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Full Length Research Paper

Field collection, preservation and large scale DNA extraction procedures for cassava (*Manihot esculenta* Crantz.)

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Some genetic studies using molecular methods such as diversity assessment or marker-assisted selection require collection of a large number of samples from fields located in the vicinity or in remote areas, followed by isolation of good quality DNA in a short time span. In the present study, different tissue preservation methods were compared for subsequent DNA extraction using a modified CTAB method in two 96-well plates, following grinding of leaf tissues with a GenoGrinder 2000. We found that preservation of leaf tissues in NaCl-CTAB-azide buffer (as described in Rogstad, 1992) at 4°C is a better storage procedure than preservation at -20°C to obtain good quality DNA. Comparison of DNA extraction with or without use of phenol revealed that the quality of DNA was not drastically affected when non-phenol extraction protocol was used and did not affect PCR amplification. Thus, the recommended DNA extraction procedure allowed us to process 192 samples per day at a cost of $0.80 per sample, with an average yield of 1.8 µg, suitable for both PCR and genotyping.

Key words: Cassava, NaCl-CTAB-azide solution, phenol, genogrinder 2000.

INTRODUCTION

In breeding studies, numerous populations are sampled to detect candidate genes or molecular markers associated with economically important traits (Chao et al., 2005; Schenkel et al., 2005). Similarly, a large number of accessions are sampled to determine the genetic diversity present in germplasm collections (Slotta et al., 2005, 2006; Reusch, 2006). Advances in DNA technology along with contemporary genetic and genomic studies (such as Marker-Assisted Selection, DNA fingerprinting, TILLING, and diagnostics) also necessitate the isolation of DNA from a large number of samples. In addition, widespread application of MAS and molecular characterization may require collection of tissue samples from sites distant from laboratories, hence impeding immediate processing of samples. Therefore, identification of a suitable technique for collection of samples in the field and subsequent preservation in the laboratory is imperative. A range of preservation techniques have been described in the past that include drying of samples at ambient temperature, cryopreservation with dry ice or liquid nitrogen, ethanol or isopropanol, or buffer solutions that yield sufficient quantities of DNA for genetic studies (Kilpatrick, 2002; Wehausen et al., 2004; Goolsby et al., 2006). However, some of these preservation methods can be cumbersome when collecting leaf samples from remote locations if dry ice, or liquid nitrogen or freeze drying is unavailable. Breeders or conservation geneticists often collect samples from locations that are far away from the laboratories and generally depend on silica gel to dry leaf samples (Chase and Hillis, 1991; Arnaud-Haond et al., 2005;

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Abbreviations: CTAB, Cetyltrimethylammonium bromide,; **EDTA**, hexadecyltri-methylammoniumbromide; **PVPP**, polyvinyl polypyrrolidone; **PEG**, polyethylene glycol; **PCR**, polymerase chain reaction; **MAS**, marker-assisted selection.

Reusch, 2006). This method is effective for many species with rapid rates of desiccation that prevents DNA degradation. However, improper use of silica gel (too little for a large amount of tissue or insufficiently dry) may result in poor desiccation of leaf tissue and DNA degradation. Furthermore, it may not be always convenient to carry a large amount of silica gel while collecting samples in remote locations and keep it dry. In the present study, we report experiments to determine a methodology for collecting and preserving cassava leaf samples from remote geographical areas for low-cost, high-throughput DNA extraction suitable for genotyping.

Several DNA extraction protocols are available with varying scale, amounts of tissue required, duration and cost. In plants, in particular, a vast array of methods has been published (reviewed in Varma et al., 2007). With a reduction in the cost of DNA sequencing and genotyping, larger numbers of samples are being genotyped than before for large scale diversity assessments and marker-assisted breeding. In cassava, the long duration required to develop varieties by conventional methods fails to meet the urgency of tackling the threat posed by a multitude of constraints including drought, pests and diseases, and poor nutritional qualities. Marker Assisted Selection (MAS) holds a promise to enhance the improvement of traits of economic importance. The availability of proper tissue preservation techniques and cost-effective high-throughput DNA extraction procedures pave the way for the deployment of MAS in cassava improvement. Several high-throughput DNA extraction procedures have been reported for sorghum, millet, groundnut, pigeonpea and chickpea (Mace et al. 2003), and for cocoa (Bhattacharjee et al., 2004). Here we report a protocol for the low-cost DNA extraction from cassava in 96-well format, a modification of the extraction procedure reported by Doyle and Doyle (1987). Two plates (192 samples) are generally processed per person per day resulting in DNA of sufficient quality and quantity for genotyping.

MATERIALS AND METHODS

Collection and preservation of leaf samples

Young, unfolded leaf samples (1.2 cm^2 or 20 - 30 mg) were collected directly from the field and were kept on: (1) 12 x 8 well 1.2 ml polypropylene strip tubes with strip caps (Marsh BioMarket, USA) in two 96-well deep well plate together with two 4 mm stainless steel grinding balls (Spex CertiPrep, USA) placed on ice; and (2) in 2.0 ml eppendorf tubes containing 1.8 ml of saturated NaCl-CTAB-azide solution (70 g NaCl, 3 g CTAB, 0.04 g Na azide dissolved in 200 ml distilled water). In the laboratory, samples in 96-well plates were stored at -20°C and samples in eppendorf tubes were stored at 4°C until DNA extraction was performed. Each of these methodologies is appropriate in different circumstances method (1) where the field is close to the point of extraction, and method (2) where dry ice or ice cannot be used, in remote locations. Preservation in 70% and absolute ethanol was attempted, but yielded low levels of DNA, and was thus deemed unsuitable for the collection of cassava samples.

Reagents for DNA extraction

DNA extraction buffer (CTAB buffer) consisting of 7% CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 2 M NaCl, 2% PVP (Mr. 40,000) and 0.2% β-mercaptoethanol. The other reagents required are:

i.) TE buffer, consisting of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)
ii.) Low-salt TE buffer consisting of 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA (pH 8.0)
iii.) Phenol: chloroform: isoamyl-alcohol (25:24:1 v/v)
iv.) Isopropanol stored at -20°C
v.) CI consisting of chloroform: isoamylalcohol (24:1 v/v)
vi.) Sodium acetate (3 M) stored at room temperature
vii.) Ethanol 70% and 100% stored at 4°C
viii.) Polyethylene glycol (PEG) (4%)
ix.) RNase A (10 mg/ml).

Initial sample preparation

Leaf samples collected on ice were stored at -20°C for one week, two weeks and one month before DNA extraction was carried out. Grinding was done before extraction buffer was added. Similarly, leaf samples collected in NaCl-CTAB-azide buffer (Rogstad, 1992) were stored at 4°C for a week, two weeks and one month before DNA extraction was carried out. In the latter case, leaf samples were removed from eppendorf tubes using forceps; washed vigorously in distilled water; and placed inside 1.2 ml polypropylene strip tubes with strip caps (Marsh Biomarket), containing 2 pre-chilled 4 mm chrome-plated grinding balls (Spex Certiprep, as described by Mace et al., 2003; Bhattacharjee et al., 2004). Grinding was done after adding the extraction buffer (Note: Preferably, the extraction buffer should be freshly prepared).

DNA extraction protocol

For samples collected on ice:

1. Incubate the extraction buffer at 65°C for 30 min.
2. Remove the bases of two 96 well plates containing the leaf samples, and chill them in liquid nitrogen for about 2 min.
3. Replace the bases of the plates and process them in a GenoGrinder 2000 (Spex CertiPrep, USA), following the manufacturers instructions at 500 strokes/minute for 2 minutes.
Repeat steps 2 and 3 to ensure thorough grinding (Note: Repeat grinding procedure to ensure sufficient disruption and homogenization of leaf tissue. However, it is important to note that too much of grinding could result in shearing of DNA).
4. Centrifuge the plates for 2 minutes at 2250 g (Eppendorf centrifuge model 5810 with Swing-bucket rotor model A-2-DWP).
5. Add 450 µl of preheated (65°C) extraction buffer to each well. Re-place the strip caps and vortex until the tissue is resuspended. Proceed to step 6.

For samples collected in NaCl-CTAB-azide solution:

1. Incubate the required amount of extraction buffer at 65°C for 30 min.
2. Add 450 µl of preheated CTAB buffer to each sample and close the strip caps tightly (Note: Strip caps should be properly tightened before grinding or mixing).
3. Load two 96-well plates onto GenoGrinder 2000 after balancing the boxes (Note: Two 96-well plates should be properly balanced before grinding or centrifuging).

4. Process the samples following the manufacturer's instructions, at 500 strokes/min for 10 min. Repeat this step till the samples are well ground. Proceed to step 6.

Steps common for both sampling methods

6. Remove the bottom of the plates and incubate for 30 min in a 65°C water bath with occasional mixing. Care should be taken to ensure that the caps do not pop open.
7. Remove plates from the water bath and add 400 µl of CI to each well. Tightly cap the tubes and mix well by inverting the box 2 - 3 times. Hold the boxes tightly to ensure that the caps do not pop open.
8. Centrifuge plates at 2250 g for 20 min.
9. Transfer about 300 µl of the supernatant into freshly labeled strip tubes without disturbing the interface (Note: Use of multi-channel pipettes is recommended for faster transfer of samples). Remove the chrome-plated balls and discard the strip tubes.
10. Add 0.7 volumes (210 µl) of ice-cold isopropanol to the supernatant and mix by inverting the tubes 2 - 3 times to precipitate the DNA.
11. Centrifuge plates at 2250 g for 20 min.
12. Decant the supernatant carefully without disturbing the pellet and air-dry for 20 min.
13. Add 200 µl of low-salt TE and 3 µl of RNase A per well and incubate at 37°C for 1 h (or overnight at room temperature).

Solvent extraction

14. Add 200 µl phenols: chloroform: isoamylalcohol (25:24:1) to each sample and invert 2-3 times to mix well. (Note: Due to the hazardous nature of phenol, solvent extraction was also carried out with-out this step for each of the samples mentioned above to determine whether phenol could be omitted without compromising DNA quality).
15. Centrifuge tubes at 2250 g for 15 min.
16. Transfer a fixed volume of 180 µl of the supernatant to freshly labeled strip tubes (Note: Use of multi-channel pipettes is recommended).
17. Add 200 µl of CI and mix the samples well by inverting the tubes 2 - 3 times. Centrifuge at 2250 g for 15 min.
18. Transfer a fixed volume of aqueous layer (approximately 180 µl) to freshly labeled tubes. (Make sure you do not disturb the interface layer as this is where proteins, polyphenolics and polysaccharides accumulate).

Purification

19. Add 315 µl of ethanol-acetate-PEG solution (30 ml of cold 100% ethanol, 1.5 ml of 3 M sodium acetate [pH 5.2] and 4% PEG) to each sample. Place the samples at -20°C for 10 min.
20. Centrifuge plates at 2250 g for 20 min.
21. Carefully decant supernatant from each sample and wash pellet with 200 µl of 70% ethanol.
22. Centrifuge plates at 2250 g for 10 min and carefully decant supernatant and air dry the pellets for 1 h.
23. Resuspend pellet in 100 µl low-salt TE buffer and store at 4°C or -20°C for medium term storage or -80°C for long-term storage.
24. Chrome-plated balls should be cleaned in soapy water for 1 h and then kept in 0.2 M HCl solution for 10 min. The balls should then be rinsed in distilled water and dried well to reuse.

DNA quantification and analyses

DNA concentration was determined with a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The Nanodrop uses undiluted sample in a minimum volume of 1-2 µl with a sensitivity range from 5 ng to 3700 ng. Absorbance at 260 nm (A_{260}) is measured for each DNA sample to determine the DNA quantity. Aliquots of 3 µl of freshly extracted genomic DNA was then electrophoresed on 0.8% agarose gels, stained with ethidium bromide, and visualized under an ultra-violet transilluminator for quality and yield assessment.

Twenty-five samples were randomly selected for PCR amplification with two cassava SSR primers (AT52, IITA primer, and S7, CIAT primer). The total volume of PCR reaction was 10 µl, which contained 1 µl of freshly extracted DNA (2.5 ng), 1 µl of 10x PCR buffer (Bioline), 1 µl 25 mM $MgCl_2$, 0.5 µl each of 10 pm forward and reverse primer, 0.2 µl of 10 mM dNTPs, 1U of *Taq* polymerase (Bioline). Amplifications were carried out in a gradient cycler PTC 200 (MJ Research). The PCR cycle consisted of initial denaturing at 95°C for 2 min, followed by 35 cycles at 94°C for 30 s, 65°C annealing for 20 s with 1°C reduction in temperature per cycle for 10 cycles, and 72°C for 30 s. This was followed by further primer extension at 72°C for 5 min. PCR products were stored at 4°C. Amplification products were then electrophoresed on 3.5% metaphor-agarose gels using a standard ladder marker (Hyperladder V, Bioline).

RESULTS

Preservation of leaf samples

Appropriate handling and preservation of leaf tissue material prior to extraction is critical in obtaining reasonable quantity and quality of DNA for genotyping. For most DNA extraction procedures, leaf tissues are usually collected fresh and directly used to extract DNA (Aljanabi and Martinez, 1997) and sometimes, they are stored at -20°C or -80°C to freeze the tissues before extracting DNA. Freezing prevents the activity of the nucleases that degrades the DNA while in thawed condition (Jofuku and Goldberg, 1988). In the present study, we collected samples from the field on ice and preserved at -20°C for one week, two weeks and one month. Similarly, samples were collected and kept in NaCl-CTAB-azide buffer solution and stored at 4°C for one week, two weeks and one month, to standardize a preservation technique for cassava. The results of DNA extracted from cassava leaves that had been collected on ice and stored at -20°C or preserved in NaCl-CTAB-azide buffer at 4°C with data for DNA extracted from freshly collected leaf tissues for comparison, are summarized in Table 1. Preservation of leaf samples at -20°C yielded intact, high molecular weight DNA from samples stored up to two weeks, however, the samples that were stored for one month resulted in low molecular weight DNA. It has been described by Ribeiro and Lovato (2007) that long term preservation of leaf tissues at low temperature has a major influence on DNA quantity and quality. For samples preserved in NaCl-CTAB-azide buffer, high quantity and quality DNA was obtained for most of the samples (stored for one week, two weeks and one month), although the leaves showed some discoloration with time (mainly after 48 h of storage at 4°C). In some cases, the DNA looked yellowish or

Table 1. Effect of tissue preservation and storage conditions on DNA extracted (with or without phenol) from young leaves of cassava (*Manihot esculenta* Crantz.).

Conditions	Quality and Quantity of extracted DNA				PCR amplification
	With phenol	Color	Without phenol	Color	
Fresh leaves with liquid nitrogen	High	White	High	White	Reliable
Stored at -20°C					
One week	High	White	High	White	Reliable
Two weeks	Moderate	White/Yellow	Moderate	Yellow	Poor
One month	Very low	Yellow/Brown	Very low	Yellow/Brown	Very poor/Unreliable
NaCl-CTAB-azide solution					
One week	High	White	High	White/Yellow	Reliable
Two weeks	Moderate	White/Yellow	Moderate	White/Yellow	Reliable
One month	Moderate	White/Yellow	Moderate	White/Yellow	Reliable

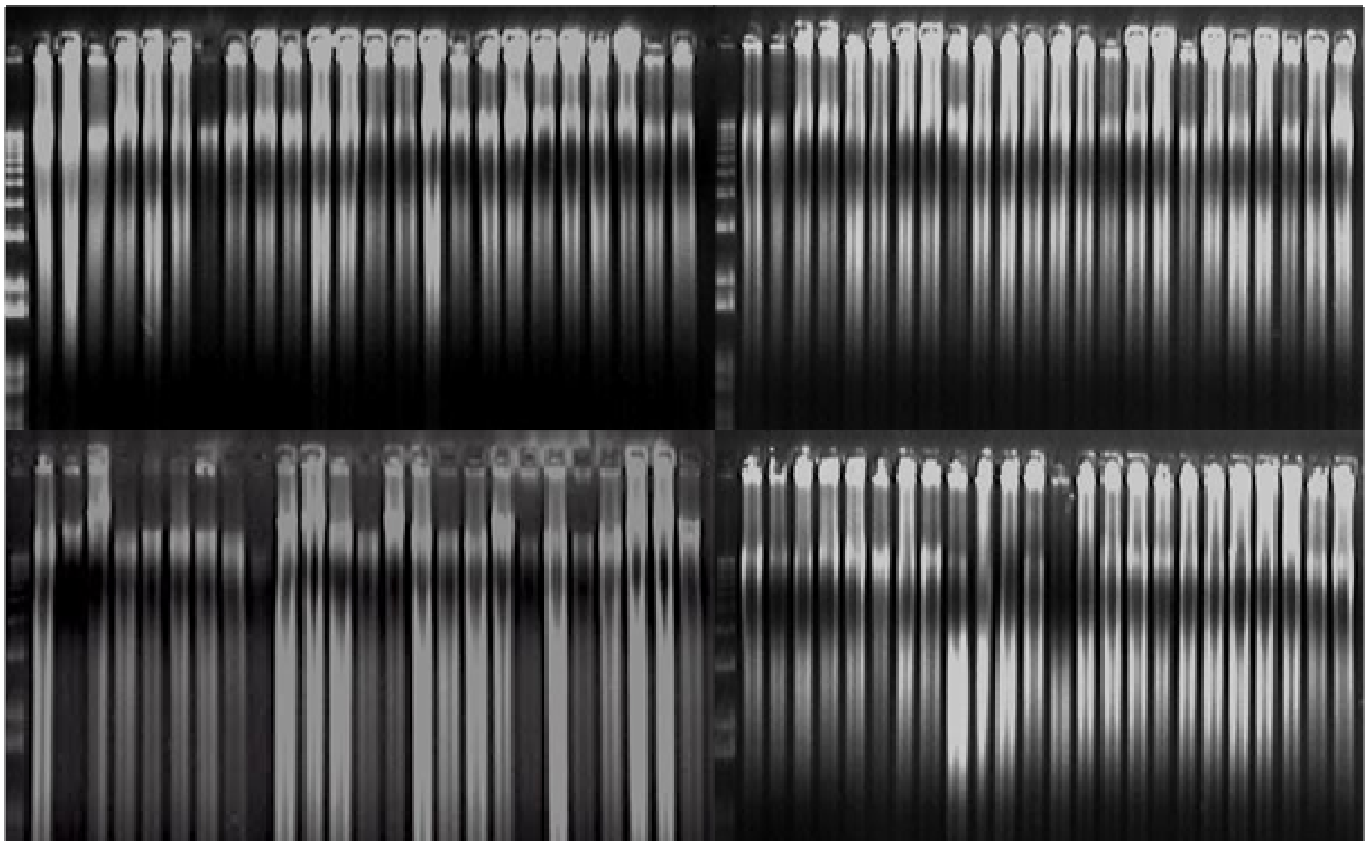

Figure 1. Agarose gels (0.8%) of genomic DNA extracted from 96 cassava accessions.

yellowish-brown which may be due to presence of phenolic compounds. The use of liquid nitrogen was omitted as storage in saturated NaCl-CTAB-azide buffer helped in softening the leaf tissues and facilitated subsequent grinding in the extraction buffer. The results, therefore, confirmed that both preservation methods could be fol-lowed in cassava. However, for collection of samples from remote locations, it is feasible to preserve tissues in NaCl-CTAB-azide solution prior to extraction. The high quality of genomic DNA obtained is also evident from high molecular weight bands (Figure 1). In addition, this method reduced the usage of liquid nitrogen and storage at -20°C, thus reducing the cost involved in preservation of leaf samples for longer time. The

bactericidal and detergent properties of CTAB also helped in avoiding bacterial and fungal proliferations (Rogstad, 1992; Štorchová et al., 2000). Some degree of DNA shearing was observed, which might be due to grinding of tissues in the GenoGrinder using steel balls, however, the quantity obtained was high and quality was adequate for PCR amplification (Figure 2). The use of different type and size of beads may reduce the degree of DNA shearing. In both cases, a modified CTAB extraction procedure that included the use of 4% PVP and 2% PEG (Sharma et al., 2008) was used to remove the polysaccharides, polyphenols and proteins present, thus reducing the interference of these compounds with PCR amplification.

DNA extraction with or without phenol

In most DNA extraction procedures of cassava, it is recommended that solvent extraction is done using phenol: chloroform: isoamylalcohol (25:24:1) (Dellaporta et al., 1983; Doyle and Doyle, 1987; Sharma et al., 2008). In the present study, we followed solvent extraction for purification of DNA with or without the use of phenol: chloroform: isoamylalcohol (25:24:1) to determine if the quality of DNA was affected. There is no doubt that purification of DNA using phenol was significantly better because it denatures proteins well. However, the major disadvantage of using phenol is its caustic and toxic properties that can pose health hazards. There is also a considerable loss of DNA sample after phenol extraction. Many researchers do not recommend the use of phenol or suggests not using it while extracting DNA from phenolic-rich plant species (Richards, 1990; Williams and Ronald, 1994; Aljanabi and Martinez, 1997). The results from PCR amplification confirmed that there is no difference in the quality of amplified products while DNA was extracted with or without phenol (Figure 2). At IITA, we routinely follow a low throughput method of DNA extraction from cassava, using manual grinding, that does not require phenol-chloroform.

Quality and quantity of DNA

The quality of DNA is generally assessed by agarose (0.8%) gel electrophoresis or spectrometric analysis or through PCR amplification of DNA samples. In the present study, samples were first electrophoresed on 0.8% agarose gel to assess the quality and quantity of DNA (Figure 1). The NanoDrop spectrophotometer was then used to determine the quantity and quality of DNA. It uses the same absorbance principle as in any other spectrometric analysis in which the ratio of absorbance at A_{260}/A_{280} is 1.8 for a pure DNA sample and a decrease in this value indicate the presence of contamination by mostly proteins, while an increase in the ratio indicates the presence of RNA (Sambrook and Russell, 2001).

DNA from six randomly selected samples from each extraction procedure (samples stored at -20°C for one week, two weeks and one month; and in NaCl-CTAB-azide solution at 4°C for one week, two weeks and one month) with or without phenol was subjected to PCR amplification using two pairs of cassava SSR primers. The results indicated that SSR profiles obtained on high resolution agarose gels with DNA from fresh samples or samples stored at -20°C or in NaCl-CTAB-azide solution with or without use of phenol (Figure 2) were essentially the same indicating that the established protocol for preservation of leaves in NaCl-CTAB-azide buffer and DNA extraction without the use of phenol can be routinely used in cassava. However, it is noteworthy that, if large numbers of primer pairs are assayed, the amplification result may vary depending on the robustness of individual primer pairs. Some primers, depending on the assay, may tend to be sensitive to the quality of the template DNA.

DISCUSSION

The main objective of the present study was to establish and simplify the preservation technique and DNA extraction procedure in cassava so that sufficient amount of high quality DNA can be obtained for large scale genotyping studies. The method described in the present study is not only rapid but also cost-effective when large number of samples is to be collected from remote locations and DNA needs to be extracted. The key modifications that were made in the present study are (1) use of NaCl-CTAB-azide buffer to preserve the leaf samples at 4°C, (2) use of GenoGrinder 2000 to grind leaf tissues, (3) use of PVP (2%) and PEG to remove polyphenolic com-pounds and proteins completely (4) purification of DNA without use of phenol.

Following the extraction procedure described here, processing time was reduced by about 40% compared to other recommended procedures in cassava, making it possible to extract DNA from 192 samples per day. The extracted DNA was of good quality and quantity sufficient for about 1000 PCR reactions. In addition, the cost of extraction (material cost) including labor cost, was reduced by five-to-six fold, making the cost per sample up to $0.80. This simple, cost-effective, rapid and high-throughput sample preservation and extraction protocol can there-fore be used for large scale DNA extraction in cassava, wherein the leaf tissues are collected from remote locations.

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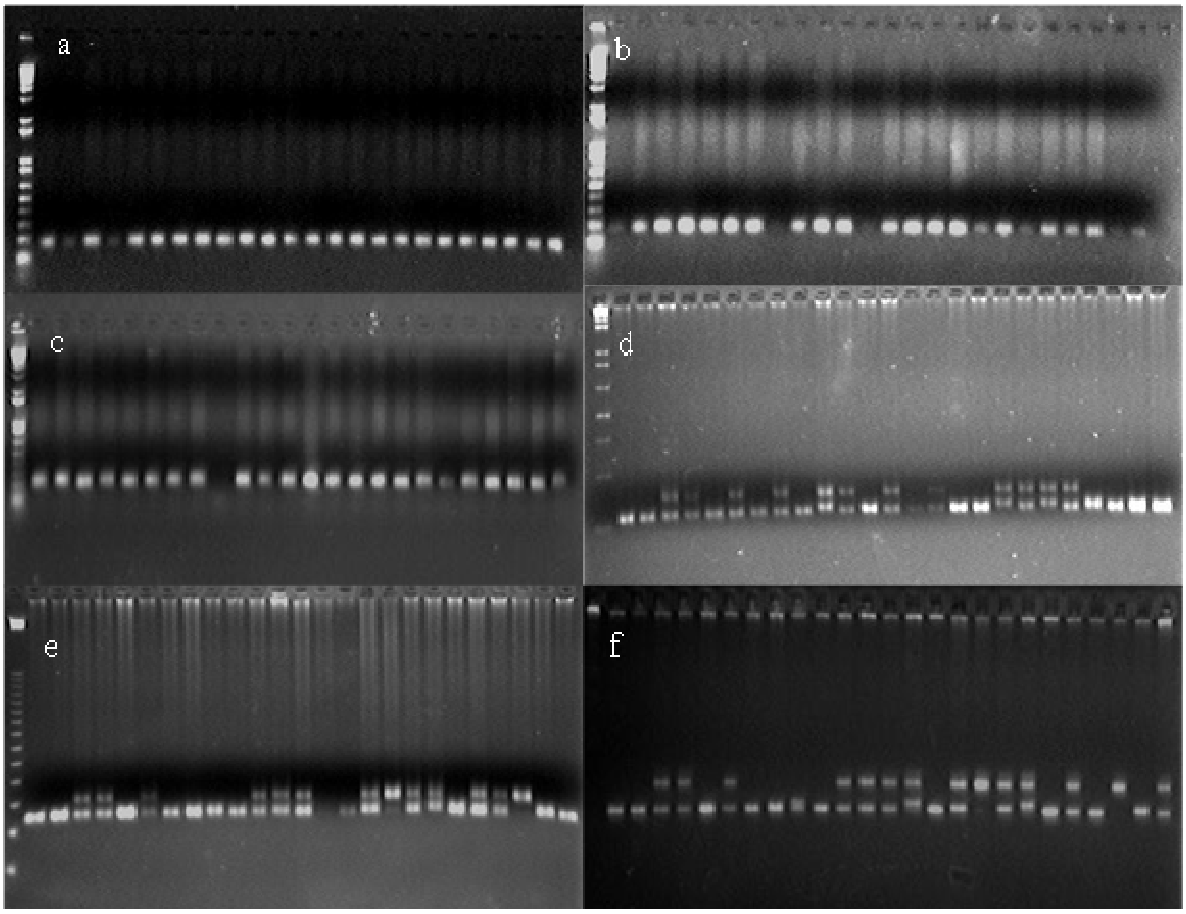

Figure 2. PCR amplification of DNA isolated from six cassava genotypes with two oligonucleotide primers (AT52: a, b, c; S7: d, e, f). The first and second lane in all the figures represents DNA standard and DNA obtained from fresh leaves, respectively. In 2a, 3rd-8th lane represents samples stored at -20°C for one week and extracted with phenol; 9th-14th lane represents samples stored at -20°C for two week and extracted without phenol; 15th-20th lane represents samples stored at -20°C for 2 weeks and extracted with phenol; 21st-26th lane represents samples stored at -20°C and extracted without phenol. In 2b, 3rd-8th lane represents samples stored at -20°C for one month and extracted with phenol; 9th-14th lane represents samples stored at -20°C for one month and extracted without phenol; 15th- 20th lane represents samples stored in CTAB buffer for one week and extracted with phenol; 21st-26th lane represents samples stored in CTAB buffer for one week and extracted without phenol. In 2c, 3rd-8th lane represents samples stored at CTAB buffer for 2 weeks and extracted with phenol; 9th -14th lane represents samples stored in CTAB buffer for 2 weeks and extracted without phenol; 15th-20th lane represents samples stored in CTAB buffer for one month and extracted with phenol; 21st-26th lane represents samples stored in CTAB buffer for one month and extracted without phenol. The Figures 2d, 2e and 2f represent similar conditions for the second primer.

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