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Research Article

Exploration of Markers associated with Heat Stress Tolerance in *Pennisetum Glaucum* (L.) R. Br.

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Abstract

Development of high-temperature stress-tolerant Pennisetum glaucum (L.) R. Br. genotypes are essential for growing these as an irrigated summer crop for enhanced production. Identification of molecular markers associated with heat stress tolerance is a pre-requisite for developing tolerant P. glaucum through molecular breeding strategies. In the present research, 59 expressed sequence tag (EST) derived microsatellite markers [50 EST-Simple Sequence Repeats (SSR) + 9 Sequence Tagged Sites (STS)] were developed from the available heat stress-responsive transcriptome data in pearl millet and validated in 24 diverse genotypes of pearl millet. Among them, four EST-SSRs markers (15.38%) and one STS markers (16.66%) displayed polymorphism respectively. The polymorphic information content (PIC) of newly developed EST-SSRs was in the range from 0.207 to 0.662 with an average value of 0.435. Based on the five polymorphic markers, the 24 pearl millet genotypes were clustered into 5 main and 11 sub-clusters. Annotation of the five transcripts for which polymorphism was detected, was found to code for genes related to stress response and signaling pathway. The EST-SSRs and STS markers thus analyzed would augment the existing SSR marker resource and find their applications in diversity assessment and marker-aided breeding programs for genetic improvement of pearl millet.

Keywords: Pearl Millet; Abiotic Stress Tolerance; SSR Markers; Validation; Polymerase Chain Reaction

Abbreviations

SSR: Simple Sequence Repeats; RFLP: Restriction Fragment Length Polymorphism; DArT: Diversity Arrays Technology; QTL: Quantitative Trait Locus; PCR: Polymerase Chain Reaction; STS: Sequence-Tagged Sites

Introduction

Pennisetum glaucum (L.) R. Br. (Pearl millet) is the staple food in semi-arid tropics and ranks as the sixth most important food grain crop of the world. Being a highly climate-resilient crop, it is well adapted to the area where several abiotic stresses such as drought, high salinity, low pH and high temperature are prevalent [1]. Moreover, its nutritional qualities have made it one of the most popular millet to be consumed as food grain in recent times [2]. Pearl millet is now being cultivated as an irrigated summer season (February - June) crop in India, where high temperature incidences are common during flowering. High temperature (>42°C) during summer season coincides with flowering and causes spikelet sterility, leading to a drastic reduction in grain yield. Therefore, it has become essential to develop pearl millet genotypes having tolerance to high-temperature stress.

Molecular breeding is being employed for developing stress-tolerant pearl millet genotypes using Marker-assisted selection (MAS) approach. The linkage map of pearl millet was expanded with SSR markers, integrated to develop a consensus map of 353 RFLP and 65 SSR markers using four different crosses [3], and subsequently, with the addition of DArT markers [4], the relation of pearl millet with the foxtail millet and rice genomes were established [5]. QTLs for drought tolerance and components of drought adaptation such as flowering time, grain yield, stover yield, were mapped in pearl millet [6-8]. Some of these developed molecular markers have already been applied for MAS- breeding programs for the improvement of pearl millet. Efforts are still underway for the development and application of molecular markers in pearl millet to make it stand with other cereals like rice, sorghum, maize, wheat

and barley [9]. However, breeding programs based on DNA markers for improving abiotic stress tolerance in pearl millet are comparatively few even in recent years due to the limited availability of PCR-compatible co-dominant markers that can be used readily in breeding programs. Of late, RNA-seq or transcriptome sequencing through next-generation sequencing platforms has been able to provide a reliably large amount of information on the functions and expression of genes at a given time point [10,11]. Expressed sequence tag databases have become particularly attractive resources for *in silico* mining of molecular markers. The EST-SSRs (Expressed Sequence Tags – Simple Sequence Repeats) have an intrinsic advantage over genomic SSRs in terms of its origin from coding regions of the genome and transferability to other related species [12]. Sequence-Tagged Site (STS), molecular markers are of a relatively short sequence (around 200-500 bp) having a single occurrence in the genome, whose location and sequence is known. STS locus containing polymorphisms become valuable genetic markers for molecular breeding. Therefore, we sought to develop additional EST-SSR markers for pearl millet research from the available heat stress-responsive transcriptome pearl millet data (SRP151237). These markers were validated in 24 diverse genotypes. Out of the 50 EST-SSRs markers studied here, only 4 markers (15.38%) displayed polymorphism. In case of 9 STS markers, only one (16.66%) was detected to be polymorphic. Based on the five polymorphic markers, the 24 pearl millet genotypes could be clustered into 5 main clusters and 11 sub-clusters. The five transcripts for which polymorphism was detected, were found to code for genes related to stress response and signaling pathway. This study is the first report regarding the development of heat stress associated SSR markers based on transcriptome data in pearl millet, EST-SSRs and STS markers analyzed in the present study would add to the existing SSR marker resource which will be useful in diversity assessment analysis, strengthening the genetic maps and marker-aided breeding programs for genetic improvement of pearl millet.

Materials and Methods

Plant material

Seeds of twenty-four diverse genotypes of *Pennisetum glaucum* (L.) R.Br. were obtained from Division of Genetics, I.A.R.I., New Delhi on request (Table 1). Seeds similar in size and visibly free of insect/fungal infection were selected and sown as line sowing on a well drain soil at a depth of about 3-4 cm in the month of June –July (Kharif season) in the "Genetics Block B" field of IARI, New Delhi. The plants were allowed to grow and leaves were collected as plant samples at the 20-days-old seedling stage. Care was taken to collect tender young leaves preferably from the top most plant parts. The collected plant samples were wrapped in aluminum foil and immediately frozen in liquid nitrogen followed by storing in -80°C deep freezer for further use.

Sl. No.	Genotype	Sl. No.	Genotype	Sl. No.	Genotype	Sl. No.	Genotype
1	ICM B 92777	7	J108	13	411B	19	PPMI 69
2	PPMI 872	8	TT-1	14	576B	20	PPMI 627
3	H 77/29-2	9	TT-6	15	PPMI 720	21	DPR-7
4	PRLT-2/853	10	KSU 13/2	16	PPMI 575	22	TPR-8
5	ICM B 841	11	Tift 23B	17	PPMI 597	23	WGI-100
6	J2340	12	7042S	18	PPMI 59	24	Coloured

Table 1: List of *P. glaucum* genotypes used in the present study.

Membrane stability test by electrolyte leakage measurement

Electrolyte leakage was measured as per the protocol given by Howarth., *et al.* 1997 (13).

Software used in the study

Primer3 Input (version 0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/) software was used for designing primers [14]. The following parameters were considered for primer designing and the same was given as input to the software: primer length 18-23 bp, with optimum value 20 bp; Tm 57°-62°C, with optimum value 60°C; GC content 30-70%, with the optimum value 55%; and product size range 120-300 bp optimum value 150 bp for EST-SSR and product size range 300 – 500 bp optimum value 400 bp for STS markers, Max 50 self-complementarity 5.0 and Max 30 self-complementarity 0. Diversity and phylogenetic analysis based on evolutionary dissimilarities were carried out by DARwin5 software tool [15]. Power Marker V3.25 (https://brcwebportal.cos.ncsu.edu/powermarker/) software was used for calculating the PIC value of developed markers [16]. For functional annotation of sequences related to heat stress tolerance, Blast2GO software tool was used (https://www.blast2go.com/).

Genomic DNA isolation from *P. glaucum* genotypes

Genomic DNA was isolated from leaf tissue of 20 days old seedling (24 different *P. glaucum* genotypes) using a modified CTAB based method [17]. Optical density (O.D.) of DNA samples was measured at 260 nm and 280 nm using Nanodrop 2000 (Thermo Fisher Scientific, USA) and was used to calculate the quality and quantity of the DNA isolated. The extracted genomic DNA of all genotypes was also quantified by agarose gel electrophoresis.

PCR Amplification using EST-SSR and STS specific primer.

DNA amplification with the designed EST-SSR and STS specific primer (Supplementary material Table S1) was carried out by Polymerase Chain Reaction (PCR) in 25 µL reaction mixtures containing 2 µl of template DNA (25ng/µl), 5 µM of each forward and reverse primers, 1 U of Taq polymerase (D1806- Sigma Aldrich, USA), 10X Taq Reaction buffer with $MgCl_2$, 2.5 mM of each dNTPs (dTTP, dGTP, dCTP, dATP), sterile nuclease-free water to make up the volume of the reaction. PCR amplification was carried out in 96 well Thermal Cycler (Applied Biosynthesis, Waltham, Massachusetts, USA) as per the following program: an initial denaturation step of 5 minutes; followed by a loop of 35 cycles each consisting of denaturation (95°C for 30 sec), annealing (50°C - 60°C for 30 sec) and extension (72°C for 1 min); the final extension was performed at 72°C for 10 minutes. The PCR products were removed from the thermal-cycler and stored at 4°C. 5 µL of 6X DNA loading dye (98% formamide, 10 mM EDTA, 25% xylene cyanol) was added to the product obtained after PCR amplification of microsatellite markers and loaded on 3% metaphor agarose gel made using 1X TAE. To separate the microsatellite markers, electrophoresis was carried out at 30 V for 1-1.5 hours. The PCR bands were visualised under UV light in a gel documentation system [18].

Scoring for markers and annotation

The clear and reproducible alleles amplified by each EST-SSR and STS markers among *Pennisetum glaucum* were scored according to their fragment size (bp) corresponding to the 100 bp and 1 kb plus DNA molecular weight marker as viewed on 3% agarose (Metaphor) gel electrophoresis. The binary scoring format was done to check the amplification pattern of the polymorphic bands and score was given based on amplification with the EST-SSR/STS primers as '0' for the absence of expected amplicon size, '1' for the presence of expected amplicon and 5 for non-amplification. The scoring data was used to analyze the diversity within the studied genotypes and Polymorphic Information Content (PIC) using DARwin software [15] and Power marker V3.25 [19] software respectively. A phylogenetic tree was constructed using a pairwise distance matrix computed by calculating a dissimilarity matrix using a shared allele index with DARwin software [19]. An unweighted neighbour-joining tree was constructed using the calculated dissimilarity index. The genetic distance between accessions was estimated using NEI coefficient [20] with the bootstrap procedure of resampling (1000) across markers and individuals from allele frequencies. The unigene, which contain the EST-SSR motif showing polymorphism were searched for homology with known genes using Blastn program of Blast2GO for identification of the gene function and their role in metabolic pathway [20].

Results and Discussion

Membrane Stability Index (MSI) assay

The different genotypes of pearl millet were analyzed for the extent of membrane injury under heat stress by membrane stability index (MSI) assay. It was observed that the MSI value for the different genotypes ranged from 32.24% to 71.54% with genotype PPMI 59 having the lowest MSI (32.24%) and genotype WGI 100 having the highest MSI (71.54%) (Figure 1). The average value of MSI for all the 24 genotypes was found to be 51.8%. Of the 24 genotypes studied the MSI value for thirteen genotypes (92777B, PPMI872, 477/29-2, J108, TT1, TT6, KSU13/2, Tift23, 576B, PPMI720, PPMI575, PPMI59, Coloured) was observed to be less than the average MSI (51.8%), whereas eleven genotypes (PRLT 2/853, J2340, 841B,7042S, 411B, PPMI597, PPMI69, PPMII629, DPR7, TPR8, WGI100) were found to have MSI value of more than 51.8%.

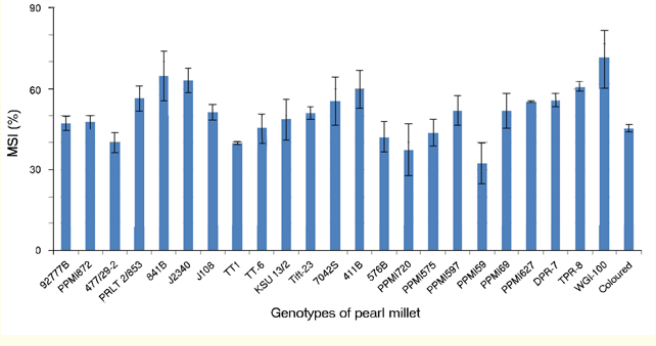


Figure 1: Membrane stability index of different pearl millet genotypes.

Designing of primers for EST-SSRs and STS markers from highly expressed unigene.

A set of 59 highly expressed unigenes with a threshold P-value of less than 0.005 and an expression upregulation of a fold change of greater than 2 were selected from the available heat-responsive transcriptome data. Of these selected unigenes, 50 unigenes were the ones which had SSR motifs while 9 were used for the development of STS markers. (Supplementary material Table S1).

Distributions of repeats motif of the SSR markers developed

The EST-SSR markers for which the primers were developed consisted of 26 SSRs with trinucleotide motifs, 20 SSRs with dinucleotide repeat motif, 2 SSRs with tetra-nucleotide repeat motif and 1 each from penta and hexanucleotide repeat motif. For the development of STS markers, 9 highly expressed unigenes were randomly selected (Figure 2).

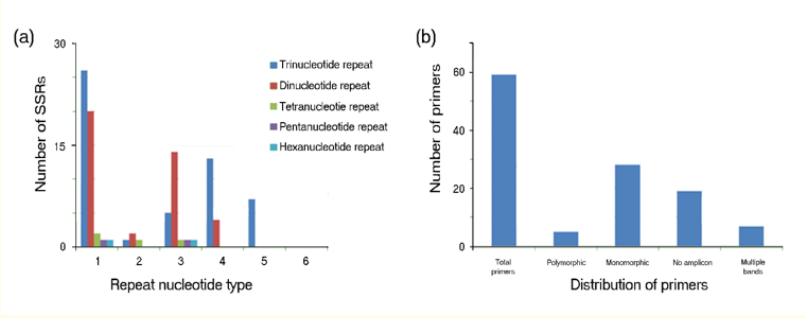


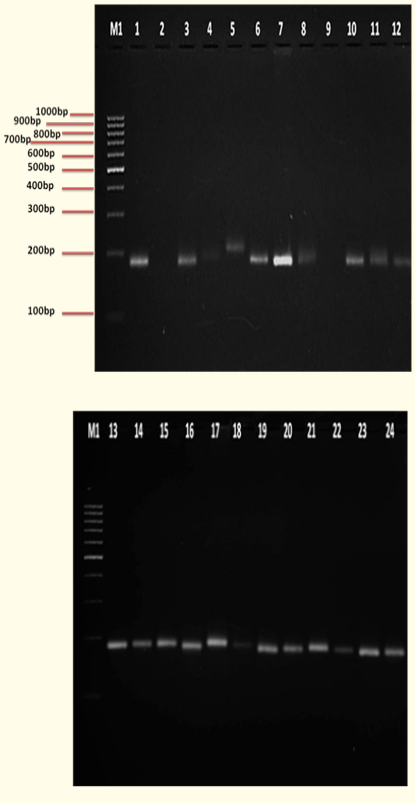
Figure 2: a- Distribution of monomorphic, polymorphic, unamplified and multiple band over the tri, di, tetra, penta and hexa nucleotide repeats, b-Overall distribution of primers

Validation of EST-SSRs and STS polymorphism

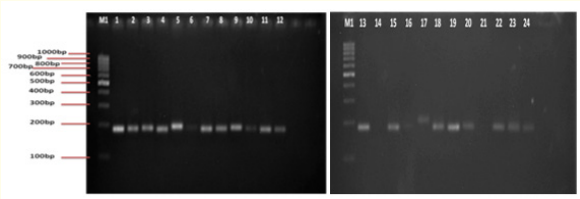
Out of the 50 EST-SSRs markers, 26 markers displayed successful amplification resulting in an amplicon of the expected size. No amplification was observed in 17 markers whereas 7 markers were found to result in multiple undesirable amplicons. Only 4 EST-SSRs markers (15.38%) (SSRPgCL8220Contig1, SSRPgCL2813Contig2, SSRPgCL4537Contig1, SSRPgCL118Contig3) were found to be polymorphic among the 24 genotypes tested (Supplementary material Figure S1, Table S1). In the case of STS markers, out of nine markers designed, six were found to be successfully amplified with an amplicon of the expected size. Only one polymorphic STS marker (SSRPgCL4894Contig1) was found in the present study (Supplementary material Figure S2) and two markers did not give any amplification (Supplementary material Table S1). The PIC of newly developed EST-SSRs was calculated by using Power Marker and was found in the range of 0.207 to 0.662 with an average value of 0.435.

Diversity analysis

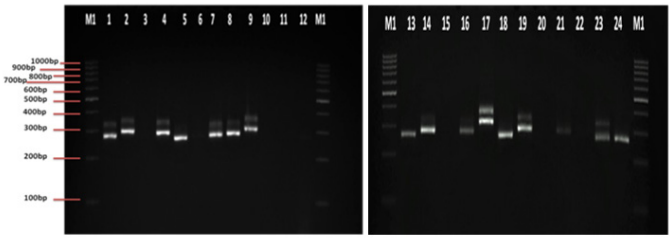
Based on the data score given on the amplicons obtained for EST-SSR and STS tested on the 24 panels of genotype diversity analysis was carried out. The analysis revealed 5 main clusters and 11 subclusters. It was observed that the genotype PPMI69 had a distinct pedigree compared to the other genotypes (Figure 3).



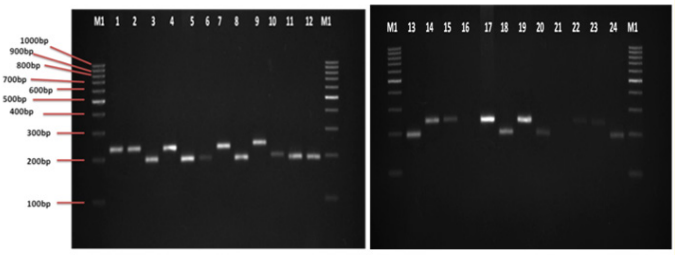
a. primer 13SSRPgCL8220Contig1



b. 42SSRPgCL2813Contig2

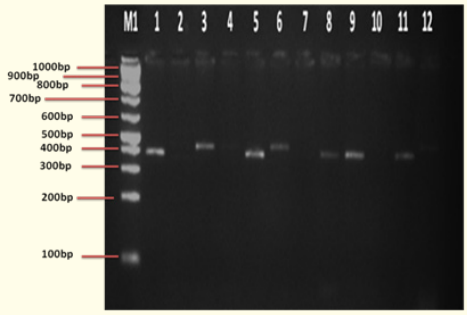


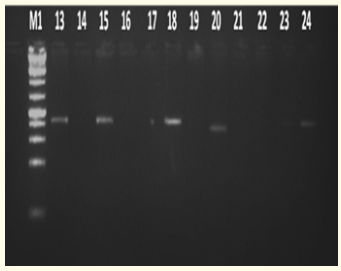
c. primer 45SSRPgCL4537Contig1



d. primer 47SSRPgCL118Contig3

Figure S1: Polymorphism resultant of 4 EST-SSR markers for 24 pearl millet genotypes run on 3% metaphor agarose: Lane 1 to 24 represents the genotypes of pearl millet; 1- 92777B; 2- PPMI872; 3- 477/29-2; 4- PLRT-2/853; 5- 841B; 6-J2340 ; 7-J108; 8-TT-1; 9-TT-6; 10- KSU 13/2; 11- Tift-23; 12- 7042S; 13-411B; 14-576B; 15-PPMI720; 16-PPMI575; 17-PPMI597; 18- PPMI59; 19- PPMI69; 20-PPMI629; 21-DPR-7;22-TPR-8; 23-WGI-100; 24-PPMI683, M1-100 bp DNA ladder.





Primer 56STSPg CL4894Contig1

Figure S2: Polymorphism resultant of 1 STS markers for 24 pearl millet genotypes run on 3% metaphor agarose: Lane 1 to 24 represents the genotypes of pearl millet; 1- 92777B; 2- PPMI872; 3- 477/29-2; 4- PLRT-2/853; 5- 841B; 6-J2340 ; 7-J108; 8-TT-1; 9-TT-6; 10- KSU 13/2; 11- Tift-23; 12- 7042S; 13-411B; 14-576B; 15-PPMI720; 16-PPMI575; 17-PPMI597; 18- PPMI59; 19- PPMI69; 20-PPMI629; 21-DPR-7;22-TPR-8; 23-WGI-100; 24-PPMI683, M1 is the 100 bp ladder in the following image, M1- 100 bp DNA ladder.

Sl. no.	Name	Primer sequence	Size in bp	Tm (°C)	Amplification
1	SSRPgCL10115Contig1F SSRPgCL10115Contig1 R	CACGAGATCGCTCTCTACA ATGAGCATGTAGACCACCAC	224	55	Nil
2	SSRPgCL6349Contig1 F SSRPgCL6349Contig1 R	CAAAGCTCTTTCGGGAAC CTTCAAAGCCGCCACCTC	231	59	multiple band
3	SSRPgCL8Contig1 F SSRPgCL8Contig1 R	ACATGATGCTCCATGTCG TATAGCTCTCGGCAGGTG	244	55	Nil
4	SSRPgCL13196Contig1 F SSRPgCL13196Contig1 R	CACTCAGTGTCTTCCAGGTG CCCTCCTCCGCTTTTCTC	235	58	Monomorphic
5	SSRPgCL3974Contig1 F SSRPgCL3974Contig1 R	ATGGTCTTAAGCCGTCGT GTAGTGCGAGGAGGATCAG	245	55	Nil
6	SSRPgCL5868Contig1 F SSRPgCL5868Contig1 R	GGTACACGTCGAGGTCACT AATTCTGACGGGGACGAG	247	55	Nil
7	SSRPgCL3358Contig1 F SSRPgCL3358Contig1 R	AAGGTAGCCTCCTCTGCTC GAAGAGGCTGTCCA TGAG	218	55	Nil

8	SSRPgCL565Contig2 F SSRPgCL565Contig2 R	TATAGGAGGAGAGGAGAGAGC GTAGTCCACCGTATCAGGAGT	202	55	multiple band
9	SSRPgCL3058Contig1 F SSRPgCL3058Contig1 R	CTGGTCCTGCTCTACCTCT CTG-GTCCTGCTCTACCTCT	202	54	Unspecific band
10	SSRPgCL573Contig2 F SSRPgCL573Contig2 R	CGGTTGAAGGGAA TCCTC GAGCA TGAAGGCCTTGTC	223	57	multiple band
11	SSRPgCL6324Contig1 F SSRPgCL6324Contig1 R	CTAAAACCCTCCCCAAAGA TCCCTTCTCCTTCTTCTCC	250	55	Nil
12	SSRPgCL6637Contig1 F SSRPgCL6637Contig1 R	CATCAGGTGGTGCATAGAC GATCTGCTAGTGCTGGAATC	203	54	multiple band
13	SSRPgCL8220Contig1 F SSRPgCL8220Contig1 R	GAGAATGGGAGGAACTCG CTTCCTCGTGCTTACCTTC	225	55	Polymorphic
14	SSRPgCL8813Contig1 F SSRPgCL8813Contig1 R	CGCCTTCTTCTTCTTTCC ATTCTCACGCCCTGAAAC	208	55	multiple band
15	SSRPgCL13665Contig1 F SSRPgCL13665Contig1 R	GGCTAGAAGGTAGTGGAAGC GAAATGGACGAGGGGTAT	231	55	multiple band
16	SSRPgCL2061Contig1 F SSRPgCL2061Contig1 R	CTCTCTCCCGAATTCTCAC AGGGGTTGAGGAGGGTAG	217	55	Nil
17	SSRPgCL7317Contig1 F SSRPgCL7317Contig1 R	CCTGAGCGCCA TGCAGGA GCTA-CACTTGGCTCTCCA	239	60	monomorphic
18	SSRPgCL1139Contig1 F SSRPgCL1139Contig1 R	CACACCGGCGAACATCTG CTCTCTCTCCTCGCACCT	244	58	Nil
19	SSRPgCL8803Contig1 F SSRPgCL8803Contig1 R	GAGGTGAGGTGGGGTTAT AGT-CAGACTGGAGGATTGG	165	54	Nil
20	SSRPgCL7350Contig1 F SSRPgCL7350Contig1 R	GCGAGGAGGTAGTCGTAGA CTTCCTCCAACCCCTGAG	207	56	Multiple band
21	SSRPgCL4518Contig1 F SSRPgCL4518Contig1 R	GGTGTAGTTGAAGAGGATGC AAGGAGGAGATAAGGTCTGG	377	54	Nil
22	SSRPgCL455Contig2 F SSRPgCL455Contig2 R	AAGAACTCGCATGGAGAAG AGACTGCA TCAGCGACAG	198	55	Nil
23	SSRPgCL3173Contig1 F SSRPgCL3173Contig1 R	GCTGGGTATTAGAGGTAGGG CTT-TAACGCCATGGTCAG	155	55	Nil
24	SSRPgCL5271Contig1 F SSRPgCL5271Contig1 R	CTCGTCCTTCTTGCTCACT GTTTC-GACGACCAGCAGT	194	56	Nil
25	SSRPgCL6602Contig1 F SSRPgCL6602Contig1 R	TCGTCGTCTACGACTACTCC TCGT-GCAGGTAAAGGATG	160	55	monomorphic
26	SSRPgCL6416Contig1 F SSRPgCL6416Contig1 R	ACCA TGTGGAACGTCTTG GGGACTCTGATCTGTTGTTCT	190	55	monomorphic
27	SSRPgCL2359Contig1 F SSRPgCL2359Contig1 R	CTCCCTGCTTTCTCGAA TG-GAAAGAATTCAGCACAATC	208	55	monomorphic
28	SSRPgCL12633Contig1 F SSRPgCL12633Contig1 R	TATCCTACTCCTTCCGCAAC CCCT-GTAGAGGACCCTACTC	246	55	Nil
29	SSRPgCL10708Contig1 F SSRPgCL10708Contig1 R	TGATGTACGAGTTCAAGGTG GAGCCAGAGCTCGAAGGT	218	56	Nil
30	SRPgTRINITY_DN39409 F SRPgTRINITY_DN39409 R	GTGTCCTATTCCCAAGAACA AT-GCTGCTAGCTCTCCTCT	183	55	monomorphic
31	SSRPgCL8937Contig1 F SSRPgCL8937Contig1 R	GCTCGGTCTTCTGTTTAAGA GCAGCTCTTCTGGTAGAAGTT	230	55	monomorphic
32	SSRPgCL2262Contig1 F SSRPgCL2262Contig1 R	GAGGAAAACTCAGCAGAGAA GGACCCGTAGATAGTCAGGT	227	55	Nil

33	SSRPgCL2739Contig1 F SSRPgCL2739Contig1 R	GGTCCCTGTAGTGACAAAAA TGT-GTGTTTGAACTCACCTG	224	55	monomorphic
34	SSRPgCL6300Contig1 F SSRPgCL6300Contig1 R	GATCTGTACAGTGGCAGCTT TCCTGGATTGTTCTTTCTTG	222	55	monomorphic
35	SSRPgCL5084Contig1 F SSRPgCL5084Contig1 R	AGATCTGACACCGGTACG CTCCC-GAGTGTGAGTGAC	166	56	monomorphic
36	SSRPgCL12463Contig1 F SSRPgCL12463Contig1 R	CCACCATCAACTTGTTTTTAC TGATCTGATGACCATCTTCTT	206	54	monomorphic
37	SSRPgCL10183Contig1 F SSRPgCL10183Contig1 R	ACCACAGGTGGGGGAAGAG CGC-GACTTCTACCTGGAG	226	59	monomorphic
38	SSRPgCL5668Contig1 F SSRPgCL5668Contig1 R	AACAACAGTTGAAGCCTAACATG-TATGTTGAAATGAGCAGA	174	54	monomorphic
39	SSRPgCL1649Contig1 F SSRPgCL1649Contig1 R	CGCCTCCTTCTGCTGCTG CAA-GATTTGCCACCTATAACC	233	59	Nil
40	SSRPgCL10804Contig1 F SSRPgCL10804Contig1 R	AGGTCACCTCAACATAGCATA TCTCTGTACAAAGGGTTGAAA	244	55	monomorphic
41	SSRPgCL5956Contig1 F SSRPgCL5956Contig1 R	GTTCAAGCTTCACACA TTGAC AACTACCTAGACCTCGACCTG	219	55	monomorphic
42	SSRPgCL2813Contig2 F SSRPgCL2813Contig2 R	TTGGAGAGAGAAATAGTGCTG CTTTGAGGTGCTTGTTACTTG	183	55	polymorphic
43	SSRPgCL2721Contig1 F SSRPgCL2721Contig1 R	CAATACCTTTTTCAGTTCAGC TTCTTTCTGCTCTGAACACTC	190	54	monomorphic
44	SSRPgCL10812Contig1 F SSRPgCL10812Contig1 R	CCAAAACTTAACTCCTGTTTCT CACCAGGGTCAGGAGAAC	165	55	monomorphic
45	SSRPgCL4537Contig1 F SSRPgCL4537Contig1 R	CCACAACAGCCTATTAGAAAC AAAA TTGTGGAGGAAGAACTG	197	55	polymorphic
45	SSRPgCL4537Contig1 F SSRPgCL4537Contig1 R	GCGAGATGAAGGAGTTCTAC GGCGGTAGAACTTGGAGT	197	55	polymorphic
46	SSRPgCL9292Contig1 F SSRPgCL9292Contig1 R	GTGTGGGTGTGTGACTGAC GACTCCACCTCCTGATACTG	219	55	monomorphic
47	SSRPgCL118Contig3 F SSRPgCL118Contig3 R	CTGACACGTAGTCTCAGAACC GATCGATGGTCTAGCTGTCTC	243	55	polymorphic
48	SSRPgCL6490Contig1 F SSRPgCL6490Contig1 R	CAGAGCTGGATAAGCTGGT CCAGCACA TCGTCAAGTC	218	55	monomorphic
49	SSRPgCL10497Contig1 F SSRPgCL10497Contig1 R	CAGTTTCTCCAGA TTTGACTG TCTACTTCTGATGAAGCCAAG	249	55	monomorphic
50	SSRPgCL730Contig2 F SSRPgCL730Contig2 R	CCAACGTGGTTGTCTTCGTA AAATGCGGCGAACTTGGA	210	55	monomorphic
51	STSPgCL3095Contig1 F STSPgCL3095Contig1 R	TCTCCCTGCTTTCTCGAATC AGAGACCGTAAACGCAGCAT	470	61	Nil
52	STSPg CL2359Contig1F STSPg CL2359Contig1R	A TCGAA TCCA TTCCCCA TTT TTGTGCACCTCAACTTTGCT	493	60	monomorphic
53	STSPgCL2061Contig1F STSPg CL2061Contig1R	CACGAGCCGAGAGAATCAAT CATGAGCGGTGAAGAAGGAT	483	60	Nil
54	STSPgCL10837Contig1F STSPgCL10837Contig1R	CAACTGCTTCGGTCGGTTAT TGGTGA TGAGGA TGA TCTGG	440	60	monomorphic
55	STSPgTRINITY_DN39409F STSPgTRINITY_ DN39409R	TCATGGAACCGGAGACAAAT AGGAA TGCAGCTGCTCAA	435	60	monomorphic
56	STSPgCL4894Contig1F STSPgCL4894Contig1R	CGCTCGGTCTTCTGTTTAAGA TGCCGACAGAGTAAATGCAA	484	60	polymorphic

57	STSPgCL8937Contig1F STSPgCL8937Contig1R	ATCGTAAAGGCGGATGACCT CTCCTTGAAGCGGATGATGT	381	60	monomorphic
58	STSPgTRINITY_DN41652F STSPgTRINITY_ DN41652R	TCGATGAGGACGAGGAAGAT TG-GTCGCACCATCAAGATTAA	528	60	monomorphic
59	STSPgCL3490Contig1F STSRPgCL3490Contig1R	CACGAGATCGCTCTCTACA AT-GAGCATGTAGACCACCAC	482	60	monomorphic

Table S1: List of primers used and status of amplicon obtained.

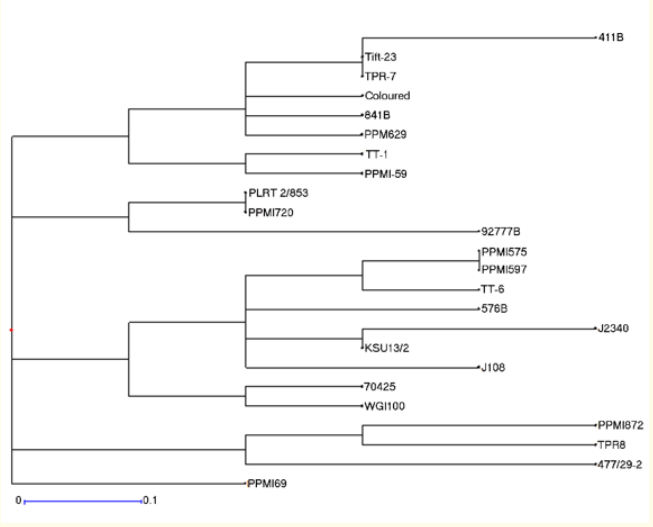


Figure 3: Diversity of the genotypes based on alleles of SSRs markers.

Annotation of identified unigenes

The 59 unigenes used for EST-SSR/STS marker development in the present study were subjected to annotation analysis to understand the role of the genes/markers in heat tolerance. Annotation analysis using BLAST2GO was carried out with the sequences of the candidate unigenes to identify the homology of the unigenes to the known genes/ESTs in the database. The five polymorphic markers were present on contigs, CL8220Contig1, CL2813Contig2, CL4537Contig1, CL118Contig3, and CL4894Contig1. Annotation of polymorphic SSR containing unigene revealed: CL8220Contig1 showed similarity (79.1%) to the crocetin chloroplastic having a role in glucosyltransferase activity; CL2813Contig2 showed similarity (74.1%) to zinc-finger homeodomain 4 isoform X2 having a role in DNA binding and transcriptional regulation; CL118Contig3 showed similarity (71.2%) to CCA tRNA nucleotidyltransferase 2 having a role in transferase activity. One of the polymorphic SSR containing unigene CL4537Contig1 did not show any match to the existing database. The only STS containing polymorphic unigene CL4894Contig1 showed similarity (79.78%) to probable LRR receptor-like serine-threonine- kinase having a role in ATP binding and amino acid metabolism (Table 2).

Primer	SSR/STS motif	No. of alleles	Product size (bp)	PIC value	Description	Functions
SSRPgCL8220Contig1	(CGC)5	3	195-225	0.4199	cyanidin 3-O-rutinoside 5-O glucosyltransferase	Having role in salt tolerance
SSRPgCL2813Contig2	(GA)11	2	183-205	0.2078	Zinc finger homeodomain 4 isoform	Role in abiotic stress tolerance via Na^+/K^+ pump regulation
SSRPgCL4537Contig1	(CT)7	3	280-308	0.5213	No significant homology	N/A
SSRPgCL118Contig3	(AGAT)6	4	196-268	0.6628	putative CCA tRNA nucleotidyl transferase	Involved in small RNA metabolic pathways under various stress condition
STSPgCL4894Contig1	(A)11	2	402-424	0.3680	probable LRR receptor-like serine threonine- kinase At1g34110	integral component of membrane, ATP binding; amino acid metabolic process

Table 2: Annotation of selected contigs showing polymorphism.

Global warming affects the seed germination, plant growth and development resulting in a drastic reduction in agricultural production. Thus, there is a need for growing crops that are tolerant to various abiotic stresses. Pearl millet is known to be tolerant to heat stress but further improvement for growing it as irrigated summer crop is of instant need. A lot of genetic variabilities is available for high-temperature stress tolerance in pearl millet which can be utilized in breeding programs. However, there is a need to develop/identify markers associated with high-temperature stress tolerance. In the present study, 59 molecular markers identified from high temperature-responsive transcriptome data of pearl millet (developed in a previous study) were validated in 24 diverse genotypes.

The 24 genotypes were assessed for their high-temperature stress tolerance ability by measuring membrane stability index (MSI). Variation in MSI (32.24% to 71.54%) was observed among the 24 genotypes, indicating variation in the sensitivity to high-temperature stress. Similar variations (52.58% to 71.21%) in MSI have been observed in a previous study with 38 pearl millet varieties [21]. The MSI value for WGI 100 was the maximum (71.54%) indicating its inherently better membrane stability and consequently better tolerance to biotic and abiotic stresses. PPMI59 genotype with the lowest MSI (32.24%) is considered to be the most sensitive to abiotic stress. Our findings are in accordance with those of the previous workers [22,23]. The variation in the MSI content found in the present study indicates the suitability of the material for the polymorphism survey and diversity analysis.

Of the 59 primers designed for amplification of EST-SSR and STS motifs, 32 primers (54.24%) resulted in successful amplification indicating that the parameters employed during designing of primers were appropriate. A total of four polymorphic loci were identified with 1.32 average number of allele per EST-SSR locus tested across the surveyed panel of twenty-four genotypes. Out of the four polymorphic EST-SSR markers, SSRPgCL118Contig3 produced 4 alleles, SSRPgCL2813Contig2 produced two alleles and three alleles each were produced each by SSRPgCL8220Contig1 and SSRPgCL4537Contig1 across the panel of twenty-four genotypes.

The PIC values of markers provided an estimate of their discriminating power in a set of accessions by taking not only the number of alleles but also the relative frequencies of each allele. Although the PIC values (an average value of 0.435) were slightly less than the polymorphism percentage of the pearl millet SSR markers reported in earlier studies, it has been well documented that EST-derived SSRs are less polymorphic than those derived from genomic libraries [24,25]. The previous studies using EST-SSR markers for genetic diversity analysis in cereals like wheat and barley exhibited a PIC value of 0.44 and 0.45 respectively [26]. The average PIC value was lower compared to the PIC values of genomic SSR (0.4-0.9) markers in pearl millet, sorghum, foxtail millet [27]. The EST-SSR markers are generally less polymorphic and provide fewer alleles and lower PIC values as compared to the genomic SSR markers [28]. In the present study, tri-nucleotide repeat based markers were more polymorphic than the di-nucleotide, tetra-nucleotide and pentanucleotide repeat-based markers. Similar results have also been reported in previous studies on pearl millet [24,25].

Estimation of genetic diversity and identification of superior genotypes are the major objectives of any crop improvement program. For genetic diversity assessment, molecular markers are preferred in recent times as they offer considerable advantages over morphological markers. DNA markers have also been used to evaluate genetic diversity in pearl millet [22]. Though we could identify only five polymorphic markers in the present study, we still examined their utility in clustering the 24 genotypes according to their genetic distance and the analysis revealed 5 main clusters and 11 sub-clusters.

The annotation of the identified 4 SSR sequence and one STS sequence revealed homology with genes involved in the stress-responsive mechanism or signaling pathway as *Setaria italica* cyanidin 3-O-rutinoside 5-O-glucosyltransferase, which is known to have a role in salt stress tolerance [29], *Setaria italica* zinc-finger homeodomain protein 4-like, which has a role in abiotic stress tolerance via Na/K pump regulation [30], *Sorghum bicolor* putative CCA tRNA nucleotidyl transferase which is known to be involved in small RNA metabolic pathways under various stress condition, leucine-rich repeat (LRR) receptor-like serine-threonine-kinase with a role in transferring phosphorus-containing groups and thus involved in different metabolic pathways including plant defense mechanism [31]. Homology search with one of the transcript resulted in "No homology" with any known gene/transcript. Further analysis needs to be done to link the polymorphic markers with their genes. For a comprehensive linking of the maker to the trait, the large number of molecular markers are needed to be developed and validated in a large number of genotypes or mapping populations.

Conclusion

Fifty-five microsatellite markers were developed from the available heat stress-responsive transcriptome data in pearl millet available and validated in the 24 diverse genotypes. Out of the

50 EST-SSRs markers studied, only 4 markers (15.38%) displayed polymorphism. In the case of 9 STS markers, only one (16.66%) was able to detect polymorphism. Based on the five polymorphic markers, the 24 pearl millet genotypes could be clustered into 5 main and 11 sub-clusters. The five transcripts for which polymorphism was detected, were found to code for genes related to stress response and signaling pathway. The polymorphic markers developed can be used in marker-assisted breeding for heat stress tolerance in pearl millet, for association mapping, genetic diversity assessment, genome mapping and saturation of pearl millet genetic map. The EST-SSR and STS markers can be employed for preparing a ''transcript map'' and can also be utilized for transferability in other minor millets for which less genetic resources are available.

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Conflict of Interest

The authors declare no conflicts of interest.

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