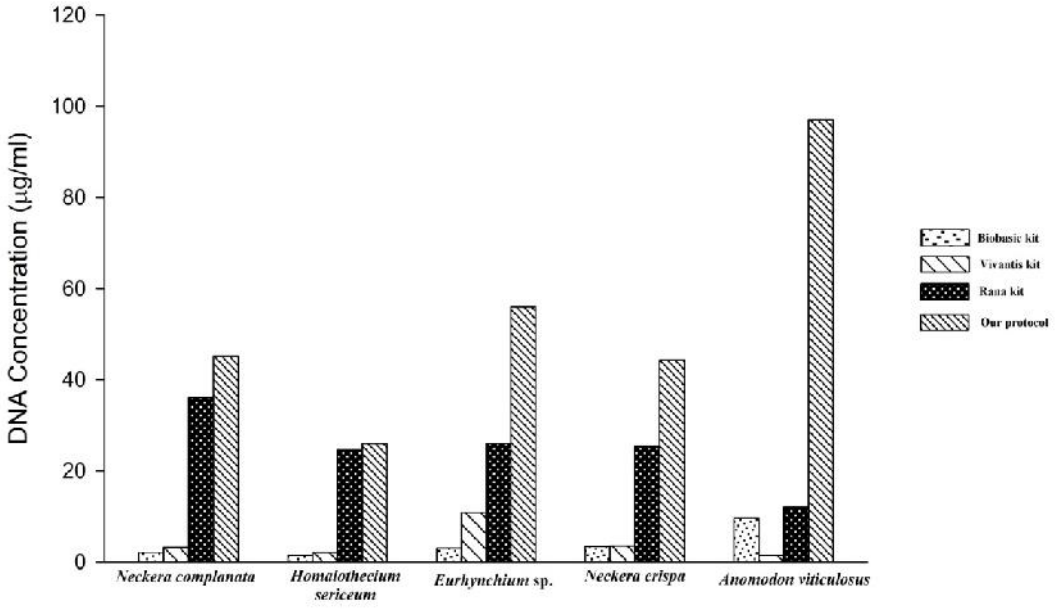

Fig. 1. Comparing of DNA concentration (µg/ml) in different studied methods in selected species (in accordance with table 2).

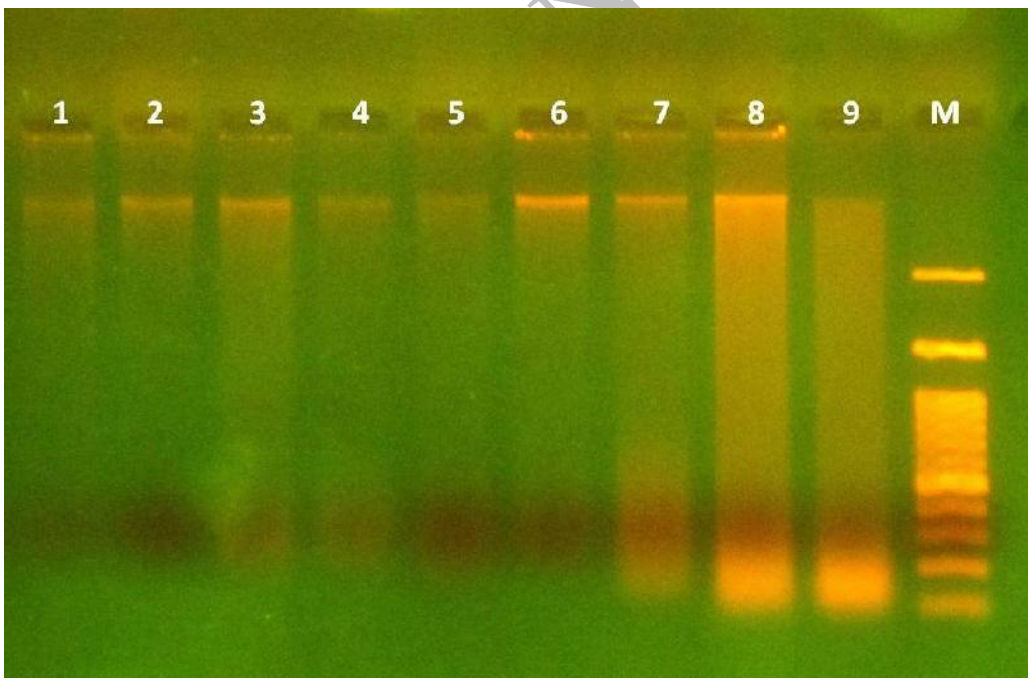

Fig. 2. Agarose gel electrophoresis of DNA samples .Undigested DNA extracted with the method described here (M. 100 bp molecular-weight size marker, fermentas). The numbers are in accordance with table 1.

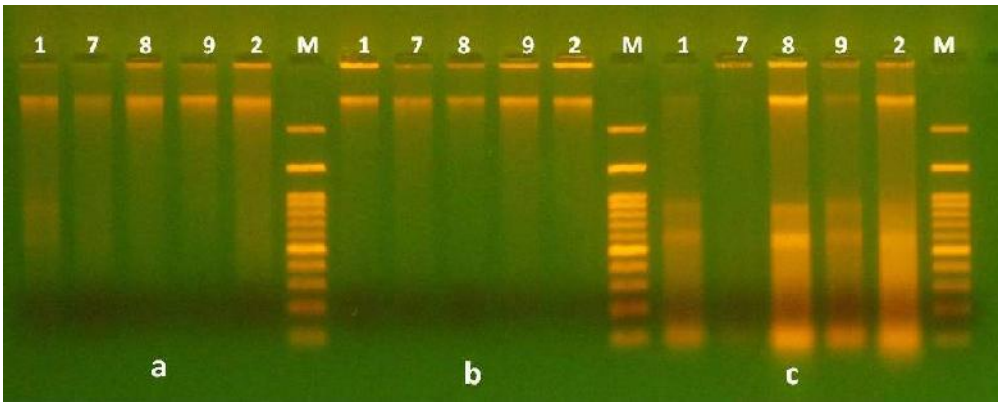

Fig. 3. Agarose gel electrophoresis of some extracted DNA samples, following three extraction kits (M. 100 bp molecular-weight size marker, fermentas) (a. Bio Basic kit, b. Vivantis kit, c. Rana kit). The numbers are in accordance with table 1.

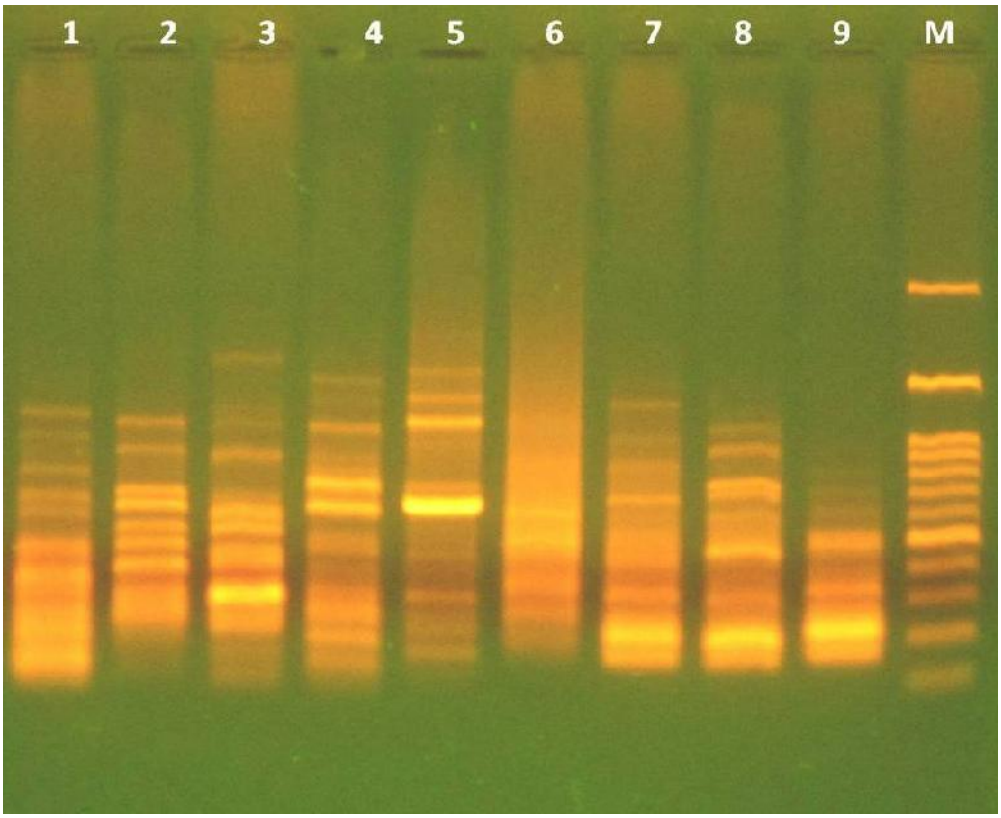

Fig. 4. Gel photograph of ISSR amplified products using (primer UBC 834) (1–9) (M. 100 bp molecular-weight size marker, fermentas). The numbers are in accordance with table 1.

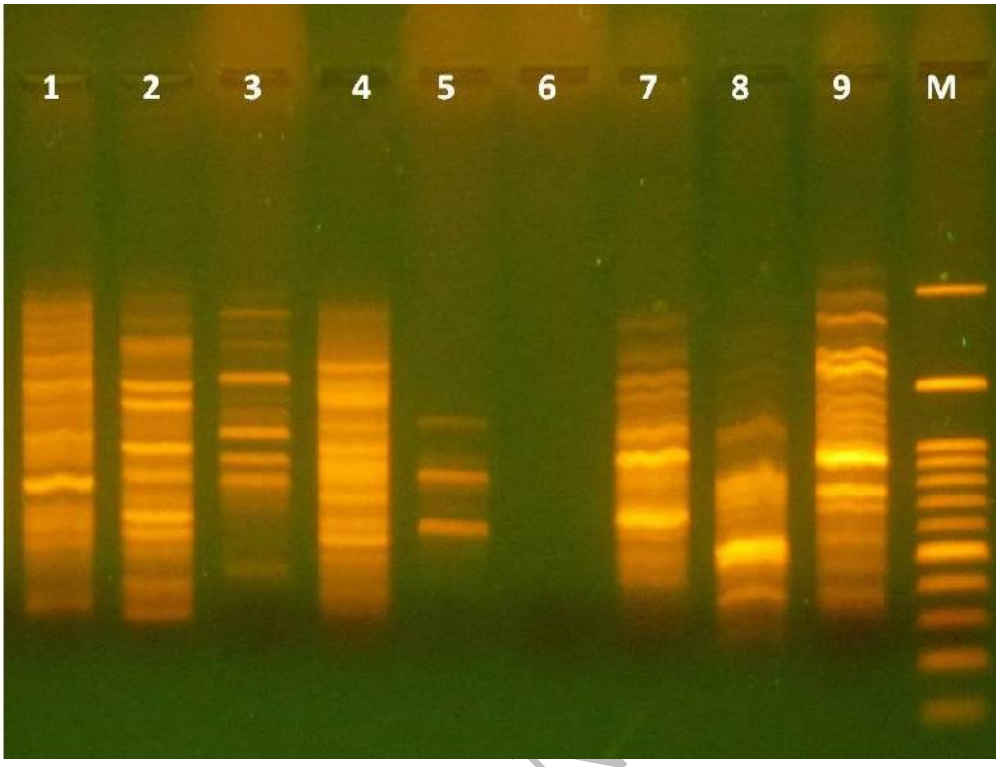

Fig. 5. Gel electrophoresis of PCR products SCoT marker (1–9) (M. 100 bp molecular-weight size marker, fermentas). The numbers are in accordance with table 1.

DNA isolated by this method yielded reproducible and consistent amplification products proving its compatibility for the PCR applications using the ISSR and SCoT markers (Figs 4–5). All genomic DNA samples produced a clear, sharp and reproducible PCR product pattern. The PCR experiment was repeated several times and an identical banding pattern was obtained.

Different values of materials used in this protocol were compared to Križman *et al.* (2006) and Sahu *et al*. (2012) in table 4.

Table 3. Function of solutions/chemical materials

Material	Function
Activated charcoal	Polyphenol-binding agent removing polysaccharide and polyphenol
Triton X-100, SDS, PVP	Removing proteins and polyphenol (instead of phenol and proteinase K)
N-lauroyl sarcosine	Removing proteins
Ammonium acetate	Removing polysaccharide

Table 4. Comparing different concentration of material used in this protocol with Sahu *et al.* (2012) and Krizman *et al.* (2006)

Condition / Method	Sample	Activated charcoal	N-lauroylsarc-osine	SDS	β-mercaptoethanol	NaCl (precipitation step)	Acetate solution	Final DNA solution
Sahu *et al.* 2012	Mangroves/ Saltmarsh	-	-	-	0.2%	-	Sodium acetate	TE buffer
Krizman *et al.* 2006	*Foeniculum vulgare, Origanum vulgare, Cannabis sativa, Humulus lupulus*	0.5%	-	-	-	-	Ammonium acetate	TE buffer
Our protocol	Nine moss species	0.3% optimized	2% optimized	1% optimized	0.1%	5 M	Ammonium acetate optimized	dd H2O
		0.5%	1.5%	0.5%			Sodium acetate	
		1%	1%	0.8%			Potassium acetate	

Discussion

Based on previous studies performed on lower plants, CTAB-DNA extraction protocol of Doyle & Doyle (1990) had several shortcomings in various steps. The presence of the secondary metabolites such as polyphenols and polysaccharides in these plants could influence the quality and/or quantity of the extracted DNA. To overcome these drawbacks, a variety of materials and chemicals with different concentration were utilized (e.g. β-mercaptoethanol, sodium N-lauroyl sarcosine, phenol, sodium acetate and proteinase K) (Tables 3 & 4). The phenol and proteinase K used for removing the proteins are known to be toxic and expensive, respectively. Though it is deemed to be a risky material, liquid nitrogen has also been applied in most of the studies. These compounds have proven to be challenging and costly; hence, for overcoming these challenges, various materials and methods have been employed in the present study. For example, keeping samples in –20 ºC before the extraction and mechanical grinding, could be more cost-effective and a proper replacement for liquid nitrogen; hazardous phenol was substituted with Triton X-100 and SDS. Interestingly, the addition of activated charcoal as a polyphenol-binding agent staved off the irreversible interactions of DNA and polyphenols and strongly removed the contaminants during the first step centrifuge. It could be eliminated from the final buffer simply due to its insolubility in all solutions [This material was already used in higher plants (Krizman *et al.* 2006)]. Even though, proteinase K could be very impressive in extraction steps, three times washing with chloroform/isoamylalcohol diminished the protein contaminant adequately. Another advantage presented by this method would be requirement for less plant material (0.05 g compared with 0.1 g in a commercial kit and 1 g in Sahu *et al.* 2012) and no need of centrifuge with a refrigerating function. More than half of the pellets were white with no visible discoloration that showed low quantity of polyphenol contamination. In contrary with Sahu *et al.* (2012), we advantaged some new materials like N-lauroyl sarcosine and SDS that could be improved DNA yield in the studied materials (mosses). Sahu *et al.* (*l.c.*) replaced the liquid nitrogen step with keeping the samples in –40/–80 ºC in higher plants with secondary metabolites.

Maintaining the samples in –20 ºC also demonstrated the same results in our survey and was therefore amenable to moss experiments. Other advantageous step in compare with above technique (Sahu *et al*. 2012) in this protocol was addition of NaCl (with ammonium acetate) to precipitation buffer that influenced on purity of extracted DNA. Acceptable amplification and clear banding pattern of extracted DNAs indicated the reliability of this protocol. Various acetate solutions like sodium acetate, potassium acetate and ammonium acetate were tested for the polysaccharide removal; among them, ammonium acetate was more remarkable.

Conclusion

In this study, a safe, cost-efficient and reliable DNA purification procedure was explained. The results indicated the relevance of applying this method for the DNA extraction in mosses. High efficiency and lack of toxic organic solutions make our protocol as a desirable substitute for the commercial kits. Furthermore, high quality DNA produced with our method allows us to propose this protocol as an alternative not only in the molecular markers analysis but also in any other downstream applications based on the polymerase chain reaction, sequencing technologies and bioinformatics tools.

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