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ASSOCIATION OF PHENOTYPIC CHARACTERS WITH GENETIC MARKERS BY USING SIMPLE SEQUENCE REPEATS IN FINGER MILLET (*ELEUSINE CORACANA* L. GAERTN)

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ABSTRACT

Finger millet [*Eleusine coracana* (L.) Gaertn] is an important crop used for food, fodder and industrial purposes. With the objective of increasing the utilization of finger millet germplasm in crop improvement, a composite collection consisting of 1000 accessions was developed, evaluated in three environments for 15 agronomic traits and profiled using 20 SSR markers. This study has reported the marker-trait associations by using Simple Sequence Repeats markers. Allelic data on 959 accessions and 20 markers based on quality index was used for further statistical analysis. A total of 231 (121 common and 110 rare) alleles were detected in the composite collection. Significant variation of all the agronomic traits was observed. Marker UGEP8 in LG3 and UGEP56 in LG9 showed strong association with days to 50% flowering in composite collection in over all the tree environments. Several other markers were associated with the traits but were not consistent across environments.

Key words : Finger millet, Phenotypic characters, Simple sequence repeats, Molecular variation

Introduction

Finger millet (*Eleusine coracana* L. Gaertn) is an important crop in several countries of Asia and Africa used for food, fodder, and industrial purpose. Finger millet is a highly self-fertilized allotetraploid (2n = 4x = 36) derived from the wild tetraploid progenitor *E. coracana* subsp. *africana*. The A genome donor is believed to be *E. indica*. Both *E. floccifolia* or E. *tristachya* have been considered as potential B genome donors to *E. corancan* based on rDNA restriction pattern (Hilu *et al*., 1992) and genomic *in-situ* hybridization (Bisht and Mukai, 2001).

In finger millet the diversity has been studied using morphological characters like growth habit, leaf architecture or floral morphology (Rachie and Peter, 1997). At molecular level, DNA markers such as RFLP (Muza *et al*., 1997), RAPD (Das *et al*., 2007), SSRs (Dida *et al*., 2007) have been used to determine genetic diversity. Comparative analysis of finger millet genetic map with rice genetic map was a novel attempt that reported high level of conserved co-linearity between the two genomes (Srinivasachary *et al*., 2007). Low molecular variation was reported in the cultivated finger millet in the past as the results were based on limited number of germplasm and markers. With the discovery of large numbers of genomic SSR markers (Dida *et al*., 2007), it is now possible to conduct extensive molecular diversity and QTL analysis in finger millet. Population structure using 79 finger millet accessions and 45 SSR markers have been reported (Dida *et al*., 2008). The present study aimed to assess the genotypic diversity, to

dissect the population structure of global composite collection and to find marker- trait associations in global finger millet composite collection.

Materials and Methods

All the 1000 accessions of the finger millet composite collection including four internal checks (VR708, VL149, PR202 and RAU8) were grown in the field. The DNA was extracted from single seedling of each accession by high throughput 96- well plate mini preparation method. From the preliminary screening of 31 SSR markers (Dida *et al*., 2007) on an eight diverse finger millet genotypes (IE4709, IE6082, IE2921, IE5177, IE4057, IE4443, IE2564 and IE3025), 20 polymorphic SSR markers were selected to genotype the composite collection and list of markers are mentioned in Table 1. Of these, 19 SSRs belong to dinucleotide repeats and one to trinucleotide repeats. The 20 SSR markers used for genotyping were mapped on nine chromosomes.

Polymerase chain reaction (PCR)

The PCR reactions were conducted in 96-well and 384-well micro-titer plates in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) Thermal Cycler. The PCR reactions were performed in 5 ml volume in 384-well PCR plates. The reaction mixtures contained 10 pmol of primer, 25 mM $MgCl_2$, 2mM dNTP, 0.3 unit of *Taq* polymerase and 1x PCR buffer (Applied Biosystems, Foster City, CA, USA). The touch down PCR protocol was used for the following reaction of following: three-minute denaturation cycle, followed by first five cycles of 94°C for 20 seconds, 60°C for 20 seconds and 72°C for 30 seconds, then by 30 cycles of 94°C for 20 seconds. After completion of 30 cycles, a final extension of 20 min at 72°C to ensure amplification to equal lengths of both DNA strands. The amplified PCR products were tested on 1.2 per cent agarose gel to check for the amplification of the products.

Table 1 : Marker, chromosome number and position repeat motif, forward and reverse primer sequence.

Marker name	Chromosome no	Position _cm	Repeat motif	Forward Primer	Reverse Primer
UGEP56	9A	7.4	(GT)12	CTCCGATACAGGCGTAAAGG	ACCATAATAGGGCCGCTTG
UGEP8	3B	65.2	(GA)13	ATTTCCGCCATCACTCCAC	AGACGCAAATGGGTAAATGTC
UGEP11	5Ab	63.5	(CT)12	CCTCGAGTGGGGATCCAG	AAGACGCTGGTGGAAATAGC
UGEP15	3 A	6.5	(CT)22	AAGGCAATCTCGAATGCAAC	AAGCCATGGATCCTTCCTTC
UGEP3	3A & 3B	IIIA(75.8), IIIB(64)	(CA)7N 12(GA)15	CCACGAGGCCATACTGAATAG	GATGGCCACTAGGGATGTTG
UGEP81	6B	2.9	(GT)12	AAGGGCCATACCAACACTCC	CACTCGAGAACCGACCTTTG
UGEP5	9B	29.1	(TC)12 AC(TC)4	TGTACACAACACCACACTGATG	TTGTTTGGACGTTGGATGTG
UGEP107	1A	9.5	(GA)15	TCATGCTCCATGAAGAGTGTG	TGTCAAAAACCGGATCCAAG
UGEP31	3A	75.8	(GA)12	ATGTTGATAGCCGGAAATGG	CCGTGAGCCTCGAGTTTTAG
UGEP104	3B	124.7	(CT)11	TCAGCACCACCTGAATAGG	AATAGGGAGGGCGAAGACTC
UGEP90	6B	23.3	(CT)11/ (CT)8	GGCCTTTGCAGTCATGTGAG	CGACTCCAGGTGTTGTTGG
UGEP18	1B	70.3	(CT)12	TTGCATGTGTTGCTTTTTGC	TGTTCTTGATTGCAAACTGATG
UGEP68	9B	0.0	(CT)14	CGGTCAGCATATAACGAATGG	TCATTGATGAATCCGACGTG
UGEP65	8A	31.6	(CT)19	AGTGCTAGCTTCCCATCAGC	ACCGAAACCCTTGTCAGTTC
UGEP1	5Ab	25.9	(TC)11	TTCAGTGGTGACGGAAGTTCT	GGCTCCATGAAGAGCTTGAC
UGEP10	8A	52.2	(GA)19	AAACGCGATGAATTTTAAGCTC	CTATGTCGTGTCCCATGTCG
UGEP102	10	3.7	(TG)17	ATGCAGCCTTTGTCATCTCC	GATGCCTTCCTTCCCTTCTC
UGEP26	5B	121.1	(CGG)7	ATGGGGTTAGGGTTCGAGTC	TGTCCCTCACTCGTCTCCTC
UGEP77	4B	4.8	(CT)19	TTCGCGCGAAATATAGGC	CTCGTAAGCACCCACCTTTC
UGEP12	8B	50.8	(CT)22	ATCCCCACCTACGAGATGC	TCAAAGTGATGCGTCAGGTC

Genotyping

The PCR products were size-separated by capillary electrophoresis using an ABI 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA). The PCR products of 4 primer pairs labeled with different dyes (FAM, VIC, NED and PET) could be pooled (post-PCR), because of the different signal spectra of the fluorophores used. The products of the same fluorophore-labeled primers were also pooled, when they had non-overlapping amplicons in terms of size. The pooled PCR products were mixed with 0.25 µl of the GeneScan 500™ LIZ® internal size standard and 7 µl of Hi-Di™ Formamide (Applied Biosystems, Foster City, CA, USA). The final volume was made up to 12 µl with sterile double-distilled water. This mixture was denatured for 5 minutes at 95°C and cooled immediately on ice.

Fragment size fractionation

After denaturation, the plate with samples was placed into the sequencer machine (ABI Prism 3700 DNA analyzer). The capillary run was performed using the "GeneScan2_POP6 Default" run module and "G5" filter-set. The analysis module used was "GS500 analysis". The fragments were separated in a 50 cm capillary array using POP6 (Performance Optimized Polymer) as the separation matrix.

Data processing

After the capillary runs were over, the raw data were processed with Genescan 3.1 software (Applied Biosystems) to size the peak patterns in relation to the internal size standard GeneScan 500™ LIZ®. The principle behind this is that standards are run in the same lane or capillary injection as the samples, which contain fragments of unknown sizes labeled with different fluorophores. Genescan® analysis software automatically calculates the size of the unknown DNA sample fragments by generating a calibration sizing curve based upon the migration times of the known fragments in the standard. The unknown fragments are mapped onto the curve and the sample data is converted from migration times to fragment size. Genotyper 3.7 (Applied Biosystems) was used for allele calling. The peaks were displayed with base pair values and height (amplitude) in a chromatogram and the allelic data were exported in to Excel spread sheet for further analysis.

Association mapping

Phenotyping

Phenotyping of composite collection along with four check cultivars (VR708, VL149, PR 202 and RAU 8) was carried out in three environments, *viz*., 2005-'06 post rainy at Tamil Nadu Agricultural University, Coimbatore (E1), 2006 rainy (E2) and 2007 rainy (E3) at ICRISAT, Patancheru. This experiment was conducted in augmented design with one of the four control cultivars repeated after every nine entries in all the environments. Data on 15 quantitative traits [days to 50% flowering (DF), plant height (PH), number of basal tillers (BTN), culm branching (CB), flag leaf blade length (FLBL) and width (FLBW), flag leaf sheath length (FLSL), peduncle length (PL), panicle exertion (PE), ear head length (EHL) and width (EHW), length and width of longest finger (LLF and WLF), number of fingers per ear head (NF) and plot yield (PY)] were recorded following finger millet descriptors. Mean, range and broad sense heritability were calculated for all traits to study the variability present in the germplasm material.

Population structure analysis

The model-based software STRUCTURE Version 2.2 (Pritchard *et al*., 2000) was used to infer population structure. The most appropriate K value was determined using a burn-in of 10,000 to 100,000, K = 1 to 10 and five runs per K value using a model allowing for admixture and correlated frequencies. The basis of this kind of clustering method is the allocation of individual genotypes to K clusters in such a way that Hardy – Weinberg equilibrium and linkage equilibrium are valid within clusters, whereas these kinds of equilibrium are absent between the clusters. The range of possible tested K was from 2 to 5. The most stable and high average likelihoods for population stratification were obtained with 3 or 4 groups, while the standard deviation was more stable with $K = 4$. Population structure consisted of a Q – matrix that describes the present cluster parentage for each line in the analysis.

Association of markers with traits

All association tests were run with the mixed linear model (MLM) method in TASSEL 1.9.4 (http://www.maizegenetics.net/), a recently developed unified mixed-model method simultaneously taking into account multiple levels of both gross level population structure (Q) and finer scale relative kinship (K). The population structure matrix (Q) was identified by running STRUCTURE at K = 4. Only markers with an allele frequency of 5% or greater were included in the association analysis.

Results and Discussion

Population structure

The composite collection was dissected in to four populations by using software STRUCTURE and mentioned in Fig. 1. Although the population subgroups

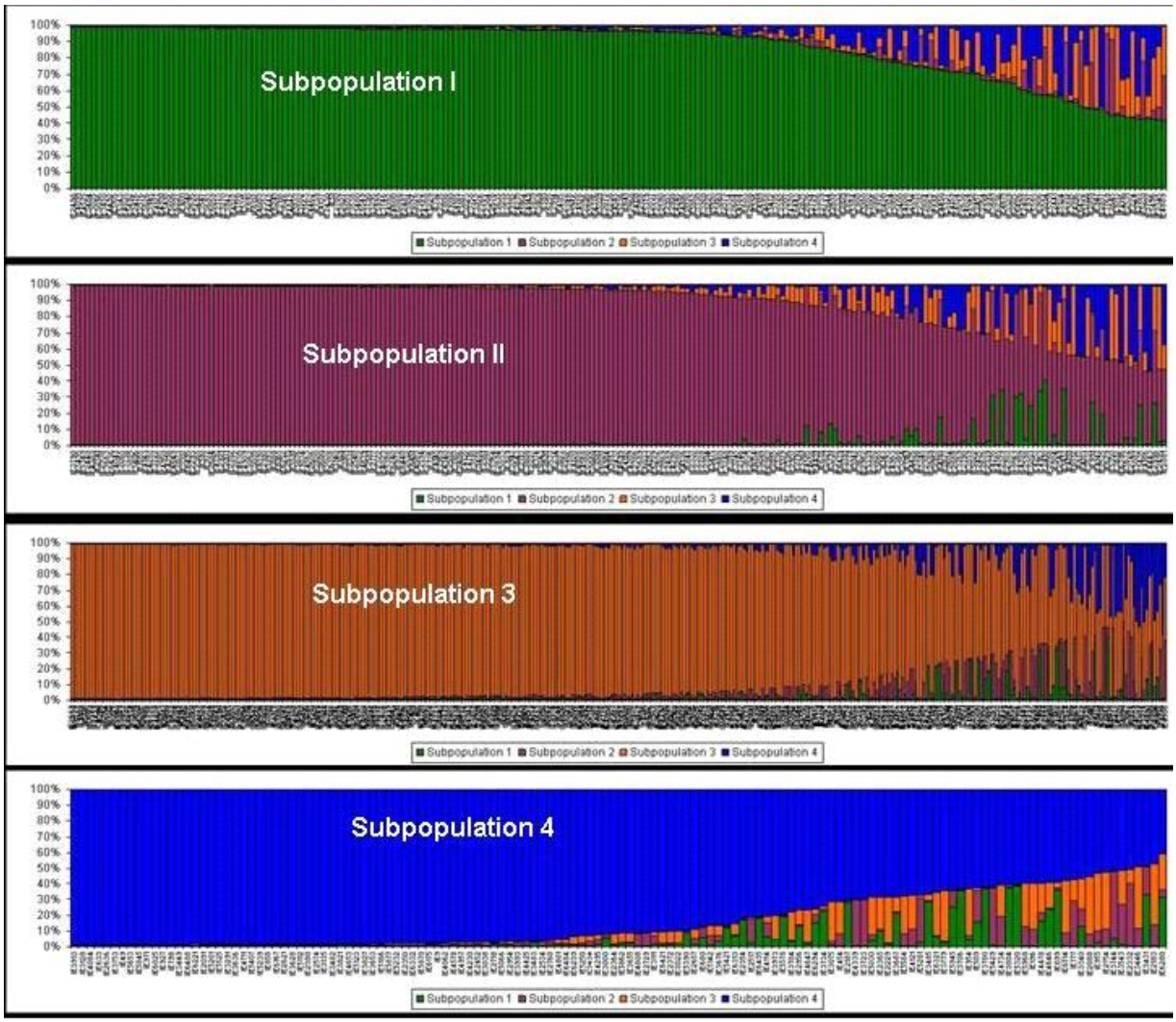

Fig. 1 : Classification of finger millet composite collection based on four major, geographical regions and accessions with mixture of colour were percentage of admixtures from the respective population.

corresponded largely to geographic regions, there were some notable exceptions. Excluding some accessions that showed evidence of admixture, most of the African accessions belonged to the subpopulation 1 and Asian accessions belonged to subpopulation 2. Most of the accessions in subpopulation 3 were having 10 -60% admixture, which might be due to the result of hybridization between adapted African and Asian (especially Indian) accessions and these are called as Indaf varieties. Subpopulation 4 had more admixtures (10 – 60%) of accessions from all geographical regions. Respective Q matrix outputs four subpopulations were generated for structure based association analysis, which minimize the spurious association between genotype and phenotypic associating.

Association mapping

Substantial variation was observed for all traits and high heritability showed greater importance of the traits in revealing marker trait associations. The marker trait association of composite collection data was validated with reference set data. It was observed that the marker-trait association varied with the environments and population used. In the present study, association analysis resulted inconsistent association between the traits and markers for most of the traits mainly due to limited number of random and non trait specific markers mentioned in Table 2. However, in the present study, QTL for days to 50 per cent flowering had consistent association with UGEP8 in LG3 (E2, E3 and pooled for both composite collection and reference set) and UGEP56 in LG9 (E2 and E3 in composite collection and E1 in reference set). It indicated relatively tight linkage between the trait and marker. Also the association varied in different sample size consisting of composite collection with 959 accessions and reference set with 300 accessions. It has been suggested that large numbers of molecular markers are needed to better cover the entire nuclear genome for

Table 2 : Association of 20 SSR loci with agronomic traits in finger millet composite collection in three environments and pooled.

Traits	Environment	Marker	Linkage Group	Position(cM)	*P*
Days to 50% flowering	E1	UGEP11	5A	63.5	0.034
	E2	UGEP56	9A	7.4	0.028
		UGEP8	3B	65.2	0.001
	E3	UGEP56	9A	7.4	0.025
		UGEP8	3B	65.2	0.016
	Pooled	UGEP8	3B	65.2	0.048
Plant height	E1	UGEP3	3A & 3B	75.8 & 64	0.013
		UGEP65	8A	31.6	0.044
	E2	UGEP68	9B	0	0.046
		UGEP104	3B	124.7	0.042
Basal tiller number	E2	UGEP3	3A & 3B	75.8 & 64	0.039
		UGEP8	3B	65.2	0.003
Culm branching	E2	UGEP8	3B	65.2	0.047
		UGEP26	5B	121.1	0.017
Flag leaf blade length	E2	UGEP90	6B	23.3	0.035
	Pooled	UGEP26	5B	121.1	0.038
Flag leaf blade width	E2	UGEP56	9A	7.4	0.019
		UGEP65	8A	31.6	0.001
	Pooled	UGEP3	3A & 3B	75.8 & 64	0.028
Flag leaf sheath length	E2	UGEP26	5B	121.1	0.016
		UGEP18	1B	70.3	0.001
Peduncle length	E1	UGEP18	1B	70.3	0.024
	E2	UGEP65	8A	31.6	0.028
	Pooled	UGEP11	5A	63.5	0.02
Ear head length	E1	UGEP56	9A	7.4	0.001
	E2	UGEP8	3B	65.2	0.001
	E3	UGEP107	1A	9.5	0.001
	Pooled	UGEP26	5B	121.1	0.032
Length of longest finger	E2	UGEP68	9B	0	0.054
	E3	UGEP3	3A & 3B	75.8 & 64	0.001
Plot yield	E3	UGEP104	3B	124.7	0.03

such association studies (Jensen, 1989). However, in our study only 20 markers were used. The marker UGEP3 on LG3 was associated with seven traits (PH, DF, BTN, CB, EHL, FLBL, FLBW) in composite collection and (BTN, FLBL, PL, EHW, PY, FL, PH) in reference set. Also majority of the markers were found to be associated with more than one trait, such an association may arise due to pleotropic effect of the linked QTL on different traits (Culp *et al*., 1979).

Conclusion

The global finger millet composite collection showed rich allelic diversity (231 alleles, 11.6 alleles per locus, 121 common alleles and 110 rare alleles at 1%). The markers UGEP8 and UGEP56 were consistently associated with days to 50 per cent flowering indicating relative strong association between marker and traits. Extensive study of these markers in mapping population would be helpful for confirmation of QTL.

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