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A simple ethanol wash of the tissue homogenates recovers high-quality genomic DNA from *Corchorus* species characterized by highly acidic and proteinaceous mucilages

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Abstract A simple Miniprep based on early elimination of highly acidic and proteinaceous mucilages through ethanol washing of the tissue homogenates has been developed for the extraction of genomic DNA from mature leaves and seeds of *Corchorus* spp. As compared to high cetyltrimethylammonium bromide (CTAB)-NaCl DNA extraction followed by ethanol-based removal of remnant mucilages from the DNA pellet, this simple miniprep consistently and reproducibly recovers high amounts of DNA with good spectral qualities at A_{260}/A_{280} and A_{260}/A_{230}. The purified DNA is efficiently digested by restriction endonucleases, and is suitable for PCR amplification of nuclear microsatellites with expected allele sizes.

Keywords: *Corchorus,* DNA extraction, microsatellite, mucilage, PCR, restriction digestion

INTRODUCTION

The hydrocolloid mucilage is a complex mucopolysaccharide, with a highly branched structure containing varying proportions of L-arabinose, D-galactose, L-rhamnose, D-xylose as well as galactouronic acid (Sepúlveda et al. 2007). It is typically characterized by high viscosity that imparts a slimy consistency when released in solutions. Mucilages, therefore, represent one of the most unwanted contaminants affecting extraction and purification of high-quality genomic DNA in a large number of crop species across plant families (Varma et al. 2007). They cause the maceration of tissues with a small volume of extraction buffer highly cumbersome, even with prior freezing of tissues in liquid nitrogen or at -80ºC. They also make the extracted DNA unmanageable in pipetting and produce either an extremely viscous DNA pellet or a brownish slimy consistency during isopropanol precipitation and in the final elution in the tris ethylenediaminetetraacetic acid (TE) buffer. This results in not only low DNA yield, but also poor-quality DNA, which is neither suitable for digestion with restriction endonucleases nor amenable to amplification by Taq polymerase chain reaction *(*PCR) because the polysaccharides prevent access of enzymes to DNA molecules (Fang et al. 1992; Barnwell et al. 1998).

Mucilages of *Corchorus* spp. including *C. capsularis* L. (the white jute) and *C. olitorius* L. (the tossa jute), which are an important source of bast fibre, are highly acidic and proteinaceous (Stephen et al. 2006). They are rich in uronic acid (65%) and consisted of rhamnose, glucose, galactouronic acid and glucuronic acid in a molar ratio of 1.0:0.2:0.2:0.9:1.7 in addition to the acetyl (3.7%) group (Khan et al. 2006). The yield of this hydrocolloid from leaves of *C. olitorius* has been estimated to be as high as 4.5% (w/w) based on dry mass (Yamazaki et al. 2009). In *Corchorus* spp., the DNA is most often found to be contaminated with protein, possibly due to highly proteinaceous nature of mucilages. In addition, mature leaves of *Corchorus* spp. produce increased quantities of mucilages due to the presence of

wide mucilage canals, which are formed as the surrounding mucilage cells dissolve (Kundu et al. 1959). Because of conformational changes, there occurs an irreversible increase in viscosity of the hydrocolloid from leaves of *C. olitorius* at >60ºC (Yamazaki et al. 2009), a temperature usually used for cell lysis during DNA isolation from plant tissues. Therefore, extraction of genomic DNA from mature *Corchorus* leaves in the absence of young leaves during the specific time of collection vis-à-vis developmental stage is virtually impossible.

There are several modifications of the basic cetyltrimethylammonium bromide (CTAB) protocol adapted for isolation of genomic DNA from mucilaginous plant tissues (Barnwell et al. 1998; Echevarría-Machado et al. 2005; Cota-Sánchez et al. 2006). In essence, these adaptations rely on avoiding co-precipitation of polysaccharides with the DNA by keeping them in solution while precipitating the DNA using a selective precipitant, such as CTAB with (Cota-Sánchez et al. 2006) or without (Barnwell et al. 1998) a high concentration of NaCl. Other methods, which are based on DNA extraction using silica (Rogstad, 2003; Echevarría-Machado et al. 2005), are not only expensive, but also tedious and technically demanding. For jute species, an increased volume of CTAB extraction buffer combined with dissolving crude nucleic acid pellet in 1 M NaCl has been reported to reduce the viscosity of the mucilage (Ghosh et al. 2009).

For mucilages, however, ethanol shows the highest solubility (Iturriaga et al. 2009). It is not only cheap and readily available, but also atoxic as compared to the other solvents. This led us to develop a very simple, quick and cost-effective DNA miniprep from mature leaves and seeds of *Corchorus* species based on ethanol-based early removal of proteinaceous mucilages from the tissue macerates followed by CTAB extraction and isopropanol precipitation of high-quality DNA suitable for PCR amplification and restriction enzyme digestion. This method has been compared with a DNA miniprep based on high CTAB-NaCl extraction followed by ethanol-based removal of leftover mucilages after isopropanol precipitation of the DNA.

MATERIALS AND METHODS

Plant material

Eight *Corchorus* spp., viz., *C. aestuans* L. (accession no. WCIJ-037), *C. capsularis* L. (the white jute) cv. JRC-212 and mutant CMU-010, *C. fascicularis* Lam. (accession no. WCIJ-004), *C. olitorius* L. (the tossa jute) cv. JRO-524 and mutant PPO-4, *C. pseudo-capsularis* Schweinf. (accession no. WCIJ-031), *C. pseudo-olitorius* Islam & Zaid. (accession no. WCIJ-034), *C. tridens* L. (accession no. WCIJ-047)

Table 1. Information on eight SSRs characterized in *Corchorus* spp. to assess the quality and performance of the DNA extracted from leaves and seeds using two different DNA minipreps.

Accession no.	nSSR motif	Primer sequence 5' - 3'	Allele size (bp)	T_a (ºC)
MJM 432	$(ac)_8$	CAAGCTTCTGCAGGTATGCTC GGACTGAGATGGCAACAGTCT	186	57
MJM 513	$(ac)_{12}$	TCTTGTGCCAAGGTATCCATC TCACGAGAAGAGCGAATTGAT	356	56
MJM 519	$(gt)_6$	AGCATGCTAACTTGAAGACGC TGGAAGATCAGAGGGTCAACA	182	56
MJM 561	$(gt)_{16}$	AGTGCAAACACGAGAGCAAAT ATGGCATCCTCTCATCTTCCT	311	56
MJM 615	$(tc)_{15}$	AGGATCCATGGGAAATAATCG CTTGTCGACACCCTTCAATGT	218	56
MJM 623	$(ct)_{16}$	TTCTGCAGTTGTCTCCCTGTT ACGAGAAGACACAGTGGTGCT	319	56
MJM 630	$(ta)_9$	AGAACACGTTCACCAGACAGC TTCTTGATTCCAAACTGGGTG	389	57
MJM 652	$(tc)_{23}$	ATTCTTAGATCCGGCCATGTT CCGGTCTTAATTCAGGCCTAT	236	56

MJM (Meerut jute microsatellites).

and *C. trilocularis* L. (accession no. WCIJ-022) were used in the present study. The plants were grown in the experimental field of the Central Research Institute for Jute and Allied Fibres (CRIJAF), Kolkata, India (22.45ºN, 88.26ºE; 3.14 m above msl) during the summer (March-September; mean day/ night temperature: 32.7-24.2ºC; RH: 68.8-93.7%) following the recommended cultural practices.

Genomic DNA minipreparation

The 4th leaves from the top of 60-day-old field-grown plants and one-year-old seeds stored (6ºC) at the active collection of the institute germplasm repository were used for DNA extraction.

Fresh leaf material (0.2-0.3 g) and seeds (0.2-0.3 g) were finely macerated, without freezing in liquid nitrogen/ -80ºC, in a pre-chilled (-20ºC) mortar using CTAB extraction buffer (100 mM Tris-HCl and 20 mM EDTA, pH 8.0) with varying salt concentrations (see below). All chemicals and reagents, except for ethanol (Merck KGaA, Darmstadt, Germany), used in the study were of molecular biology-grade from Sigma-Aldrich (St. Louis, Missouri, USA).

Miniprep I

The tissue was macerated in 1.0 ml of 1.4 M NaCl-2% CTAB and then transferred into a 2.0 ml Eppendorf microcentrifuge tube followed by the addition of 1.0 ml of 100% ethanol. The mixture was gently vortexed, centrifuged at 5000 x *g* for 6 min, and the supernatant was discarded. After adding 1.0 ml of 1.0 M NaCl-5% CTAB, the mixture was incubated at 60ºC for 1 hr in a water bath, allowed to cool, and 1.0 ml dichloromethane was added (gently mixed) followed by centrifugation at 5000 x *g* for 15 min. The aqueous phase was carefully transferred into a 1.5 ml microcentrifuge tube, and 1.0 ml of 2-propanol was added to precipitate the DNA, which was collected by centrifugation at 5000 x *g* for 4 min. The DNA pellet was washed once with 500 µl of 100% ethanol, centrifuged at 5000 x *g* for 4 min and stored overnight suspended in 500 µl of 100% ethanol (ice cold) at -20ºC. The next day, after centrifugation at 5000 x *g* for 4 min, the DNA pellet was washed with 70% ethanol, dried at room temperature inside a Laminar flow clean air work station, dissolved in 60 µl of TE buffer (pH 8.0) at 60ºC for 2 hrs and kept at 4ºC for immediate use or at -20ºC for long-term storage.

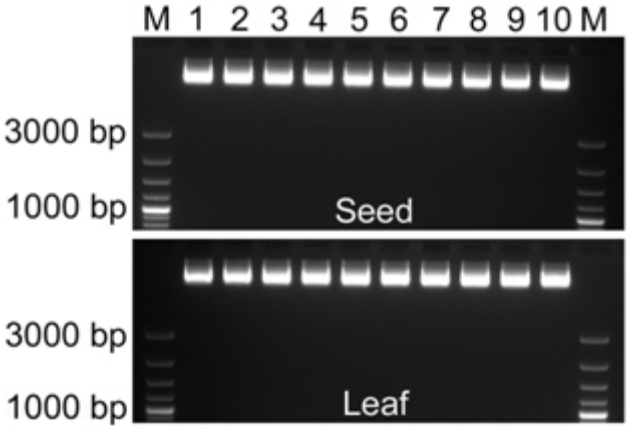

Fig. 1 Agarose (0.9 %) gel of high molecular weight genomic DNA isolated form mature leaves and seeds of *Corchorus* species using DNA Miniprep I. M: 100-bp DNA ladder. 1: *Corchorus aestuans*. 2: *C. capsularis* mutant CMU-010. 3: *C. capsularis* cv. JRC-212. 4: *C. fascicularis*.5: *C. olitorius* cv. JRO-524. 6: *C. olitorius* mutant PPO4. 7: *C. pseudo-capsularis*. 8: *C. pseudo-olitorius*. 9: *C. tridens*. 10: *C. trilocularis*.

Miniprep II

The tissue was macerated in 1.0 ml of 2.5 M NaCl-5% CTAB, transferred into a 2.0 ml Eppendorf microcentrifuge tube and incubated at 60ºC for 1 hr in a water bath. The DNA was finally precipitated by dichloromethane-propanol steps following the same method as in DNA miniprep I. The DNA pellet

was incubated with 500 µl of 100% ethanol at room temperature for 30 min followed by centrifugation at 5000 x *g* for 4 min. The DNA was stored and processed, according to miniprep I.

Analyses of DNA yield and quality

For each sample, 2 µl of DNA was drawn and diluted 1:50 in ultrapure water. DNA concentration was measured twice at 260 nm in a UV spectrophotometer (BioPhotometer; Eppendorf AG, Hamburg), with absorbance also recorded at 230 and 280 nm. DNA yield was calculated by multiplying the mean concentration and hydration volume. The A_{260}/A_{280} and A_{260}/A_{230} ratios were used to assess DNA quality in terms of protein and carbohydrate/ polyphenolics contamination, respectively. DNA was also run on a 0.9% agarose gel against a known molecular weight marker and stained with ethidium bromide (0.5 µg ml^{-1}) for visualization.

Restriction analysis

The DNA extracted from leaves as well as seeds were digested by three restriction endonucleases, *viz.,* EcoRI, HpaII and MspI (New England BioLabs Inc., Ipswich, USA). The restriction mix (20 µl:1 x restriction buffer, 10-20 U restriction enzyme and 2-20 µg genomic DNA) was incubated overnight at 37ºC. The digested DNA was fractionated in 0.9% agarose gels in 0.5 x neutral electrophoresis buffer (0.1 M Tris, 0.9 mM EDTA and 12.5 mM sodium acetate; pH 8.1) with 0.5 µg ml^{-1} ethidium bromide and visualized under a UV trans-illuminator.

Microsatellite analysis

The eight simple sequence repeats (SSRs) developed from SSR-enriched genomic library of *C. olitorius* cv. JRO-524 (Table 1) were characterized to assess the quality and PCR-compatibility of the DNA extracted from leaves and seeds of *Corchorus* species. The PCR reaction (20 µl:1 x assay buffer, 2.5 mM $MgCl_2$, 200 µm of each dNTP, 1.0 U Taq polymerase (Bangalore Genei, Bangalaru, India), 0.2 µM of each primer and 50.0 ng of template DNA) was of 30 cycles at 94ºC for 1 min, T_aºC for 1 min and 72ºC for 1 min followed by a final extension at 72ºC for 5 min (MyCyclerä, Bio-Rad, Hercules, USA). Amplified products were separated in 3% Metaphor agarose (Cambrex Bio Science Rockland, Inc., Rockland, USA) gels in 1.0 x TBE buffer with 0.5 µg ml^{-1} ethidium bromide at 4 V cm^{-1} constant voltage and visualized under a UV trans-illuminator.

Table 2. Spectral assessment of the quality and quantity of the DNA extracted from highly mucilaginous mature leaves of *Corchorus* spp. using two different DNA minipreps.

Genotype	DNA amount (µg g^{-1} fresh mass)		A_{260}/A_{280}		A_{260}/A_{230}	
	Miniprep I	Miniprep II	Miniprep I	Miniprep II	Miniprep I	Miniprep II
C. aestuans	306.8	376.6^{ns}	1.9	1.8^{ns}	1.7	1.5*
C. capsularis cv. JRC-212	166.4	431.1^{ns}	1.8	2.0^{ns}	1.7	1.2*
C. capsularis mt. CMU-010	548.9	500.2^{ns}	2.0	1.9*	2.1	1.9^{ns}
C. fascicularis	271.0	322.0^{ns}	1.8	1.8^{ns}	1.5	1.5^{ns}
C. olitorius cv. JRO-524	278.6	360.2^{ns}	2.0	1.9^{ns}	2.0	1.8^{ns}
C. olitorius mt. PPO-4	358.7	482.8^{ns}	2.0	1.9	1.9^{ns}	1.9^{ns}
C. pseudo-capsularis	132.3	348.5**	1.8	2.0	1.6*	1.7^{ns}
C. pseudo-olitorius	315.9	224.8^{ns}	1.8	1.7^{ns}	1.7	1.5^{ns}
C. tridens	163.3	293.2^{ns}	1.8	1.8^{ns}	1.3	1.3^{ns}
C. trilocularis	162.3	316.1*	1.6	1.7^{ns}	1.2	1.3

*,** and ns = significantly different at $P \leq 0.05$ and 0.01, respectively and not significant at $P \leq 0.05$, according to Student's unpaired t-test.

Statistical analysis

For each genotype, there were three replications per miniprep. Data on DNA yields and absorbance ratios at A_{260}/A_{280} and A_{260}/A_{230} were analyzed by Student's unpaired *t*-test using the statistical software SigmaStat Version 2.03 (SPSS, Inc., Chicago, USA).

RESULTS AND DISCUSSION

Using basic CTAB miniprep, genomic DNA could not be extracted from mature leaves and seeds of *Corchorus* species because of the presence of gelling mucilages rendering the entire extraction process including handling and pipetting unmanageable. Even when the viscous DNA pellet was dissolved in the TE buffer, it was found to be unsuitable for endonuclease restriction digestion as well as microsatellite-based PCR amplification (results not shown). Both the minipreps tested in this study yielded large amounts of mucilage-free genomic DNA from mature leaves and seeds of *Corchorus* species (Table 2 and Table 3). As high as ~549 and ~500 µg DNA g^{-1} fresh leaf mass were extracted using minipreps I and II, respectively. As expected, seeds, which are characterized by comparatively low mucilage content, yielded higher amounts of DNA using both the minipreps (Table 3). However, miniprep I based on ethanol-based early removal of mucilages from the tissue macerates prior to CTAB extraction was found to be more reproducible and consistent than miniprep II in terms of DNA yield (Figure 1). Miniprep II based on late ethanol-based removal of remnant mucilages from the DNA pellets resulting from high CTAB-NaCl extraction did not produce reproducible DNA yields over minipreparations. Early removal of mucilages might have made the miniprep more manageable in terms of handling and pipetting, thus resulting in less loss of DNA at the early stages of the extraction process vis-à-vis more consistent DNA yields over minipreparations.

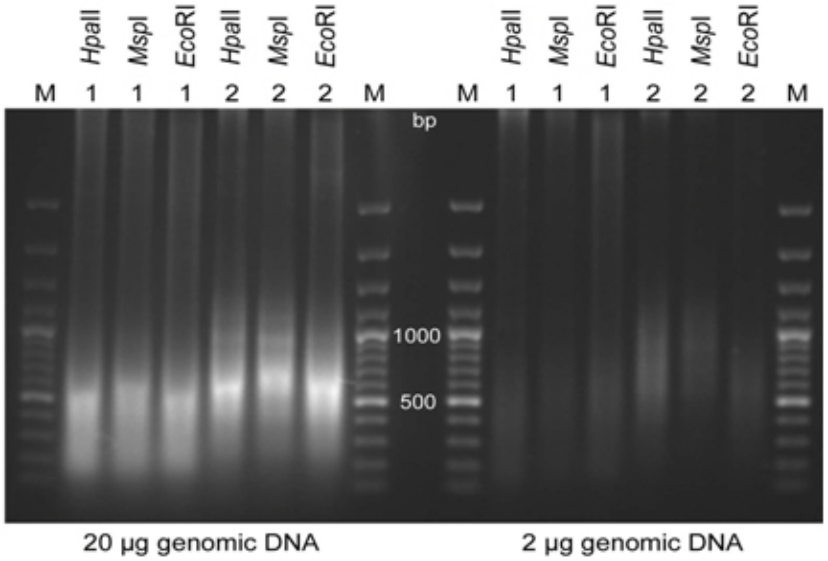

Fig. 2 Restriction endonuclease digest of genomic DNA extracted from mature leaves of *Corchorus* species using DNA Miniprep I. M: 100-bp DNA ladder. 1: *Corchorus pseudo-olitorius*. 2: *C. olitorius*.

Spectral analysis showed that absorbance ratios at A_{260}/A_{280} were between 1.8 and 2.0 for all *Corchorus* species, except for *C. trilocularis* when the DNA was extracted from highly mucilaginous mature leaves using miniprep I (Table 2). In contrast, A_{260}/A_{280} ratios of the DNA extracted from mature leaves using miniprep II were found to be <1.8 in *C. capsularis* cv. JRC-212, *C. pseudo-olitorius* and *C. trilocularis*. For miniprep I, absorbance ratios of the leaf DNA at A_{260}/A_{230} were between 1.5 and 2.1 for all *Corchorus* species, except for *C. pseudo-capsularis*, *C. tridens* and *C. trilocularis*. Similar to A_{260}/A_{280} ratios, in some *Corchorus* species, A_{260}/A_{230} ratios were significantly lower for the DNA fraction extracted by miniprep II than by miniprep I. The DNA extracted from seeds using both minipreps I and II showed good spectral quality at A_{260}/A_{280} (Table 3). However, in general across

Table 3. Spectral assessment of the quality and quantity of the DNA extracted from highly mucilaginous seeds of *Corchorus* spp. using two different DNA minipreps.

Genotype	DNA amount (µg g^{-1} fresh mass)		A_{260}/A_{280}		A_{260}/A_{230}	
	Miniprep I	Miniprep II	Miniprep I	Miniprep II	Miniprep I	Miniprep II
C. aestuans	815.0	694.5^{ns}	1.9	1.8^{ns}	1.4	1.3^{ns}
C. capsularis cv. JRC-212	1262.6	1051.2*	1.8	2.0^{ns}	1.5	1.9**
C. capsularis mt. CMU-010	1013.9	817.2^{ns}	2.0	1.9^{ns}	2.0	1.5^{ns}
C. fascicularis	1148.8	650.2*	2.0	1.8**	2.2	1.3**
C. olitorius cv. JRO-524	1093.2	609.6**	2.0	1.9^{ns}	2.0	1.7^{ns}
C. olitorius mt. PPO-4	960.6	613.9*	2.0	1.9**	1.9	1.6^{ns}
C. pseudo-capsularis	1455.2	846.9^{ns}	1.8	1.7*	1.6	1.1**
C. pseudo-olitorius	297.1	638.3**	1.7	1.7^{ns}	1.0	1.1^{ns}
C. tridens	1165.2	850.9^{ns}	1.9	1.8^{ns}	1.8	1.5^{ns}
C. trilocularis	482.1	432.1^{ns}	1.9	1.8^{ns}	1.7	1.8^{ns}

*,** and ns = significantly different at P ≤0.05 and 0.01, respectively and not significant at P ≤0.05, according to Student's unpaired t-test.

species, seed DNA extracted by miniprep I was of better spectral quality at A_{260}/A_{230} than that extracted by miniprep II. Good spectral qualities of the DNA isolated from both leaves and seeds at A_{260}/A_{280} suggested very little contamination of the DNA fraction by proteins (Barnwell et al. 1998; Michiels et al. 2003). Similarly, A_{260}/A_{230} ratios indicated relatively less contamination of the DNA fraction by polysaccharides and aromatic compounds (Michiels et al. 2003) when the DNA was extracted by miniprep I. In general, *Corchorus* species, particularly wild species, are rich source of polyphenolics, aromatic compounds and other characterized or uncharacterized secondary metabolites including glycosidic compounds referred to as corchorin (Khan et al. 2006). This may be the reason for low A_{260}/A_{230} values of the DNA fractions in some wild *Corchorus* species, such as *C. fascicularis*, *C. pseudo-capsularis*, *C. trilocularis*, etc. Polyphenolics and secondary metabolites are known to detrimentally affect DNA extraction in many plant species (Michiels et al. 2003; Weising et al. 2005).

DNA extracted from mature leaves and seeds of *Corchorus* species using both the minipreps was suitable for endonuclease restriction digestion. Using the three different endonucleases (EcoRI, HpaII and MspI), complete restriction digestion was ensured for small and large quantities of leaf DNA extracted by both minipreps I and II (Figure 2). Except for *C. pseudo-olitorius*, seed DNA extracted by miniprep I was suitable for endonuclease restriction digestion. In comparison, seed DNA extracted by miniprep II was found to be less amenable to endonuclease restriction digestion (results not shown). This may be due to the presence of interfering compounds preventing access of endonucleases to DNA (Barnwell et al. 1998; Michiels et al. 2003). Similar results were obtained for microsatellite-based PCR amplification analysis. Genomic DNA isolated from mature leaves of *Corchorus* species using both the minipreps were PCR-compatible, as revealed by nuclear microsatellite analysis based on eight simple sequence repeats (Table 1). For all nuclear SSRs, expected alleles (sizes in bp) were amplified (Figure 3).

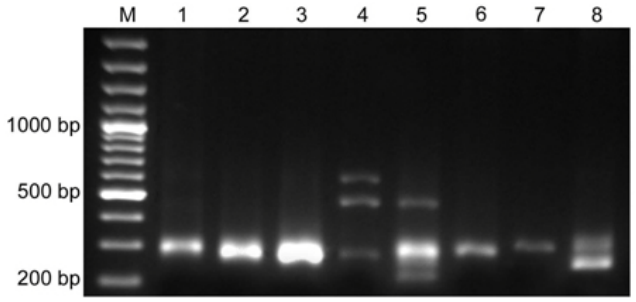

Fig. 3 Microsatellite (primer MJM 561)-based PCR amplification of an expected 311 bp product, from genomic DNA isolated from mature leaves of *Corchorus* species using DNA Miniprep I. M: 100-bp DNA ladder. 1: *Corchorus aestuans*. 2: *C. capsularis* mutant CMU-010. 3: *C. capsularis* cv. JRC-212. 4: *C. fascicularis*. 5: *C. olitorius* cv. JRO-524. 6: *C. olitorius* mutant PPO4. 7: *C. pseudo-capsularis*. 8: *C. pseudo-olitorius.*

In summary, we report that early elimination of mucilages from the tissue homogenates by ethanol wash is a simple and cost-effective method for DNA extraction from highly mucilaginous mature leaves and seeds of *Corchorus* species. Besides ensuring consistent DNA yields, this method recovers high-quality genomic DNA for downstream applications involving restriction endonuclease digestion and/or PCR-based amplification.

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