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A Modified Protocol for Isolation of High-Quality DNA Suitable for Molecular Marker Analysis and Genome Sequencing in *Moringa oleifera* Lam. and Wild Relative *Moringa concanensis* Nimmo ex Dalzell & A. Gibson

Garima[1], R. Geeta [1, 2] and Priya Panjabi*[1]
[1]*Department of Botany, University of Delhi, Delhi–110 007, India*
[2] *Present address: Besant Nagar, Chennai–600 090, India*

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ABSTRACT

DNA extraction is the preliminary step in plant molecular biology experiments. The quality and quantity of extracted DNA plays a crucial role in the success of further experimental steps. In the present study, owing to the high content of polysaccharides and polyphenols in tissue (leaves, fruit flesh) of *Moringa oleifera* and its wild relative *M. concanensis,* both the quality and quantity of DNA extracted using the commonly available DNA isolation protocols was found to be low and degraded under storage, and therefore not suitable for further molecular biological studies. A CTAB-based protocol was modified using higher levels of CTAB, NaCl, PVP and βME, , to extract high quality DNA from smaller quantities of tissue of cultivated and wild forms of *M. oleifera,* and *M. concanensis*. The modified protocol enabled isolation of DNA from different samples of *M. oleifera* including fresh leaf samples harvested from seedlings and mature plants, dried leaf samples preserved in silica gel or herbarium specimens and also from fresh/ frozen fruit pulp. Both the quality and the yield of the extracted DNA, estimated by NanoDrop, was better using the modified protocol when compared to previous protocols. The isolated DNA did not degrade for at least 3 years when stored at -20°C. The DNA quality was seen in successful PCR amplifications and whole genome sequencing. The DNA extraction protocol reported here will help researchers to isolate high quality, RNA-free DNA in good quantities for molecular research applications in species of *Moringa*.

Key words: CTAB, genomic DNA, Moringa, polyphenols, polysaccharides, PVP.

***Author for correspondence: Priya Panjabi; e-mail: ppanjabi09@gmail.com**

Abbreviations: βME: beta-mercaptoethanol; CI: chloroform: Isoamyl alcohol; CTAB: cetyltrimethylammonium bromide; EDTA: ethylenediaminetetraacetic acid; MOC: cultivated *Moringa oleifera*; MOW: wild *Moringa oleifera*; MC: *Moringa concanensis*; PCI: phenol: chloroform: isoamyl alcohol; PVP: polyvinylpyrrolidone.

Introduction

The tree *Moringa oleifera* Lam. (family Moringaceae) is a nutritionally important angiosperm that is rich in bioactive phytochemicals of potential medicinal value (e.g., Saini et al., 2016; Abd Rani et al., 2018; Chodur et al., 2018; Fahey et al., 2018). The tree is cultivated in the tropics worldwide, but also grows wild in eastern Pakistan, north-western parts of India; the closely related wild relative, *M. concanensis*, occurs across India, Bangladesh and Pakistan (Olson 2002, 2017).

There is a great deal of interest in the genetic diversity of these species that has led to a range of DNA-based studies. In most genetic and genomic studies in *M. oleifera*, isolation of genomic DNA has been done using diverse CTAB methods including Doyle and Doyle (1987) (Ferdous, 2012; Rufai et al., 2013; Shahzad et al., 2013; Ganesan et al., 2014; Rajalakshmi et al., 2017; Singh et al., 2019; Rajalakshmi et al., 2019; Paul et al., 2021)). Our attempts to use these methods were only partially successful as, using them, we were able to isolate pure DNA from cultivated, but not wild forms of *M. oleifera*, or from *M. concanensis* (Fig. 1). In the case of wild *M. oleifera* and *M. concanensis*, the crushed tissue samples were highly mucilaginous and the isolated DNA was viscous and colored unlike in the cultivated forms. This could be indicative of higher content of polysaccharides and polyphenols in these accessions (Fang et al., 1992). Apart from this problem, we found that the DNA isolated using either of the protocols degraded within three to five weeks of storage at -20°C. We therefore modified the

method of Doyle and Doyle (1987) and found that our modified DNA isolation method overcomes the problems associated with existing methods and gave high yields of pure and stable DNA that could be used in genome sequencing, PCR amplification and restriction digestion.

Materials and Methods

Plant material

Leaf samples of *M. oleifera* (wild and cultivated) and *M. concanensis* were obtained from accessions collected in Delhi, Punjab, Himachal Pradesh, Bihar and Tamil Nadu states of India or grown from seed [PKM-1 variety procured from Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India] (Table 1). Tender fruits of some accessions of *M. oleifera* were obtained from the National Bureau of Plant Genetic Resources (NBPGR) Centre, Issapur, Delhi. A single accession of *M. peregrina*, collected at the International Moringa Germplasm Collection, Jalisco, Mexico, was also tested. Leaf and fruit samples were cleaned with distilled water to remove dust particles and biological materials (fungus, insects etc.). All tissue materials (collected fresh and dried in silica gel) were stored at -80°C. Frozen samples were taken out for extraction at the time of DNA isolation. Moringa seeds were grown in small pots in the laboratory, and leaves harvested at the seedling stage.

Modifications and DNA isolation protocol

We modified the extraction buffer of Doyle and Doyle (1987) by increasing the concentrations of cetyltrimethylammonium bromide (CTAB) from 2 to 2.5% w/v and NaCl from 1.4 M to 1.5 M in the extraction buffer. The final constitution of the modified DNA extraction buffer was -100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.5 M sodium chloride (NaCl) and 2.5 % w/v CTAB. We powdered 0.5 g of tissue with 0.3 g PVP-40 in liquid nitrogen, in a mortar and pestle. To this, we added 10 ml of preheated modified DNA extraction buffer. The frozen mixture was thawed and transferred to a 50 ml Oakridge tube, to which we added βME to a final concentration of 0.3%. In the modified protocol, concentrations of both PVP (3% w/v) and βME (0.3% v/v) were also higher in comparison with the existing protocol (Doyle and Doyle, 1987), which contains 1% w/v PVP and 0.2% v/v βME. The tubes were incubated in a water bath at 65°C for 1 hr with intermittent mixing. Post-incubation, the tubes were allowed to cool to room temperature, and 10 ml of chloroform: isoamyl alcohol (CI, 24:1) was added per tube. The mixture was vortexed and centrifuged at 10,000 rpm for 15 min. The supernatant (aqueous phase) was transferred to fresh tubes. For DNA precipitation, an equal volume of ice-cold isopropanol was added to the supernatant, the contents mixed gently by inverting the tubes, which were then incubated at -20°C for 2 hr. Following this, the tubes were centrifuged at 12,000 rpm and the pellet obtained was air dried and dissolved in 500 µl TE buffer. For removal of RNA (optional step) from the samples, 1/10 volume of RNase A (Sigma-Aldrich; 10 mg/ml) was added and the tubes incubated at 37°C for 30 min. Equal volume of phenol: chloroform: isoamyl alcohol mixture (PCI, 25:24:1) was added to the tubes which were then centrifuged at 10,000 rpm for 10 minutes. The supernatant (aqueous phase) was transferred to fresh tubes and mixed with an equal volume of CI, the contents gently mixed by inverting the tubes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected in fresh tubes and 0.2 volume of 3M sodium acetate (pH 5.2) was added. To this mixture an equal volume of absolute alcohol was added. The contents were gently mixed and tubes incubated overnight at -20°C for DNA precipitation. Subsequently, the tubes were centrifuged at 12,000 rpm for 10 min. The DNA pellet was washed with 1 ml of 70% ethanol, air dried and re-suspended in 200 µl of nuclease free water. The dissolved DNA was stored at -20°C until further use. The quality and quantity of the genomic DNA was checked using 0.8% agarose gel electrophoresis and evaluated spectrophotometrically (NanoVue, GE Healthcare, USA).

Our modified protocol was initially established using fresh leaf material harvested from a locally cultivated tree of *M. oleifera*. The protocol was further used for different cultivated and wild accessions of *M. oleifera* and of *M. concanensis*. In case of these samples, which have high mucilage and appear to be rich in polyphenols, we had to make further modifications, such as reducing the amount of tissue (sequentially from 0.5 g to 0.1 g) and increasing the number of rounds (one to two) of CI purification till desirable DNA quality was achieved.

PCR amplification of plastid gene, *atpB* and *cytochrome P450* gene-based markers (Inui et al., 2000; Shahzad et al., 2013) was performed using 50 ng of template *Moringa* genomic DNA in a total reaction volume of 25 µl. The reaction mix comprised of 1X

PCR buffer (NEB), 2.5 mM $MgCl_2$ (ProMega), 160 µM dNTP mix (Biotools), 1 µM each of the forward and reverse primers, and 1 U of *Taq* DNA polymerase (Genei, no longer in operation). The PCR conditions were: initial denaturation at 94°C for 3 minutes followed by 35 cycles of 94°C for 1 minute, primer annealing for 1:30 min at 59 or 56°C, respectively for *atpB* and *cytochrome P450*, DNA extension at 72°C for 3 minutes and a final extension at 72°C for 10 minutes.

Results and Discussion

The optimised protocol was highly effective in extracting high quality DNA from a wide range of accessions – *M. oleifera* (both cultivated and wild) and *M. concanensis*, as well as from diverse plant parts – leaves of seedlings and mature trees, and fruit pulp. The protocol was found to be equally efficient in isolating DNA from both dried leaf material stored in silica gel and herbarium specimens (Fig. 1).

The DNA yield from the modified protocol was 8.6-51.0 µg/g in MOC, 4.6-20.8 µg/g in MOW and 6.2-33.6 µg/g in MC, which, although variable, was generally higher as compared to Doyle and Doyle (1987) protocol in which the yield was 4.4-16.6 in MOC, 1.1-8.9 µg/g in MOW and 3.7-16.2 µg/g in MC (Table 1, Fig. 2).

Sample absorbance ratio values (A260/A280 and A260/A230) were used as a measure of purity for nucleic acids, with ideal values of ~1.8 (A260/A280) and ~2.0 (A20/A230). Ratios of A260/A280 and A260/A230 for DNA samples extracted using our protocol ranged from 1.77-1.96 and 1.92-2.14 respectively, as compared to 1.30-1.95 and 1.52-2.66 in the case of Doyle and Doyle (1987), indicating that higher levels of DNA purity were achieved with the modifications made (Table 1).

The most important benefit of the modified protocol was that the DNA integrity (based on degradation observed on 0.8% agarose) was maintained even upon long term storage at -20°C. With other protocols including Doyle and Doyle (1987) and Ferdous et al. (2012) (results not shown), the DNA isolated from both wild and cultivated *Moringa* accessions was found to degrade within 30-45 days of extraction despite additional steps of CI or PEG purification and storage at -20°C (Fig. 3), suggesting precipitation of impurities such as polyphenols and polysaccharides with the DNA. The collections were done during the period Feb-Oct, 2016 and in July, 2017 and DNA was isolated from 95 accessions in 2017 for a genetic diversity study (results not shown) using the method of Doyle and Doyle (1987). After a month, most samples were degraded when checked on 0.8% agarose. On the other hand, all the 95 DNA samples (isolated by the present protocol in several batches a year after collection in 2018), were found to be intact when checked in 2020, three years after isolation and storage at -20°C. We show this in a few representative examples (Fig. 1). Despite the long period of storage, all samples retained their amenability to PCR amplification (see below).

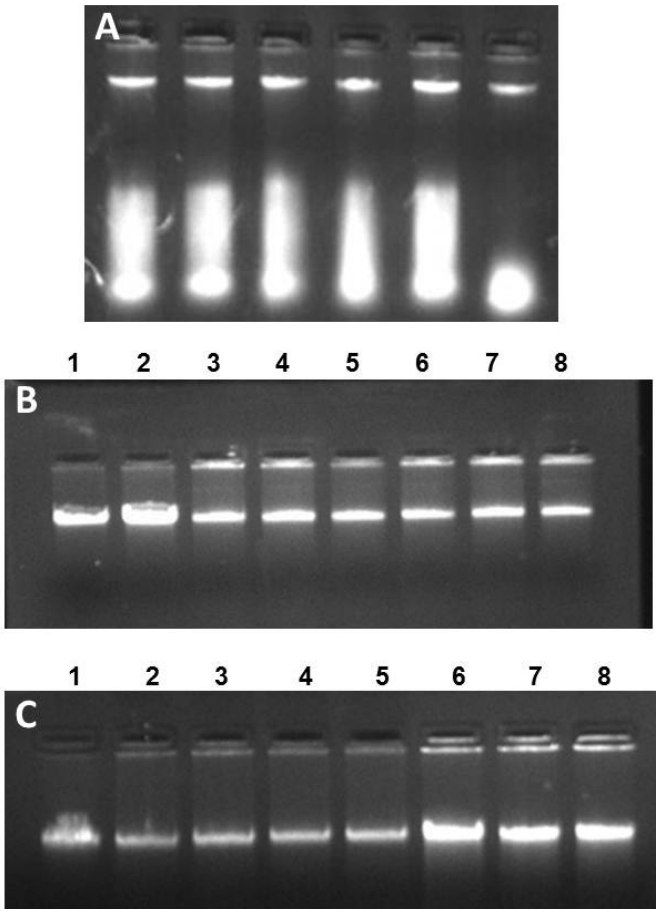

Figure 1: Agarose gel electrophoresis of *Moringa* genomic DNA isolated following the modified protocol. (A) DNA samples without RNAse treatment. Lanes 1-2: cultivated *M. oleifera*; lanes 3-4: wild *M. oleifera*; lanes 5-6: *M. concanensis*, lanes 1-2: fresh leaf tissue; lane 3, 5, 6: leaf tissue dried in silica gel and stored at -80°C; lane 4: fruit pulp stored at -80°C. (B, C) Isolated DNA with RNAse treatment (B) Lane 1-2: 200 and 400 ng of Lambda DNA respectively; lanes 3-8: cultivated *M. oleifera*; lanes 3-7: leaf tissue dried in silica gel and stored at -80°C; lane 8: fruit pulp stored at -80°C. (C) Lane 1: 300 ng Lambda DNA; lanes 2-4: wild *M. oleifera*; Lanes 5-8: *M. concanensis* (leaf tissue dried in silica gel and stored at -80°C).

Table 1. Comparative analysis of the yield and quality of DNA obtained using the modified protocol and that of Doyle and Doyle (1987) from 19 leaf accessions of three Moringa entities. Also given are details of collection. Yield (ug/g) and absorption ratios, A260/A280 and A260/A230, were estimated by NanoDrop Spectrophotometer (NanoVue, GE Healthcare, USA). All material was stored at -80°C after collection, except samples 1 and 3. Abbreviations; Species MOC: *Moringa oleifera* cultivated; MOW: *Moringa oleifera* wild; MC: *Moringa concanensis*; States: BI: Bihar; DL: Delhi; HP: Himachal Pradesh; PB: Punjab; RJ: Rajasthan; TN: Tamil Nadu.

Collection and Experiment Information							Our Protocol				Doyle and Doyle, 1987			
S.No.	Species	Sample Name	Place of Coll.	Form of Coll.	Date of Coll.	Date of Expt.	Amt. Tissue (g)	Yield (ug/g)	A260/ A280 ratio	A260/ A230 ratio	Amt. Tissue (g)	Yield (ug/g)	A260/ A280 ratio	A260/ A230 ratio
1	MOC	MO0046	DL	Fresh	2018, Jun	2018, Jun	0.46	35.6	1.82	2.07	2.62	9.8	1.36	2.41
2	MOC	MO0046*	DL	Fresh	2018, Apr	2018, Jul	0.49	8.6	1.81	1.96	0.5	4.4	1.95	1.85
3	MOC	PKM-1	DL^	Fresh	2018, Jun	2018, Jun	0.6	51	1.83	2.01	2.03	14	1.44	2.22
4	MOC	PKM-1*	DL^	Fresh	2018, Apr	2018, Jul	0.49	11.4	1.83	1.92	0.5	5.6	1.62	1.71
5	MOC	MO0104	BI	Si gel	2016, Jul	2018, Jun	0.58	45.6	1.82	2.14	1.84	16.6	1.64	1.83
6	MOC	MO0104*	BI	Sigel	2016, Jul	2018, Jul	0.46	10.7	1.77	1.94	0.5	5.9	1.78	1.63
7	MOW	MO0066	BI	Sigel	2016, Jul	2018, Jun	0.48	4.6	1.9	2.09	1.9	1.1	1.45	2.66
8	MOW	M00140	PB	Sigel	2016, Sep	2018, Jun	0.43	6.5	1.86	2.14	1.9	1.1	1.36	1.85
9	MOW	M00140*	PB	Si gel	2016, Sep	2018, Jul	0.19	20.8	1.78	1.97	0.2	8.9	1.32	1.52
10	MOW	MO0200	HP	Si gel	2017, Jul	2018, Jun	0.48	8.1	1.84	2.04	1.56	5	1.3	1.84
11	MOW	MO0200*	HP	Si gel	2017, Jul	2018, Jul	0.22	18.8	1.8	1.92	0.3	5.6	1.76	1.55
12	MC	MC0013	RJ	Si gel	2016, Jul	2018, Jun	0.44	6.2	1.83	2.06	2.37	9.7	1.31	1.83
13	MC	MC0020	RJ	Si gel	2016, Jul	2018, Jun	0.51	29.4	1.82	2.05	1.96	7.6	1.43	2.26
14	MC	MC0025	RJ	Si gel	2016, Jul	2018, Jun	0.53	26	1.81	2.04	2.32	7.4	1.89	1.82
15	MC	MC0026	RJ	Si gel	2017, Jul	2018, Jun	0.49	11.5	1.85	2.09	1.67	3.7	1.59	1.63
16	MC	MC0035	TN	Si gel	2017, Jul	2018, Jun	0.45	27.5	1.93	2.03	1.5	6.8	1.73	1.84
17	MC	MC0035*	TN	Si gel	2017, Jul	2018, Jul	0.19	27.3	1.82	2.01	0.2	16.2	1.78	1.63
18	MC	MC0042	TN	Si gel	2017, Jul	2018, Jun	0.55	33.6	1.96	2.07	1.76	6.1	1.71	1.93
19	MC	MC0042*	TN	Si gel	2017, Jul	2018, Jul	0.18	18.5	1.8	2.03	0.2	11.9	1.61	1.8

^seedlings; seeds from TNAU

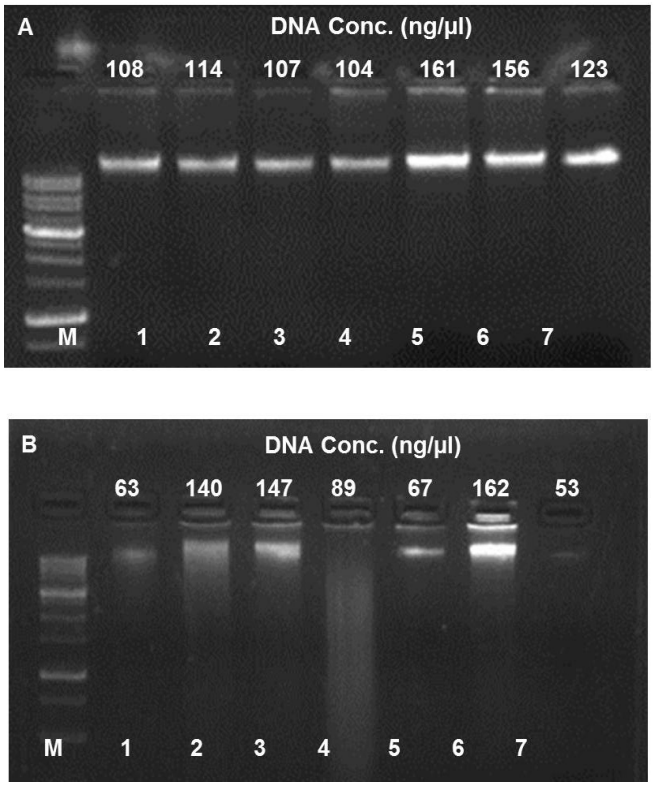

Figure 2: Comparison of *Moringa* genomic DNA isolated by two protocols. (A) The present protocol, and (B) Doyle and Doyle (1987). Lanes 1-3: cultivated *M. oleifera*; lanes 4-5: wild *M. oleifera*; lanes 6-7: *M. concanensis*. M: 1 kb DNA ladder. All samples were collected in 2016 or 2017 (except lane 1 and 2, in 2018), DNA was isolated in 2018 and re-analyzed for quality on 0.8% agarose in 2020, three years after iosations and storage at -80°C. The details of the accessions used (marked with *) and the DNA isolated are given in Table 1.

Additionally, the DNA isolated from wild *M. oleifera* and *M. concanensis* accessions using published protocols, was highly viscous (as assessed based on ability to pipette) and coloured. With the present modifications, we were able to resolve these issues. A summary of the properties of DNA obtained in our protocol compared with that of Doyle and Doyle (1987), is presented in Table 2.

The genomic DNA samples extracted following the newly developed protocol were subjected to PCR amplifications, to test their suitability for use in PCR based genetic diversity analysis involving DNA marker systems. PCR amplification of plastid gene, *atpB* and *cytochrome P450* gene-based markers (Inui et al., 2000; Shahzad et al., 2013) was performed. All *Moringa* accessions tested amplified well, as visualised in a 1% agarose gel (Fig. 4). Further, the DNA quality was found suitable for whole genome sequencing using

Table 2.Summary of properties of DNA isolated using the modified protocol and that of Doyle and Doyle (1987). Qualitative properties of DNA isolated from *M. oleifera* cultivated (MOC), *M. oleifera* wild (MOW) and *M. concanensis*(MC) are summarised.

DNA properties	Moringa accessions	Our protocol	Doyle & Doyle, 1987
Colour of the isolated DNA	MOC	White/ translucent	White/translucent
	MOW	White/ translucent	Yellow-brown
	MC	White/ translucent	Yellow-brown
DNA viscosity (as assessed based on ability to pipette)	MOC	Low	Moderate
	MOW	Low to moderate	High
	MC	Low	High
DNA Integrity Stored at -20°C (degradation as observed on 1% agarose)	MOC	At least 3 years	30-45 days
	MOW	At least 3 years	Complete degradation at 30 days
	MC	At least 3 years	Complete degradation at 30 days

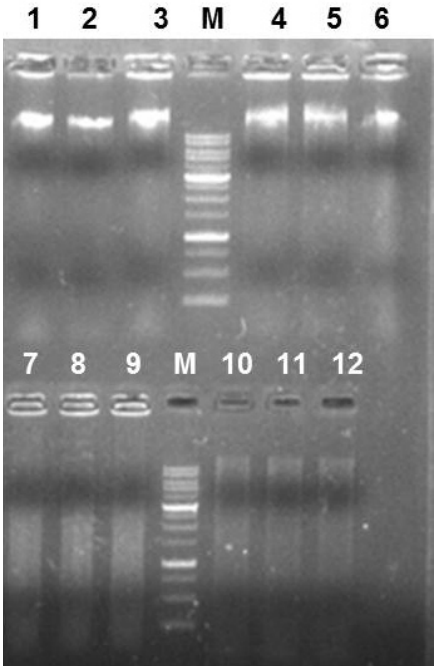

Figure 3: Gel electrophoresis analysis of the stability of genomic DNA after storage at -20°C Genomic DNA isolated using the present method was found intact (Top panel: lanes 1-6), while those isolated using the CTAB method of Doyle and Doyle (1987) (Bottom panel: lanes 7-12) were found to degrade within 45 days of isolation. Lanes 1-3, 7-9: wild *M. oleifera*; lanes 4-6, 10-12: cultivated *M. oleifera*; lane 4: 1 kb DNA ladder.

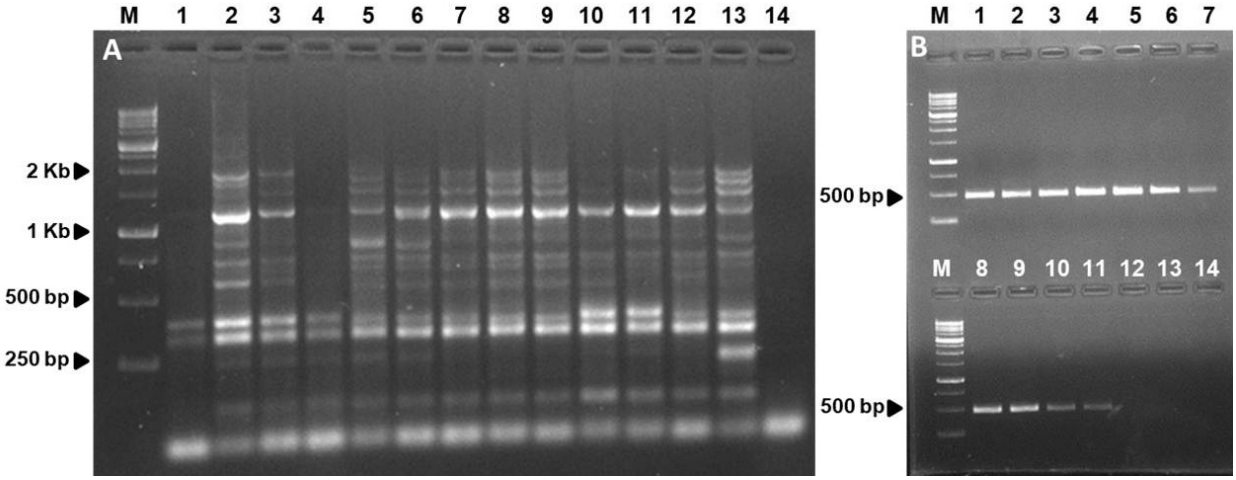

Figure 4: PCR amplification profiles of *Moringa* genomic DNA using primers from *cytP450* and *atpB* genes. (A). Fragments amplified by *cytP450* (250-1800 bp) based primer pair CYP2B6 (forward primer- 5' GAC TCT TGC TAC TCC TGG TT 3', reverse primer- 5' CGA ATA CAG AGC TGA TGA GT 3'; Inui et al., 2000); lane 1-3: cultivated *M. oleifera*; lane 4-6: wild *M. oleifera*; lane 7-12: *M. concanensis*; lane 13: *M. peregrina*; lane 14: negative control (PCR mix with no DNA). (B) Fragments amplified by *atpB* (475 bp) based primer pair (forward primer- 5' GGC CGT ATT GCT CAA ATC AT 3', reverse primer- 5' TTT CCT CCA CGA CGA TAA GG 3'; Shahzad et al., 2013); lane 1-3: cultivated *M. oleifera*; lane 4-6: wild accessions of *M. oleifera*; lane 7-11: *M. concanensis*; lane 12: negative control (PCR mix with no DNA); lane 13-14: blank. M: 1 kb DNA ladder.

Illumina based next generation sequencing, which was done for one of the accessions (unpublished results). Overall, the results showed that DNA extracted by this protocol could successfully be used for a variety of applications and downstream analyses.

Our modification of the existing protocol of Doyle and Doyle (1987) was found to yield high amounts of pure DNA from across a wide range of accessions of *M. oleifera,* and *M. concanensis*, both cultivated and wild (Fig. 2). With the high levels of DNA purity achieved with this protocol, DNA stability under long term storage was remarkably enhanced.

High levels of polyphenols, polysaccharides and other secondary metabolites in plants, as in *Moringa* spp., affect the DNA extraction process (e.g., Sahu et al., 2012; Mishra & Singh 2013). In such cases, fresh leaves from seedlings or young plants (suitably grown in dark conditions) with reduced levels of polyphenols and polysaccharides are recommended (e.g., Lodhi et al., 1994). However, this is not possible in the case of field exploratory studies where leaf samples are collected from remote geographical locations and seeds may not be available in all seasons. In such cases modifications are made to standard protocols, which may be effective in particular species or groups of species (e.g., Khanuja et al., 1999; Sahu et al., 2012). Our protocol was equally efficient in extracting DNA from mature field grown leaves, fruit pulp, dried leaf samples stored in silica gel, and in herbarium specimens, thereby circumventing these limitations.

As noted above, several modifications of the Doyle and Doyle (1987) method have been made that may be appropriate for particular groups of species. Researchers working with *Moringa* spp. (Rufai et al., 2013, Shahzad et al., 2013, Mishra and Singh 2013, Ganesan et al., 2014, Rajalaksmi et al., 2017, Paul et al., 2021) appear to have been successful using modified methods of Doyle and Doyle (1987). In our studies, existing CTAB methods resulted in low DNA yield in accessions of wild forms (*M. oleifera* and *M. concanensis*) and generally, the DNA degraded within 45 days of storage. The presence of polysaccharides in extracted DNA hinders restriction digestion and downstream applications that require enzymatic reactions (Furukawa & Bhavanandan, 1983; Demeke & Adams 1992; Lodhi et al., 1994). Optimization of NaCl concentrations can significantly inhibit co-precipitation of polysaccharides with the DNA (Fang et al., 1992). Similarly, increasing the concentration of CTAB, PVP-40 and or βME has been shown to remove or reduce polyphenols in DNA isolated from different plant species (Maliyakal, 1992; Khanuja et al., 1999; Sá et al., 2011; Sahu et al., 2012; Mishra & Singh 2013).

In the present protocol, we increased the concentrations of CTAB (2.5X), NaCl (1.5 M), PVP-40 (3%) and βME (0.3%). In addition, we used less tissue in the case of wild forms (0.1-0.3 g) compared with cultivated ones (0.5). Smaller amounts of tissue were also used by Mishra and Singh (2013), but their method involves 2-3 washes with PVP, which prolongs isolation time. We believe that in our shorter protocol, the combination of changes –higher concentrations of CTAB, PVP, βME with a lower amount of tissue – was important in overcoming the problems of DNA isolation in a wide range of accessions of different types of Moringa. The high purity of DNA achieved with the protocol circumvents the need to use commercially available DNA extraction kits with purification columns, which are suggested for plants rich in polyphenols and other secondary metabolites. Use of such kits would not be economical for genetic diversity studies involving large numbers of samples.

The protocol developed by us is efficient, works across species of *Moringa* including wild forms, and yields large amounts (15-25 µg/g) of clean, colourless, non-viscous, pure and RNA-free genomic DNA. This protocol would be useful in isolating pure genomic DNA for diverse applications in molecular biology.

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Conflict of interest: The authors declare that they have no conflict of interest.

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