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Full Length Research Paper

A quick DNA extraction protocol: Without liquid nitrogen in ambient temperature

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Marker assisted selection is an effective technique for quality traits selection in breeding program which are impossible by visual observation. Marker assisted selection in early generation requires rapid DNA extraction protocol for large number of samples in a low cost approach. A rapid and inexpensive DNA extraction protocol has been described for different tissues of color rice and other plant species which contain pigment and polyphenolic compound. This method has been modified from well known cetyltrimethylammonium bromide (CTAB) method where CTAB is used for DNA extraction. This protocol is simple and fast compared to other methods and no liquid nitrogen is required. Only inexpensive chemicals and ordinary laboratory equipments are enough for DNA extraction. The quantity of total genomic DNA from different tissues was almost similar which was extracted from 10 mg samples. The extracted DNA is stable and applicable to marker assisted selection, DNA fingerprinting, quantitative traits loci analysis, screening of transformants and enzymatic digestion.

Key words: Different plant tissues, inexpensive, rice flour, rapid DNA extraction.

INTRODUCTION

Marker assisted selection, diversity assessment, germplasm identification, quantitative traits loci analysis and transformants screening are the important techniques in the molecular study. Hundreds to thousands samples need to be processed for the above-mentioned analysis and rapid DNA extraction with expectable quality is the prerequisite for such kind of experiments (Post et al., 2003). Several authors (Muray and Thompson,1980; Dellaporta et al., 1983; Doyle and Doyle, 1990) described DNA extraction methods which are widely used in plant molecular biology, but most of the protocols are time consuming, comparatively expensive and requires liquid nitrogen for grinding (Sharma et al., 2003; Allen et al, 2006). The cetyltrimethylammonium bromide (CTAB) method is one of the most popular protocol for rice DNA isolation, including other plants, bacteria (Caccavo et al., 1994), fungi (Thuan et al., 2006) and animals (Shahjahan et al., 1995). A number of modifications have been made on CTAB method (Kang et al., 1998; Allen et al., 2006). Some methods have been reported to minimize the DNA extraction steps but they need a large amount of plant tissue and liquid nitrogen (Tussell et al., 2005).

Usually, leaf tissues are frequently used for DNA extraction from different plants. Many researchers suggested using the fresh tissue, but it has some limitations such as the glass house or field required for plantation as well as liquid nitrogen is essential for collection and storage. Continuous liquid nitrogen supply is a problem in many developing countries because purchasing time is

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Abbreviations: CAPs, Cleaved amplified polymorphic sequence; **CIP,** chloroform, isoamyl alcohol and phenol; **CTAB,** cetyltrimethylammonium bromide; **EDTA,** ethylenediaminetetraacetic acid; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase; **ITS1,** internal transcribed spacer1; **SDS,** sodium dodecyl sulfate; **SSR,** simple sequence repeat.

Table 1. List of primers used in the study.

Primer name	Primer sequence	Product size (bp)	Type of marker
Wx	F: ACCATTCCTTCAGTTCTTTG R: ATGATTTAACGAGAGTTGAA	530	CAPs
RSODA	F: ATGGTGAAGGCTGTTGTTGT R: TCAGCCTTGAAGTCCGATGA	359	Specific for copper/zinc-superoxide dismutase
RM1	F: GCGAAAACACAATGCAAAAA R: GCGTTGGTTGGACCTGAC	82-126	SSR
RM536	F: TCTCTCCTCTTGTTTGGCTC R: ACACACCAACACGACCACAC	211-249	SSR
OPA-7	GTGATCGCAG	250-1170	RAPD
GAPDH			Housekeeping
ITS1	F: GAAGTAAAAGTCGTAACAAG R: CCTCCGCTTATTGATATGC	530	Species specific

unpredictable from overseas. In addition, to store fresh tissue in -80°C fridges is another constrain. Moreover, it needs few weeks to few months from plantation to fresh tissue collection and also requires more attention for management practices. To overcome these problems Kang et al. (1998) developed a DNA extraction method using the dry half seeds of rice, and Ahmadikhah (2009) described a rapid method for DNA isolating from rice seed. But rice flour has not been used extensively for DNA extraction. Rice flour can be used any time and minimizes the step at grinding phase. This protocol is also applicable to other plant species such as oil palm, banana, *Moringa* and fungus. The objective of this study was to develop a simple and rapid method to isolate DNA under normal laboratory condition (room temperature) from small amount of tissue for large number of samples.

MATERIALS AND METHODS

Rice flour, seeds and leaves of two high yielding varieties BR16 and MR219 were used for DNA extraction and leaves of *Moringa*, oil palm, banana and fungus (*Fusarium proliferatum*) were used to evaluate the efficiency of this protocol. All leaves samples were collected in polyethylene beg on ice and stored at 4°C.

Primers

Extracted DNA was amplified with simple sequence repeat (SSR), cleaved amplified polymorphic sequence (CAPs), random amplified polymorphic DNA (RAPD) markers and specific primer (Table 1).

Solutions and buffers

The solutions used were: 1M Tris-HCl (prepared using 121.14 g Tris-HCl dissolved in 800 ml deionized water and adjudged to pH 8.0 using concentrated HCl. Then top up the total volume to 1 L with de-ionized water) and 0.5M EDTA (prepared using 186.12 g of EDTA dissolved in 800 ml de-ionized water). Ten molar (10 M) NaOH solution was used to adjust the pH to 8.0. Then top up the total volume to 1 L with de-ionized water. EDTA alone will not dissolve unless NaOH is added. Other solutions include: 3.5M NaCl (204.54 g NaCl added into 800 ml of de-ionized water and adjudged the final volume to 1 L with de-ionized water); 5% SDS: (5 g SDS dissolved into 100 ml de-ionized water); 2% CTAB (2 g CTAB dissolved into 100 ml de-ionized water); 1% PVP: (1 g PVP added into 100 ml 2X CTAB solution); and 70% ethanol (71.5 ml 95% ethanol mixed with 28.5 ml de-ionized water.

The buffers used include: Extraction buffer, 2X CTAB solution, chloroform: isoamyl alcohol (24:1) with 5% phenol (CIP), 5X Tris-borate-EDTA (TBE) buffer, 1X TBE buffer and 1X TE buffer. Their composition and preparation are shown in Tables 2 to 7, respectively.

DNA extraction procedure

Four hundred microliters (400 µL) extraction buffer and 400 µL of 2 X CTAB solution were added into 2 ml Eppendorf tube containing 0.01 g of rice flour. In case of leaf and seed, 0.01 g tissues were ground with 600 µL extraction buffer by mortar and pestle and poured into 2 ml Eppendorf tube. Then 400 µL of 2X CTAB solutions and 400 µL chloroform: isoamyl alcohol: phenol (24:1:5%) mixture were added in the same tube. The mixture was mixed well by vortex mixture and centrifuged at 8,400 × *g* in microcentrifuge at room temperature for 10 min. The supernatant was transferred into new tubes. Two third volume of isopropanol was added and mixed gently by inverting the Eppendorf tube. The tubes were incubated at room temperature for 10 to 15 min and centrifuged at 8,400 × *g* for 5 min. Supernatant was removed and the DNA pellet was washed with 70% ethanol. Afterward the pellet was air-dried and re-suspended into 50 µL TE buffer. The quality and quantity of extracted DNA were measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Polymerase chain reaction (PCR) amplification

Polymerase chain reaction (PCR) reaction was carried out in a

Table 2. Composition and preparation of the extraction buffer.

Reagent	Stock Solution	Final concentration	100 ml preparation
Tris HCl (pH 8)	1 M	200 mM	20 ml
EDTA (pH 8)	0.5 M	25 mM	5 ml
NaCl	3.5 M	200 mM	5.7 ml
SDS	5%	0.5% SDS	10 ml
De-ionized water	-	-	59.3 ml

Table 3. Composition and preparation of the 2X CTAB solution.

Reagent	Stock solution	Final concentration	100 ml preparation
Tris HCl (pH8)	1 M	100 mM	10 ml
EDTA (pH8)	0.5 M	20 mM	4 ml
NaCl	3.5 M	1.4 M	40 ml
CTAB	-	2% (w/v)	2 g
PVP	-	1% (w/v)	1 g
De-ionized water			46 ml

Table 4. Composition and preparation of the chloroform: isoamyl alcohol (24:1) with 5% phenol (CIP).

Reagent	100 ml preparation
Chloroform	91.2 ml
Isoamyl alcohol	3.8 ml
Phenol	5 ml

Table 5. Composition and preparation of the 5X TBE buffer.

Reagent	1 L preparation
Tris HCl (pH 8)	54 g
EDTA (pH 8), 0.5 M	20 ml
Boric acid	27.5 g
Water	Up to 1 L

volume of 25 µL, including 1 µL template DNA directly used after extraction. Five microliters 5X Green GoTaq® Flexi Buffer, 3 µL $MgCl_2$ solution (25 mM), 0.5 µL PCR nucleotide mix (10 mM each), 0.2 µL primers (0.4 µmol), and 1.0 U of *Taq* DNA polymerase were used according to the company instruction (Promega) for both SSR and RAPD markers. The optimum annealing temperature was determined for specific primer. The following condition was performed for PCR amplification of SSR marker: initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing (temperature varies with primer) for 1 min, and 2 min extension at 72°C. Final extension was performed for 7 min at 72°C (Ahmadikhah, 2009).

In random amplified polymorphic DNA (RAPD) analysis, the following condition was used: initial denaturation at 94°C for 1 min

Table 6. Composition and preparation of the 1X TBE buffer.

Reagent	1 L preparation
5X TBE	200 ml
De-ionized water	800 ml

followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 34°C for 1.5 min and extension at 72°C for 2 min and a final extension at 72°C for 5 min (Resmi et al., 2007).

Gel electrophoresis

Three microliters of genomic DNA was subjected to electrophoresis with 1% (w/v) agarose gel at 75 volt for 40 min to check the DNA quality and the amplified PCR products were further subjected to electrophoresis on 3% (w/v) MetaPhor agarose gel for 70 min. 1× TBE running buffer were used to prepare and run the gel. Finally, the gel was stained with ethidium bromide and visualized under ultraviolet (UV) light.

Restriction digestion

Two hundred nanograms of PCR product (amplified with Wx primer) were digested with 1 µL of FastDigest AccI (Xmil) restriction enzymes at 37°C for 1.5 h following the manufacturer's recommendation (Fermentas). The digested DNA (10 µL) was subjected to electrophoresis on 1% agarose gel at 75 volt for 40 min and viewed under ultraviolet (UV) light.

RESULTS

The quality and quantity of extracted DNA were

Table 7. Composition and preparation of the 1X TE buffer.

Reagent	Stock solution (M)	Final concentration (mM)	100 ml preparation
Tris HCl (pH 8)	1	10	1 ml
EDTA (pH 8), 0.5M	0.5	1	0.2 ml
De-ionized water	-		97.8 ml

Table 8. Quantity and quality of genomic DNA extracted from different tissues measured by Nanodrop spectrophotometer.

Tissue	Concentration (ng/µL)	Purity	
		$A_{260/280}$	$A_{260/230}$
BR16 leaf	327.22 ± 30.88	2.09 ± 0.05	2.11 ± 0.03
BR16seed	315.85 ± 28.65	2.09 ± 0.04	2.17 ± 0.01
BR16 flour	262.14 ± 8.92	2.02 ± 0.05	2.26 ± 0.1
BR16 flour (with kit)	14.11 ± 3.79	1.97 ± 0.05	1.85 ± 0.03
MR219 leaf	307.70 ± 21.72	2.04 ± 0.03	2.13 ± 0.04
MR219seed	321.07 ± 18.58	2.06 ± 0.02	2.14 ± 0.03
MR219 flour	313.56 ± 41.19	2.01 ± 0.09	2.16 ± 0.02
MR219 flour (with kit)	30.25 ± 3.43	1.83 ± 0.02	1.95 ± 0.07
Oil palm leaf	85.98 ± 5.63	2.03 ± 0.04	2.35 ± 0.07
Banana leaf	108.86 ± 12.02	2.19 ± 0.01	2.45 ± 0.05
Maringa sp	624.57 ± 18.45	1.88 ± 0.02	2.01 ± 0.02
Fusarium sp	215.94 ± 20.21	2.13 ± 0.03	2.05 ± 0.02

Values are mean (±SE) (n= 3).

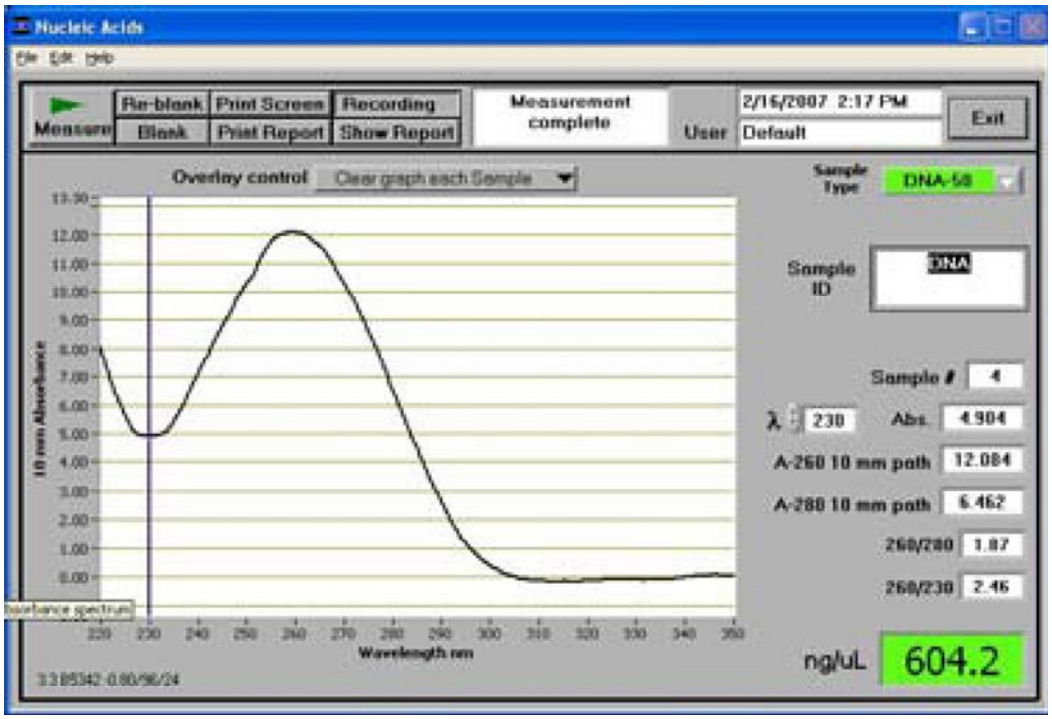
measured by NanoDrop ND-1000 spectrophotometer V5.3 2 (NanoDrop Technologies, Wilmington, USA). The yields of extracted DNA ranged from 85.98 - 624.5 ng/µL. The ratio of 260/280 and 260/230 were 1.88 to 2.19 and ≤ 2, respectively (except DNA extracted by commercial kit) (Table 8). A ratio of absorbance 260/280 and 260/230 are used to assess the purity of nucleic acid and secondary measurement, respectively. The accepted range of 260/280 and 260/230 ratios are commonly in ~1.8 and 2 - 2.2, respectively (Thermo Scientific, 2011). The spectral structure of extracted DNA (Figure 1B and C) using this protocol was similar to typical spectral pattern (Figure 1A) (Thermo Scientific, 2011).

The quality of extracted genomic DNA was also checked by 1% agarose gel electrophoresis (Figures 2A and B). To verify the suitability of extracted DNA for PCR amplification, simple sequence repeat (SSR), cleaved amplified polymorphism sequence (CAPs) markers and specific primers were used (Figure 3A, B and D). PCR was also performed with RAPD markers OPA-7 using DNA of *Moringa* and was successfully amplified (Figure 3C). The species specific primer ITS1 was used to amplify fungus (*F. proliferatum*) DNA. To assess the genetic diversity of rice genotypes, RM1 and RM536 were used and polymorphic products were amplified at 82 - 126 and 211 - 249 bp, respectively. A PCR-*Acc*I CAPs marker (Wx) was used to examine the amylose content of rice genotypes which amplified at 530 bp (Figure 4A). The PCR product was completely digested with *Acc*I and two DNA fragments were obtained (405 and 125 bp), indicating high amylose content of BR16 (Figure 4B) (Liu et al., 2006).

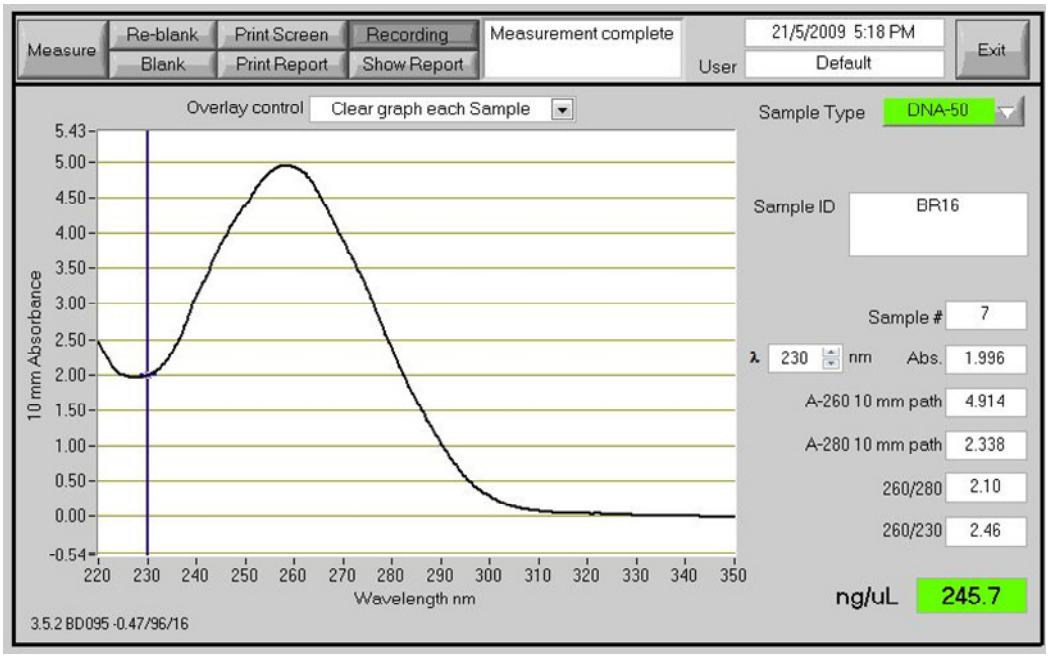
Furthermore, to check the suitability of the extracted DNA, one-year old DNA was amplified with RM536 (Figure 5A); the DNA was amplified same as before. PCR product was also used to test the stability. Extracted DNA was amplified with RM1 (SSR marker) just after DNA extraction (Figure 5B) and stored at 4°C. The same PCR products were also subjected to electrophoresis after 1 year and the same results were obtained as before (Figure 5C).

DISCUSSION

In the described protocol, no liquid nitrogen was required for the storage and grinding of the tissues. In addition, the expensive chemicals have not been used. To trim down the time, the extraction buffer was directly added to the rice flour, while rice seed and leaf tissues of different plants were ground with extraction buffer. No incubation has been required for DNA extraction in both cases, but



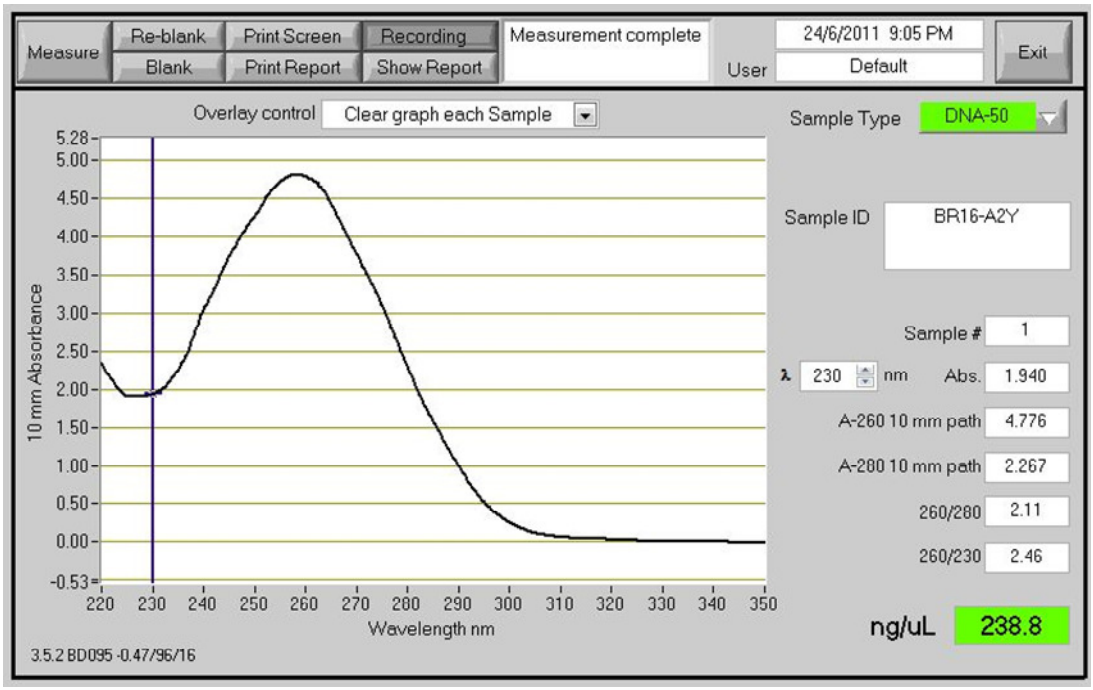
A



B

Figure 1. Comparison of Nanodrop spectrophotometry measurements of extracted DNA from rice flour to determine the quality of DNA. (A) Typical spectral pattern of nucleic acid supplied by Thermo scientific. (B) Spectral pattern of BR16 rice flour measured immediately after extraction. (C) Same DNA (BR16 rice flour) measured after two years.

quality and quantity of extracted DNA were similar with or without incubation in extraction buffer (data not shown). However, Rajendrakumar et al. (2011) reported that DNA degradation occurred when seed was ground before incubation in the buffer which disagrees with the finding of our study. Moreover, DNA extraction buffer, CTAB



C

Figure 1. Contd.

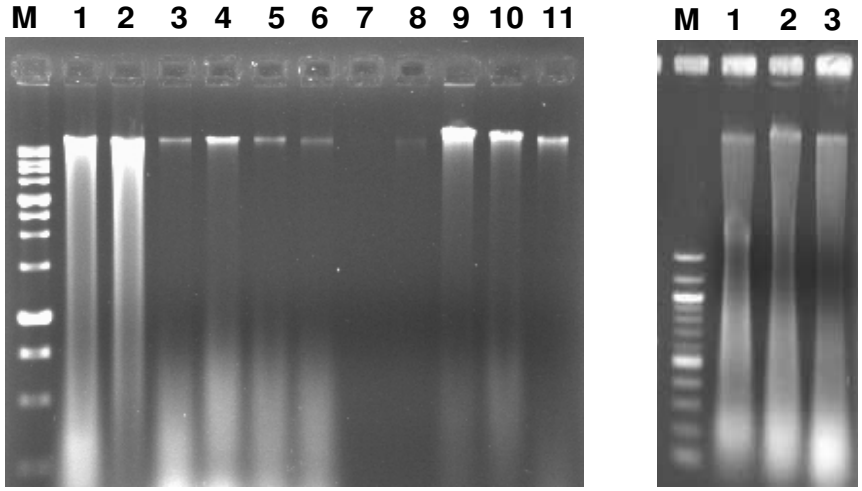


Figure 2. Quality test of DNA samples from different plant tissues on 1% (w/v) agarose gel. (A) Lane M, 1 kb DNA ladder; lane1, BR16 leaf; lane 2, BR16 seed; lane 3, BR16 flour; lane 4, MR219 leaf; lane 5, MR219 seed; lane 6, MR219 flour; lane 7, BR16 flour extracted with kit; lane 8, MR219 flour extracted with kit; lane 9, oil palm leaf; lane 10, banana leaf; lane 11, *Fusarium proliferatum*. (B) Lane M, 100 bp DNA ladder; lanes 1 to 3, *Moringa* from different genotypes.

solution and CIP were added in the same step to reduce the time. The DNA extraction from rice flour required approximately 50 min for 20 - 25 samples and 80 min for seed and leaf which indicates that 2.5 - 3 min is required for each sample.

In this study, very common chemicals were used for DNA extraction instead of costly chemicals. Several authors used expensive chemicals such as RNase

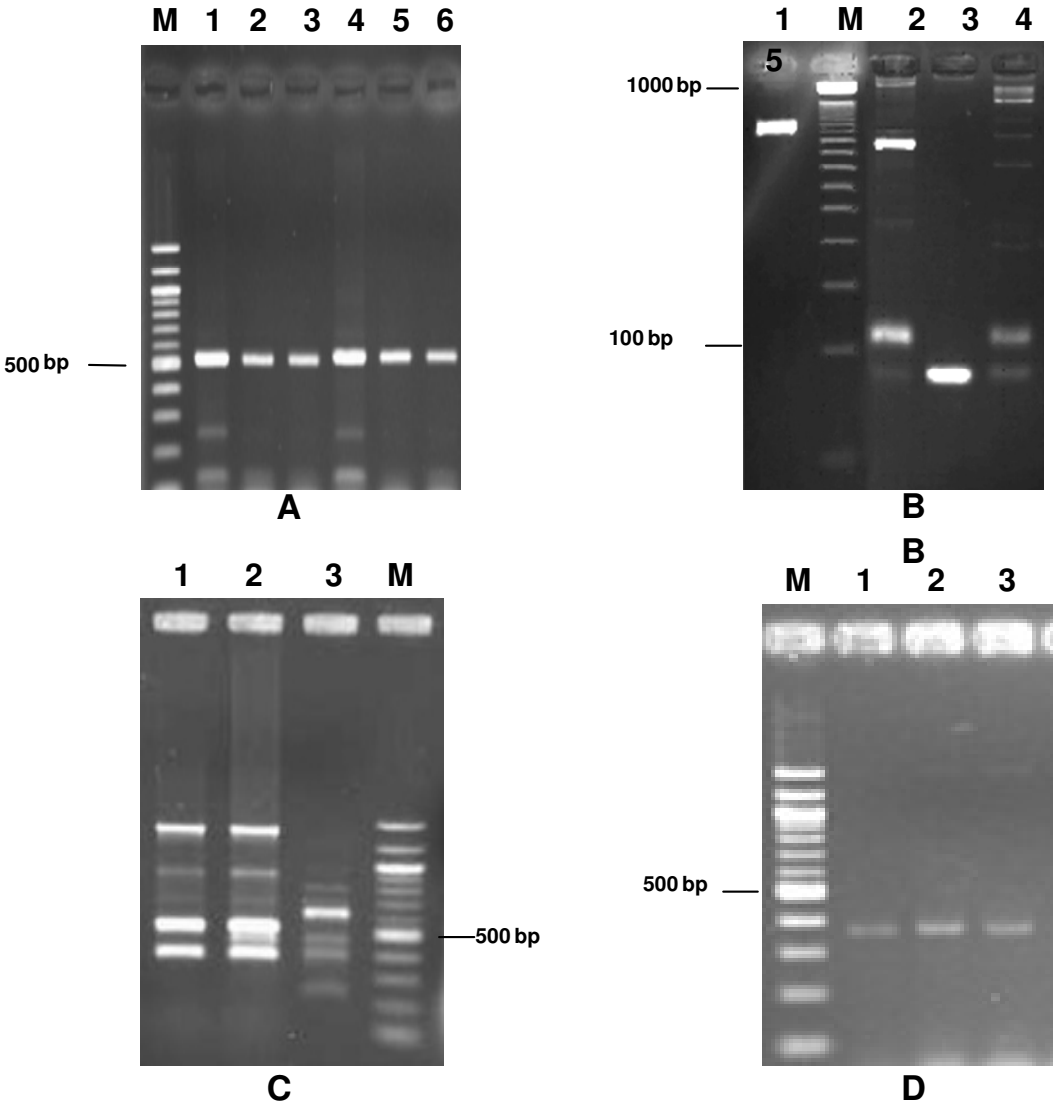


Figure 3. PCR amplification with extracted DNA on 3% MetaPhor agarose gel. (A) CAPs marker for Wx gene amplification with different tissues of two rice genotypes. Lane M, 100 bp DNA ladder; lane1, BR16 leaf; lane 2, BR16 seed; lane 3, BR16 flour; lane 4, MR219 leaf; lane 5, MR219 seed; lane 6, MR219 flour. (B) Lane 1, *Fusarium proliferatum* with specie specific marker (ITS1); lane M, 50 bp DNA ladder; lane 2, oil palm with specific primer (Wx) for waxy gene; lane 3, oil palm with housekeeping gene (GAPDH); lane 4, banana with specific primer (Wx) for rice waxy gene. (C) Lanes 1 to 3: *Moringa* with OPA-7 RAPD marker; lane M, 100 bp DNA ladder. (D) Lane 1, 100 bp DNA ladder; lane 2, DNA from rice flour amplifies with specific primer of RSODA gene.

(Ahmadikhah, 2009) and proteinase K (Kang et al., 1998) for rapid and simple DNA extraction. And the quality of extracted DNA was high enough to PCR amplification for marker assisted selection and genetic diversity analysis (Figures 5A to C) without RNase and proteinase K. The amplified PCR products of rice flour DNA showed similar banding patterns and intensity like seed and leaf tissues (Figure 3A). The amplification of expected bands with SSR, CAPs, RAPD and specific primer were evident of good quality genomic DNA without RNase and proteinase K. The complete digestion of PCR product with *Acc*I indicated that the extracted DNA is also useful for genetic manipulation. The extracted DNA samples and PCR products in the present study were stable more than 1 year, but extracted DNA was unstable in a rapid DNA extraction protocol developed by Warner et al. (2001). Commercial DNA extraction kit is not economic for marker assisted selection or diversity analysis in which large number of samples is used. DNA was extracted by Gene√All™ Plant SV mini DNA extraction kit and it was observed that concentration was too low to amplify (Figure 2 and Table 8). Therefore, this protocol was

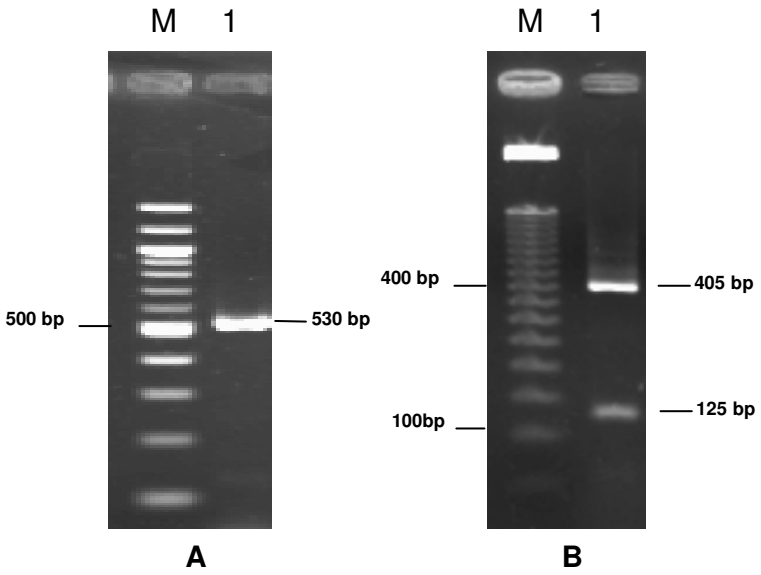


Figure 4. Undigested and digested PCR product of waxy gene (Wx). (A) Amplified PCR product (530 bp) of Wx gene. Lane M, 100 bp DNA ladder; lane 1, PCR product .(B) Digestion with *Acc*I from amplified PCR product of Wx gene. Lane M, 50 bp DNA ladder; lane 1, PCR product digested with *Acc*I and after electrophoresis, two DNA fragments were obtained (405 and 125 bp).

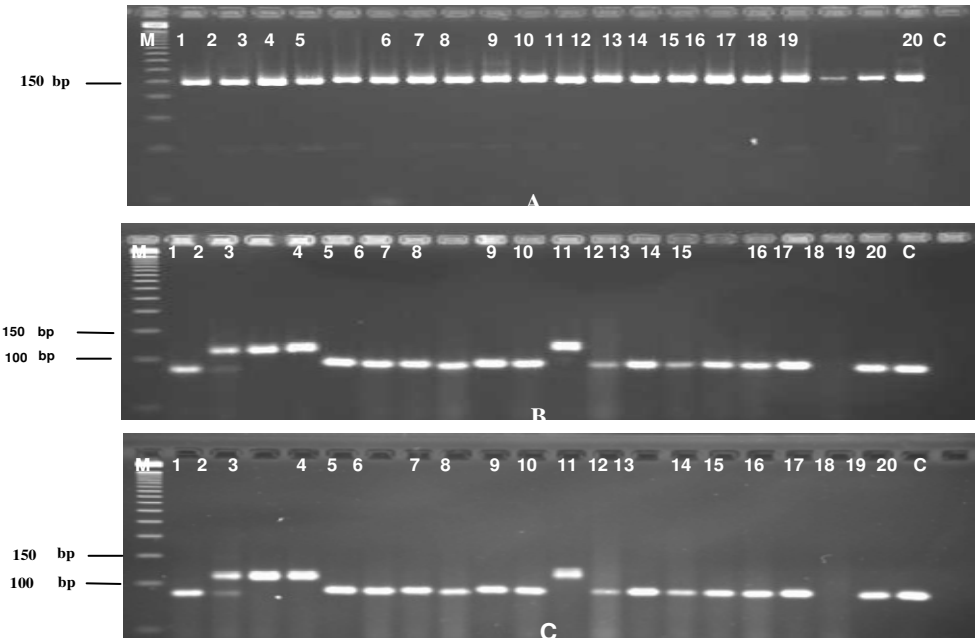


Figure 5. Amplification of extracted DNA from flour of different color rice using SSR marker. (A) Lane M, 50 bp DNA ladder; lanes 1 to 20, DNA of different rice genotypes using after one year with RM536; C, negative control. (B) Lane M, 50 bp DNA ladder; lanes 1 to 20, PCR product amplified with RM1 immediate after DNA extraction; C, negative control. (C) Lane M, 50 bp DNA ladder; lanes 1 to 20, PCR product amplified with RM1 after one year of DNA extraction; C: negative control. Lane 1= BR16; lane 2= BR29; lane 3 = MR219; lane 4 = MR220; lane 5 = Bukit Garam582; lane 6 = Bukit Garam753; lane 7 = Bukit Garam1334; lane 8 = Bukit Garam1449; lane 9 = NERICA7; lane 10= Tenom; lane 11= Karibang; lane 12= Karingam; lane 13= Padi Hijau Menis; lane 14= Padi Beleong; lane 15= Padi Durak B; lane 16= Padi Kalopak; lane 17= Bukit Merah; lane 18= Bukit Hitam; lane 19 = Bukit Kelakak; lane 20= Padi Padi.

found to be potential for DNA extraction using different tissues of rice and other plants.

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