International Journal of Agriculture Innovations and Research
Volume 4, Issue 6, ISSN (Online) 2319-1473
Manuscript Processing Details (dd/mm/yyyy) :
Received : 10/05/2016 | Accepted on : 17/05/2016 | Published : 25/05/2016

Exploitation of Simple Sequence Repeat Markers to Assess the Genetic Diversity in Sudan Pearl Millet [*Pennisetum Glaucum* (L.) R. Br.] Collections

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Abstract – **Environmental factors can radically influence plant morphology and therefore, classification depending only on visible characteristics is inherently flawed. The use of molecular markers provides a much more reliable approach to explore genetic diversity in crops. Studies on genetic diversity in *Pennisetum glaucum* L. germplasm suggest promising opportunities for the use of undomesticated materials for improving pearl millet varieties. This study therefore, aimed at the exploitation of simple sequence repeat markers (SSRs) for assessing the genetic diversity of 36 accessions of Sudan collections of pearl millet using 10 SSRs markers. The 10 markers produced 30 alleles with an average of 3 alleles per locus. The number of alleles amplified by each primer varied from 1 to 6. The size of the Polymerase Chain Reaction (PCR) products ranged from 138 to 264 bp. Polymorphism Information Content (PIC) values of microsatellite markers varied from 0 to 0.68 with an average of 0.33. Cluster analysis with the SSR markers resulted in five major groups. The geographical distribution of accessions did not fit with the genotypic cluster. The results provide a good starting point for molecular characterization of pearl millet collections and selection of parental lines for genetic improvement in pearl millet.**

Keywords – **Pearl Millet Accessions, Pennisetum Glaucum, Genetic Diversity, SSRs Markers.**

I. Introduction

Pearl millet [*Pennisetum glaucum*(L.) R. Br.] belongs to the genus Pennisetum, of the Poaceae family and the Paniceae tribe. The crop is mostly grown under harsh environmental conditions on infertile soils of low water – holding capacity, where other cereal crops generally be unsuccessful [1].These harsh conditions are expected to magnify, therefore, the future appropriateness of pearl millet cultivation is expected to spread out to other geographic regions under various climate change scenarios [2]-[3]. It is suggested that a stretching belt from Sudan to Senegal would exhibit the greatest genetic diversity in the region which represent the center of origin for pearl millet [4]-[5]. Pearl millet is an excellent forage and, because of its low hydrocyanic acid content, is the best annual grazing crop in the southern USA [6]-[7]) and an important summer forage crop in Australia and south America as well [8]–[7]. Despite the good attributes of pearl millet as irrigated forage crop [9] however; it is not grown for forage production in Sudan. Nevertheless, Sudan is pearl millet germplasm rich country as recently, more than 1600 pearl millet accessions were collected from different parts of Sudan and are available in the Agricultural Plant Genetic Resource Conservation and Research Centre (APGRC) of the Agricultural Research Corporation (ARC), Wad Medani, Sudan (El Tahir Ibrahim, Personal communication, 2012). Phenotypic variations among 100 Sudan pearl millet accessions were already verified [10]. Genotypic variations, however, need to be studied as well.

Pearl millet is derived from a narrow gene pool. It has a relatively small diploid genome (2n = 2x = 14) with a basic chromosome number of seven, and a haploid DNA content of 2.5 pg and genome size of 2450 M bp [11]-[7]. Molecular marker technologies can greatly facilitate the crop improvement programs in several ways [12]. Recently, the use of molecular marker technology for the genetic improvement of pearl millet has made some headway, and pearl millet has been elevated to the status of a molecular crop. A number of Simple Sequence Repeat (SSR) primers are available in pearl millet [13]-[7] and these markers have been used for genetic diversity analysis [14]-[7]-[15].

Studies of genetic diversity in *Pennisetum* germplasm suggest promising opportunities for the use of undomesticated materials for improving pearl millet varieties. The objective of this study, therefore, aimed at the exploitation of simple sequence repeat markers for assessing the genetic diversity of 36 accessions of pearl millet for possible development of forage type varieties.

II. Materials And Methods

Thirty six pearl millet accessions were obtained from the genebank of the APGRC, Sudan (Table I). All accessions were originally collected from western parts of Sudan, where pearl millet is traditionally grown. Thirty three accessions were from Darfur region, while only three accessions were from Kordofan region. The molecular diversity analysis was done at the Biotechnology and Biosaftely Research Center, ARC, Shambat, Sudan.

DNA Extraction

Four hundred ml of buffer AP_1 (lysis buffer) and 4 µl of RNase a stock solution (100 mg/ml) were added to 20 mg of grounded seeds. The mixture was incubated for 10 min at 65˚C. One hundred thirty ml of the buffer AP_2 (precipitation buffer, contains acetic acid) was added to the lysate, mixed and incubated for 5 min in ice. The lysate was centrifuged for 5 min at 14000 rpm. The aqueous phase was applied to QIA shredder Mini spin column (Lilac) placed in a 2 ml collection tube and centrifuged for 2 min at 14000 rpm.

The flow–through fraction was transferred to a new tube. Following that, 675 µl of buffer AP_3/E (binding buffer, contains guanidine hydrochloride) was added to the cleared lysate and mixed by pipetting. A volume of 650 µl of the mixture was applied from the last step and centrifuged for 1 min at 8000 rpm and flow–through was discarded. This step was repeated with remaining sample. DNeasy Mini spin column placed in a new 2 ml collection tube. 500 µl of AW (wash Buffer, contains ethanol) was added to DNeasy Mini Spin column and was centrifuged for 1 min at 8000 rpm. The flow–through was discarded and 500 µl AW buffer was added to the DNeasy Min Spin column and was centrifuged for 2 min at 14000 rpm. Then DNeasy Mini Spin column was transferred to 2 ml microcentrifuge tube and 100 µl of buffer AE (elution buffer) was pipetted directly into the DNeasy membrane. The mixture was incubated for 5 min at room temperature (15 – 25˚C). After that it was centrifuged for 1 min at 8000 rpm to elute the DNA. The quantity and quality of the extracted genomic DNA was determined by loading 15µl of the extract on a 0.8% agarose gel 1 X TBE buffer (Tris base, Boric acid, EDTA), then stained in ethidium bromide (10%) for 30 min and visualized by UV transillumination.

PCR primers

A set of 10 Simple Sequence Repeats (SSRs) markers were used to assess the genetic diversity among 36 pearl millet accessions (Table 2). These markers were developed by [16] for pearl millet.

PCR amplification

PCR amplification was performed in a volume of 10 µl containing approximately 50 ng of template DNA solution (0.5 µl), 0.25 µl of each primer, 1 µl of PCR buffer, 2 µl of Q solution, 0.2 µl of dNTPs, ,0.05 µl of Taq DNA polymerase and 5.75 µl of doubled distilled H_2o. Reaction were run in an Eppendorf PCR Master cycler gradient with an initial denaturation step for 5 min at 94°C; followed by 40 cycles of 94°C for 30 sec, 54°C to 57°C (depending on the annealing temperature of each primer (Table II) for 30 sec, and 72°C for 1 min; followed by a final extension at 72°C for 5 min and held at 4°C.

In order to obtain a precise estimate of fragment size, PCR products were electrophoresed on 3% Metaphore gels for 3 hours and stained in ethidium promide for 30 min. Amplified fragments were visualized and the size of the PCR product was determined using BioCapt Software which could automatically recognize the lane and band (the peaks region) and measure the fragment size. Only the reported allele size of loci [17]-[18] as an amplification product of SSR was used for genotyping.

Statistical Analysis

For the molecular data, binary matrices were analyzed by DARwin5 (version 5.0 158. CIRAD Research Unit Genetic Improvement of Vegetative Propagated Crops (http:// darwin.cirad .fr /darwin). Jacard's coefficients were clustered to generate dendrogram using the unweighted pair group method with arithmetic average (UPGMA). Five genetic parameters were calculated; namely PCR product, allele frequency, number of observed alleles, gene diversity and polymorphism information content (PIC) using Power Marker version 3.25 (http://www.powermarker.net).

Table 1. Sites of collection of the pearl millet accessions used *

Accession	Collection Site/Village	Region
HSD2020	Zaleingi-Jebel Marra	Darfur
HSD2023	Zaleingi-Jebel Marra	Darfur
HSD2031	Zaleingi-Jebel Marra	Darfur
HSD2035	Zaleingi-Jebel Marra	Darfur
HSD2044	Zaleingi-Jebel Marra	Darfur
HSD2052	Zaleingi-Jebel Marra	Darfur
HSD2055	Zaleingi-Jebel Marra	Darfur
HSD2058	Zaleingi-Jebel Marra	Darfur
HSD2062	Zaleingi-Jebel Marra	Darfur
HSD2064	Zaleingi-Jebel Marra	Darfur
HSD2065	Zaleingi-Jebel Marra	Darfur
HSD2095	Zaleingi-Jebel Marra	Darfur
HSD2098	Zaleingi-Jebel Marra	Darfur
HSD2100	Zaleingi-Jebel Marra	Darfur
HSD2113	Zaleingi-Jebel Marra	Darfur
HSD2121	Zaleingi-Jebel Marra	Darfur
HSD2140	Habila	Darfur
HSD2142	Habila	Darfur
HSD2144	Habila	Darfur
HSD2151	Habila	Darfur
HSD2163	Boose	Darfur
HSD2175	Goase Badeen	Darfur
HSD2176	Goase Badeen	Darfur
HSD2180	Birk Saira	Darfur
HSD2190	Tawila	Darfur
HSD2194	Hilladeabi	Darfur
HSD2198	Hilladeabi	Darfur
HSD2205	Um-marabeek	Darfur
HSD2238	Bendis	Darfur
HSD2240	Bendis	Darfur
HSD2243	Bendis	Darfur
HSD2251	Bendis	Darfur
HSD2257	Bendis	Darfur
HSD2262	Um-Sharaya	Kordofan
HSD2264	Um-Sharaya	Kordofan
HSD2304	El Jikka	Kordofan

*Information was obtained from the APGRC, Wad Medani, Sudan

International Journal of Agriculture Innovations and Research
Volume 4, Issue 6, ISSN (Online) 2319-1473

Table 2. Simple sequence repeated marker names, repeated type and length, primer sequences and annealing temperature

Primer pair	Repeated type and length	Forward sequence (5'-3')	Reverse sequence (5'-3')	Annealing temp.
ICMP3050	$(TA)_8$	ATG TCC AGT GTT GAC GGT GA	CGGGGAAGAGACAGGCTACT	60 – 64
ICMP3063	$(GTG)_5$	TCC GGT AGA GAC CGT AAT GG	GGCACTCCCTAGCAAAATGA	60 – 62
ICMP3078	$(TGCCA)_3$	TCC AGA CAG TTC AGC AGG TG	CCACACGAGACAGAGCACAC	62 – 64
ICMP3085	$(TCA)_5$	CTG AAG CTG AAG AGG CCT TG	GGCGGAGATCAGAGTTCG	58 – 62
ICMP3086	$(CAT)_5$	ACC AAA CGT CCA AAC CAG AG	ATATCTCTTCGCTGCGGTGT	60 - 60
ICMP3088	$(TCC)_8(TCTA)_4$	TCA GGT GGA GAT CGA TGT TG	TTACGGGAGGATGAGGATG	58 – 60
ICMP3092	$(TAG)_5$	GTT GCT GTC ATG TCG TCT GG	CATCATGCCTGTGAGCAATG	60 – 62
CTM-9	$(CT)_{20}$	GCC TCC TCT TGA TAC CAT ATT	TAGCCTTGGCTGCTATATTC	58 – 60
CTM-60	$(CT)_{23}$	AAG CCC CGA TCA CAT CAA	AGCCGAGCCTCATCCC	54 - 54
CTM-25	$(CT)_{34}$	GCG AAG TAG AAC ACC GCG CT	GCACTTCCTCCTCGCCGTCA	64 – 66

III. Results

The 10 Simple Sequence Repeats (SSRs) markers produced 30 alleles with an average of 3 alleles per locus (Table 3). The number of alleles amplified by each primer varied from 1 to 6. Among the 10 primers, only ICMP 3063 detected a single allele. The size of the PCR products ranged from 138 (ICMP3086) to 264 bp (ICMP 3092). Polymorphism information content (PIC) values of microsatellite markers varied from 0 to 0.68 with an average of 0.33 (Table III). Representative amplifications product of the primers, ICMP 3088 and CTM-25 are shown in Fig. 1. Most of the primer pairs produced a maximum of two bands per genotype; one primer pairs (CTM-25) produced more bands than expected.

Cluster analysis with SSRs markers resulted in five major clusters, which were designated as clusters 1 to 5 starting from the top of the page (Fig. 2). The geographical distribution of accessions did not fit with the genotypic clustering pattern. Cluster one consisted of nine accessions, 67% of themwere from Jebel Mara, the remaining 33% were from Um-sharaya (Kordofan), Bendis and Habila. Cluster two consisted of eight accessions, 50% of them were from Goase Badeen and Hilladeabi, and the other 50% were from Birk Saira, Boose, Tawila and Bendis. Cluster threewas the smallest one and constituted of two accessions, one was from Jebel Marra and the other was from Bendis. Cluster four consisted of 9 accessions,78% of them were from Jebel Mara and the other 22% were from Bendis and Um Marabeek. On the other hand, 38% of the accessions in cluster five were from Habila, 25% were from Jebel Mara and the remainders (37%) were from Um-sharaya (Kordofan), Eljikka (Kordofan) and Bendis (Fig. 2). Six accessions in cluster one and two accessions in cluster four showed 100% genetically similarity. The six accessions in cluster one included five from Jebel Marra *viz*, HSD 2062, HSD 2100, HSD 2098, HSD 2095 and HSD 2065 and one from Um-sharaya, Kordofan *viz*,HSD 2262 .The two accessions of cluster 4 were accession HSD 2238 from Bendis and accession HSD 2205 from Um-marabeek.

Table 3. Simple sequence repeat marker names, observed PCR product, number of observed alleles and polymorphism information content (PIC) value

Primers	Observed PCR	No. of	PIC
ICMP3050	216-229	2	0.3207
ICMP3063	177-229	1	0.0000
ICMP3078	234-256	4	0.4441
ICMP3085	206-210	2	0.1411
ICMP3086	138-157	6	0.6840
ICMP3088	164-206	2	0.3623
ICMP3092	245-264	2	0.1411
CTM-9	218-248	3	0.3308
CTM-60	214-230	5	0.6372
CTM-25	256-334	3	0.2854
Total		30	3.3467
Average		3	0.3347

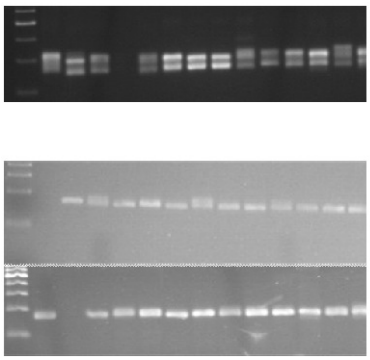

Fig. 1. Polymerase chain reaction amplification of pearl millet genomic DNA from 36 accessions

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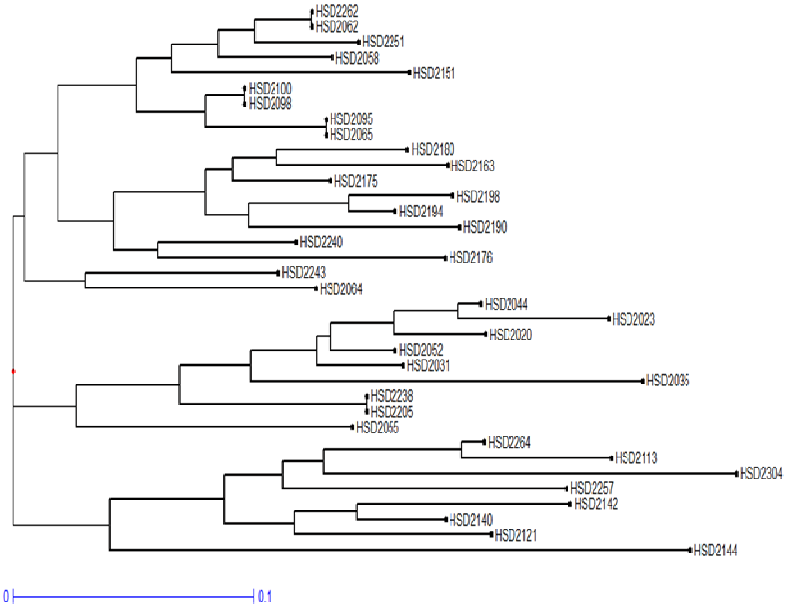

Fig. 2. Dendrogram displaying genetic relationships among the studied 36 pearl millet accessions using UPGMA cluster analysis of Jaccard genetic similarity coefficients generated from 10 SSR markers.

IV. Discussion

The apparently low genetic variability among Sudan pearl millet collections in this study could be explained by the general narrow gene pool of pearl millet on one side. On the other hand, the accessions used in this study were obtained from the plant genetic resources units which were in turn collected from farmers' fields. Those latter, tend to select and save high yielding heads to improve their varieties. Moreover, about 92% of those accessions were collected from Darfur region, of which 50% were from Jebel Marra area in Darfur region, where pearl millet is grown under more or less similar agro-ecological conditions, which are different from those in Kordofan area. Consequently however, the narrow genetic variation could be expected.

Most of the primer pairs produced a maximum of two bands per genotype. CTM-25 primer pairs produced more bands than expected according to the diploid constitution of pearl millet. This probably imply the duplication of some loci; in line with [19] who reported that pearl millet genome carries at least one and probably two duplications between linkage groups 1 and 4.Allprimerpairs (10 SSRs) were examined for polymorphisms among the 36 accessions of pearl millet. The average number of alleles found in this study *viz*, 3 was lower than that reported by [7]-[20] for pearl millet. Compared with other cereals, our results are comparable or a little lower than what has been observed in sweet sorghum with an average of 3.22 [21], maize with an average of 3.17 [22], sorghum with an average of 9 [23] and foxtail millet with an average of 6.16 [24]. Similarly, the polymorphism information content (PIC) found in this study is lower than that reported by [7]-[20] for pearl millet. Compared to other cereals, PIC found in this study is lower than that reported for sweet sorghum [21], maize[22], sorghum [23] and foxtail millet [24], but generally the primers that contain the short repeats displayed very low PIC value [25].

The genotypic diversity among the 36 selected pearl millet accessions was verified through SSR markers evaluation. Pearl millet collections in the PGR are recognized by accessions name in their area of collection. This could lead to confusion within the PGR collection if the same accession is collected under different names depending on the locality responsible for its collection. We found relatively few examples where such confusion might exit. For example, accession HSD 2262 grown in the Um sharaya (Kordofan) appears to be genetically similar to HSD 2062 which is grown in Jebel marra (Darfor). Similarly, accession HSD 2238 grown in the Bendis (Darfor) is genetically similar to HSD 2205 which grown in the Um-marabeek (Darfor). These observations are indicative of germplasm exchange among different geographical regions. Also could be attributed to the few markers used in this study. In fact, about 300 markers are needed to cover all the genome of pearl millet [12].

Plant morpho-agronmic traits can at least be partially influenced by environmental factors. Therefore, relying only on visible characteristics could result in inconsistent classification of the same materials studied under different environments. The use of molecular markers provides a much more reliable approach to study genetic diversity in crops that complement the data resulting from comprehensive and more detailed morpho-agronomic characterization. The results provide a good starting point for the characterization of pearl millet collections and selection of parental lines for genetic improvement in pearl millet this would result in the production of pearl millet genotypes that would suit the harsh environments of the Sudan.

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