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An efficient and simple CTAB based method for total genomic DNA isolation from low amounts of aquatic plants with a high level of secondary metabolites

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ABSTRACT

An efficient DNA isolation protocol specifically modified to get pure quality DNA required for molecular studies has been reported in this paper. Some aquatic plants (*Potamogeton* spp., *Ceratophyllum demersum* and *Myriophyllum spicatum*) were used for the study. The protocol developed will be useful in getting high and pure DNA. Instead of using the available DNA extraction kits, this protocol can be used to get pure quality DNA, free from proteins and polysaccharide compounds. The absorbance rate A260/A280 was 1.92 ± 0.069 and A260/A230 was 1.73 by spectrophotometer and NanoDrop machines which showed the sample genomic DNA is pure, free from contaminant proteins and polyphenolics/polysaccharides compound. The highest concentration of DNA was 640 ± 340.58 ng/µl when measured at 260 nm. When we run on agarose gel also, the isolated DNA gave a clear and sharp band. Thus, the DNA does not need any additional purification before proceeding for molecular analysis of the isolated DNA samples. This protocol is very simple and economical which will find wide applications in genomic studies of aquatic plants.

Keywords: DNA extraction; *Potamogeton*; *Ceratophyllum*; Contaminations; Purity.

Introduction

Many protocols have been used in plant DNA isolation, but because of chemical heterogeneity, many of them could be applied to a limited number of species, or even closely related species in some cases fail to respond to the same protocol (1). Molecular techniques require isolation of genomic DNA of suitable purity that is the prerequisite for molecular research. Also, successful using of PCR based downstream applications requires efficient recovery of good quality and quantity of DNA. To isolate pure and intact DNA from plant tissues, numerous protocols have been reported (1-7). However, plant

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species belonging to the same or related genera can exhibit enormous variability in the complexity of pathways of dispensable functions. Thus, these DNA extraction protocols cannot be reproduced for all plant species (8). The cetyl trimethylammonium bromide (CTAB) method and their modifications are reported by various authors (9-11). Some studies for DNA extraction from aquatic plants used CTAB method (12, 13). These protocols sometimes need modification to obtain good quality total DNA for polymerase chain reaction (PCR). Various commercial extraction kits such as DNeasy Plant Mini kits (Qiagen Valencia, CA, USA) are available, but the main problem with these commercially available kits is their high cost per sample (14). There are many major methods for DNA isolation from plant and marine species. One of them is phenol-chloroform-based extraction. Using of phenol-chloroform isoamylalchol, would remove protein impurities successfully without affecting DNA yield. Some researchers also indicated that phenol in the extraction buffer significantly increased the yield of DNA (15). The disadvantages of this method include generation of toxic products (16) and differentiation of some amount of extracted DNA among analyses (17). Another method is based on Chelex-100, which is effectively applied for bacteria, *Chlamydomonas* and animals and rarely used for plant species due to the need for additional time consuming and tedious steps (16, 18). Salt-based protocols are universal and rapid for extraction of high quality DNA (19). Heat based procedures make cell lysis in different tissues necessary (20). Aquatic plants are reported to have great economical values and health benefits. They have low amounts of DNA. The current protocol development aimed to make this technique readily available in poorly equipped laboratories. In the present study, there is no need for commonly used expensive extraction kits. This method leads to getting very high concentrations of permanent DNA from aquatic plants. It also bypasses RNAase and using of poisonous materials such as phenolchloroform and PVP treatment. The protocol was efficiently employed in aquatic plants including *Potamogeton* spp. (Potamogetonaceae), *Ceratophyllum* (Ceratophyllaceae) and *Myriophyllum* (Holarogaceae). The method does not require expensive and hazardous reagents. The quantity and the quality of the DNA extracted by this method are high enough for long distance transport and performing thousands successful PCR-based reactions, RAPD (Random amplified Polymorphic DNA), ISSR (Inter simple sequence repeat), SSR (Simple sequence repeat), SRAP (Sequence-related amplified polymorphism), and amplification of plant barcode genes (ITS, *trnH-psbA*, *matK* and *rbcL*), with reduced cost. The efficiency and requirement of less expensive as well as non-hazardous chemicals make the present method an attractive alternative to the existing methods of genomic DNA isolations in aquatic plants. Using this protocol we were able to isolate DNA even from herbal leaves. One of the advantages of this protocol is physical grinding treatment undertaken with either dry ice or liquid nitrogen that can prevent DNA oxidation (21) The current study was taken up to gain quality DNA from aquatic plants for molecular biology studies with some modifications in the CTAB method.

Materials and Methods

We used several protocols for DNA extraction from plants:

1) Phenol-chloroform isoamyl alcohol: 50 mg of lyophilized material was mixed with 900 µl of CTAB lysis buffer. All samples were incubated at 65°C for 60 min before being centrifuged at 12000 g for 5 min at 4°C. Supernatants were transferred to 2-ml microfuge tubes and 900 µl of phenol: chloroform: isoamyl alcohol (25: 24: 1, pH = 6.7) added for each extraction. Samples were mixed thoroughly prior to being incubated at room temperature for 10 min. Phase separation occurred by centrifugation at 12000 g for 15 min at 4°C, and the upper aqueous phase was re-extracted with a further 900 µl of phenol: chloroform:isoamyl alcohol. Next, samples were centrifuged at 12000 g for 10 min at 4°C, and the upper aqueous phases were transferred to fresh 2-ml microfuge tubes. The final extraction was performed with 900 µl of chloroform: isoamyl alcohol (24: 1), and layer separation occurred by centrifugation at 12000 g for 15 min at 4°C. Precipitation of DNA was achieved by adding the upper phase from the last extraction step to 450 µl of isopropanol containing 50

µl of 7.5 M ammonium acetate. Samples were incubated at 20°C overnight. Samples were centrifuged at 7500 g for 10 min at 4°C, and supernatants were discarded. Finally, DNA pellets were washed three times in 1 ml of 70% (v/v) ethanol. The final pellet was air-dried and re-suspended in 200 µl of 75 mM TE buffer (15).

2) Chelex-based isolation for transgenic plant and algal species: In a 1.5-ml microfuge tube, shoot or leaf tissue (10-15 mg; one or two leaf discs with a diameter of 1 cm) is homogenized with a pestle for 1 min in 150-300 µl of 5% Chelex 100 (Bio-Rad, USA). The tissue is vortexed for 10 s, incubated in boiling water for 5 min, then vortexed again for 10 s, and finally centrifuged at 13000 rpm for 1 min. The supernatant can then be used as template for PCR amplification (18).

3) A protocol for DNA extraction from plants with high amount of polysaccharides and secondary metabolites: 1 g of plant tissue was submerged in 5 ml of absolute alcohol for 5 min and alcohol to was allowed to evaporate. The tissue was grinded in presence of 1% PVP and the extraction buffer was pre-warmed by using a pre-chilled mortar and pestle (−40°C/−80°C) at room temperature. The ground material was transferred into 2-ml centrifuge tubes and incubated in water bath at 60°C for 1h. The tubes were centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was collected in 1.5 ml centrifuge tube using bored tip. To the supernatant we added equal volume of chloroform: isoamyl alcohol (24:1) and then mixed it by inversion for 15 min. The tubes were centrifuged at 10000 rpm for 10 min at 4°C and the supernatant was collected in 1.5 ml tubes. The previous step was repeated. The tubes were centrifuges at 10000 rpm for 10 min at 4°C and the supernatant was collected. To the supernatant we added twice the volume of chilled isopropanol to precipitate the DNA and incubate it at −20°C for 30 min. The tubes were centrifuge at 10000 rpm for 10 min at 4°C and the pellet was collected. The pellet was washed with 70% ethanol and air dried in room temperature. 50-100 µl of TE buffer was added to dissolve the DNA (14). The DNA was stored for further use at −20°C (22).

4) A universal protocol for DNA extraction from marine species and Human blood using CTAB: The leaves were crushed with help of mortarpestle using PVP to remove phenolic contamination, then we added adequate amount of CTAB into leaves to make fine slurry, and took 1.5 ml of sample into 2ml eppendorf tube. 10µl of β-mercaptoethanol was added into each eppendorf tube, shaken well to mix properly. Incubate at 65°C for 1h in water bath. Centrifuge at 13000 rpm for 15 min, collect supernatant in new eppendorf tube and add equal amount of Chroloform: isoamyl alcohol (CIA) to the supernatant. We collected the upper layer in a fresh eppendorf tube then added equal volume of chilled isopropanol. The mixture was incubated for 20 min at 20°C so as to precipitate the DNA. Centrifuge at 13000 rpm for 15 min. The supernatant was discarded and 500µl of 70% ethanol was added to the pellet obtained for washing. Mix well and centrifuge at 8000 rpm for 5 min. The supernatant was discarded and 70 µl of TE buffer was added (23).

5) A simple DNA extraction protocol for plant biological systems: WTake half of a young dry leaf and cut it into small pieces then grind it using a porcelainmortar and pestle in 400 µl of the extraction buffer (1% SDS, 0.5 M NaCl (no EDTA, Tris-HCl). Add more buffer until it reaches a final volume of 1200 µl. Harvest the homogenate into 1.7 ml microfuge tubes. Spin (13500 rpm, 4 min, RT) using a microfuge. Transfer the supernatant into a new microfuge tube and add an equal volume of isopropanol (500 µl in our study) and mix gently by inversion. Place the mixture on ice for 5 min. Spin (13500 rpm, 4 min, RT) using a microfuge. Discard the supernatant and wash the DNA pellet with 500 µl 70% (v/v) ethanol. Spin (13,500 rpm, 2 min, RT). Discard the ethanol. Let the pellet air-dry. Dissolve the DNA in 50 µl ddH_2O and store it at 4°C for immediate use or −20°C (24).

Plant material

Representatives of some common genera of aquatic plants including *Potamogeton*, *Ceratophyllum* and *Myriophyllum* were collected from a vast area of Iran. *Potamogeton* comprise 14 species in Iran (25). In this research we extracted DNA from 12 species of them

(*P. pectinatus* L., *P. perfoliatus* L., *P. nodosus* Poir., *P. lucens* L., *P. crispus* L., *P. natans* L., *P. amblyphyllus* L., *P. friesii* Rupr., *P. filiformis* Pers., *P. pusillus* L., *P. berchtoldii* Fieber and *P. alpinus* Balb.). We identified the species according to credible resources (26, 27). Also the species checked by the specimens in herbarium of VUB (Vrije Universiteit Brussel). *Ceratophyllum* comprises one species in Iran (28). We tested *C. demersum* L. for this experiment. *Myriophyllum* comprises two species in Iran (29) and we used *M. spicatum* L.

Required solutions:

- CTAB Extraction buffer: 2% CTAB (4 g), 100 mM Tris-HCL (20 ml Tris-HCL, pH8), 1.4 M NaCl (56 ml NaCl, 5 M), 20 mM EDTA (16 ml EDTA, 0.25 M) adding distilled water to 200 ml with pH8).

Tris-Hcl 1 M: 12.1 g Tris-base, 800 ml water, 35 ml HCL 1 M.

EDTA 0.5 M: 10 g NaCl, 800 ml water, 186.1 g EDTA.

Chroloform:isoamyl alcohol (CIA) (Merck, Darmstadt, Germany) 24:1

Protocol for DNA extraction from aquatic plants:

1- Pour Extraction Buffer (CTAB: 1-1.5 ml for each sample+2-3 µl of Beta mercaptoethanol for each sample in falcon tube and heated and incubated at 65°c.

Note: If you want to extract from 4 samples you should pour 4 ml of CTAB and 8 µl of Beta mercaptoethanol in falcon tube and put it in water bath).

2- Grind leaf tissue (1 g) in liquid N_2 with a mortar and pestle or with a grinding mill.

Note: all extractions done from a homogenized pool of the exact same tissue of the same individuals.

3- Add ground leaf to a 2 ml tube with a spatula and add 1.5 ml of hot Extraction Buffer from water bath.

4- Put the tube in water bath.

5- Continue for other samples.

6- After 3 h, take the samples from water bathand add 750 µl Chloroform/Isoamylalchol (24:1) to each tube and invert many times for 15 min by hand.

Note: in this stage don't use vortex because it induces DNA denaturation.

7- Centrifuge the tubes for 5 min at 10000 g.

8-Take the supernatant phase carefully and pour to another tube

9- Add 1 ml of Isopropanol and 700 µl of sodium acetate 3 M (pH=5.5).

10- Put the sample tubes on the ice and put them in the refrigerator overnight.

11-Centrifuge for 10 min at 10000 g

12-Take out the upper phase and wash the DNA pellet in ethanol 70% then put invert the tube for drying DNA pellet during 1 day

13- Dissolve DNA pellet in distilled water.

Note: in this stage do not use TE buffer for DNA dilution because TE results in DNA chelation that can prevent from PCR performance a long time after extraction.

We used 11 ISSR primers for PCR of all species of *Potamogeton*, *Ceratophyllum* and *Myriophyllum*. The primer sequences used in this study are listed in Table 1.

ISSR PCR: The PCRs were carried out in a 15 µl volume with 250 nM of each primer (Table 2), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 U Taq polymerase, and 50-100 ng of genomic DNA. After 4 min at 95°C, PCR was followed by 40 cycles of 1 min at 95°C, 1 min at annealing temperature (Table 2), 2 min at 72°C, followed by a final extension step of 10 min at 72°C. PCR products were detected by 2 % agarose and ethidiumbromide staining under UV light (Fig. 2.4).

We also used 10 pairs of primers of SRAP for all of species of *Potamogeton* named above, *Ceratophyllum demersum* and *Myriophyllum spicatum*. The primer sequences for examined taxa are showed in Table 2 and 3, respectively.

Table 1. Sequences and annealing temperatures of primers used for ISSR in selected aquatic plants

Primer ID	Sequence (5'→ 3')	Tm
ISSR 807	AGAGAGAGAGAGAGAGT	50
UBC 872	GATAGATAGATAGATA	38
ISSR 823	TCTCTCTCTCTCTCTCC	52
ISSR 826	ACACACACACACACACC	52
ISSR 811	GAGAGAGAGAGAGAGAC	52
ISSR 812	GAGAGAGAGAGAGAGAA	50
UBC 873	GACAGACAGACAGACA	48
ISSR 2	AGAGAGAGAGAGAGAGG	52.6
ISSR 4	CTCTCTCTCTCTCTCTGG	45.7
ISSR 810	GAGAGAGAGAGAGAGAT	54.3
ISSR 3	AGCAGCAGCAGCAGCAGCG	52.6

Table 2. Sequences of used SRAP primers for *Potamogeton* spp. in this study

Primer ID	Sequence (5'→ 3')
Me 1	TGAGTCCAAACCGGATA
Me 2	TGAGTCCAAACCGGATA
Me 3	TGAGTCCAAACCGGAAT
Me 4	TGAGTCCAAACCGGACC
Me 5	TGAGTCCAAACCGGAA
Me 6	TGAGTCCAAACCGGACA
Em 2	GACTGCGTACGAATTTGC
Em 3	GACTGCGTACGAATTGAC
Em 4	GACTGCGTACGAATTTGA
Em 17	GACTGCGTACGAATTCCA

Table 3. Sequences of used SRAP primers for *Ceratophyllum demersum* and *Myriophyllum spicatum*

Primer ID	Sequence (5'→3')
Me 2	TGAGTCCAAACCGGATA
Me 4	TGAGTCCAAACCGGACC
Me 5	TGAGTCCAAACCGGAA
Me 6	TGAGTCCAAACCGGACA
Em 2	GACTGCGTACGAATTTGC
Em 3	GACTGCGTACGAATTGAC
Em 4	GACTGCGTACGAATTTGA
Em 6	GACTGCGTACGAATTGCA
Em 17	GACTGCGTACGAATTCCA

SRAP PCR: The PCRs were performed in 25 µL reaction volumes containing Taq 2× Master Mix Red (Amplicon), 0.1 µM of each forward and reverse primer, 50 ng DNA template, and nuclease-free water to 20 µL. The PCR program conducted with the following cycle profile in a Eppendorf Thermal Cycler (Mastercycler Gradient): 5 min of initial denaturation at 94°C followed by 5 cycles of 1 min denaturing, 1 min annealing at 35°C and 1 min of elongation at 72°C, after these, 35 cycles of 1 min denaturing, 1 min annealing at 50°C ending with an elongation step of 5 min at 72°C. The PCR products were 2 % agarose and ethidiumbromide staining under UV light (Fig 2,3,5).

SSR analyses were down only for all populations of *P. pectinatus* with 9 primers. The primer sequences used for study are indicated in Table 4.

Table 4. sequences of SSR primers for *Potamogeton pectinatus*

Locus	Primer sequence	Repeat motif
Potpect 24	F Ned- TCAGTGAAAGAAAGCCAGGA	(GA)n
	R GGGCTTATGGCGTTATCAA	
Potpect 26	F Fam-GTATAGGCGAGGTGCGAGAG	(CT)*n*
	R CTTCATGTCGACCACCTTCC	
Potpect 28	F Fam-TCGTTTCCTCCATTCGTAGG	(GA)*n*
	R AATAAAAAGGGCCCAGACC	
Potpect 32	F Hex-CAGCAAACGAAACAACCAAA	(GA)*n*
	R AAAAGAAGCCGTTGTTTACAGAG	
Potpect 34	F Fam-GTAAGGCAAGCAGCGTCAAC	(GA)*n*
	R GTTTGTGAGCTAGCGGGAAG	
Potpect 37	F Hex-CACTTCCTCTGTGCTGCTTG	(CT)*n*
	R GCGTGCTCTTCCTGAGTTCT	
Potpect 39	F Hex-TCACAACACCTCACCCAGAA	(GA)*n*
	R CCATTTCCATTCCTCACTGC	
Potpect 40	F Ned-AAATCTCCAAATATTTCCACTGTTG	(GA)*n*
	R CAAAGATTGAGCTCCCCAAA	
Potpect 42	F Ned-TTAGCAAGTGGGTGGGTTTC	(CT)*n*
	R TGCACTCGTGTGTCTCTTCC	

This single amplification was made possible by the use of the QIAGEN Multiplex PCR Kit (QIAGEN) in a final volume of 10.5 µl, as follow: 25 ng of DNA template, 5 µl 2× QIAGEN Multiplex PCR Master Mix [QIAGEN Multiplex PCR Buffer, pH 8.7, containing dNTPs, QIAGEN HotStar Taq DNA Polymerase, and 6 mM $MgCl_2$ (for a final concentration of 3 mM)], 1 µl Q-Solution (59 concentrated proprietary QIAGEN PCR additive), 1 µl of a primer mix with 2 µM of each primer (for a 0.2 µM final concentration of each primer) and 1 µl of highly pure water obtained from a Milli-Q Synthesis A10 (Millipore, Molsheim, France). PCR were carried out in 96-well plates on a MyCycler TM thermal cycler (BIO-RAD) under the following conditions: 15 min denaturing at 95°C, [3000 denaturing at 94°C, 1.5 min annealing at 57°C and 1 min extension at 72°C] × 30 cycles and a final extension step at 72°C for 10 min. PCR were carried out in 96-well plates on a MyCycler TM thermal cycler (BIO-RAD) under the following conditions: 4 min denaturing at 94°C, [3000 denaturing at 94°C, 1 min annealing at 57°C and 1 min extension at 72°C] × 30 cycles and a final extension step at 72°C for 30 min.

For barcoding the species and study of interspecific diversity of *Potamogeton* species in Iran, we used four fragments ITS, *trnH-psbA*, *matk* and *rbcL*. The primer pairs used for amplifying each locus were as follows: rbcl-a-F and rbcl-a-R; *matK*, 390F and 1326R; ITS1, ITS2, ITS3, ITS4, trnHf-05, psbA3-f. The PCR amplification for ITS was performed in 30 µl reaction mixture containing 3 µl DNA (50 ng), 17.8 µl water, 6 µl PCR buffer 5 mM, 0.6 µl dNTP 10mM, 1.8 µl $MgCl_2$ 25 mM, 0.06 µl forward primer 0.1 mM, 0.06 µl reverse primer 0.1 mM, 0.6 µl BSA (10mg/ml), 0.2 µl Taq (5u/ µl). The PCR amplification for ccmp10, ccmp2 and *trnH-psbA* was performed in 12.5 µl reaction mixture containing 2.5 µl water, 6.25 µl MasterMix, 1.25 µl primer mix and 2.5 µl DNA. The PCR amplification for *rbcL*, *trnH-psbA* and *matK* was performed in 25 µl reaction mixture containing 5 µl water, 12.5 µl MasterMix, 2.5 µl Primer Mix, 5 µl DNA. The PCR amplification conditions for the ITS region were as follows: an initial predenaturation step at 95ºC for 4 min, followed by 35 cycles of 1 min at 95ºC, 1 min at 54ºC, and 1 min at 72ºC, with a final extension step of 10 min at 72ºC. The PCR amplification conditions for the *TrnH-psbA* region were as follows: an initial predenaturation step at 95ºC for 4 min, followed by 35 cycles of 1 min at 94ºC, 1 min at 50ºC, and 1´.30´´ at 72ºC, with a final extension step of 20 min at 72ºC. The PCR amplification conditions for the matK region were as follows: an initial predenaturation step at 95ºC for 4 min, followed by 35 cycles of 1 min at 94ºC, 1 min at 52ºC, and 1´.30´´ at 72ºC, with a final extension step of 20 min at 72ºC. The PCR amplification conditions for the rbcl region were as follows: an initial predenaturation step at 95ºC for 4 min, followed by 35 cycles of 1 min at 94ºC, 1 min at 50ºC, and 1´.30´´ at 72ºC, with a final extension step of 20 min at 72ºC.

Results

Purity of DNA as checked using Thermo NanoDrop and Spectrophotometer is shown in Table 5. Absorbance ratio A260/A280 was 1.92 ± 0.069 and A260/A230 was 1.7. DNA yield was 640 ± 340.58. The extracted DNA was analyzed on 1% agarose gel and was visualized by staining with ethidium bromide and transillumination under short-wave UV light of BioRad gel doc system (Fig. 1). In this study we also tested fresh samples after collection and dried those using liquid N_2 but total DNA extracted was not adequate and we did not get any PCR product (Fig. 1). So we used herbarium samples instead of fresh leaves in this protocol. Furthermore, when we used no liquid N_2 in our protocol we were not able to get any band for neither DNA nor PCR products (Fig. 1). So using of liquid N_2 is needed for our protocol.

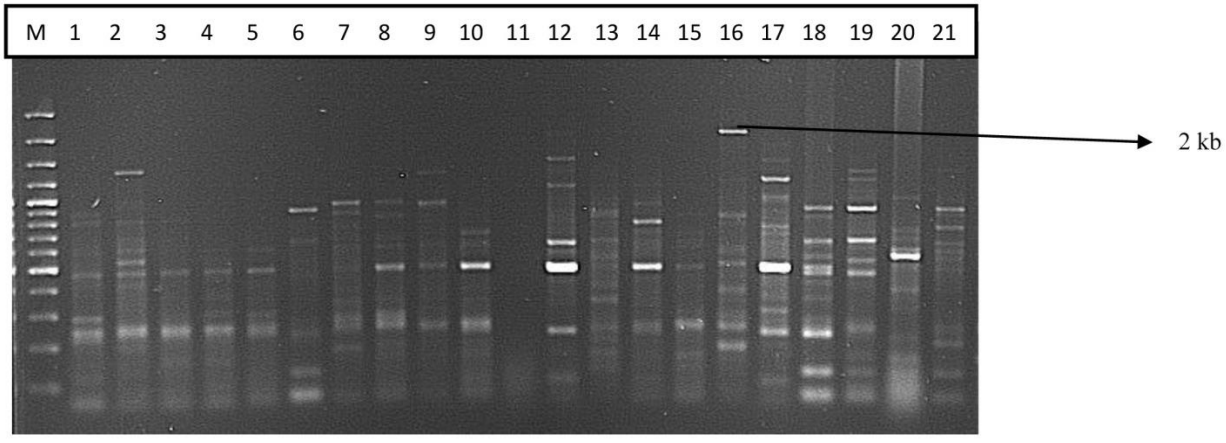

Figure 1. Total DNA extracted from aquatic plants on 0.8 % agarose gel. A. 1: *Ceratophyllum demersum*, 2: *Myriophyllum spicatum*, 3. *Potamogeton pectinatus*, wells of 4 and 5 are DNA extracted from our protocol but without liquid nitrogen and without extracted DNA and no PCR product, 6: *P. pectinatus* from another location, 7: *P. amblyphyllus*, well number 8 is with fresh leaves without any band and no PCR product, 9: *P. nodosus*. (all observed bands from our protocol). B. 1: *P. natans*, 2: *P. alpinus*, 3: *P. perfoliatus*, 4: *P. lucens*, 5: *P. friesii*, 6: *P. pusillus*, 7: *P. berchtoldii*, 8: *P. filiformis*, 9: *P. crispus*, 10: *P. pectinatus* from another location (all bands are from our protocol).

Table 5. Purity of total DNA extracted from aquatic plants in this study (the values are mean value in triplicates). Mean values and standard deviation were also calculated in triplicates

Taxon	A260/A280	A260/A230	DNA concentration (ng)?
Potamogeton pectinatus	1.92 ± 0.069	1.7	640 ± 340.58
P. perfoliatus	1.82 ± 0.067	1.7	640 ± 340.58
P. nodosus	1.92 ± 0.069	1.7	640 ± 340.58
P. lucens	1.92 ± 0.069	1.7	640 ± 340.58
P. crispus	1.92 ± 0.069	1.7	640 ± 340.58
P. natans	1.92 ± 0.069	1.7	640 ± 340.58
P. amblyphyllus	1.92 ± 0.069	1.7	640 ± 340.58
P. friesii	1.92 ± 0.069	1.7	640 ± 340.58
P. filiformis	1.80 ± 0.050	1.7	640 ± 340.58
P. pusillus	1.92 ± 0.069	1.7	640 ± 340.58
P. berchtoldii	1.92 ± 0.059	1.7	640 ± 340.58
P. alpinus	1.92 ± 0.069	1.7	640 ± 340.58
Ceratophyllum demersum	1.82 ± 0.070	1.7	400 ± 340.58
Myriophyllum spicatum	1.88 ± 0.060	1.7	540 ± 340.58

Discussion

DNA extraction from plants with high secondary metabolits is very challenging (30, 31). Some methods for DNA extraction from aquatic plants used CTAB method (12). There is another method for DNA extraction from plants with high concentration of secondary metabolites (32). It is however expensive and time consuming because of using suspension buffer with extraction buffer. There are only few modifications from the CTAB method by Doyle & Doyle (2, 33) methodology. Here we have added up sodium acetate for DNA purification. Our protocol does not need proteinase in isolation step. Some aquatic plant plants have relatively high polyphenol such as *Potamogeton* spp.and *Myriophyllum* spp. (34, 35). We can remove the polyphenols by using high levels of β-mercaptoethanol as in other protocols (7). The addition of NaCl with concentration higher than 0.5 M to CTAB is known as removing factor for polysaccharides during extraction (36). In this study we used higher concentration of NaCl (1.4 M). Abu-Romman used PVP in CTAB extraction for removing polysaccharides and polyphenol (37). We used it but in case of our experiment we had not adequate amount of good quality DNA. In our protocol DNA is precipitated by using isopropanol and sodium acetate, because isopropanol gives rise to precipitation of DNA and other phenolic and secondary metabolites solved in sodium acetate. Simultaneous using of two components (isopropanol and sodium acetate) can make a DNA in an intact form. After this step DNA pellet washed with 70% ethanol to remove salts. Previously we used only isopropanol without sodium acetate for precipitation of DNA but in final step we had a black pellet of DNA. These secondary metabolites provide colorful DNA that can inhibit PCR. Sodium acetate was also used previously in isopropanol step for efficient DNA extraction from *Passiflora foetida* (38). DNA yield is important in molecular studies. DNA extracted from aquatic plants in our study was pure with adequate concentration which can be stored for further use in molecular studies like polymerase chain reaction amplifications (Figs. 2-5) and long distance transport. Also Absorption ratio (A260/A280) of extracted DNA samples was 1.92 ± 0.069 indicating that the DNA was free from proteins and polyphenols. A260/A230 was 1.7 which showed the sample genomic DNA is pure; free from contaminants protein, polyphenolic and polysaccharide compounds. The highest length PCR product of DNA extracted by our protocol was 2-3 kb for ISSR and SRAP (Figs. 3-5). This method can be considered as a universal one, because in addition to its effective application in many aquatic plant genera, it can be used for DNA extraction from microbial organisms such as cyanobacteria (*Oscillatoria*).

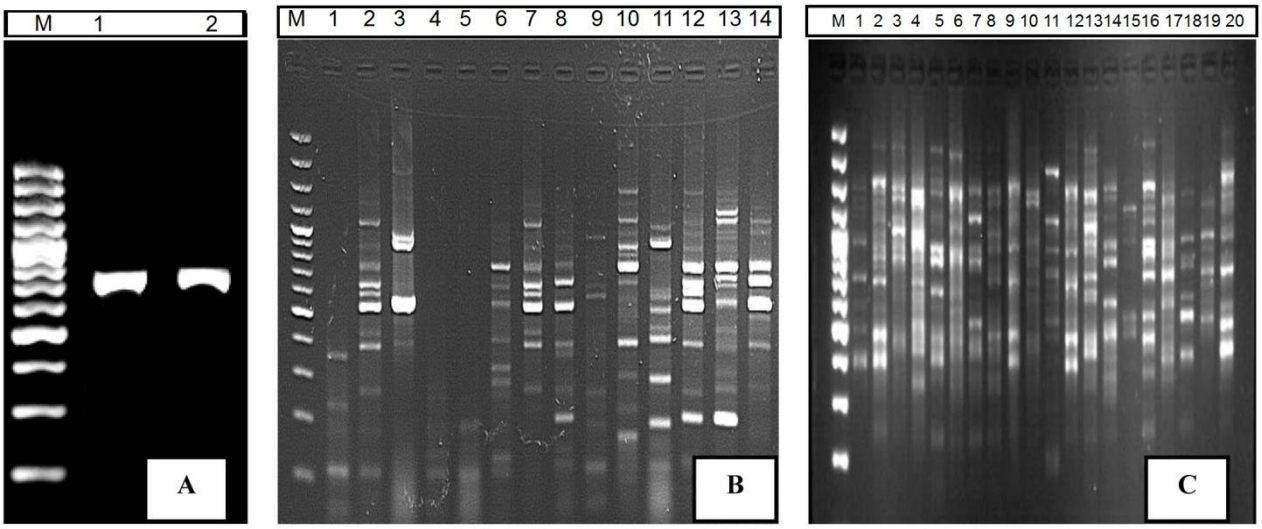

Figure 2. PCR products used extracted DNA by our protocol; A: DNA was amplified with barcoding markers, M: 100 bp DNA Ladder 1: *Potamogeton pectinatus*, 2: *P. nodosus*; B: with SRAP markers using primer of Me2-Em 4; M: 100 bp DNA Ladder, 1: *P. natans*, 2: *P. alpinus*, 3: *P. perfoliatus*, 4: *P. lucens*, 5: *P. friesii*, 6: *P. pusillus*, 7: *P. berchtoldii*, 8: *P. filiformis*, 9: *P. crispus*, 10: *P. pectinatus*, 11. *Ceratophyllum demersum*, 12. *Myriophyllum spicatum*; C: with ISSR markers using primer of ISSR 810, M: 100 bp DNA Ladder, 1-20 are different populations of *P. pectinatus*.

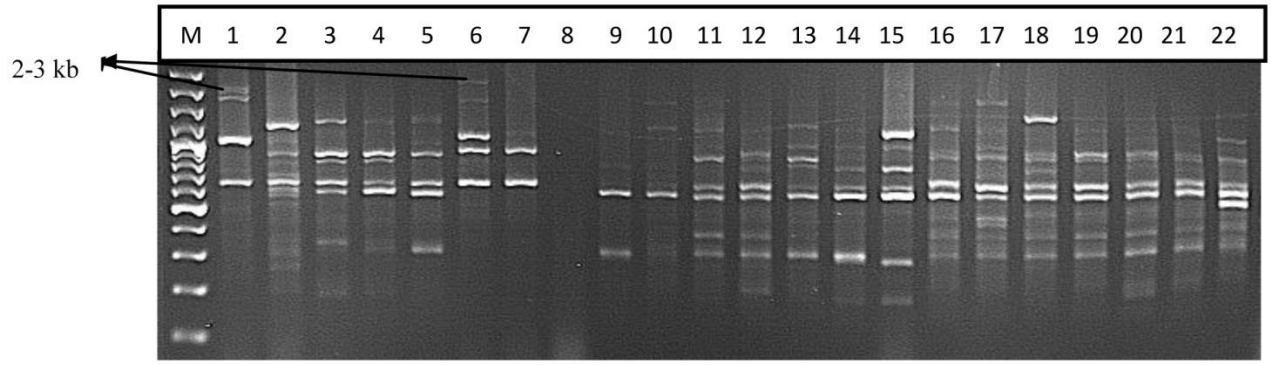

Figure 3. PCR products of SRAP marker with primer of Me 1, Em 17 used extracted DNA by our protocol M: 100 bp DNA ladder, 1: *Potamogeton natans*, 2: *P. alpinus*, 3: *P. perfoliatus*, 4: *P. lucens*, 5: *P. friesii*, 6: *P. pusillus*, 7: *P. berchtoldii*, 8: *P. filiformis*, 9: *P. crispus*, 10: *P. pectinatus*, 11. *Ceratophyllum demersum*, 12. *Myriophyllum spicatum*; 13-22: other populations of *P. pectinatus*.

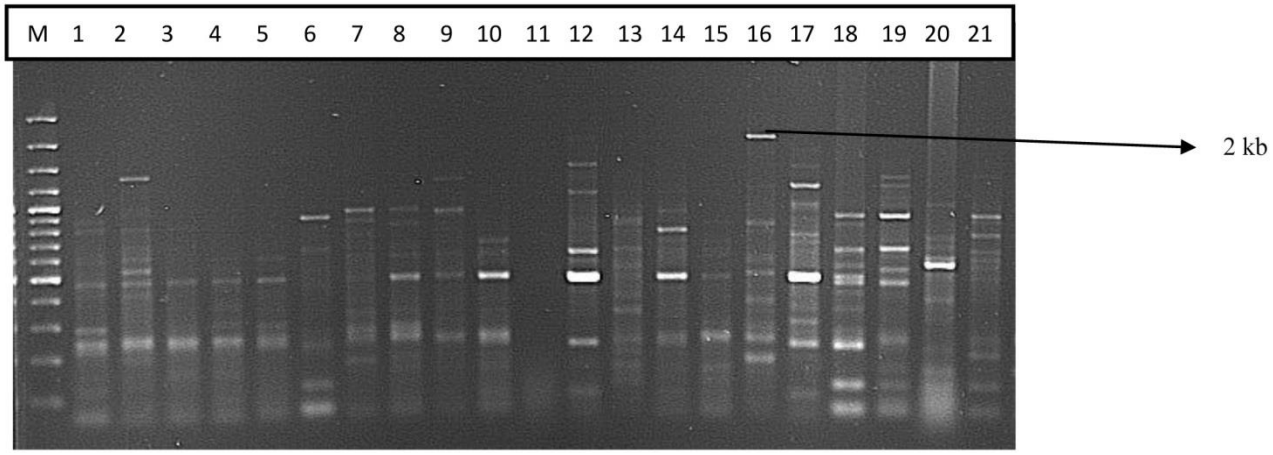

Figure 4. PCR products of ISSR marker with primer of ISSR 4 used extracted DNA by our protocol, 1: *Potamogeton natans*, 2: *P. alpinus*, 3: *P. perfoliatus*, 4: *P. lucens*, 5: *P. friesii*, 6: *P. pusillus*, 7: *P. berchtoldii*, 8: *P. filiformis*, 9: *P. crispus*, 10: *P. pectinatus*, 11. *Ceratophyllum demersum*, 12. *Myriophyllum spicatum*; 13-21: other populations of *P. pectinatus*.

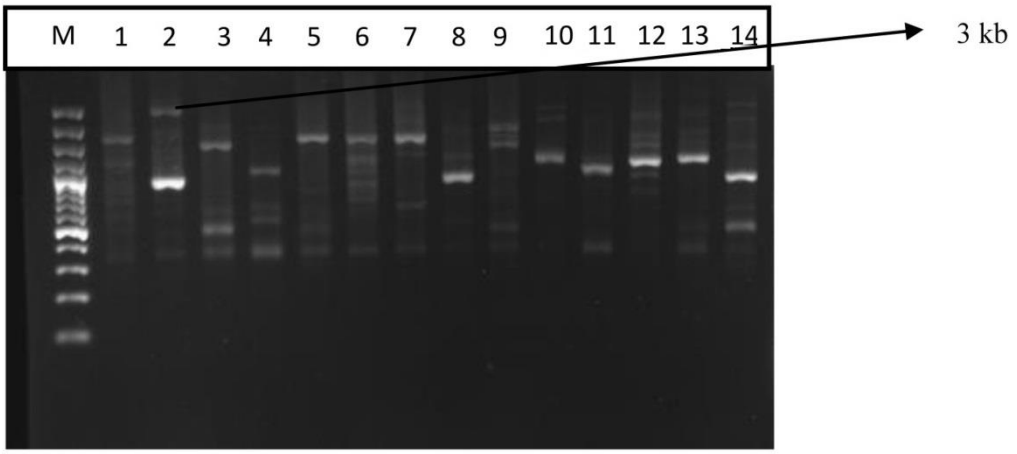

Figure 5. PCR products of SRAP marker with primer of Me5,Em 17,1: *P. natans*, 2: *P. alpinus*, 3: *P. perfoliatus*, 4: *P. lucens*, 5: *P. friesii*, 6: *P. pusillus*, 7: *P. berchtoldii*, 8: *P. filiformis*, 9: *P. crispus*, 10: P. *pectinatus*, 11. *C. demersum*, 12. M. spicatum; 13,14: other populations of P. pectinatus.

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