PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol x(x): xx–xx
https://doi.org/10.14719/pst.2850

HORIZON
e-Publishing Group
HePG

RESEARCH ARTICLE

A modified CTAB method for extracting high-quality genomic DNA from aquatic plants

Valiya Thodiyil Jaseela, Edathumthazhe Kuni Sinisha & Nediyaparambu Sukumaran Pradeep*

KSCSTE-Malabar Botanical Garden and Institute for Plant Sciences, Kozhikode 673014, India

***Email:** drnspradeep@gmail.com

OPEN ACCESS

ARTICLE HISTORY
Received: 07 August 2023
Accepted: 08 December 2023

Available online
Version 1.0 : 16 February 2024

Check for updates

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

Reprints & permissions information is available at https://horizonepublishing.com/journals/index.php/PST/open_access_policy

Publisher's Note: Horizon e-Publishing Group remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS,UGC Care etc. See https://horizonepublishing.com/journals/index.php/PST/indexing_abstracting

CITE THIS ARTICLE

Jaseela V T, Sinisha E K, Pradeep N S. A modified CTAB method for extracting high-quality genomic DNA from aquatic plants. Plant Science Today (Early Access). https://doi.org/10.14719/pst.2850

Abstract

This study introduces a streamlined approach for extracting high-quality DNA from aquatic plants using CTAB, catering to molecular studies. Seven aquatic plant species (*Hygrophila auriculata*, *Limnophila repens*, *Crinum malabaricum*, *Lagenandra ovata*, *Ludwigia peruviana*, *Eichhornia crassipes* and *Ipomoea aquatica*) spanning six orders were subjected to DNA extraction. The method combines mechanical lysis and chemical treatments to effectively disrupt cells, coupled with RNase treatment and phenol extraction to mitigate RNA and protein contamination. The optimized CTAB protocol facilitates the extraction of high-quality genomic DNA, suitable for amplifying plant barcode genes such as ITS and rbcL, as well as markers like RAPD and ISSR, thereby enhancing the efficiency and reliability of genomic studies in aquatic plants.

Keywords

Plant DNA extraction; CTAB based DNA extraction; genomic DNA; PCR amplification; RAPD primers

Introduction

Within the expansive and diverse realm of aquatic plants lies a trove of untold stories inscribed within their genomes. Flourishing in dynamic environments, these aquatic plants harbour genetic treasures with profound implications for research, conservation, and biotechnology. Amid the dynamic landscape of molecular biology, DNA extraction emerges as a foundational pillar, empowering scientists to delve into the very essence of life. In plant molecular biology research, the extraction of genetic material from plant tissues is a fundamental and critical step. Alongside extraction, purification of plant DNA becomes essential for various downstream analyses. Efficient DNA isolation from any plant holds significance across diverse scientific fields and applications, encompassing taxonomy, biodiversity assessment, conservation endeavors, ecological research, evolutionary studies, biotechnology, and molecular investigations (1). This process yields valuable genetic information that aids researchers in comprehending and safeguarding these pivotal components of aquatic ecosystems.

Several protocols (2–7) have been reported for extracting pure DNA from plants; however, the effectiveness of these protocols can vary significantly depending on the plant species. Plants within the same or related genera may exhibit different dispensable functions, posing challenge for certain species to benefit from standard DNA isolation methods. Consequently, these DNA extraction protocols cannot be universally applied to all plant species (5), often requiring modifications to attain high-quality total DNA for polymerase chain reaction (PCR). The cetyltrimethylammonium bromide (CTAB) method and its various modifications have been reported by various authors. Extracting high-quality DNA from plants presents numerous challenges (5–7) often associated with varying levels of polysaccharides, polyphenols, and other secondary metabolites in

different plant species. These components commonly hinder the DNA purification process, limiting its utility in molecular studies (6). The structural similarity of these plant components to nucleic acids allows secondary metabolites and polysaccharides to impede total DNA isolation (8). They strongly bind with nucleic acids during DNA extraction, adversely impacting the quality of isolated DNA from higher plants (9). Moreover, these metabolites influence the quantity and purity of isolated nucleic acids (5).

Polysaccharides present a significant challenge in separation from DNA (3). During DNA extraction, polysaccharides forms complexes with nucleic acids, creating a sticky and gelatinous mass and thus hinders the action of DNA-modifying enzymes such as restriction enzymes, DNA polymerase, ligase, and others (8, 10). Another significant challenge in isolating high-quality DNA is posed by polyphenols, as they often co-purify with DNA. Upon reaction with proteins, polyphenols undergo oxidation, leading to the formation of various products (11). Released during cell lysis and oxidized by cellular oxidases, polyphenols irreversibly interact with nucleic acids, covalently binding to DNA. This interaction results in enzymatic browning of the DNA pellet, rendering it unsuitable for most downstream processes (5, 12).

Optimal DNA extraction is achieved by favouring fresh, young, and healthy tissues, with leaves being particularly advantageous due to their higher cell count and lower secondary metabolites, polysaccharides, and contaminants (2, 3, 13). In contrast, DNA extracted from mature leaves is reported to be of lower quality and yield. The abundance of polyphenols, tannins, polysaccharides, and other secondary metabolites in mature leaves may result in DNA being embedded in a sticky, gelatinous matrix or produce undesirable brown-coloured products that are unsuitable for use (14). Besides leaves, various plant parts like stem (15), bark (7), seeds (10), roots (16), embryo (17), tubers (18), and callus (19) have also been investigated for DNA extraction.

Conventional methodologies for DNA extraction from plants often encounter challenges, particularly when applied to aquatic plants. The unique biochemical composition, environmental context, and the presence of complex polysaccharides, tannins, and secondary metabolites in aquatic plant tissues hinder the isolation process, resulting in suboptimal yield and degraded genetic material quality (20, 21). Recognizing the need for improved DNA isolation protocols for aquatic plants, this work presents an efficient CTAB-based method tailored to address the specific challenges posed by these samples. The aim is to ensure the extraction of high-quality genomic DNA for various genetic analyses and downstream applications. The study focuses on seven aquatic plant species: *Hygrophila auriculata*, *Limnophila repens*, *Crinum malabaricum*, *Lagenandra ovata*, *Ludwigia peruviana*, *Eichhornia crassipes* and *Ipomoea aquatica* (Table 1). Through modifications to the conventional CTAB method, the extraction process is optimized to mitigate challenges associated with the presence of secondary metabolites, polysaccharides, and other inhibitors commonly found in aquatic plant tissues. To validate the quality of the DNA extracted using the optimized protocol, PCR amplification of genomic DNA was carried out, employing techniques such as RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeat), ITS (Internal Transcribed Spacer), and rbcL (Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit).

Materials and Methods

Plant Materials Used

For this study, leaf tissue was selected to ensure higher level of homogenization. Fresh, young, and healthy leaves were collected from the natural habitats of the following plants: *Hygrophila auriculata*, *Limnophila repens*, *Crinum malabaricum*, *Lagenandra ovata*, *Ludwigia peruviana*, *Eichhornia crassipes,* and *Ipomoea aquatica* (Table 1). Immediately after plucking, the leaf samples were placed in sealable polythene bags and promptly transported to the laboratory for DNA extraction. Fresh samples are recommended but samples stored in freezer for 1 or 2 days are also appropriate for DNA extraction . The leaf samples were washed with distilled water and dried using sterile filter paper.

Reagents and Chemicals

The reagents and chemicals used for the extraction of DNA were: CTAB extraction buffer [2% CTAB (Cetyl Trimethyl Ammonium Bromide) (w/v), 100 mM Tris Buffer (pH 8.0), 20 mM Na_2EDTA (pH 8.0), 1.4 M NaCl]; Polyvinylpyrrolidone (PVP); Chloroform: Isoamyl alcohol (24:1); Chloroform; Isopropanol; 70% Ethanol; Phenol: Chloroform: Isoamyl alcohol (25:24:1); RNase A; 3M Sodium acetate; TE buffer [10 mM Tris buffer (pH 8) and 1mM EDTA (pH 8)] and liquid nitrogen.

DNA Extraction Protocol

1g portion of tender, healthy leaf sample was meticulously collected. The collected sample underwent thorough washing with distilled water and was subsequently dried using sterile filter paper. Following the drying process, the sample was precisely cut into smaller pieces. To ensure optimal homogenization, the leaf pieces were pulverized in liquid nitrogen using a pre-cooled mortar and pestle. This method

Table 1. Details of plants used in the study.

Sl.No.	Scientific Name	Common Name	Habitat	Order
1	*Hygrophila auriculata*	Marsh barbel	Emergent	Lamiales
2	*Limnophila repens*	Creeping marshweed	Submerged	Lamiales
3	*Crinum malabaricum*	Malabar river lily	Rooted floating	Asparagales
4	*Lagenandra ovata*	Malayan sword	Emergent	Alismatales
5	*Ludwigia peruviana*	Peruvian primrose-willow	Emergent	Myrtales
6	*Eichhornia crassipes*	Water hyacinth	Free floating	Commelinales
7	*Ipomoea aquatica*	Water spinach	Emergent	Solanales

helps to maintain the integrity of the sample while facilitating efficient processing for subsequent DNA extraction.

The extraction buffer was prepared by preheating it in a water bath at 65°C. Just before use, Polyvinylpyrrolidone (PVP) was added to the extraction buffer at a ratio of 0.2 g PVP for 0.5 mL of extraction buffer. To initiate the DNA extraction process, 500–1000 µL of warm CTAB extraction buffer with PVP was added to the pulverized leaf sample, creating a slurry. This slurry was then transferred into 2 mL microcentrifuge tubes, with the quantity of buffer adjusted based on the size of the leaf sample. The tubes were incubated at 65°C for 45–60 min in a water bath, and throughout the incubation period, the mixture was regularly mixed by gently inverting the tubes.

Following the incubation at 65°C, the mixture was cooled to room temperature. To facilitate phase separation, 700 µL of a Chloroform: isoamyl mixture (24:1) was added. The components were thoroughly mixed to form an emulsion, and the mixture was then subjected to centrifugation at 12,000 rpm for 15 min at 25°C.

The upper aqueous phase, containing the extracted DNA, was carefully collected and transferred to a fresh micro centrifuge tube. To further purify the DNA, an equal amount of chloroform was added, and the mixture was centrifuged at 12,000 rpm for 10 min at 4°C. The upper layer, now devoid of impurities, was transferred to another fresh micro centrifuge tube. For DNA precipitation, 100% chilled isopropanol was added along the sides of the tube. The tube was gently inverted to mix the contents, and the resulting mixture was then incubated at -20°C for 2 hrs. It's worth noting that the duration of chilled incubation directly influences the precipitation efficiency, with longer durations leading to increased DNA precipitation.

Subsequent to the chilled incubation, the mixture was centrifuged at 10,000 rpm for 15 min at 4°C. The resulting pellets, containing the precipitated DNA, were carefully collected. To remove residual contaminants, the pellets were subjected to two washes with cold 70% ethanol. Following the ethanol washes, the pellets were left to air dry.

The collected pellets were carefully suspended in 200 µL of TE buffer. To eliminate any residual RNA, 4 µL of RNase A was added, and the mixture was incubated at 37°C for 2 hrs in a water bath. Following this, 500 µL of Phenol: Chloroform: Isoamyl mixture (24:25:1) was added to the suspension. The components were gently mixed and then subjected to centrifugation at 10,000 rpm for 10 min at 4°C.

The supernatant, containing the treated DNA, was meticulously collected and transferred to a fresh micro centrifuge tube. To ensure thorough purification, 500 µL of chloroform was added to the collected supernatant, and the components were gently mixed. The mixture underwent centrifugation at 10,000 rpm for 10 min at 4°C. The resulting supernatant, now free of impurities, was carefully collected and transferred to another fresh 1.5 mL micro centrifuge tube.

To precipitate the DNA, a double volume of chilled isopropanol and 1/10 volume of 3M sodium acetate were added to the supernatant, and the mixture was left overnight at -20°C. The resulting pellets were collected by centrifugation at 10,000 rpm for 15 min at 4°C. Afterward, the pellets underwent two washes in cold 70% ethanol and were allowed to air dry. The dried pellets were then carefully re-suspended in 50 µL of TE buffer, ensuring the preparation of purified DNA suitable for subsequent analyses.

Qualitative and Quantitative Analysis of Extracted DNA

The DNA yield was quantified by measuring absorbance at 260 nm (A260) using a Multiskansky™ spectrophotometer (Thermo Scientific, USA). To assess the purity of the DNA, absorbance ratios at A260/A280 and A260/A230 were determined. Additionally, the quality of the extracted DNA was further evaluated through electrophoresis separation for all DNA samples. For this, 5 µL of DNA was mixed with 1µL of gel-loading dye and loaded onto a 0.8% agarose gel stained with 1µg/mL ethidium bromide. Electrophoresis was conducted using 1x TAE (Tris-Acetate-EDTA) buffer at 100 V until the dye front migrated to the gel's bottom. Gel visualization and imaging were performed using the Gel Doc XR+ Imaging system (Bio-Rad, USA).

Inter Simple Sequence Repeats (ISSR) Analysis

PCR amplification of DNA samples was performed using fifteen ISSR primers (Table 2) obtained from the University of British Columbia (UBC) and synthesized by Bio serve Biotechnologies (India). The PCR reaction mixture (25 µL) comprised 50-100 ng of DNA, 1 µL of each primer, 12.5 µL of master mix (Takara Bio Inc., Japan, containing PCR buffer, dNTPs, Taq polymerase, $MgCl_2$), and PCR-grade water to achieve a final volume of 25 µL. ISSR-PCR was conducted in a thermal cycler (Bio-Rad S1000, USA) through 40 cycles, including 1 cycle of 2 min at 95°C, 2 min at 53°C, and 2 min at 72°C, followed by 39 cycles of 30 sec at 94° C, 1 min at 53°C, and 2 min at 72°C. A final extension step was performed at the same temperature for 10 min. The amplified products (5µL) were electrophoresed in 1.5% agarose in 1x TAE buffer, stained with ethidium bromide, and visualized using the Gel Doc XR+ Imaging system (Bio-Rad, USA). The size of the amplicons was determined by comparing them with the Bench top 1 Kb DNA ladder (Promega).

Random Amplified Polymorphic DNA (RAPD) Analysis

PCR amplification of genomic DNA extracted from different plant leaves was conducted using twenty RAPD decamer primers (Operon Technologies) from the Operon series (Table 2). The PCR reaction mixture (25 µL) consisted of 50-100 ng DNA, 1 µL of each primer, 12.5 µL of master mix (Takara Bio Inc., Japan), and PCR-grade water to reach a final volume of 25 µL. For RAPD amplification, the protocol involved 1 cycle of 2 min at 95°C, 2 min at 35°C, and 2 min at 72°C, followed by 39 cycles of 1 min at 93°C, 1 min at 36°C, and 2 min at 72°C. The final cycle included a 7 min extension at 72°C. Amplifications were carried out in a Bio-Rad S1000 thermal cycler (USA). Amplicons were separated on a 1.5% agarose gel with 1 µg/mL ethidium bromide in 1x TAE buffer and visualized using the Gel Doc XR+ Imaging system (Bio-Rad, USA). The size of the amplicons was estimated using the Bench top 1Kb DNA ladder (Promega).

ITS and rbcL Amplifications

PCR amplification of ITS (22) and rbcL (23) (Table 2) was carried out in a 25 µL reaction mixture, including 50-100 ng DNA, 1 µL

Table 2. Sequences of primers used for the study.

Sl. No.	Primer		Sequence
1	ITS	ITS 1-F	TCCGTAGGTGAACCTGCGG
		ITS 4-R	TCCTCCGCTTATTGATATGC
2	rbcL	rbcL-F	ATGTCACCACAAACAGAGACTAAAGC
		rbcL-R	GTAAAATCAAGTCCACCRCG
3	ISSR	ISSR 1	AGAGAGAGAGAGAGAGT
		ISSR 2	AGAGAGAGAGAGAGAGC
		ISSR 3	AGAGAGAGAGAGAGAGG
		ISSR 4	GAGAGAGAGAGAGAGAT
		ISSR 5	GAGAGAGAGAGAGAGAC
		ISSR 6	GAGAGAGAGAGAGAGAA
		ISSR 7	CTCTCTCTCTCTCTCTT
		ISSR 8	CTCTCTCTCTCTCTCTG
		ISSR 9	CACACACACACACACAA
		ISSR 10	CACACACACACACACAG
		ISSR 11	TCTCTCTCTCTCTCTCG
		ISSR 12	AGAGAGAGAGAGAGAGYT
		ISSR 13	GAGAGAGAGAGAGAGAYC
		ISSR 14	ACACACACACACACACYG
		ISSR 15	BDBCACACACACACACA
4	RAPD	OPA 02	TGCCGAGCTG
		OPA 03	AGTCAGCCAC
		OPA 05	AGGGGTCTTG
		OPA 10	GTGATCGCAG
		OPB 01	GTTTCGCTCC
		OPB 04	GGACTGGAGT
		OPB 06	TGCTCTGCCC
		OPB 07	GGTGACGCAG
		OPB 08	GTCCACACGG
		OPB 10	CTGCTGGGAC
		OPB 17	AGGGAACGAG
		OPB 18	CCACAGCAGT
		OPC 08	TGGACCGGTG
		OPC 11	AAAGCTGCGG
		OPC 17	TTCCCCCCAG
		OPD 05	TGAGCGGACA
		OPD 11	AGCGCCATTG
		OPG 02	GGCACTGAGG
		OPG 03	GAGCCCTCCA
		OPG 17	ACGACCGACA

Y= C,T; B= G,C,T; D= A,G,T

of each primer (forward and reverse), 12.5 µL of master mix (Takara Bio Inc., Japan), and PCR-grade water to reach a final volume of 25 µL. The amplified products were separated by electrophoresis in a 1% agarose gel using 1x TAE containing 1 µg/mL ethidium bromide. DNA bands were visualized, and images were acquired using the Gel Doc XR+ Imaging system (Bio-Rad, USA). The amplification consisted of 35 cycles and was performed in a Biorad S1000 thermal cycler (USA).

For ITS, the PCR program involved an initial denaturation for 5 min at 97°C, followed by 34 cycles of 1 min at 97°C, 1 min at 48°C, and 2 min at 72°C, with a final extension of 5 min at 72°C. The sequential steps for rbcL included an initial denaturation of 3 min at 94°C, followed by 34 cycles of 45 sec at 94°C, 45 sec at 55°C, and 2 min at 72°C, with a final extension at 72°C for 3 min. The Bench top 1 Kb DNA ladder (Promega) was utilized to estimate the size of the amplicons.

Notes

It is crucial to refrain from thawing leaf tissue before grinding. Although it is necessary to crush the leaves before adding the extraction buffer, caution should be exercised to avoid grinding them into a fine powder, as this may lead to shearing of the DNA.

To achieve high yields of intact DNA, it is essential to use young leaves from healthy plants.

Ensuring thorough tissue suspension in the extraction buffer is crucial for achieving maximum yield.

Care should be taken not to disturb the white layer formed while removing the aqueous phase when chloroform is used to eliminate soluble proteins from the sample.

Proper air drying of the pellet is essential to ensure the complete removal of any traces of ethanol.

When transferring the aqueous phase, it is recommended to use wide-bore tips to avoid causing any harm to the DNA.

Results and Discussion

Aquatic plants, thriving in diverse and challenging environments, harbour genetic resources with substantial implications for scientific research, conservation, and biotechnology. DNA isolation plays a crucial role in unravelling the intricacies of aquatic plant biology, ecology, genetics, evolution, and conservation. The presence of specific metabolites poses challenges to DNA isolation processes and downstream reactions. Given the diversity in chemical compositions among species, a one-size-fits-all approach may not suffice, emphasizing the importance of tailored extraction protocols to ensure the desired quality of DNA. The DNA extraction method presented in this study demonstrated high efficiency, as reflected in the results (Fig. 1a). The technique yielded a higher amount of pure DNA from minimal quantities of leaf samples. The extracted DNA was of sufficient purity and concentration to be stored for subsequent molecular analyses. The purity of the extracted DNA is evident from the A260/A280 ratio ranging from 1.76 to 1.82 and the A260/A230 ratio ranging from 1.95 to 2.2 (Table 3). These ratios indicate that the DNA preparations were adequately free of proteins, polyphenolics, and polysaccharides. The A260/A280 and A260/A230 ratios are reliable indicators of DNA purity. A sample with an A260/A280 ratio around 1.8 is considered free from contaminants such as proteins, and an A260/A230 ratio around or greater than 2 indicates the absence of polysaccharides, phenol, or other organic compounds (24). The DNA concentration achieved through the presented protocol reached a maximum of 930 ± 340 ng/µL (Table 3). Gel images exhibited high-quality DNA, as evidenced by minimal to no fluorescence observed in the loading wells. This lack of fluorescence indicates low levels of polysaccharides, proteins, or phenol contamination, further supporting the effectiveness of the DNA extraction method. Successful PCR amplifications were achieved using primers ITS, rbcL, ISSR, and RAPD, as demonstrated by the distinct bands observed in the gel images (Fig. 2–5). These results indicate that the isolated DNA is of sufficient quality and purity to support PCR amplification, affirming the efficacy of the DNA extraction method.

Various DNA isolation protocols, including methods 2, 3, 14, and 17, as well as the utilization of a plant genomic DNA extraction kit and DNA zol, were employed in an attempt to extract DNA from aquatic plants such as *Hygrophila auriculata*, *Limnophila repens*, *Crinum malabaricum*, *Lagenandra ovata*, *Ludwigia peruviana*, *Eichhornia crassipes* and *Ipomoea*

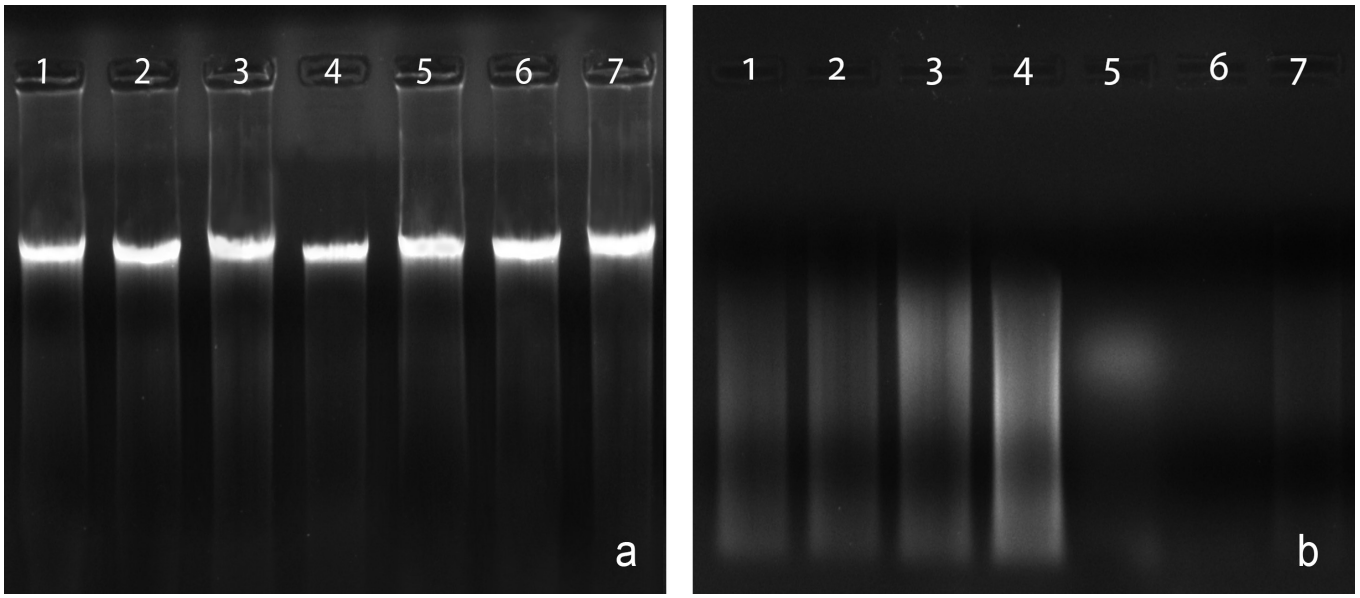

Fig. 1. Agarose gel image of genomic DNA extracted from aquatic plants resolved on 0.8% agarose gel. 1. *Hygrophila auriculata*; 2. *Limnophila repens*; 3. *Crinum malabaricum*; 4. *Lagenandra ovata*; 5. *Ludwigia peruviana*; 6. *Eichhornia crassipes*; 7. *Ipomoea aquatica* (a) Current protocol (b) Doyle & Doyle (1987) method.

Table 3. Yield and purity of genomic DNA extracted from seven aquatic plant species using the CTAB procedure modified in this study.

Scientific Name	A260/A230	A260/A280	DNA Yield (ng/µL)
Hygrophila auriculata	1.97	1.8	934
Limnophila repens	2.00	1.81	1270
Crinum malabaricum	1.94	1.77	746
Lagenandra ovata	2.00	1.76	955
Ludwigia peruviana	2.1	1.8	784
Eichhornia crassipes	1.95	1.82	841
Ipomoea aquatica	2.2	1.78	587

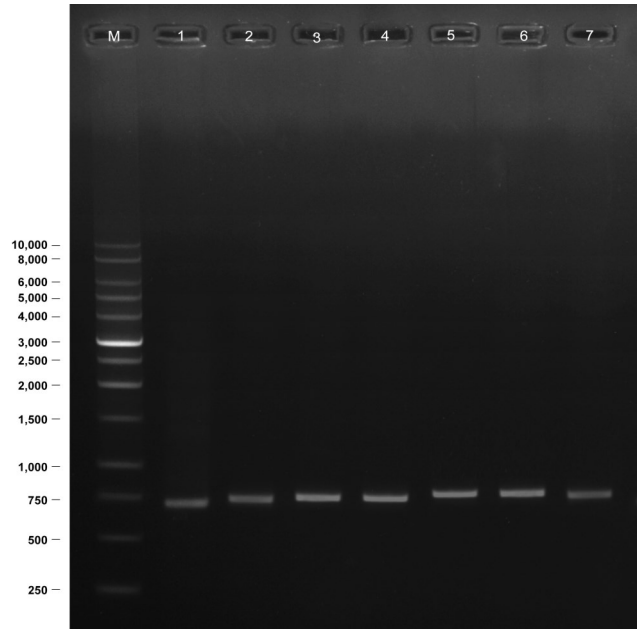

Fig. 2. PCR profile of DNA samples amplified with primer ITS. Lane M represent Benchtop1Kb DNA ladder (Promega); Lane 1–7 represents plant samples viz. *1. Hygrophila auriculata* 2. *Limnophila repens* 3. *Crinum malabaricum 4. Lagenandra ovata* 5. *Ludwigia peruviana 6. Eichhornia crassipes* and 7. *Ipomoea aquatica*

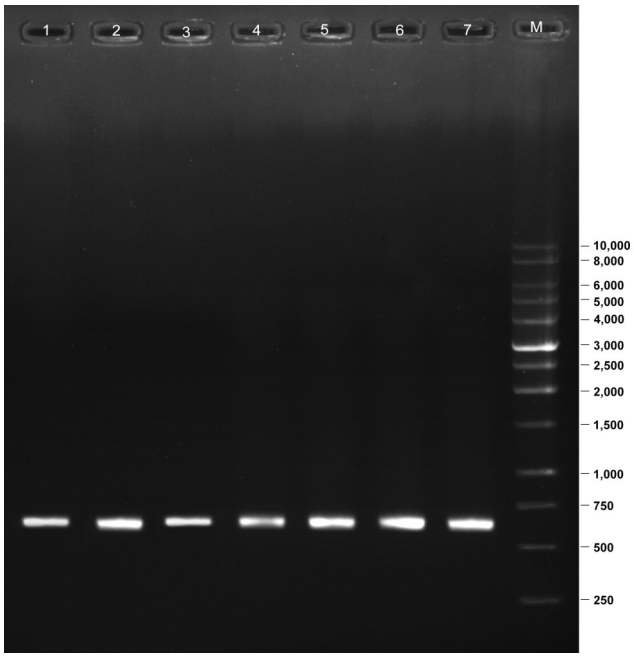

Fig. 3. PCR amplification of the samples using the primer rbcL. Lane 1–7 represents plant samples viz. 1. *Hygrophila auriculata* 2. *Limnophila repens* 3. *Crinum malabaricum, 4. Lagenandra ovata*, 5. *Ludwigia peruviana, 6. Eichhornia crassipes* and 7. *Ipomoea aquatica*; Lane M: Benchtop1Kb DNA ladder (Promega).

aquatica. Unfortunately, these methods proved to be ineffective, as indicated by the presence of sticky polysaccharides in the pellet, discoloration of the DNA, and the appearance of sheared DNA bands in the gel image (Fig. 1b). When multiple techniques were employed for DNA isolation, numerous challenges were encountered from the initial stage through the entire process. The encountered challenges, including highly viscous and sticky pellets contaminated with phenolic compounds (25), resulted in limited DNA yield, subpar DNA quality, and poor results in PCR amplification reactions. The extracted DNA was deemed unsuitable for molecular studies due to a low A260/A280 ratio, falling below the ideal limit of 1.8 (26). The motivation for this study stemmed from the necessity to devise improved DNA extraction methods capable of yielding substantial amounts of high-quality DNA from the specified plant tissues for use in molecular marker analyses. The optimized CTAB protocol presented here effectively addresses these challenges by eliminating contaminants such as polysaccharides, polyphenols, and

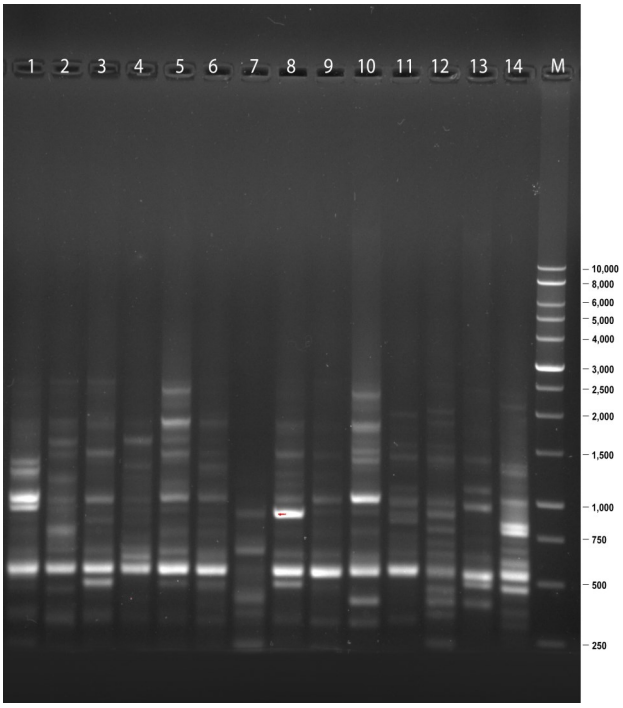

Fig. 4. ISSR profile (ISSR1) of 14 accessions of *Hygrophila auriculata*. Lane M: Benchtop1Kb DNA ladder (Promega).

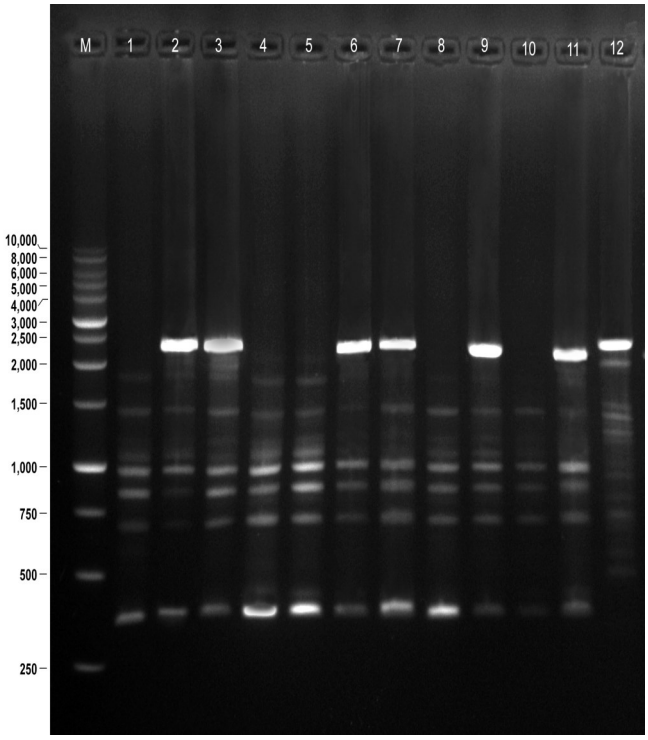

Fig. 5. RAPD (OPB 01) banding pattern of 12 accessions of *Limnophila repens*; Lane M: Benchtop1Kb DNA ladder (Promega).

proteins. This method has proven successful and applicable for extracting high-yield, high-purity DNA from seven different plant species representing six distinct plant orders.

The extraction of DNA from plant cells is a challenging process primarily due to the complex and rigid structure of the plant cell wall. Cetyltrimethylammonium bromide (CTAB), a cationic detergent, plays a crucial role in disrupting the integrity of the cell wall. It interacts with negatively charged components, solubilizing them and facilitating the release of DNA. CTAB is a commonly used detergent in plant DNA extraction (2–4), and higher concentrations have been reported to effectively eliminate polysaccharides (2). Moreover, CTAB can form complexes with cellular proteins and other contaminants, preventing their interference with the DNA isolation process.

In our method, we employed 2% CTAB and 1.4 M NaCl in the extraction buffer, which proved effective in overcoming the presence of high levels of polysaccharides. The integration of a high concentration of CTAB and a high molar salt has demonstrated effectiveness in removing polysaccharides from the extracted DNA. This approach contributes to the success of the DNA isolation process from plant tissues (2, 27). Polyvinylpyrrolidone (PVP) has been incorporated into the protocol to assist in the removal of polyphenols from the samples. PVP forms intricate hydrogen bonds with polyphenolic compounds, and these compounds can be effectively eliminated from DNA through centrifugation (28). The presence of polysaccharides and polyphenols in plants can vary significantly between species, presenting challenges during DNA extraction due to potential interference in the isolation process (6).

Polysaccharide contamination in extracted DNA has the potential to impede enzymatic reactions, such as Taq DNA polymerase amplifications (29). However, the successful amplification observed in the present study serves as an indication of the DNA purity achieved through our method, highlighting the effectiveness of incorporating PVP in mitigating polyphenol-related challenges during DNA extraction.

The proper selection of leaf samples is crucial for the successful extraction of DNA, as demonstrated in our study. We attempted to isolate DNA from various leaf sources, including fresh leaves, silica-dried leaves, and leaves stored in deep freezer for an extended duration. The results revealed that only fresh, tender, and healthy leaf samples yielded satisfactory outcomes. This aligns with previous studies emphasizing the preference for fresh, young leaves in DNA extraction due to their lower content of secondary metabolites, polysaccharides, and contaminants (2, 3).

Attempts to extract DNA from mature leaves resulted in poor-quality DNA with minimal yield, as mature leaves are characterized by higher accumulations of secondary metabolites. Consistent findings in earlier studies, such as those by Ahmed (14), support this observation. The incorporation of liquid nitrogen in the protocol facilitated the rapid disintegration of tissue into fine powder. Notably, extraction without liquid nitrogen in our protocol led to the absence of any distinct DNA bands. Therefore, the use of liquid nitrogen is deemed essential for the success of our protocol, underscoring its crucial role in the efficient isolation of DNA from leaf samples. Electrophoretic separation of the DNA extracted by the present protocol showed intense bands (Fig. 1a), signifying high degree of purity and high molecular weight intact DNA. DNA samples extracted by the present protocol were assessed for further downstream molecular analyses by PCR amplification with ISSR, RAPD, ITS, and rbcL primers. The presence of clear and well-differentiated band patterns (Fig. 2–5) reveals the effectiveness of the protocol in producing genomic DNA with high purity suitable for molecular studies based on PCR techniques. This protocol can likely be extended to other aquatic plant species.

Conclusion

The described method represents an effective and straightforward approach for extracting high-quality DNA from aquatic plant leaves using CTAB. The protocol involves a combination of mechanical lysis and chemical treatment to break down cells, high salt concentrations to remove polysaccharides, PVP to eliminate polyphenols, and RNase treatment and phenol extraction to prevent contamination from RNA and proteins. This updated CTAB protocol enables the extraction of genomic DNA of high quality, suitable for various downstream applications, including the amplification of plant barcode genes such as ITS and rbcL, as well as RAPD and ISSR. The versatility of this method makes it applicable to different aquatic plant species, providing a valuable tool for molecular studies in plant biology, ecology, and genetics.

Acknowledgement

The authors express their gratitude to the Director of KSCSTE-Malabar Botanical Garden & Institute for Plant Science for providing facilities and support. The first author acknowledges the Council of Scientific and Industrial Research (CSIR), while the second author thanks the University Grants Commission (UGC), Government of India, for financial assistance.

Authors' contributions

All the authors equally contributed to the work presented in this paper.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None.

References

1. Wilkie S, Clark MS, Leroy P, Merlino M, Nègre S, Caissard JC, Sourdille P, Lu YH, Bernard M. Genomic DNA isolation, southern blotting and hybridization. In: Clark MS, editor. Plant Molecular biology—A Laboratory Manual. Springer. Springer, Berlin, Heidelberg. 1997;p. 3-53. https://doi.org/10.1007/978-3-642-87873-2_1
2. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research. 1980;8(19):4321-26. https://doi.org/10.1093/nar/8.19.4321
3. Doyle JJ, Doyle JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry Bulletin. 1987;19:11-15.
4. Allen G, Flores-Vergara M, Krasynanski S, Kumar S, Thompson W. A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide. Nature Protocols. 2006;1(5):2320-25. https://doi.org/10.1038/nprot.2006.384
5. Porebski S, Bailey LG, Baum BR. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Molecular Biology Reporter. 1997;15:8-15. https://doi.org/10.1007/BF02772108
6. Khanuja SP, Shasany AK, Darokar MP, Kumar S. Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. Plant Molecular Biology Reporter. 1999;17:1-7. https://doi.org/10.1023/A:1007528101452
7. Novaes RML, Rodrigues JG, Lovato MB. An efficient protocol for tissue sampling and DNA isolation from the stem bark of Leguminosae trees. Genetics and Molecular Research. 2009;8 (1):86-96. https://doi.org/10.4238/vol8-1gmr542
8. Shioda M, Marakami-Muofushi K. Selective inhibition of DNA polymerase by a polysaccharide purified from slime of *Physarumpolycephalum*. Biochemical and Biophysical Research Communications. 1987;146(1):61-66. https://10.1016/0006-291x(87)90690-5.
9. Scott KD, Play ford J. DNA extraction technique for PCR in rain forest plant species. Bio techniques. 1996;20(6):974-78. https://doi.org/10.2144/96206bm07
10. Sharma AD, Gill PK, Singh P. DNA isolation from dry and fresh samples of polysaccharide-rich plants. Plant Molecular Biology Reporter. 2002;20:415-415f.https://doi.org/10.1007/BF02772129
11. Loomis WD. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. In: Methods in enzymology. Academic Press. 1974; Vol. 31: p. 528-44. https://doi.org/10.1016/0076-6879(74)31057-9
12. Lodhi MA, Ye GN, Weeden NF, Reisch BI. A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis*species. Plant Molecular Biology Reporter. 1994;12:6-13. https://doi.org/10.1007/BF02668658
13. Sika KC, Kefela T, Adoukonou-Sagbadja H, Ahoton L, Saidou A, Baba-Moussa L, Baptiste LJ, Kotconi SO, Gachomo EW. A simple and efficient genomic DNA extraction protocol for large scale genetic analyses of plant biological systems. Plant Gene. 2015;1:43-45. https://doi.org/10.1016/j.plgene.2015.03.001
14. Ahmad SM, Ganaie MM, Qazi PH, Verma V, Basir SF, Qazi GN. Rapid DNA isolation protocol for Angiospermic plants. Bulgarian Journal of Plant Physiology. 2004;30:25-33.
15. Wong LM, Silvaraj S, Phoon LQ. An optimized high-salt CTAB protocol for both DNA and RNA isolation from succulent stems of *Hylocereus* sp. Journal of Medical and Bioengineering. 2014 Dec;3(4).https://doi.org/10.12720/jomb.3.4.236-240
16. Khan S, Qureshi MI, Alam T, Abdin MZ. Protocol for isolation of genomic DNA from dry and fresh roots of medicinal plants suitable for RAPD and restriction digestion. African Journal of Biotechnology. 2007;6(3):175.
17. Rogers SO, Bendich AJ. Extraction of DNA from plant tissues. Plant Molecular Biology Manual. 1989;73-83. https://doi.org/10.1007/978-94-009-0951-9_6
18. Tapia-Tussell R, Quijano-Ramayo A, Rojas-Herrera R, Larque-Saavedra A, Perez-Brito D. A fast, simple and reliable high-yielding method for DNA extraction from different plant species. Molecular Biotechnology. 2005;31(2):137-40. https://doi.org/10.1385/mb:31:2:137
19. Kang T, Yang M. Rapid and reliable extraction of genomic DNA from various wild-type and transgenic plants. BMC Biotechnology. 2004;4(1):20. https://doi.org/10.1186/1472-6750-4-20
20. Panova M, Aronsson H, Cameron RA, Dahl P, Godhe A, Lind U, Ortega-Martinez O, Pereyra R, Tesson SV, Wrange AL, Blomberg A, Johannesson K.DNA extraction protocols for whole-genome sequencing in marine organisms. Methods of Molecular Biology. 2016;1452:13-44. https://doi.org/10.1007/978-1-4939-3774-5_2
21. Saeed, Afsharzadeh.,Shabnam, Abbasi. An efficient and simple CTAB based method for total genomic DNA isolation from low

amounts of aquatic plants leaves with a high level of secondary metabolites. Progress in Biological Sciences. 2016;6(1):95-106. https://doi.org/10.22059/PBS.2016.59012

22. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetic. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR Protocols: A Guide to Methods and Applications. Academic Press, New York. 1990;p.315-22. https://doi.org/10.1016/B978-0-12-372180-8.50042-1
23. Kress WJ, Erickson DL. A two-locus global DNA barcode for land plants: The coding rbcL gene complements the non-coding trnH -psbA spacer region. PLoS one. 2007;2(6):e508. https://doi.org/10.1371/journal.pone.0000508
24. Wilson K, Walker J. Principles and techniques of biochemistry and molecular biology. Cambridge University Press. 2005. https://doi.org/10.1017/CBO9780511813412
25. Moreira PA, Oliveira DA. Leaf age affects the quality of DNA extracted from *Dimorphandramollis* (Fabaceae), a tropical tree species from the Cerrado region of Brazil. Genetics and Molecular Research. 2011;10(1):353-58. https://doi.org/10.4238/vol10-1gmr1030
26. Sambrook J, Russell DW. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, New York, USA. 2001.
27. Paterson AH, Brubaker CL, Wendel JF. A rapid method for extraction of cotton (*Gossypium*spp.) genomic DNA suitable for RFLP or PCR analysis. Plant Molecular Biology Reporter. 1993;11:122-27. https://doi.org/10.1007/BF02670470
28. Maliyakal EJ. An efficient method for isolation of RNA and DNA from plants containing polyphenolics. Nucleic Acids Research. 1992;20:2381. https://doi.org/10.1093/nar/20.9.2381
29. Fang G, Hammar S, Grumet R. A quick and inexpensive method for removing polysaccharides from plant genomic DNA. Bio techniques. 1992;13(1):52-56. https://doi.org/10.2144/97231bm09