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Full Length Research Paper

A simplified, cost- and time-effective procedure for genotyping pearl millet in resource-limited laboratories

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Procedures for DNA extraction and genotyping of large plant populations are cumbersome and expensive for resource-limited laboratories. Through eliminating or changing several steps used in DNA extraction, PCR amplification and PAGE electrophoresis in pearl millet [*Pennisetum glaucum* (L.) R. Br.], we developed a modified procedure that reduced the cost of consumables and required less time without compromising data quality. In the revised procedure, DNA was extracted by incubating 0.5 - 0.7 g ground young leaf tissue in 2% CTAB/β-mercaptoethanol followed by refrigerated differential centrifugations with phenol:chloroform:isoamylalcohol. Steps such as additional phenol/chloroform treatments, DNA pellet drying followed by RNase treatments and incubation were eliminated, reducing use of costly and corrosive chemicals and saving time. DNA produced from 174 genotypes exhibited an average concentration of 640 ng/µL and average optical density ratio of 1.9. PCR amplification of SSR markers with this DNA produced clear and scorable bands following ethidium bromide stained agarose and silver stained polyacrylamide gel eletcrophoresis. Post PCR duplexing of two or more microsatellites based on different lengths of base pairs reduced the time and cost per unit data generation by up to half as compared to single marker per PAGE. Cluster analysis performed on the marker data generated 11 SSR primers following these procedures formed two main groups from genotypes of the U.S. origin. In summary, the procedures reported are simplified, shortened and economical and well suited for resource limited laboratories engaged in molecular breeding requiring large volume of genotyping.

Key words: Pearl millet, DNA extraction, genotyping, mapping population, SSR markers, cluster analysis.

INTRODUCTION

Pearl millet is grown primarily as a staple food crop in the hot and dry regions of Africa, the Indian subcontinent and elsewhere in the world. Its popularity as a multipurpose crop is expanding beyond agricultural systems of Africa and Asia to Australia, Brazil, Canada and the United States (Burton, 1995; Hanna, 1996; Bonamigo, 1999; Gulia et al., 2007). In arid and semi-arid regions, however, pearl millet succumbs to certain insect pests and diseases and prolonged droughts that cause considerable losses to yield of grain and biomass (FAO and ICISAT, 1996). Understanding the genetic complexities of these traits and characterizing genotypes by conventional means alone is difficult and thus necessitates plant breeders to apply DNA markers in routine breeding programs for traits identification and integration. Among available DNA markers systems, PCR based co-dominant SSRs (also known commonly as microsatellites) are preferred for genotyping large mapping populations because their reproducibility, abundance, amenability to high throughput screening. A number of SSR primers are available in pearl millet (Allious et al., 2001; Budak et al., 2004) and these markers have been used for genetic diversity analysis (Chowdari et al., 1998; Budak et al., 2004; Kapila et al., 2008), linkage and QTL mapping (Qi et al., 2004; Gulia, 2004; Gulia et al., 2007).

DNA extraction is the most time consuming and expensive step in molecular breeding experiments (Mace et al., 2003). It alone constitutes 30 - 60% of total time required for sample processing from leaf tissue collection

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to microsatellite genotyping (Ragot and Hoisington, 1993; Dilworth and Frey, 2000). The DNA extraction procedure is primarily based on the DNA marker system to be used. PCR based markers (e.g. microsatellites) produce desirable results with lower DNA concentrations of average quality while Non-PCR based (e.g. RFLP) markers require high DNA concentration of the highest possible quality. Several maxi-prep plant genomic DNA isolation procedures have been reported (Dellaporta et al., 1983; Murray and Thompson, 1984; Sharp et al., 1988; Tai and Tanksley, 1990). Later, modifying Doyle and Doyle (1987) and Sharp et al. (1988), Mace et al. (2003) extracted DNA from five tropical crops including pearl millet using high-throughput (mini-prep, 96-microplate) technology for genotyping large mapping populations using PCR-based markers. Though this procedure is expeditious, set up of a high throughput laboratory is not always possible especially in resource-limited countries because of the prohibitive instrumentation cost. In spite of these timely modifications, these DNA isolation techniques remain expensive and tedious involving several cycles of purification and use of expensive enzymes, corrosive chemicals and costly equipments.

In our present study, we focused on simplifying procedure and reducing the cost and time for microsatellite genotyping so that researchers in semi-arid tropics could use these markers in resources-limited laboratories more economically in their pearl millet breeding programs. In addition, this step-wise well explained procedures will help students and researchers learn and experience applications and methodologies of DNA marker technology. Our extraction procedure was based on the premise that SSR markers require a relatively low concentration (5 – 50 ng) of average quality DNA. Therefore, modifying existing procedures could cut more time and cost right from DNA extraction to PCR amplification to separation of PCR product using PAGE electrophoresis.

MATERIALS AND METHODS

Plant material

DNA was extracted, using this modified procedure (given below), from progenies of RIL mapping population based on cross Tift 454 x Tift 99B used for studying linkage mapping and from a set of 90 diverse genotypes for genetic diversity (Annex. 1). These genotypes were planted in the greenhouse in 15 cm pots each containing a minimum of five seeds. Young leaf tissues from two to three weeks old seedlings from each pot were harvested for DNA isolation. Collected tissues were quick-frozen in liquid nitrogen and ground to a fine powder manually using pestle and mortar and stored in 50 ml propylene tubes at -80°C.

Chemical solutions

Most of the chemical solutions used were laboratory made that consisted of 2% CTAB/β-mercaptoethanol DNA extraction (CTAB (w/v) (20 g), 1M Tris (pH 8.0) (200 mL), 5M NaCl (280 mL), 0.5M EDTA (pH 8.0) (40 mL), 0.2% β-mercaptoethanol), phenol:chloroform:isoamyl alcohol (IAA) (25:24:1), chloroform:IAA (24:1), 2.5 M sodium acetate, 7.5 M ammonium acetate, 70 - 100% ethanol and $T_{10}E_1$. Bind and repel silanes were purchased from GE Life Sciences while that of 29:1 polyacrylamide:bis solution, temed and APS (ammonium per sulphate, 10% (w/v)) from Biorad and 10X TBE were laboratory prepared for casting 8 - 12% PAGE (polyacrylamide gel electrophoresis) gels. Working solutions of 0.1% CTAB (w/v), 1.0% ammonia (v/v), 1M NaOH, 0.1% silver nitrate (w/v), 1.5% sodium carbonate (w/v) and 1.5% glycerol (v/v) were prepared for staining PAGE gels. Bromophenol dye, DNA templates, primers, RNase and DNA ladder were prepared and diluted as needed.

DNA extraction

A sample of 0.5 - 0.7 g tissue was transferred into 2 mL Eppendorf microcentrifuge tubes containing 800 - 900 µL preheated 2% CTAB (+ 0.2% β-mercaptoethanol) extraction buffer. Tissues were mixed thoroughly by gentle inversions of the tubes and incubated at 65°C for 30 min following 2 - 3 intermittent stirrings. An equal amount of phenol:chloroform:IAA (25:24:1) was added and gently mixed by several inversions to form an emulsion. The emulsion was separated into an aqueous phase and cell debris by centrifugation at 6000 rpm for 15 min at 4°C using refrigerated Eppendorf centrifuge. The upper aqueous phase was transferred to a new sterile Eppendorf tube. To further purify the samples, an equal amount of chloroform:IAA (24:1) was again added, gently mixed and separated as two phases by centrifugation at 5500 rpm for 10 min maintaining temperature at 4°C. The supernatant was transferred into a clean and sterile Eppendorf microcentrifuge tube. To the final supernatant (aqueous phase), 2.5 M sodium acetate or 7.5 M ammonium acetate (one tenth volume) and up to two volumes of chilled absolute ethanol was added, mixed by gentle inversions and incubated at -20°C for 20 min. The DNA was precipitated by centrifugation at 10,000 rpm for 5 min and excess ethanol was drained out. Precipitated DNA was rinsed twice in 70% ethanol, excess ethanol was poured off and DNA pellets were air-dried in dust free conditions for at least 30 min depending upon pellet size and moisture. The DNA samples were finally suspended in an appropriate volume of $T_{10}E_1$ (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), to make a volume of 150 - 200 µL (according to the size of the individual pellets) and stored at 4°C for further dilution and use based on DNA quantification.

DNA quantification and purity check

DNA concentration of each sample was quantified by using a Smart Spec™ Plus Spectrophotometer (Bio-Rad Laboratories Inc.) at UV absorption of 260 nm, assuming 1 OD at 260 nm is equal to 50 µg of DNA. The ratio of OD_{260}:OD_{280} was calculated to check the purity of DNA samples. DNA quality is considered good with an OD_{260}:OD_{280} ratio between 1.7 and 2.0 (Maniatis et al., 1982). Programmed algorithms of this spectrophotometer calculated final DNA concentration (ng/µL) of the sample using a dilution factor 200 (5 µL DNA was added to 995 µL of DDW) and ratio of absorbance at 260 nm and 280 nm.

DNA samples were analyzed in a 0.8% TBE-agarose gel to test its integrity (Maniatis et al., 1982). Gels were stained with ethidium bromide and viewed on a UV-transilluminator, then photographed with a camera fitted with a UV filter. The quantity of DNA in samples was estimated and confirmed by comparing the fluorescent yield of the sample with that of uncut *lambda* DNA standards. A working solution of 1000 µL from each sample was prepared maintaining a uniform DNA concentration (20 ng/µL) for PCR reactions by adding X amount of DNA and Y amount of DDW, where X = [(20 ng/µL × 1000 µL) ÷ DNA concentration of stock solution], and Y = 1000 - X.

PCR amplification

The master mix reagents were reduced to 10 µL from the earlier commonly used procedure that contains total volume of 20 µL. The master mix reagents included 1.0 µL of PCR buffer (10X), 0.2-0.5 µL of $MgCl_2$ (15 mM), 1.0 µL of DNTP mix (2 mM), 0.4 - 0.5 µL of each forward and reverse primer (30 ng/µL), 0.10 - 0.15 µL of *Taq* DNA polymerase (5 U/µL). De-ionized distilled water was added to this master mix and 1.5 - 2.5 µL of genomic DNA template (20 ng/µL) to make a total volume of 10 µL. The PCR reactions for DNA-primer amplification were conducted in a Peltier Thermo Cycler DNA Dyad Engine (Bio-Rad Laboratories Inc.). The PCR conditions were optimized at various annealing temperatures for each primer separately. A majority of pearl millet genomic SSR markers exhibited excellent amplification of the primer-DNA template using a touchdown program in the thermocycler. The touchdown program involves denaturation of the samples at 95°C, for 4 min as first step, followed by 30 s as second step and thereafter annealing at 60°C for 30 s and elongation at 72°C for 30 s. Step two (denaturation at 95°C for 30 s) is repeated 29 times followed by annealing at 45°C and elongation at 72°C. Step two was repeated 29 more times followed by extension at 72°C for 7 min and finally stored at 4°C.

PCR product separation and visualization

Separation and visualization procedure remained similar to the one previously used. 2 µL of PCR amplified product from each marker was loaded along with standard size ladder (1.0 - 2.0 µL of 100 bp ladder) on 6% polyacrylamide gels [8.0 mL 10X TBE buffer, 15.0 mL of 30% acrylamide/bis-acrylamide solution [29:1 (w/w)] and 52.0 mL distilled water or 30% (w/v) urea (in case of denaturating gel), TEMED (90 µL), 400 µL of 10% (w/v) APS (ammonium persulphate)] using a 96-toothed comb (0.4 cm well-center-to-well center with thickness of 0.4 mm). The gel was run for at least three hours at 25 W to complete migration of DNA from one top to bottom. The electrophoretically separated DNA bands were visualized by silver staining (described below). Amplifications of a set of 96 genotypes were electrophoresed by either monoplexing PCR product from a single SSR marker, or duplexing or multiplexing the PCR products from more than one SSR marker depending upon their base-pair sizes.

Duplexing

PCR products from two markers that showed reasonable distinguishable base pair differences were used in single gel lane against commonly used one marker per lane. Duplexing (more than one primer/microsatellite loaded simultaneously on a single PAGE gel) was based on the information determined from the initial marker amplification and parental screening tests. While choosing the primers, it was confirmed that the amplified products of selected primers do not co-migrate. The PCR reaction of each primer pair was set up separately and these monoplex products were pooled, that is, post-amplification multiplexing or multiloading). These samples were loaded in the PAGE and bands were visualized with silver staining procedures. Alleles for each primer were scored separately by comparing banding patterns with those of parental alleles for the respective primer pairs.

Silver staining and scoring DNA bands

Electrophoresed DNA fragments were detected with silver nitrate staining based on Goldman and Merril (1982) and Gulia (2004) with minor modifications. The glass plate containing polyacrylamide gel and separated DNA templates was rinsed in double distilled water (DDW) for 3 - 5 min followed by soaking in 0.1% (w/v) CTAB for 20 min on a shaker. The gel was incubated in 0.1% ammonia solution followed by staining in 0.1% (w/v) silver nitrate solution containing 8 mL of 1 M NaOH titrated with ammonia until the solution became clear. After rinsing in DDW for 0.5 - 1 min, the gel plate was placed and stirred in developer (1.5% $NaCO_3$ containing 0.4 mL formaldehyde) until the bands became visible. After rinsing in DDW for 0.5 - 1 min to stop staining, the gel was placed in 1.5% (v/v) fixer, rinsed in water again, cleaned and scanned for imaging. A 2L volume of the above solutions was used to completely immerse the gel plate. These solutions can be used 2 - 3 times except the developer which is made fresh every time. The visible DNA bands were scored as presence (as 1) or absence (as 0) of bands from gel plates on white light illuminator from 90 US pearl millet breeding lines against 11 SSR markers (Annex. 2) and data analysis was done using Popgene and NTSys pc softwares. The dried gel was removed from the glass plate by soaking in sodium hydroxide (NaOH) solution (40 g flakes in 4 L of DDW) for several hours.

RESULTS AND DISCUSSION

Steps of a maxi-prep that took longer time (Gulia, 2004) and mini-prep based on microplate high throughput (Mace et al., 2003) that involved high-cost instrument for DNA extraction procedures were combined to develop a new simplified procedure keeping in mind the economics of genotyping large mapping population in resource-limited laboratory. Procedures used for DNA extraction can be broadly grouped into two categories based on marker system used into maxi-prep and mini-prep (high throughput), both of which have their particular limitations. Maxi-prep is slow and cost per sample is high while instrumentation cost for mini-prep is prohibitive for common laboratories although per sample processing cost is lower. Several experiments were conducted by combining steps from maxi-prep and mini-prep DNA extraction procedure used by earlier researchers (Mace et al., 2003; Gulia, 2004; Zidani et al., 2005) to develop a simpler and more economical method suitable to laboratories with moderate or limited facilities, especially semi-arid tropics. In the modified procedure, use of smaller amount of ground leaf tissue corresponded to lesser quantities of CTAB buffer, phenol:chloroform:IAA and chloroform:IAA and other counsumables. Use of 0.5 - 0.7 g ground leaf tissues in 2 mL Eppendorf tubes enabled us to centrifuge more sample tubes at a time with a small bench top refrigerated centrifuge. This procedure eliminated consumable amounts used or even steps such phenol:chloroform, $T_{50}E_1$, RNase, additional incubation, centrifugation and drying of the DNA pellet halfway during the process as used in both mini- and maxi-prep DNA extraction (Mace et al., 2003; Gulia, 2004).

DNA quality and integrity depends on the number and time of treatments (phenol:chloroform, protienase-K, RNase etc.) to remove proteins, RNA, polysaccharides and other adhering compounds, as well as by plant age at the time of tissue collection. In an earlier study (Gulia, 2004), leaves from 2 - 3 week old seedlings grown in a greenhouse yielded good quality DNA from extraction

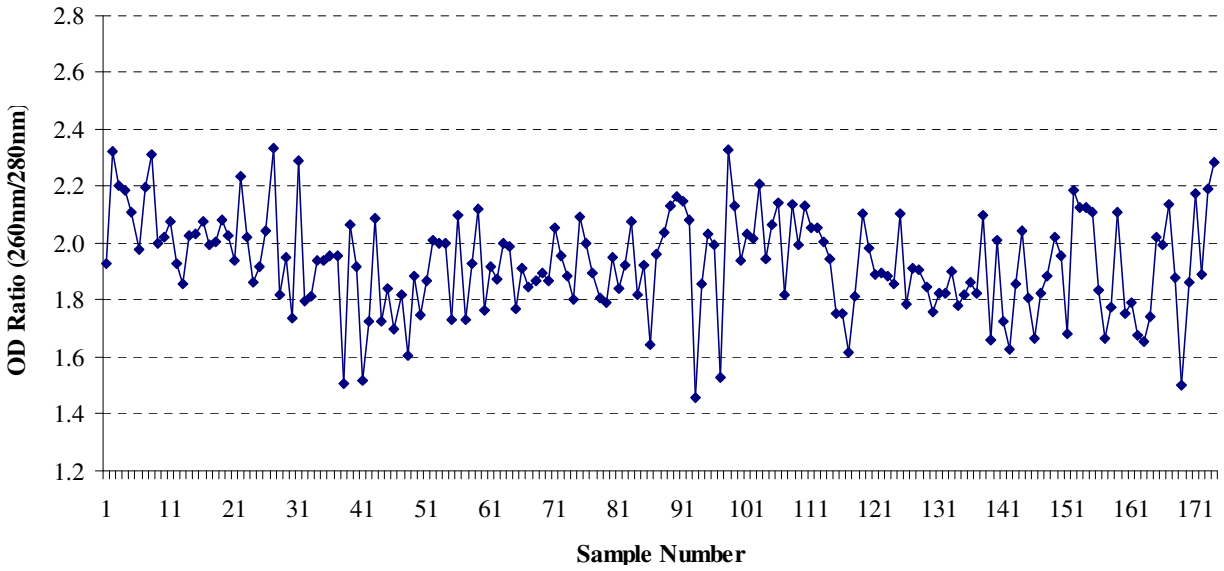

Figure 1. Optical density (OD) ratio of DNA samples extracted from 174 RIL mapping population progenies using modified DNA extraction procedure.

using CTAB maxi-prep procedure and produced excellent results from PCR amplification using SSRs, and enzymetic digestion and probe hybridization using RFLPs. But it took two days to isolate DNA from 16 - 32 samples (first day for extraction and second day for purification) and involved heavier equipment, larger volume tubes and corresponding reagents to produce DNA. This procedure was similar to one above but smaller volumes of consumables and treatments of phonol:Chroloform:IAA and RNase followed by hot water bath incubation and drying were eliminated. Thus, it was an intermediate between the max- and mini-procedures. PCR amplification of DNA samples extracted with and without RNase treatments produced similar results. However, if DNA is to be used in RFLP studies, it should preferably be treated with RNase to further purify for better results.

Optical density of DNA samples from 174 genotypes were measured by spectrophotometry (UV absorbance) at 260 and 280 nm. Most of the samples exhibited an OD ratio (260:280 nm) between 1.7 and 2.0, with a mean of 1.9 (Figure 1), which is considered to be good for DNA quality and integrity (Maniatis et al., 1982). The DNA concentration of these samples varied from 310 ng/µL to 1308 ng/µL, with a majority of the samples between 500 - 700 ng/µL (Figure 2). The concentration and quality of DNA obtained from these samples was sufficient for genotyping more than 400 SSR primers using 30 - 60 ng DNA per marker reaction. Samples were tested on 0.8 - 1.0% agarose gel electrophoresis for comparing DNA band illumination with a Lambda marker to check the DNA concentration, quality and fidelity (Figure 3). DNA produced from this revised procedure as shown in Line 1 with DNA from the max-prep with RNase treatment shown in Line 2 (Gulia, 2004). PCR amplification and PAGE electrophoresis results of DNA from modified procedure were comparable with the old procedure.

The Initial 20 µL of master mix prepared for PCR reactions using kit (Taq Polymerase, dNTPs, PCR buffer and $MgCl_2$) from Bioline Inc., SSR primers and the DNA template produced good results. However, even if PCR master mix ingredients were reduced up to half, it did not affect amplification results for most of the SSR primers. PCR amplified products produced good and clearly observable DNA bands when tested on agarose (1%) and polyacrylamide gel electrophoresis (8 - 12% PAGE) (Figures 4 and 5). Additionally, in this procedure separation of genomic SSRs by both monoplexing (simplexing) and duplexing of PCR product(s) on a single PAGE gel was employed for time saving in genotyping. Simplex PCR conditions were first optimized for each primer pair by testing appropriate thermocycling conditions and master mix contents. Two primer loci exhibiting larger base-pair differences of the PCR products were mixed (duplexing) and loaded together on the same PAGE gel (Figure 6). Primers with lesser or little base pair differences were separately loaded on the PAGE gel to make PAGE duplexes at 20 - 25 min interval. This procedure doubles the genotyping efficiency by saving time and resources up to 50% to accomplish research objectives and enhances breeding progress and success over a period of time, provided proper attention is given during electrophoresis. We used these procedures for population genotyping and characterizing genotypes for genetic diversity studies and linkage mapping using SSR markers. A cluster analysis of the marker data from 11 SSR markers against a set of 18 diverse genotypes from the U.S. was conducted and presented in this paper. It produced a dendrogram (Figure 7) that consists of mainly

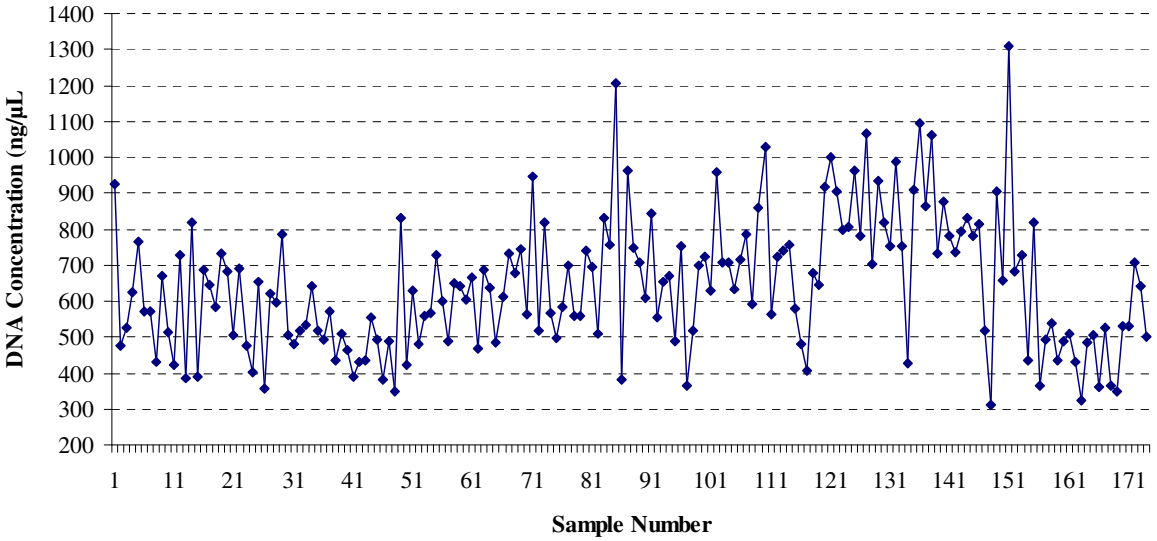

Figure 2. DNA concentration (ng/µL) of DNA samples extracted from 174 RIL mapping population progenies using modified DNA extraction procedure.

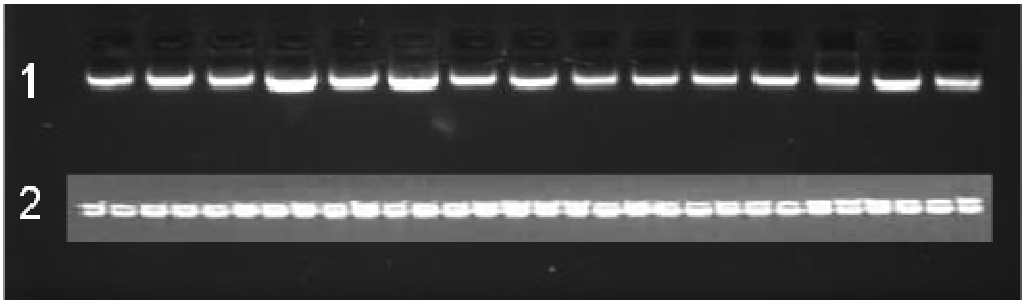

Figure 3. Comparison of DNA extracted using two different procedures by ethidium bromide stained agarose gel electrophoresis (0.8%). Line 1 indicates DNA extracted using newly developed procedure while line 2 indicates DNA extracted using old procedure (Line 2 photo taken from Gulia, 2004).

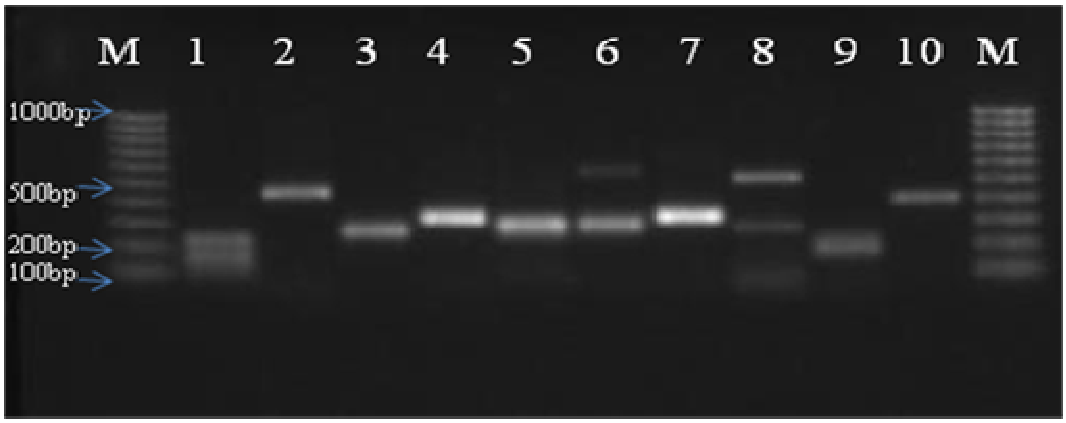

Figure 4. Amplification test of a pearl millet DNA sample using pearl millet SSR markers (CTM series) as shown on 1% agarose gel as mentioned in text. (The numbers from 1 to10 represent pearl millet genomic primer-pairs CTM 2, CTM 3, CTM 11, CTM 12, CTM 21, CTM 25, CTM 27, CTM 57, CTM10 and CTM 55, respectively).

Figure 5. Fingerprints of DNA samples from 24 diverse pearl millet genotypes amplified using SSR marker (PSMP 2074) and electrophoresis on 8% PAGE gel. (M indicates 100 base-pair ladder).

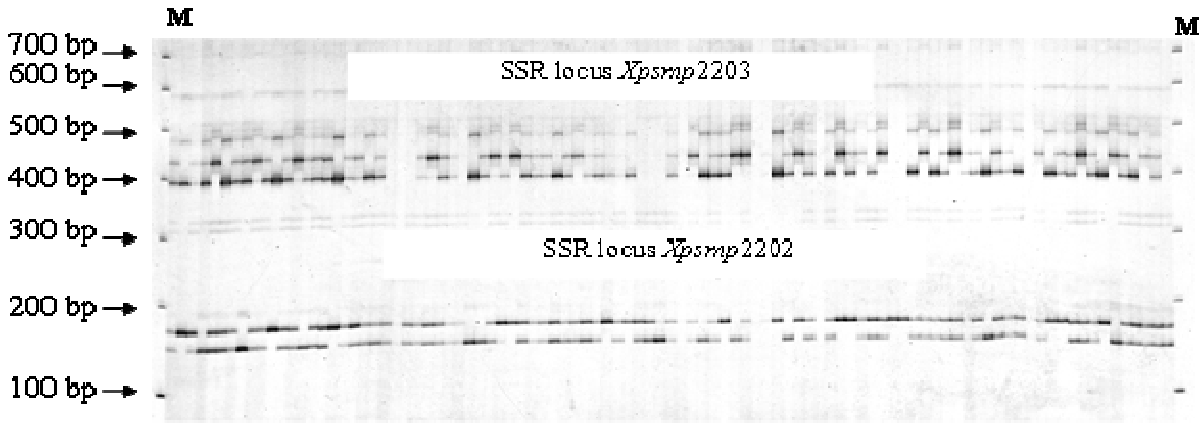

Figure 6. Post PCR product multiplexing of two primer pairs on PAGE gel electrophoresis. Two SSR markers differing from each other with more than 200 bp were loaded on one PAGE gel.

two broad groups, one clustering majority of genotypes and forming several subgroups.

In summary, this modified and simplified pearl millet DNA extraction procedure differed from earlier ones in terms of time, simplicity and resources and technology requirements, while reducing use of costly enzymes and corrosive and hazardous chemicals. Using a reduced volume of master mix and duplexing of PCR product from two or more primers reduces costs of genotyping and saved considerable time. These results will prove useful to learners and researcher in molecular breeding working in semi-arid tropics in particular. The protocols developed in this study should result in a more economical application of DNA marker technology in resource-limited laboratories.

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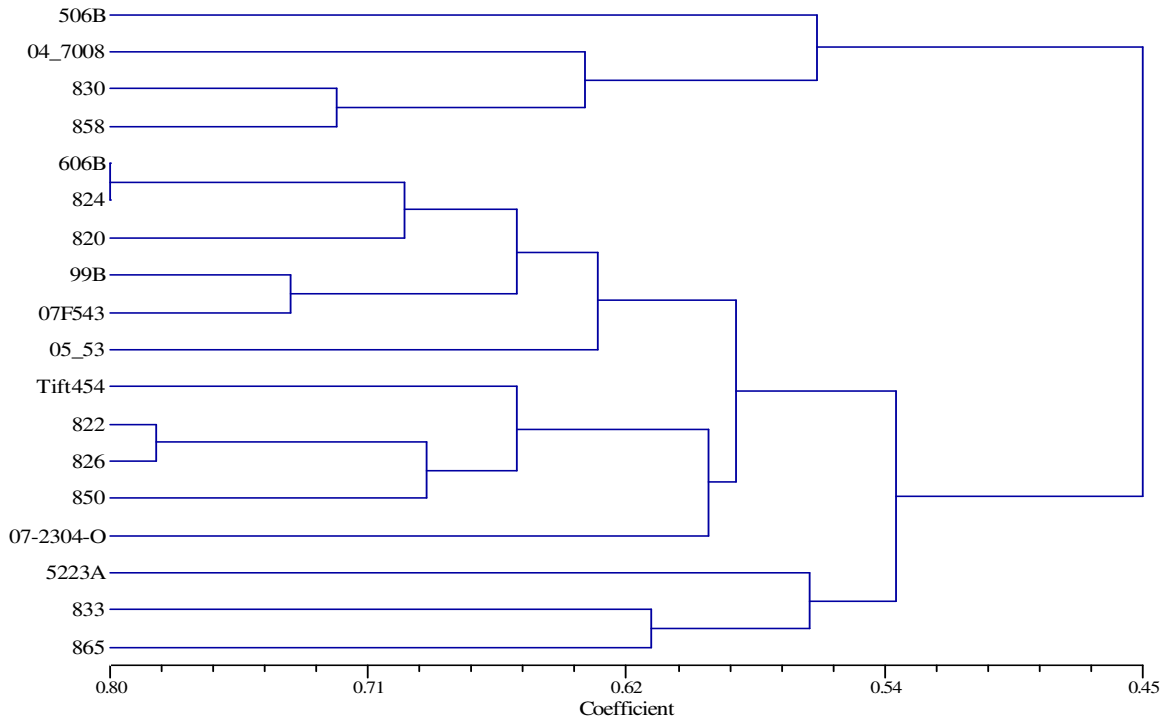

Figure 7. Dendrogram displaying genetic relationships among the studied pearl millet breeding lines from the US using UPGMA cluster analysis of Jaccard genetic similarity coefficients generated from 10 SSR markers.

Annexure 1. Plant material (germplasm/breeding material) collected from Africa, India and USA used for DNA extraction and diversity study.

African lines	African lines	Indian B lines	US line
PS 202_1_4	ICMV_IS_90311	703	606B
PS 202_1_9	Synthetic1_2000	706	99B
PS 202_1_13	Zatib	710	Tift_454
PS 202_1_16	DMR72	711	07_2304_O
PS 202_1_23	Zongo	719	5223A
PS 202_1_24	Sosat_C88	720	07F543
PS 727_4_8	ICMV_IS_89305	727	04_7008
PS 727_4_10	Gwagwa	738	05_53
PS 727_4_11	NKK	749	820
PS 727-4_18_1	Sosank	750	822
PS 727_4_19	CIVT	757	824
PS 727_4_22	Taram	765	826
PS 727_4_23	HKP_GMS	773	830
PS 727_4_26	GB_8735	782	833
PS 727_4_30	Toronio	790	850
CIVT2	Arrow	796	858
CIVT9	Bongo_short_head	801	865
CIVT10	P3Kollo	806	
CIVT14		810	
CIVT16		817	
CIVT18		818	
CIVT19		820	

Annexure 1. Contd.

CIVT20		821	
CIVT22		822	
CIVT24		506B	
CIVT25			
CIVT27			
CIVT29			
CIVT30			

Annexure 2. Pearl millet SSR genomic marker used for PCR amplification and diversity data generation for the plant material used in this.

Primers	Forward (5' to 3')	Reverse (5' to 3')
CTM 59	TCCTCGACATCCTCCA	GACACCTCGTAGCACTCC
CTM 60	AAGCCCCGATCACATCAA	AGCCGAGCCTCATCCC
PSMP 2040	CATTACACGTTTCTTCAAACGC	TCTTCGGCCTAATAGCTCTAAC
PSMP 2201	CCCGACGTTATGCGTTAAGTT	TCCATCCATCCATTAATCCACA
PSMP 2202	CTGCCTGTTGAGAATAAATGAG	GTTCCGAATATAGAGCCCAAG
PSMP 2206	AGAAGAAGAGGGGGTAAGAAGGAG	AGCAACATCCGTAGAGGTAGAAG
PSMP 2207	CAGGGCATACTTCAAGATTGATTC	GTCCACTTGTTATTCTCTATCACC
PSMP 2209	TTGGACGATTTGGAAGCATAG	GAGGAAAAGAGCCATACAGAGAC
PSMP 2214	CGCACAGTACGTGTGAGTGAAG	GATTGAGCAGCAAAAACCAGC
PSMP 2224	GGCGAAATTGGAATTCAGATTG	CGTAATCGTAGCGTCTCGTCTAA
PSMP 2235	ATAAGTGGACCCCATGCAGCAC	CGAAAGACTAGCAAAATTGCGCCTTC

REFERENCES

Allouis S, Qi X, Lindup S, Gale MD, Devos KM (2001). Construction of a BAC library of pearl millet, *Pennisetum glaucum*. Theor. Appl. Genet. 102: 1200-1205.

Bonamigo LA (1999). Pearl millet crop in Brazil: Implementation and development in the Cerrado savanahas. In: Neto AL de F, Amabile RF, Netto DAM, Yamashita T, Gocho H (Eds) Proceedings of the International Pearl Millet Workshop. EMBRAPA Cerrados: Planaltina, DF, Brazil. pp. 31-66.

Budak H, Pedraza F, Cregan PB, Baenziger PS, Dweikat I (2003). Development and utilization of SSRs to estimate the degree of genetic relationships in a collection of pearl millet germplasm. Crop Sci. 43: 2284-2290.

Burton G (1995). History of hybrid development in pearl millet. In: Proceedings of First Grain Pearl Millet Symp. Tifton, GA. 17-18 Jan, 1995. Univ. of Georgia, Tifton. pp. 5-8.

Chowdari KV, Davierwala AP, Gupta VS, Ranjekar PK, Govila OP (1998). Genotype identification and assessment of genetic relationships in pearl millet [*Pennisetum glaucum* (L.) R. Br] using microsatellites and RAPDs. Theor. Appl. Genet. 97: 154-162.

Dellaporta SL, Wood J, Hicks JB (1983). A plant DNA minipreparation: version II. Plant Mol. Biol. Rept. 1: 19-21.

Dilworth E, Frey JE (2000). A rapid method for high throughput DNA extraction from plant material for PCR amplification. Plant Mol. Biol. Rep. 18: 61.64.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf material. Phytochem. Bull. 19: 11-15.

FAO and ICRISAT (1996). The world sorghum and millet economics: Facts, trends and outlook. FAO, Rome, Italy and ICRISAT, Patancheru-502324, AP, India.

Goldman D, Merril CR (1982). Silver staining of DNA in polyacrylamide gels: linearity and effect of fragment size. Electrophoresis 3: 24-26.

Gulia SK (2004). QTL mapping for improvement of downy mildew [*Sclerospora graminicola* (Sacc.) J. Schroet.] resistance (DMR) in pearl millet [*Pennisetum glaucum* (L.) R. Br.] hybrid parental line ICMB 89111. Ph.D. Thesis submitted to CCS HAU Hisar/ICRISAT India.

Gulia SK, Hash CT, Thakur RP, Breese WA, Sangwan RS (2007). Mapping new QTLs for downy mildew [*Sclerospora graminicola* (Sacc.) J. Schroet.] resistance in pearl millet (*Pennisetum glaucum* (L.) R. Br.). In: Singh DP, Tomar VS, Behl RK, Upadhyaya SD, Bhale MS, Khare D (Eds) Crop Production in Stress Environments-Genetic and Management Options. Agrobios Publishers, Jodhpur, India. pp. 373-386.

Gulia SK, Wilson JP, Singh BP, Carter J (2007). Progress in grain pearl millet research and market development. In: J. Janick (ed.) Issues in New Crops and New Uses. ASHS Press, Alexandria, VA. pp. 196-203.

Hanna WW (1996). Improvement of millets-Emerging trends. In: Proceedings. 2nd Intl. Crop Sci. Congress, Delhi, India. pp. 139-146.

ICRISAT (2005). Pioneering market-assisted breeding results in pearl millet hybrid resistant to downy mildew. www.icrisat.org/media/2005/Media19.htm

Kapila RK, Yadav RS, Plaha P, Rai KN, Yadav OP, Hash CT, Howarth CJ (2008). Genetic diversity among pearl millet maintainers using microsatellite markers. Plant Breeding 17:33-37.

Mace ES, Buhariwalla HK, Crouch JH (2003). A high-throughput DNA extraction protocol for tropical molecular breeding programs. Plant. Mol. Biol. Rep. 21: 459-460.

Maniatis T, Fritsch ER, Sambrook J (1982). Quantification of DNA and RNA. In: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press: NY. pp. 448-469.

Masi P, Zeuli PLS, Donini P (2003). Development and analysis of multiplex microsatellite markers sets in common bean (*Phaseolus vulgaris* L.). Mol. Breed. 11: 303-313.

Murray M, Thompson WF (1984). Rapid isolation of high molecular weight plant DNA. Nucl. Acids Res. 8: 4321-4325.

Paris M, Carter M (2000). Cereal DNA: A rapid high throughput extraction method for marker-assisted select. Plant Mol. Bio. Rep. 18: 357-360

Qi X, Pittaway TS, Liu H, Waterman E, Padi FK, Hash CT, Zhu J, Gale MD, Devos KM (2004). An integrated genetic map of pearl millet, *Pennisetum glaucum*. Theor. Appl. Genet. 109: 1485-1493.

Ragot M, Hoisington, DA (1993). Moleular markers for plant breeding: comparisions of RFLP and RAPD genotyping. Theor. Appl. Genet. 86: 975-984.

Sharp PJ, Kries M, Sherry PR, Gale MD (1988). Location of β-amylase sequences in wheat and its derivatives. Theor. Appl. Genet. 75: 286-290.

Tai TH, Tanksley SD (1990). A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue. Plant Mol. Biol. Rep. 8: 297-303.

Zidani S, Ferchichi A, Chaieb M (2005). Genomic DNA extraction methods for pearl millet (*Pennisetum glaucum*) leaves. Afr. J. Biotechnol. 4: 862-866.