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Comparative study of genomic DNA extraction protocols identified CTAB as an efficient method for PCR-based genetic analysis across diverse plant species

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Abstract

Efficient and reliable DNA extraction is essential for molecular biology research. The diverse chemical composition of plant species and the possibility of secondary reactions during extraction pose obstacles to establishing a uniform protocol. This study compared the efficiency of four DNA extraction methods (CTAB, Edward, Manitol, and Sorbitol) across three distinct plant species: *Physcomitrella patens* (bryophyte), *Brassica campestris* (angiosperm), and *Pinus taeda* (gymnosperm). DNA was isolated from roots, stems, and leaves of each species, with three replicates per tissue type. The methods were assessed according to DNA yield, purity, and integrity by spectrophotometry and agarose gel electrophoresis. The CTAB method consistently yielded the highest DNA concentrations and purity ratios (A260/A280) between 1.78 and 1.84, demonstrating little protein contamination across all species and tissues, particularly in roots. Manitol and Sorbitol methods provided intermediate yields and purity, with Manitol being more effective for *B. campestris* and *P. patens*, while Sorbitol was beneficial for woody plants like *P. taeda*. The Edward method produced the lowest DNA yields and purity, particularly in samples with high concentrations of phenolic compounds and polysaccharides. Agarose gel electrophoresis revealed clear bands for all methods, with CTAB and Manitol yielding more pronounced, high-molecular-weight DNA bands. Statistical analysis using two-way ANOVA and Tukey's post hoc test showed significant effects of species and extraction protocol on DNA yield and purity, with a significant species $\times$ protocol interaction. The CTAB method demonstrated significantly higher yields and purity compared to other methods. These findings highlight the importance of selecting an appropriate DNA extraction method based on plant species and tissue type, to ensure optimal yield, purity, and integrity for subsequent molecular applications.

1 Introduction

A high-quality, pure, and intact genomic DNA extraction is crucial for numerous molecular biology applications, such as PCR restriction digestion, and next-generation sequencing (Tan & Yiap, 2009). However, contaminants, including polysaccharides, polyphenols, and a variety of secondary metabolites i.e., tannins, lignin, saponins etc., continues to pose significant problems in the extraction of purified DNA from plant tissues (Sangwan et al., 1998). These contaminants, when present in DNA, may pose particular problems while inhibiting the activity of restriction endonucleases (Pandey et al., 1996) and DNA Taq polymerase (Fang et al., 1992). Notably, polysaccharides particularly pectin, mucilage and gums co-precipitate with DNA, can increase the viscosity of DNA solutions and could hinder pipetting and quantification. Additionally, oxidized polyphenols can form covalent complexes with DNA, which can reduce its stability and long-term usability (Katterman & Shattuck, 1983). Due to the low content of these polysaccharides, polyphenols and other secondary metabolites,

the extraction of DNA from young tissue of plants is therefore preferred, and mostly the fresh leaf samples are recommended in DNA extraction protocols. In consideration of the varying compositions of polysaccharides, hydroxybenzenes, esters, and other secondary metabolites among plant species belonging to distinct phyla e.g., mosses, flowering plants and woody plants, diverse protocols may be required for their manipulation at various stages of their life cycle (Kotchoni & Gachomo, 2009). Several types of DNA extraction procedures have been established for various plant species (Li et al., 2013). The cetyltrimethylammonium bromide (CTAB) method, initially described by Doyle and Doyle in 1987 (Doyle & Doyle, 1987), is among the most commonly utilized protocols and has been modified multiple times to enhance yield and purity (Doyle & Doyle, 1987; Huang et al., 2000). The Edward method remains to be a widely utilized technique for plant DNA isolation (Edward et al., 1991). Moreover, procedures utilizing Manitol (Štorchová et al., 2000) and Sorbitol buffers (Russell et al., 2010) have shown effective in improving the quality of DNA extraction. Sodium dodecyl sulphate (SDS)-based techniques

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(Sunnucks & Hales, 1996), in addition to SDS in conjunction with NaCl buffer (Mogg & Bond, 2003) have been effectively utilized for the extraction of high-quality genomic DNA from plant tissues (Scalaferrri et al., 2013).

This study selected plant species based on their biological, industrial, and ecological importance.

Physcomitrella patens (*P. patens*), a moss with a simple body plan, the ease of targeting genes (Roberts et al., 2011), host for heterologous expression of recombinant therapeutic proteins and commercially valuable small natural products (Reski et al., 2015), this makes it a unique model organism for plant biologists' studies regarding plant functional genomics (Reinhard et al., 2004; Vesty et al., 2016). The *Brassica campestris* (*B. campestris*) specie has become a useful material among scientists during last few years, because of the genetic manipulation in it can be used to enhance traits of other transgenic plants as heat tolerance in transgenic tobacco is improved by overexpression of BcHsfA1 transcription factor from *B. campestris* (Zhu et al., 2018). CRISPR/Cas 9 genome editing is used in *B. campestris* which may assist gene functional studies and molecular breeding (Xiong et al., 2019). The loblolly pine *Pinus taeda* (*P. taeda* L.) is a crucial specie as it offers major ecological benefits regarding climate control, wildlife maintenance and carbon fixation which makes it important in genetic engineering.

The plants selected in this study belong to different phylogenetic groups and tissue types, and each of them has a unique phytochemical profile. Mosses usually have a comparatively lower concentration of lignin and can accrue distinctive phenolic acids though flowering plants have inconsistent concentrations of flavonoids, hydroxybenzenes and esters. Woody plants, including *Pinus taeda*, are particularly abundant in resin acids, terpenoids and lignin-phenolics, which are also highly recognized to make DNA isolation difficult (Porebski et al., 1997; Sharma et al., 2002). Hence, the current study aims to evaluate and contrast several genomic DNA extraction procedures across these different plant species representing separate evolutionary lineages. Due to the structural, biochemical, and genetic variations among these species, standardized DNA extraction techniques may not produce uniformly efficient or pure DNA across all groups. This comparison is essential for creating optimized, cost-efficient, and widely applicable DNA isolation method that facilitate downstream applications such as PCR, sequencing, and genomic analysis across distinct taxa.

2 Materials and Methods

2.1 Plant material collection and preservation

Young (fresh growing) plants were collected from Pakistan Forest Institute Peshawar, and stored in an ultra-cold freezer at -80 °C until use. Plants were stored in preservation buffer (Table 1). In order to standardize the extraction tests, 0.1 g of tissues were used for repetition in each protocol.

2.2 Description of extraction protocols

This study examined four distinct genomic DNA extraction protocols: the CTAB method, the Edward method, and Manitol and Sorbitol methods, using explants from three different plant species. Each experiment was carried out in triplicate for each tissue type, ensuring repeatability and statistical reliability. In all extraction methods, plant tissues were first soaked in their respective buffer solutions (Table 2), and the resulting homogenates were transferred to 1.5 ml microcentrifuge tubes. Reagents were thereafter introduced sequentially in accordance with the specific methodological requirements. The efficiency,

yield, and quality of DNA were thoroughly compared among methods, with any procedural adjustments or noticeable differences thoroughly recorded and evaluated.

2.3 Reagents and Stock Solutions

All reagents were molecular biology grade. The stock solutions were prepared according to the standard procedures: 121.1 g of Tris base was dissolved in distilled water to make a 1 L of 1 M stock solution (pH 8.0); 292.2 g of NaCl was dissolved to make a 1 L of 5 M stock solution; 186.12 g of EDTA was dissolved to form a 1 L of 0.5 M stock solution (pH 8.0). Manitol and sorbitol solutions were made by dissolving 182.17 g/L of each compound, respectively in distilled water to make a 1 L of 1 M stock solution. A 10 % (w/v) SDS solution was made by dissolving 10 g of SDS by heating the solution and adjusting the final volume to 100 mL, and a 1% (w/v) PVP-40 solution was made by dissolving 1 g of PVP-40 and making the final volume to 100 mL.

2.4 CTAB method (Doyle & Doyle, 1987)

A 0.1 g of the plant tissue was ground in a sterile mortar with 100 µL CTAB isolation buffer. After chopping, the mixture was placed into a 1.5-ml micro centrifuge tube and incubated at room temperature for 5 minutes. The tissue was crushed again with the addition of 300 µL CTAB isolation buffer, followed by incubation at 60 °C for 15 minutes, inverting gently every five minutes. To each sample 400 µL chloroform/isoamyl alcohol (24:1 v/v) was added. Being briefly vortexed, the samples were centrifuged for 10 minutes at 12,000 × g. To the 1.5 ml new micro centrifuge tubes, about 700 µL of supernatant was transferred. Then 100 % 300 µL ice-cold isopropanol was added to the tubes, and to precipitate the nucleic acid, the tubes were inverted 5 times gently. Subsequently, the samples were centrifuged for 10 minutes at 13,000 × g, and the supernatant was discarded. The pellet was air-dried for 2 hours and then resuspended in 40 µL of TE buffer (Table 2).

2.5 Edward method (Edward et al., 1991)

In a sterile mortar, 0.1 g of plant tissues were ground taking 300 µL of SDS buffer. After chopping mixture were placed into a 1.5-ml micro centrifuge tube and incubated for 5 minutes at room temperature. The tissues were crushed again with the addition of 300 µL SDS buffer. The samples were incubated at 60 °C for 15 minutes, inverting gently every five minutes. By addition of 500 µL SDS buffer, the volume of mixture was adjusted to 1000 µL. The samples were vortexed and incubated at 85 °C for 10 minutes, followed by centrifugation for 10 minutes at 4000 × g. About 700 µL of supernatant was transferred to new, 1.5-ml micro centrifuge tubes. Again, the samples were centrifuged for 10 minutes at 4000 × g. The supernatant that was obtained from the initial centrifugation was thoroughly transferred to new sterile microcentrifuge tubes. Each tube was subsequently filled with 400 µL of ice-cold isopropanol, and gently inverted five times to mix. In order to facilitate DNA precipitation, the samples were then incubated for 10 minutes at room temperature. The tubes were then centrifuged at 13,000 × g for 10 minutes, and the supernatant was discarded. The pellet was air-dried for 2 hours and after that, resuspended in 40 µL TE buffer (Table 2). Each procedure was assessed to determine its integrity (via 1% agarose gel electrophoresis), purity (A260/A280 ratio), and DNA yield (ng/µL).

2.6 Manitol method (Štorchová et al., 2000)

Plant tissues were initially crushed and thoroughly cleaned with tap water to eliminate debris. The tissues were then homogenized in a sterile mortar and pestle with 1.0 ml of chilled Manitol isolation buffer to facilitate breakdown of cells under aseptic conditions. The samples were incubated in an extraction buffer containing Manitol for 20 minutes at room temperature, followed by centrifugation $4000 \times g$ for 10 minutes. The supernatant was thoroughly removed, and 700 μL of high-salt 3 $\times$ CTAB lysis buffer was added to the pellet. The mixture was subsequently incubated at 60 °C for 1 hour to promote cell lysis and nucleic acid release. Subsequently, 700 μL of chloroform: isoamyl alcohol (24:1, v/v) was added into each sample, followed by gentle agitation. The samples were incubated at room temperature for 20 minutes to promote phase separation and protein denaturation. The solution was homogenized by inversion gently each 5 min, followed by centrifugation for ten minutes at $12,000 \times g$. The supernatant was discarded and DNA was precipitated by addition of 3 M 70 μL sodium acetate (1/10 volume) and 500 μL cold isopropanol (2/3 volume). Following incubation, the tubes were kept in a freezer at -20 °C for 1 hour to promote DNA precipitation. The samples were subsequently centrifuged at $13,000 \times g$ for 15 minutes, revealing visible DNA pellets. The pellets were then rinsed twice with 200 μL of 70% ethanol to eliminate remaining salts and contaminants. Next to washing, the ethanol was thoroughly removed, and the DNA pellets were properly air-dried to eliminate any residual ethanol prior to resuspension. Once dried, DNA was resuspended in 40 μL TE buffer (Table 2).

2.7 Sorbitol method (Russell et al., 2010)

In a sterile mortar, 1.0 ml of Sorbitol isolation buffer was used to homogenize approximately 0.1 g of plant tissue. In order to facilitate the breakdown of cells and the early separation of organelles, the homogenate was thereafter incubated for 20 minutes at room temperature. After incubation, the samples were centrifuged for 10 minutes at $4,000 \times g$ in order to separate the nuclei and cellular debris. After thoroughly discarding the supernatant, 0.35 ml of isolation buffer was added to the resulting pellet. Following the addition of 0.35 ml of lysis buffer, the mixture was thoroughly vortexed. To facilitate in cell lysis, the samples were incubated for 20 minutes at 64 °C. A 500 μL of chloroform: isoamyl alcohol (24:1, v/v) was added after incubation, and the tubes were carefully inverted every five minutes to ensure adequate homogenization. After centrifuging the samples for 10 minutes at $12,000 \times g$, the upper aqueous phase was carefully transferred to fresh, sterile microcentrifuge tubes. Immediately 1/10 (v/v) of sodium acetate and 2/3 (v/v) of ice-cold isopropanol was added to the collected supernatant. After 20 minutes of incubation at -20 °C to facilitate DNA precipitation, the tubes were centrifuged for 15 minutes at $13,000 \times g$. To get rid of any remaining salts and CTAB, the resultant DNA pellet was washed with 100 μL of 70% ethanol. The pellet was then allowed to air dry before being resuspended in 40 μL of TE buffer (Table 2).

2.8 PCR Amplification of the 18S rRNA Gene

Amplification of the 18S ribosomal RNA (rRNA) gene was performed using polymerase chain reaction to assess the integrity, purity and amplifiability of genomic DNA obtained isolated from *P. patens*, *B. campestris*, and *P. taeda*. The reactions were performed in a total 25 μL containing 2.0 mM MgCl_2 2 μL , 10 $\times$ PCR buffer 2.5 μL , 1 μL each universal primer (1391f: 5'GTACACACCGCCGCTC3'; EukBr: 5'TGATCCTTCTGCAGGTTACCTAC3'), 10 mM of each dNTP 0.5 μL , 5 U/ μL of Taq DNA polymerase 0.2 μL , 2 μL of template DNA, and 15.8 μL nuclease free water. PCR conditions were as follows:

initial denaturation step of 95 °C for 3 min, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 56 °C, and extension for 45 s at 72 °C, with a final extension for 10 min at 72 °C. The amplification was expected to produce a product of about 450bp. The resulting PCR products were then analyzed by electrophoresis on a 1.8% agarose gel to which ethidium bromide dye was added and viewed after an ultraviolet light source under gelDoc (Wang et al., 2014).

2.9 Statistical analysis

A two-way ANOVA, followed by Tukey's post hoc multiple comparisons test, was used to assess the efficiency of four DNA extraction methods (CTAB, Sorbitol, Manitol, and Edward) across three plant species (*P. patens*, *B. campestris*, and *P. taeda*). All statistical analyses were performed using SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA) and GraphPad Prism (version 12; Systat Software, Chicago, IL, USA).

Table 1 Description of the buffer solutions used in the DNA extraction protocols.

Buffer	Ingredients	Reference
Preservation buffer	3% CTAB and 35% NaCl solution	(Rogstad, 1992)
CTAB isolation buffer	2% CTAB, 0.2% β -mercaptoethanol, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% PVP, pH 8.0	(Doyle & Doyle, 1987)
SDS buffer	0.5% SDS, 25 mM EDTA, 200 mM Tris, 200 mM NaCl, pH 8.0	(Edward et al., 1991)
Manitol isolation buffer	0.35 M Manitol, 100 mM Tris, 5 mM EDTA, with 1% PVP-40 and 1% β -mercaptoethanol, pH 8.0	(Štorchová et al., 2000)
salt 3 $\times$ CTAB lysis buffer	3% CTAB, 100 mM Tris, 20 mM EDTA, 3 M NaCl, 0.2% β -mercaptoethanol and 1% PVP-40, pH 8.0	(Russell et al., 2010)
Sorbitol isolation buffer	0.1 M Tris-HCl, 0.34 M Sorbitol, 0.2% v/v β -mercaptoethanol, 5 mM EDTA, 1% PVP, pH 7.6	(Štorchová et al., 2000)
Sorbitol Lysis buffer	2% CTAB, 2 M NaCl, 0.2 M Tris-HCl, 0.05 M EDTA, pH 7.6	(Štorchová et al., 2000)

Table 2 Description of the 10 mL buffer solutions employed in the DNA extraction protocols.

Buffer	Ingredients' concentrations for 10 ml
Preservation buffer	CTAB 0.3 g, NaCl 3.5 g, dissolved in distilled water
CTAB isolation buffer	[CTAB 0.2 g, 1.4 M NaCl 2.8 ml, 100 mM Tris-HCl 1 ml, 20 mM EDTA 0.4 ml, β -mercaptoethanol 0.02 ml, (added just before use), PVP 0.2 ml], pH 8.0
SDS's buffer	[0.5% SDS 1.25 ml, 200 mM Tris HCl 1 ml, 25 mM EDTA 1 ml, 200 mM NaCl 1 ml], pH 8.0
Manitol isolation buffer	[100 mM Tris HCl 1 ml, 0.35 M Manitol 0.2 ml, 5 mM EDTA 0.4 ml, β -mercaptoethanol 0.1 ml and PVP-40 0.1 ml], pH 8.0
salt 3 $\times$ CTAB lysis buffer	[100 mM Tris HCl 0.1 ml, 3 M NaCl 0.4 ml, CTAB 0.3 g, 20 mM EDTA 0.02 ml, β -mercaptoethanol 0.02 ml and PVP-40 (0.1 ml)], pH 8.0
Sorbitol isolation buffer	[0.34 M Sorbitol 0.2 g, 0.1 M Tris-HCl 1 ml, 5 mM EDTA 0.4 ml, PVP 0.1 ml, β -mercaptoethanol 0.02 ml], pH 7.6
Sorbitol Lysis buffer	[0.2 M Tris-HCl 1 ml, 2 M NaCl 0.82 ml, 0.05 M EDTA 0.1 ml, CTAB 0.2 g, pH 7.6
TE buffer	[1.0 mM EDTA 0.0372 g, 10 mM Tris-HCl 0.1211 g, dissolved in distilled water, pH 8.0]

3 Results and Discussion

In molecular biology, high-quality genomic DNA extraction is an essential and a key step. Low-cost and time-efficient extraction protocols, however, are often hindered by two major challenges. (A) Due to the differences in compounds between different plant species i.e., varying compositions of polysaccharides, hydroxybenzenes, esters, and other secondary metabolites there is no universal extraction protocol. (B) Additionally, the crude tissue extract is reduced from secondary chemical reactions that might otherwise reduce DNA yield (**Kotchoni & Gachomo, 2009**).

Methods for purifying genomic DNA previously included phenol-chloroform treatments as described by Sambrook (**Sambrook, 1989**), and procedures based on tissue crushing in liquid nitrogen (**Li et al., 2013**). Despite their effectiveness, these methods are time-consuming, incur storage and costly to handle. Additionally, these methods utilize hazardous chemicals that require special handling and disposal.

In the present study, simple, safe, inexpensive and universal methods suited to DNA extraction in plants species of different phylum highlighted. Four DNA extraction methods were compared in this study; the CTAB method, the Edward method, Manitol and Sorbitol methods. The protocols were used to isolate genomic DNA from three distinct tissues i.e., roots, stem and leaves of three individual plants; garden moss '*P. patens*'; a native species to the bryophytes, *B. campestris*; an Angiosperm and Loblolly pine '*P. taeda*' a Gymnosperm. For each tissue type three replicates were used. In plant sciences, many researchers choose to isolate DNA from mitotically active tissues or from reproductive tissues. The reason to it is that in both circumstances, in the tissues at least some of the cells have replicated their DNA and thus, will yield higher DNA content. Hence, we tested tissues that were mitotically active, as newly grown hairy roots, newly evolved soft stem tissues and fresh growing leaves. The results showed that high-molecular weight DNA was obtained in large quantities and of good quality by all protocols.

For roots tissues, Nanodrop showed that the CTAB method consistently yielded the highest DNA across all tested species. *B. campestris* provided the most DNA with CTAB (156.9 ± 1 ng/ μ l), followed by *P. patens* (148.8 ± 1 ng/ μ l) and *P. taeda* (145.58 ± 1 ng/ μ l) (Figure 1). The DNA purity (A260/A280 ratio) for CTAB-extracted samples varied from 1.78 to 1.84, suggesting no protein contamination (Figure 2). These findings support previous research revealing that CTAB is an efficient method for plants rich in secondary metabolites (**Doyle & Doyle, 1987; Kotchoni & Gachomo, 2009**). The Manitol method provided appropriate yields, particularly effective for *B. campestris* (156.96 ± 1 ng/ μ l) and *P. patens* (143.9 ± 1 ng/ μ l) (Figure 1), with purity ratios ranging from 1.70-1.78 (Figure 2). Its compatibility with bryophytes supports earlier research emphasizing its efficacy in reducing polysaccharide contamination during lysis (**Varma et al., 2007**). The Sorbitol method produced significantly reduced DNA quantities for all

species, with *B. campestris* (151.5 ± 1 ng/ μ l), *P. patens* (146.3 ± 1 ng/ μ l) and *P. taeda* (142.3 ± 1 ng/ μ l) (Figure 1), with an A260/A280 ratio of 1.73-1.81 (Figure 2), indicating relatively small contamination. However, it is beneficial for woody plants, as Sorbitol functions as an osmotic stabilizer, facilitating nuclear separation (**Healey et al., 2014; Murray & Thompson, 1980**). The Edward protocol produced comparatively lowest DNA yields, particularly in *P. taeda* (133.5 ± 1 ng/ μ l) and *P. patens* (137.6 ± 1 ng/ μ l) however *B. campestris* showed better results (143.13 ± 1 ng/ μ l) (Figure 1), with satisfactory purity values (1.65-1.73) (Figure 2). Although it is rapid and cost-effective, its efficacy decreased significantly in samples with elevated concentrations of phenolic and polysaccharides, supporting previous findings (**Edward et al., 1991**).

The CTAB method exhibited higher effectiveness for stem tissues across all species, with *B. campestris* producing the highest DNA concentration (154.3 ± 1 ng/ μ l), followed by *P. patens* (146.7 ± 1 ng/ μ l) and *P. taeda* (144.2 ± 1 ng/ μ l) (Figure 1). The purity ratios varied between 1.80 and 1.84 (Figure 2), signifying negligible protein contamination. The findings validate previous studies indicating that CTAB efficiently eliminates polysaccharides and polyphenols from metabolically active stem tissues (**Doyle & Doyle, 1987; Porebski et al., 1997**). The Manitol method yielded relatively good results, especially in *B. campestris* (148.9 ± 1 ng/ μ l) and *P. patens* (142.5 ± 1 ng/ μ l), with purity values ranging from 1.74 to 1.79. The Sorbitol method yielded intermediate results (147.2 ± 1 ng/ μ l in *B. campestris*; 143.7 ± 1 ng/ μ l in *P. patens*; 140.8 ± 1 ng/ μ l in *P. taeda*) while exhibiting acceptable purity (1.76-1.80). These findings highlight Sorbitol's function as a stabilizer for starch-rich tissues, enhancing DNA quality in structural organs such as stems (**Healey et al., 2014**). In contrast, the Edward method yielded the lowest concentrations (137.8 - 142.5 ng/ μ l) while maintaining acceptable purity (1.68-1.73), supporting earlier studies indicating that this enhanced protocol is less efficient in complex plant specimens (**Edward et al., 1991**).

In leaf tissues, the CTAB method yielded the highest DNA concentrations in leaf tissues, measuring 152.1 ± 1 ng/ μ l in *B. campestris*, 145.6 ± 1 ng/ μ l in *P. patens*, and 143.9 ± 1 ng/ μ l in *P. taeda* (Figure 1). The A260/280 ratios (1.81-1.85) validated the high quality of DNA, demonstrating the effective use of CTAB in tissues rich in secondary metabolites including chlorophyll and phenolic compounds (**Kotchoni & Gachomo, 2009**). Manitol-based extractions produced relatively low concentrations (140 - 147 ng/ μ l across species), with purity levels between 1.73 and 1.78. The Sorbitol method consistently produced lower yields compared to CTAB, although proved effective in *B. campestris* (144.9 ± 1 ng/ μ l), indicating its appropriateness for leaf tissues where vacuolar contents may disrupt DNA extraction (**Varma et al., 2007**). The Edward method yielded the lowest concentrations (133.9 - 140.4 ng/ μ l) and average purity ratios of 1.70, indicating that, although convenient, this method is inadequate for leaves containing elevated quantities of interfering chemicals (**Edward et al., 1991; Murray & Thompson, 1980**).

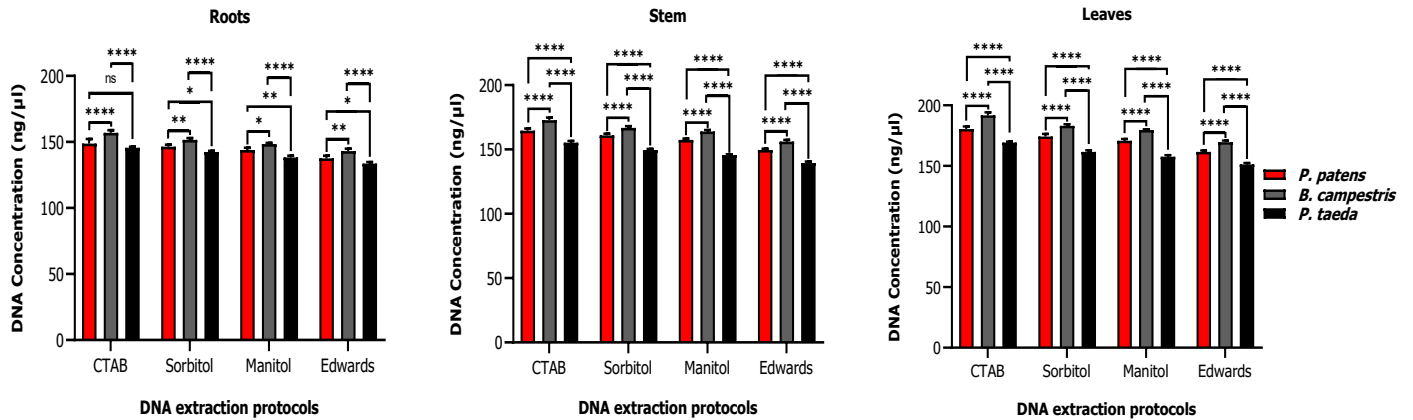


Figure 1 Comparison of DNA yield across three plant species (*Physcomitrella patens*, *Brassica campestris*, and *Pinus taeda*) using four extraction protocols (CTAB, Sorbitol, Manitol, and Edward). Tukey's multiple comparison test was applied to assess interspecies differences. Error bars indicate standard deviation. Significance is indicated as follows: ns ($p > 0.05$); * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

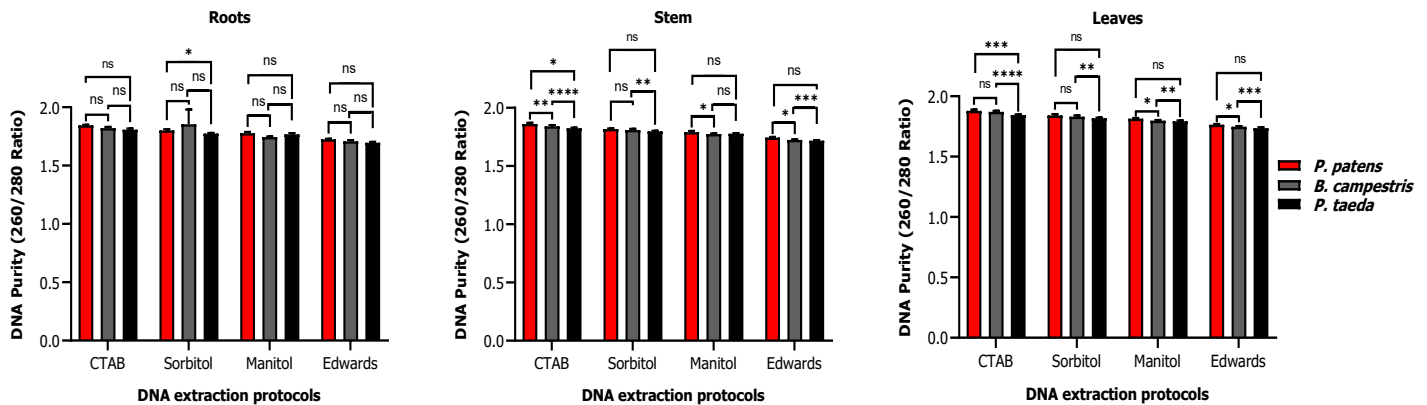


Figure 2 Comparison of DNA purity (A260/280 ratio) across three plant species (*Physcomitrella patens*, *Brassica campestris*, and *Pinus taeda*) using four extraction protocols (CTAB, Sorbitol, Manitol, and Edward). Tukey's multiple comparison test was applied to assess interspecies differences. Error bars indicate standard deviation. Significance is indicated as follows: ns ($p > 0.05$); * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

Agarose gel electrophoresis demonstrated clear bands across all methods; however, the CTAB and Sorbitol methods yielded more pronounced, high-molecular-weight DNA bands without smearing, suggesting minimal shearing. The Edward method exhibited weak bands, especially in gymnosperm samples, indicating potential degradation or inadequate extraction. Sorbitol-based extractions exhibited moderate band purity, aligning with its intermediate yield (Figure 3).

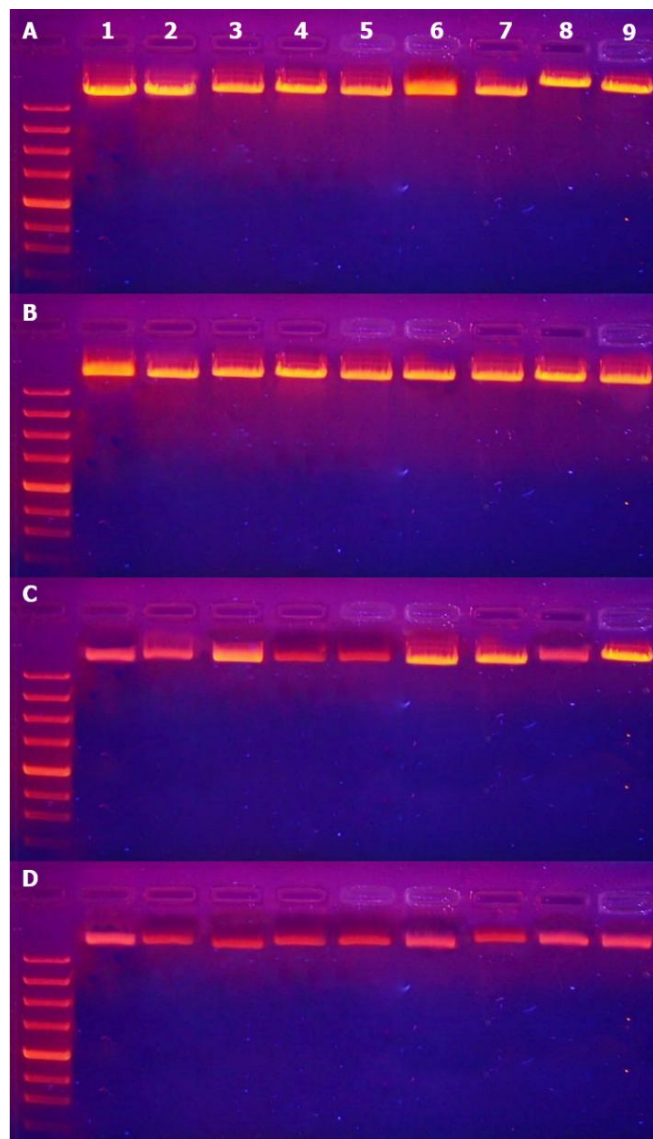


Figure 3 Agarose gel electrophoresis showing genomic DNA extracted from plant tissues using four different DNA extraction protocols. Lanes 1-3 shows DNA extracted from roots, stem, leaves of *Physcomitrella patens* lanes 4-6 from *Brassica campestris*, and 7-9 from *Pinus taeda*. A; CTAB method, B; Sorbitol method, C; Manitol method, D; Edward method. The DNA samples were run on a 1% agarose gel stained with ethidium bromide and visualized under UV light. A molecular weight DNA ladder (leftmost lane) was used to compare fragment size.

The quality and suitability of the genomic DNA isolated using the CTAB technique were assessed using PCR amplification and sequencing. The successful amplification of the target regions was confirmed by the presence of clear and distinct bands on agarose gel (Figure 4), which means that the isolated DNA was yielded enough, and it did not contain any inhibitory contaminants. The resultant sequences were used to construct a

strong phylogenetic tree (Figure 5), thus further ensured that the DNA had the required purity and concentration to be used in downstream molecular applications. Together, these findings emphasize that DNA isolated with the CTAB method is reliable to undergo PCR- and sequencing-based studies.

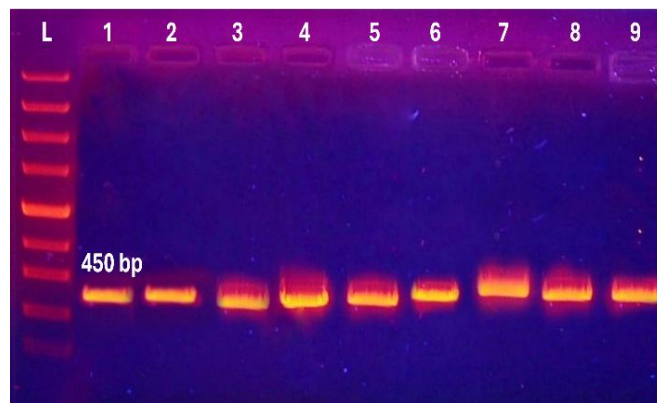


Figure 4 Agarose gel electrophoresis (1.5%) of PCR amplification of the 18S rRNA gene from genomic DNA isolated using the CTAB method. A ~450 bp amplicon was consistently obtained across all samples. The 200 bp DNA ladder is shown in the leftmost lane for size estimation. Lanes 1-3 correspond to PCR products amplified from root, stem, and leaf tissues of *Physcomitrella patens*, respectively; lanes 4-6 represent amplification from root, stem, and leaf tissues of *Brassica campestris*; and lanes 7-9 show amplification from root, stem, and leaf tissues of *Pinus taeda*. The presence of clear, single bands of the expected size indicates high-quality, PCR-amplifiable DNA across all tissue types and species.

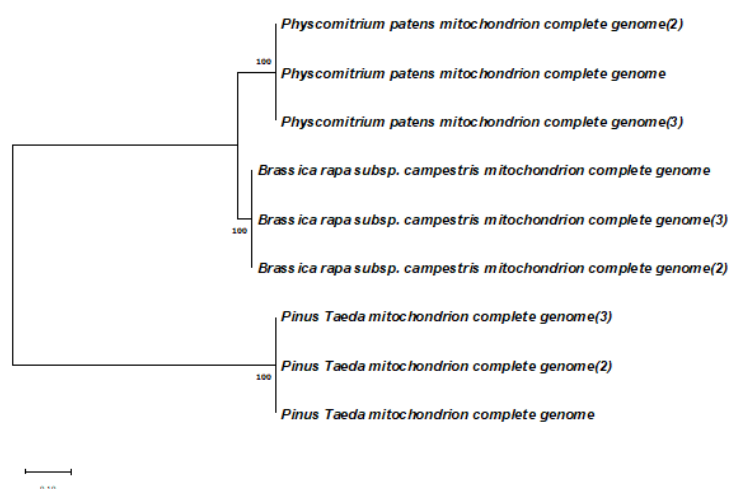


Figure 5 Phylogenetic tree inferred from complete mitochondrial genome sequences of *Physcomitrium patens*, *Brassica rapa subsp. campestris*, and *Pinus taeda*. Replicate sequences of each species cluster together into distinct clades. Bootstrap support values (expressed as percentages from 1000 replicates) are shown at the nodes, with values of 100 indicating strong support for the corresponding branches. The scale bar represents substitutions per site, indicating evolutionary distance among the taxa.

Statistical Analysis

A two-way ANOVA, followed by Tukey's post hoc multiple comparisons test, was applied to evaluate the effects of both species and extraction protocols on yield and purity of DNA isolated by four DNA extraction methods (CTAB, Sorbitol,

Manitol, and Edward) across three plant species (*P. patens*, *B. campestris*, and *P. taeda*). The findings revealed significant main effects of species ($p < 0.001$) and protocol ($p < 0.001$), along with a notable species $\times$ protocol interaction ($p < 0.05$), suggesting that the efficiency of all methods varied among plant species. Tukey's HSD post hoc comparisons indicated that the CTAB method consistently yielded considerably higher results ($p < 0.01$) in all tissues, whereas Edward demonstrated the lowest values across species. CTAB and Sorbitol yielded significantly elevated A260/A280 ratios (1.78-1.85; $p < 0.05$) for DNA purity, indicating minimal protein contamination, while Manitol and Edward exhibited marginally lower however acceptable purity values (1.69-1.75) (Table 3). A comparative study of tissues revealed that root extractions exhibited the highest overall concentrations, followed by stems and leaves, with leaves demonstrating greater variations in purity parameters. These statistical results confirm that protocol effectiveness is dependent upon tissue type and species, highlighting the advantage of CTAB for optimal DNA extraction from plants containing complex secondary metabolites.

Source of Variation	df	F-value (Concentration)	p-value (Concentration)	F-value (Purity)	p-value (Purity)
Species (S)	2	28.64	<0.001 ***	14.72	<0.001 ***
Tissue (T)	2	19.53	<0.001 ***	8.91	0.002 **
Protocol (P)	3	42.87	<0.001 ***	21.65	<0.001 ***
Species $\times$ Tissue	4	4.12	0.007 **	2.89	0.032 *
Species $\times$ Protocol	6	6.54	<0.001 ***	3.27	0.011 *
Tissue $\times$ Protocol	6	5.71	<0.001 ***	3.04	0.014 *
Species $\times$ Tissue $\times$ Protocol	12	122.36	0.018 *	1.92	0.046 *
Error	72				

Table 3. Two-way ANOVA results for DNA concentration (ng/ μ l) and purity (A260/A280 ratio) across species, tissues, and extraction protocols.

Values represent results from two-way ANOVA testing the effects of plant species, tissue type, and DNA extraction protocol on DNA concentration and purity. Significant effects are indicated as $p < 0.05$, $p < 0.01$, $p < 0.001$.

4 Conclusions

This study provides a thorough evaluation of four genomic DNA extraction protocols. The results demonstrate that while all methods provided amplifiable genomic DNA of good quality and integrity, the CTAB method consistently exceeded the other protocols in yield, purity, and reliability across all examined species. CTAB produced the highest quantities (up to 156.9 ng/ μ l) and preferable purity ratios (A260/A280 between 1.78 and 1.84), thereby demonstrating its effectiveness for plants abundant in secondary metabolites. For different plants species, the use of different ingredients and choice of protocols interfere in the result of genomic DNA extraction. Among all methods, Buffer containing CTAB was the most efficient, whereas the liquid nitrogen use was also proved to be a non-necessary factor for obtaining DNA in adequate quality and quantity.

Statements and Declarations

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Not applicable.

Conflict of interests

The author declare that they have no competing interests.

Data availability statement

All data generated and analyzed during this study are included in the main manuscript and its supplementary materials. No additional datasets were generated or deposited in external repositories.

Authors' contributions

Jawad Ahmad supervised the work, conceived and designed the study, conducted the laboratory work and analyzed the data, drafted the initial manuscript, contributed to statistical analysis and data interpretations.

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