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Development and Validation of Microsatellite Markers for Barnyard Millet Obtained by Partial Genome Assembly

Meniya V.H., Shital M. Padhiyar, Hiral Desai, Jasminkumar Kheni and Rukam S. Tomar*

Junagadh Agricultural University, Junagadh 362 001, India

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Abstract: Barnyard millet (*Echinochloa esculenta* L.) is grown for human consumption as well as fodder. Barnyard millet is the second most important small millet after finger millet. An Ion S5 Next Generation Sequencer (NGS) was used to sequence the 400bp DNA library of barnyard millet to obtain a draft genome and for mining of microsatellite markers. The *de novo* assembly yielded assembled reads of 59,67,79,933 bp with 11,39,481 contigs. A total of 46,157 SSRs were identified from 11,39,481 contigs examined. The number of sequences containing SSR were 41,591 and the number of sequences containing more than 1 SSRs were 3,867. Fifteen SSR markers were validated among the 30 accessions of barnyard millet. The average percentage polymorphism of markers was 66.54% with average Polymorphism Information Content (PIC) of 0.28 and average SSR primer index (SPI) of 0.57. These microsatellite markers can be used for linkage mapping and genes/QTLs tagging for genetic improvement of target traits in barnyard millet and related crops.

Key Words: Barnyard millet, draft genome, microsatellite, partial genome assembly.

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Praveen Kumar
R.N. Kumawat
R.K. Solanki
N.K. Jat

***Correspondence**

Meniya V.H.
rukam@jau.in

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Millets are said to be orphan crops as they stand second after staple cereals crops, but they provide food security to farmers and consumers residing in harsh hot climatic regions of Asia and Africa (Wilson and VanBuren, 2022). Pearl millet and Sorghum are the major millet crops followed by minor millets like foxtail millet, finger millet, proso millet, kodo millet, barnyard millet. India has second largest germplasm collection of barnyard millet in the world after Japan (Singh *et al.,* 2021). Barnyard millet has two species "Japanese barnyard millet (*Echinochloa esculenta*) and Indian barnyard millet (*Echinochloa frumentacea*)." The two species have different chromosome numbers (2n = 54 and 2n = 36, respectively), and hybrids between them are sterile. Barnyard millet in Japan is cultivated in areas where climatic and edaphic conditions are unsuitable for rice cultivation (Yabuno, 1987). In India, barnyard millet production of 0.147 mt and productivity 1034 kg ha^{-1}, respectively (Renganathan, 2020). Genetic improvement following conventional approach has resulted development of six high yielding varieties of barnyard millet in India. Besides conventional breeding research on application of molecular markers in millet improvement in also gaining

pace. Importance of millet as climate resilient crop and as source of genes(s) for coping with climatic adversities has drawn attention of researchers in the recent years to understand the millet genomics for genetic improvement (Wilson and VanBuren, 2022).

Molecular markers use is a highly precise approach for development of cultivars with desired traits and microsatellite markers are preferred to establish marker-trait association. Microsatellites are repetitive segments of DNA scattered throughout the genome in noncoding regions between genes or within genes (introns), like short tandem repeats (STR), simple sequence repeats (SSRs), or simple sequence length polymorphism (SSLPs) (Litt and Luty, 1989). Microsatellites are defined as 2-8 bp repeats by some researchers (Armour *et al*., 1999), whereas others classify them as 1-6 or even 1-5 bp repeats (Schlotterer *et al.,*1998). The most common types of microsatellites are repeats of mono-, di-, tri-, and tetra nucleotides; however, repeats of five (penta-) or six (hexa-) nucleotides are also frequently categorized as microsatellites. The enrichment of genomic libraries through selective hybridization or primer extension has historically served as the foundation for microsatellite identification and isolation. Finding microsatellite repeats in DNA datasets like EST sequences is an alternative strategy. The discovery of hundreds of microsatellite sites in the genome of a target species is made possible by new methods like Next Generation Sequencing (NGS), which can provide a huge number of high quality genome sequences quickly and inexpensively. The microsatellite markers can be used for QTL mapping and development of linkage map (Tomar *et al.,* 2017). Hence in the current study, partial sequence of barnyard millet genome was obtained using NGS. The contig sequences created by partial *de novo* assembly were further mined for microsatellite locations to design SSR markers which were validated on barnyard millet genotypes to classify them as crop specific SSR markers.

Materials and Methods

DNA extraction and partial genome sequencing

Barnyard millet genome sequencing was performed with DNA extracted from a genotype CO(KV)2, provided by ICAR-Indian Institute of Millets Research (IIMR), Rajendranagar (Hyderabad, Telangana, India). Fresh young leaves of the accession Co(KV)2, were used for DNA extraction as per the protocol described by Desai and co-workers (Desai *et al*., 2021**)** for little millet. The genomic DNA fragment library of around 400bp was amplified using emulsion PCR (Ion OneTouch™ 2 System, Thermofisher Scientific, USA), followed by enrichment of template positive bead recovery. The sequencing of ~400bp fragments was carried out in Ion S5 sequencer following the protocol laid down for the sequencer.

Genome assembly

The presence of contaminated DNA sequences in the barnyard reads were verified by BLASTing it against a database of potential contaminant DNA (fungi, bacteria and virus). Any kind of contaminating sequences were removed from the analysis. The CLC trimmer function (default limit = 0.05) (CLC Genomics Workbench 8.2 software, CLC Bio, Aarhus, Denmark) was used to eliminate the adapters sequence used in the Ion S5 sequencing process. Assembly was also performed on CLC Genomics Workbench software using the parameters Length Fraction (LF) and Sequence Similarity (SIM) between DNA reads, as described by the CLC Genomics Workbench software, with maximum stringency (0.50 LF and 0.80 SIM). The minimum contig length parameter was set to 100 bp.

SSR search and primer design

MISA (MIcroSAtellite; http//pgrc.ipk-gatersleben.de/misa) was employed for SSR mining and identification. The minimum number of repeats used for selecting the SSRs was six for di-nucleotide repeats, five for tri-nucleotide repeats, and three for tetra-, penta-, and hexa-nucleotide repeats. Primers for SSRs were designed using Batch primer 3.0 with the following criteria primer lengths of 14-23 bases, GC content of 45-60%, annealing temperature of 50°C to 60°C, and PCR product size of 200bp and above.

Screening and assessment of SSR

The random set of primers from genome was selected to synthesize from Merck, India. DNA isolation was carried out using DNeasy Plant Mini Kit (Qiagen,Valencia, CA) from 30 barnyard millet genotypes (Table 1). The

Table 1. Statistics of barnyard millet partial genome sequence generated with Ion Torrent S5

Parameters	
No of Contigs (,000)	1139
Total size (Mb)	596.77
Maximum length	25,500 bp
Average	524 bp
N25(Bases)	1,672 bp
N50(Bases)	848 bp
N75(Bases)	413 bp

polymerase chain reaction was performed using the following PCR components: 1 µL template DNA (50 ng), 1.0 µL forward primers, 1.0 µL reverse primers 2 µL 10x Taq buffer + $MgCl_2$ (15 mM), 2 µL dNTP (2 mM), 0.4 µL Taq polymerase (Promega 5U μL^{-1}) and 2.6 µL sterile distilled water. PCR reaction was performed in Veriti thermal cycler (Applied Biosystems, USA) under condition: Initial denaturation 94°C for 3 min, denaturation 94°C for 30 s, annealing 30 cycles 58-59°C for 30 s and final extension at 72°C for 7 min. Amplified PCR product of each primer were subjected to 3% agarose gel electrophoresis at 120V for ~1.5 to 2.0 hrs with 100bp DNA ladder (Qiagen,Valencia, CA) and Agarose gel scanning was carried out in Gel Documentation system (Syngene, India).

Results and Discussion

Genome Assembly

After the quality filtration (mean quality score >=20) using CLC workbench as described in the materials and methods section, all the reads were used for assembly preparation. Data were pooled for runs of Ion S5 and then used for assembly. Assembly was carried out by CLC workbench V8.2 *de novo* assembler. The *de novo* assembly yielded assembled reads of 59,67,79,933 bp with a number of contigs count of 11,39,481. The maximum and minimum length of contigs was 25,557 bp and 100 bp respectively, while the average contig length of 524 bp. The N25, N50 and N75 contig sizes were weighted median values and defined as the length of the smallest contig in the sorted list of all contigs where the cumulative length from the largest contig to contig is at least 25%, 50%, and 75% of the total length respectively. In the present experiment, N25, N50 and N75 contigs size were found to be 1,672 bp, 848 bp and 413 bp, respectively (Table 1).

Table 2. The statistics of genomic data assessed for SSR mining

Parameters	
Total number of sequences examined	1139481
Total size of examined sequences (bp)	596779933
Total number of identified SSRs	46157
Number of sequences containing SSR	41591
Number of sequences containing more than 1 SSR	3867
Number of SSRs present in compound formation	2444

SSR mining

A total of 46,157 SSRs were identified from 11,39,481 contigs examined using MISA tool (Table 2). The number of sequences containing SSR were 41,591 and the number of sequences containing more than 1 SSR were 3,867. Moreover, the SSRs in compound form were about 2,444. Among these potential SSRs, five types of repeated motifs dinucleotide, trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide were identified, of which dinucleotide and trinucleotide were the most abundant SSRs. The number of di, tri, tetra, penta and hexa-nucleotide SSRs were 25,191, 17,630, 2,748, 474, and 114, respectively (Table 3). Among the SSRs identified, the most dominant dinucleotide repeat motif was AG/CT (11,388) followed by AT/AT (7820) and AC/GT (5031), the most dominant trinucleotide repeat motif was CCG/CGG (4,664) followed by AGC/CTG (3,418) and AGG/CCT (1,854) while the most dominant tetra-nucleotide repeat was AGAT/ATCT (389) followed by ATCC/ATGG (Fig. 1).

SSR primer design and validation

A set of 15 primers were designed from the 46,157 SSRs identified with the product size varying between 300 to 700 bp (Table 4). The primers were designed from the different

Table 3. Distribution of di-, tri-, tetra, penta and hexa-nucleotide microsatellites on contigs of barnyard millet

Unit size	Number of SSRs
Di-nucleotide repeats	25,191
Tri-nucleotide repeats	17,630
Tetra-nucleotide repeats	2,748
Penta-nucleotide repeats	474
Hexa-nucleotide repeats	114
Total	46157

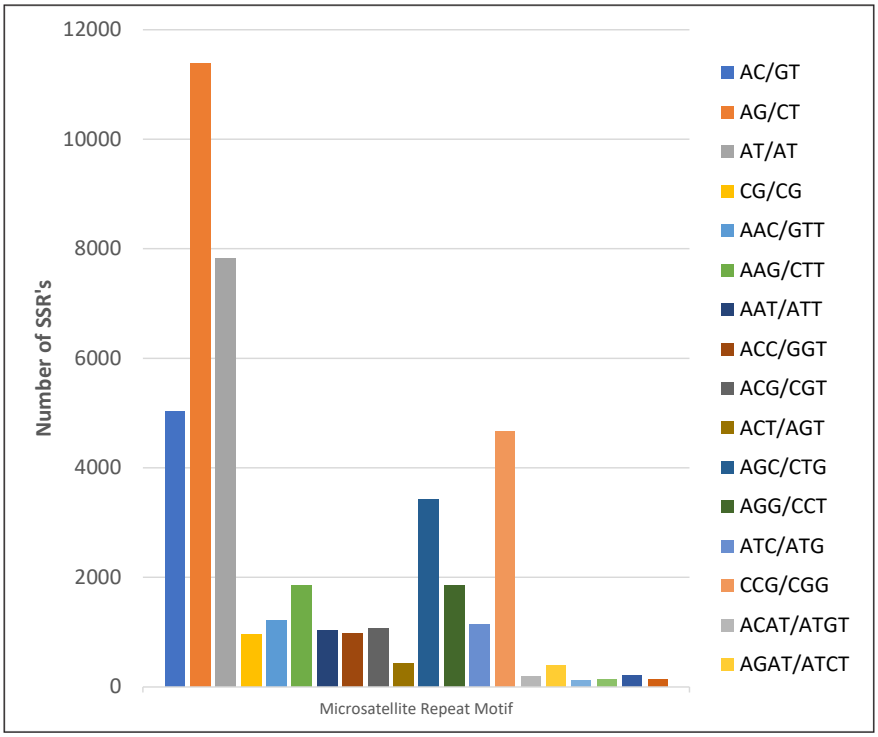

Fig. 1. Distribution of microsatellite motif types and tandem repeat numbers in Barnyard millet genome.

contigs assembled from the reads. The validation of these primers was carried out on 30 genotypes of barnyard millet (Table 5). Out of the 15 primers, 13 primers gave an amplification at the expected base pair with the total number of 21 amplified bands. Based on the sequence data produced by the *de novo* assembly technique, expected product sizes for each microsatellite marker were calculated. We verified that the polymorphic loci's size ranges included the size of the expected product. This was true for 9 out of 13 primers making 69.23% of markers to produce amplicons within the range of the predicted sizes. No markers showed amplicons that were 90% bigger or smaller than anticipated. Out of the total number of bands amplified by the 13 SSR primers, 16 were polymorphic and 5 were monomorphic with an average polymorphism of 1.23 (Table 6). The average percentage polymorphism was 66.54% with average polymorphism information content (PIC) of 0.28 and average SSR primer index (SPI) of 0.57.

The capacity to discover and create microsatellite markers for molecular breeding, is undergoing a true revolution. A new paradigm of microsatellite development has been put to the test by research teams with access to an NGS facility, and continual decline in prices for obtaining next-generation sequencing data has provided easy access for obtaining genomic sequences. Shotgun pyrosequencing of DNA or enriched libraries were primarily employed in the majority of the initial articles published reporting the use of next-generation sequencing technologies for the production of microsatellite markers. Transcriptome sequencing and assembly were the first applications of Ion S5 sequencing followed by mining of genic SSR markers [(Hamid *et al.*, 2020; Hamid *et al.*, 2019; Hamid *et al.*, 2018; Radadiya *et al.*, 2021; Rathod *et al.*, 2020a; Rathod *et al.*, 2020b)]. The genomic information generated by next-generation sequencer for microsatellite markers in millets can be used for the breeding millets against biotic and abiotic stresses (Desai *et al.*, 2021; Satyavathi *et al.*, 2022). In the present era of genomics, in year 2012 foxtail genome information was released. Later in year 2017, pearl millet, finger millet and barnyard millet genomes were announced, and lastly in year 2019 proso millet genome was released (Krishna *et al.*, 2022). The barnyard millet draft genome size is estimated to be of 1.27 Gb covering more than 90% of genome coverage, but the genome annotation is still in the early phase (Krishna *et al.*, 2022). Information on genomic sequences is limited for application purpose,

Table 4. Primers sequences of the SSR developed for banyard millet

	Seq ID	Primer Name	Seq	Product size (In-silco estimation)
1	Barnyard_Millet_Genome_contig_19	BM_GE_1F	TATTCGGAGGTGAATCATAGA	319
	Barnyard_Millet_Genome_contig_19	BM_GE_1R	ACGATGTTTCCTTGAAAAAGT	
2	Barnyard_Millet_Genome_contig_19	BM_GE_2F	GTTTTCATTAAGGTGAGTTGG	309
	Barnyard_Millet_Genome_contig_19	BM_GE_2R	TTGCAAACAAATAGGTAGAGC	
3	Barnyard_Millet_Genome_ contig_19	BM_GE_3F	TATCAATTACCAATTCGTGGA	300
	Barnyard_Millet_Genome_contig_19	BM_GE_3R	TTGGAAATAACAAGAGAACGA	
4	Barnyard_Millet_Genome_contig_117	BM_GE_4F	GACGGACTCCTATCTATTCGT	404
	Barnyard_Millet_Genome_contig_117	BM_GE_4R	CTAGTGAGAGTTTCCCCATTT	
5	Barnyard_Millet_Genome__contig_117	BM_GE_5F	CTATAGCACGAAAAACCATTC	403
	Barnyard_Millet_Genome_contig_117	BM_GE_5R	AAAGAGAGAGCTTTGCATTCT	
6	Barnyard_Millet_Genome_contig_206	BM_GE_6F	TGCTTTCTCAATCTTTCTCTG	505
	Barnyard_Millet_Genome_contig_206	BM_GE_6R	TACTGTTGCGAGACTGGTACT	
7	Barnyard_Millet_Genome_contig_206	BM_GE_7F	ATTTTATCGACCTTTCCTTTG	509
	Barnyard_Millet_Genome_contig_206	BM_GE_7R	CACTGGAGTGATCCAATATGT	
8	Barnyard_Millet_Genome_contig_206	BM_GE_8F	ATTCATTCTTGGAAAAAGAGG	501
	Barnyard_Millet_Genome__contig_206	BM_GE_8R	TTATCTCCTTCTACGACATGC	
9	Barnyard_Millet_Genome_contig_739	BM_GE_9F	TGACTTTCTTTGCTAGCTCAC	604
	Barnyard_Millet_Genome_contig_739	BM_GE_9R	ACTTAATGGGAAGTAGGCATC	
10	Barnyard_Millet_Genome_contig_2270	BM_GE_10F	GGCTAGAGATTTCTTGGAAAA	700
	Barnyard_Millet_Genome_contig_2270	BM_GE_10R	TTTAGTTGTATCGACCCAGTC	
11	Barnyard_Millet_Genome__contig_4360	BM_GE_11F	CAACGAATCTCTTGACCTAAA	600
	Barnyard_Millet_Genome_contig_4360	BM_GE_11R	TAGACACGATGTCGCTTTATT	
12	Barnyard_Millet_Genome_contig_4398	BM_GE_12F	GAGCATTTGTCGAGATAAGAA	604
	Barnyard_Millet_Genome_contig_4398	BM_GE_12R	GAAAAGCAAAGAAAAGGAATC	
13	Barnyard_Millet_Genome_contig_12460	BM_GE_13F	TATTCTGCGATTATACCTTGC	462
	Barnyard_Millet_Genome_contig_12460	BM_GE_13R	AAGGCACTGGATGTTATGTTA	
14	Barnyard_Millet_Genome_contig_24709	BM_GE_14F	TATATGCATGGTCAGTGTGAA	652
	Barnyard_Millet_Genome_contig_24709	BM_GE_14R	CTGCAAGATTACAACAACACA	
15	Barnyard_Millet_Genome_contig_16327	BM_GE_15F	ACAATCATCAGCATTAGCATC	573
	Barnyard_Millet_Genome_contig_16327	BM_GE_15R	GAAGGAATCTGGTTTGAAAAT	

for genetic improvement of the crop there is need to have set of molecular markers specific to barnyard millet. Cross transferability of SSR markers from other crops has been successful in barnyard millet, most of the EST-SSR of foxtail were transferred successfully (Kumari *et al.,* 2013), foxtail genomic SSRs were also found to show positive amplifications (Pandey *et al.,* 2013; Babu *et al.,* 2018a) observed 100% transferability of finger millet genomic-SSRs and 71% transferability of rice genomic-SSRs. Whereas, Babu *et al.,* (2018b) observed that

Table 5. List of barnyard selected for validation of the fifteen SSR markers mined

1.	BAR-1406	6.	BAR-1411	11.	BAR-1416	16.	BAR-1421	21.	BAR-1426	26.	BAR-1431
2.	BAR-1407	7.	BAR-1412	12.	BAR-1417	17.	BAR-1422	22.	BAR-1427	27.	BAR-1432
3.	BAR-1408	8.	BAR-1413	13.	BAR-1418	18.	BAR-1423	23.	BAR-1428	28.	BAR-1433
4.	BAR-1409	9.	BAR-1414	14.	BAR-1419	19.	BAR-1424	24.	BAR-1429	29.	BAR-13
5.	BAR-1410	10.	BAR-1415	15.	BAR-1420	20.	BAR-1425	25.	BAR-1430	30.	CO(KV)2

Table 6. Size, number of amplified bands, percent polymorphism and PIC obtained by 13 SSR primers in the 30 genotypes of barnyard millet

Sr. No	SSR primers	Allele/ Band Size	Total no. of allele/band	Polymorphic band	Monomorphic Band	% polymophi-sum	PIC	SPI
1.	BM_GE_1	300-350	2	2	0	100	0.47	0.94
2.	BM_GE_3	300-350	2	2	0	100	0.49	0.99
3.	BM_GE_4	500-550	2	2	0	100	0.48	0.96
4.	BM_GE_5	500-550	2	2	0	100	0.39	0.78
5.	BM_GE_6	500-600	2	2	0	100	0.40	0.80
6.	BM_GE_7	500-650	2	2	0	100	0.46	0.93
7.	BM_GE_8	500-700	1	1	1	0	0	0
8.	BM_GE_9	550-650	1	1	1	0	0	0
9.	BM_GE_10	600-700	2	2	0	100	0.49	0.99
10.	BM_GE_11	700-750	1	1	1	0	0	0
11.	BM_GE_12	600-650	1	1	1	0	0	0
12.	BM_GE_13	600-700	1	1	1	0	0	0
13.	BM_GE_14	450-550	2	2	0	100	0.49	0.99
Total			21	16	5	-	-	-
Average			1.62	1.23	0.38	61.54	0.28	0.57

S = Shared; U = Unique; T = Total Polymorphic bands; PIC = Polymorphism Information Content; SPI = SSR Primer Index = Number of Bands × PIC

maize SSR revealed more polymorphism in barnyard genotypes compared to finger millet SSRs. Crop specific SSR markers can add more to the cross species SSR markers identified in barnyard crop, more number of SSR markers are required for developing a saturated linkage map for tagging gene(s) of interest by establishing strong marker trait association.

Conclusion

Mining of SSR in crops is essentially needed for strengthening the genetic improvement programmes. Robust markers panel development leads to create a dense linkage map for establishing marker-trait association to tag genes of interest. The thirteen markers developed in the present study using the partial genome of barnyard millet cultivar CO (KV)2 can be used for knowing genetic polymorphism in the available germplasm. Further, more SSR markers can also be mined from the available partial genome of barnyard millet obtained in the study as there are 41591 sequences containing SSRs. These markers can be used in barnyard millet for the development of linkage map, QTL mapping and the characterization of the available germplasm. Above this, the transferability of these markers can also be studied in other millets crops.

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