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ORIGINAL PAPER

Exploration and mapping of microsatellite markers from subtracted drought stress ESTs in *Sorghum bicolor* (L.) Moench

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Abstract Molecular variation within defined genes underlying specific biochemical or physiological functions provide candidate gene-based markers which show very close association with the trait of interest and thus should enable to design superior genotypes. We explored microsatellite loci in a total of 9,892 subtracted drought stress ESTs of sorghum (6,295 after flowering ESTs and 3,597 before flowering ESTs) available in the NCBI dbEST database. Analysis of 9,892 ESTs identified 221 non-redundant ESTs with SSRs, from which 109 functional SSRs were developed. Among them 62 EST-microsatellites (56.8%) exhibited polymorphism for at least one sorghum genotype among the five tested and yielded a total of 161 alleles, with an average of 2.59 alleles per marker. We present a microsatellite linkage map using a RIL population derived from the cross 296B and IS18551. The map contains 128 microsatellite loci distributed over 15 linkage groups, and spanning a genetic distance of 1,074.5 cM. The map includes map positions of 28 drought EST-microsatellites developed and seven new genomic-SSRs, and are distributed throughout the map. The developed EST markers include genes coding for important regulatory proteins and functional proteins that are involved in stress related metabolism. The drought EST-microsatellites will have applications in functional diversity studies, association studies, QTL studies for drought, and other agronomically important traits in sorghum, and comparative genomics studies between sorghum and other members of the Poaceae family.

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Introduction

Drought is the most important environmental stress factor in agriculture which limits crop productivity in the arid and semi-arid regions of the world. Developing genotypes with improved drought tolerance is one of the thrust areas of current research in many crop species including sorghum. A variety of morphological and physiological changes have been identified in response to drought stress in plants. Many traits whose presence/expression is associated with plant adaptability to drought-prone environments are identified. They include root morphology and rooting depth, plant architecture, variation in leaf cuticle thickness, stomatal regulation, osmotic adjustment, antioxidant capacity, hormonal regulation, desiccation tolerance (membrane and protein stability), and maintenance of photosynthesis through persistent green leaf area (stay-green). Plants expressing a variety of genes associated with a host of these morphological and physiological traits tolerant to abiotic stresses have been demonstrated (Bohnert et al. 1995; Shinozaki and Yamaguchi-Shinozaki 1996; Bray 1997; Nguyen et al. 1997). In addition, some genes are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction in stress response (Shinozaki and Shinozaki-Yamaguchi 1997, 2000). Nevertheless, direct selection of these traits for improving yield potential under drought-stressed conditions through conventional phenotype based breeding has been hampered by their polygenic control, epistasis, significant genotype to environment (G $\times$ E) interaction and

quantitative trait loci (QTL)-to-environment (QTL × E) interaction. Therefore, breeding for drought tolerance has resulted in limited success in tailoring cultivars with high yields under variable drought conditions. Application of molecular marker technology, functional genomics, and association studies, marker-assisted selection (MAS) coupled with conventional breeding are the promising approaches for crop improvement for complex traits, such as drought tolerance.

During the last few years, emphasis has been towards the development of molecular markers from the transcribed region of the genome to associate the molecular variation of genes with phenotypic variability of agronomically important traits. Molecular variation within defined genes underlying specific biochemical or physiological functions will provide molecular markers for crops, such markers often described as 'candidate gene-based', which may show very close association with loci controlling variation for the trait of interest, allowing for the development of 'perfect markers' (Prioul et al. 1999; Thornsberry et al. 2001; Varshney et al. 2005). The construction of functional maps with genes of known function is an important component of candidate gene approach since functional map allows verification for any mapped QTL or mutation, if any, of the mapped genes is a good candidate (Aubert et al. 2006). Furthermore, the co-localization of these genic markers with QTL for target traits can be validated further through LD studies (Rafalski 2002) as well as transcriptome profiling, gene silencing (Vance and Vaucheret 2001) and induced mutagenesis (Li et al. 2001) approaches (Wilson et al. 2003). These approaches finally can facilitate gene pyramiding and accelerate the development of superior genotypes (Sorrells and Wilson 1997).

Sorghum is one of the most drought tolerant grain crops. Further, its rich genetic diversity for drought tolerance makes it an excellent crop model and choice for studying the genetic and physiological mechanisms of drought tolerance. Two distinct drought responses, namely post-flowering (after flowering) responses, when moisture stress occurs during the grain development stage, and pre-flowering (before flowering) responses, expressed when the plants are under significant moisture stress before flowering, especially from panicle differentiation to flowering, have been described in sorghum, which are controlled by different genetic mechanisms (Rosenow et al. 1983; Rosenow 1987). Staygreen trait has been considered as an important component of post-flowering drought response in sorghum (Rosenow and Clark 1981) and has been extensively studied. The genetic characterization of these two distinct drought response phases in sorghum have resulted in the identification of several genomic regions (QTL) associated with them (Tuinstra et al. 1996, 1997; Crasta et al. 1999; Subudhi et al. 2000; Xu et al. 2000; Tao et al. 2000; Kebede et al. 2001; Haussmann et al. 2002). Despite these genetic studies which focused mainly on a few component drought tolerance traits such as staygreen trait, the genetic basis of drought tolerance as a complete phenomenon in sorghum has not yet been understood, thus limiting the opportunities for its manipulation in sorghum improvement. However, the already available large collection of expressed sequence tags (ESTs) from genes which are expressed during these two drought stress phases in sorghum (Pratt et al. 2005), provides invaluable opportunity for identification of candidate genes for drought tolerance, and further development of functional markers (FM) to tag them with drought tolerance component traits so as to utilize in MAS for improving the drought tolerance in sorghum.

Many linkage maps have been developed for sorghum using different marker types, including restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs) markers (Hulbert et al. 1990; Binelli et al. 1992; Whitkus et al. 1992; Chittenden et al. 1994; Brown et al. 1996; Taramino et al. 1997; Boivin et al. 1999; Bhattramakki et al. 2000; Kong et al. 2000; Menz et al. 2002; Wu and Huang 2006, etc.). Except some markers from defined genes, many of these markers used for constructing the linkage maps represent untranscribed regions of the genome. Although they have been utilized to construct linkage maps, to detect QTL for many of the agronomically important traits and genetic diversity, they are not in general closely associated with variations in genes controlling phenotypic traits. SSRs or microsatellite markers are short tandem DNA repeats of 1–6 bp long. Because they are highly polymorphic, co-dominant and can be easily used and analyze, SSR markers have gained importance as a plant genotyping tool over other markers such as RFLP and AFLP. ESTs also contain SSR motifs which provide an inexpensive route for developing SSRs, (Scott et al. 2000; Cordeiro et al. 2001). Although SSRs derived from ESTs show lesser polymorphism than SSRs derived from genomic DNA (Cordeiro et al. 2001; Eujayl et al. 2002), they provide the potential advantage of close linkage to significant gene variants (alleles) of many genes of agronomically important traits. Furthermore, their high level of transferability among the related species, they can often be used as anchor markers for comparative mapping and evolutionary studies. The use of ESTs as a source of SSR has been documented in a number of crop species such as wheat (Nicot et al. 2004), rice (Cho et al. 2000), barley (Holton et al. 2002; Thiel et al. 2003), cotton (Han et al. 2006), sugarcane (Cordeiro et al. 2001), and grape (Scott et al. 2000). Such application is lagging behind in case of sorghum.

Our long-term goal is to develop superior genotypes for drought tolerance in sorghum through genetic analysis, association studies, functional genomic studies, and breeding efforts. The specific objectives of the present study are: (1) to develop microsatellite markers from ESTs generated from two subtracted sorghum drought-stressed cDNA libraries, namely drought-stressed after flowering (DSAF1) and drought-stressed before flowering (DSBF1) libraries; (2) to conduct a study for assessing their functional diversity using five sorghum genotypes; and (3) to map them on to the microsatellite linkage map developed using a recombinant inbred line population derived from 296B and IS18551 parental lines.

Materials and methods

Plant material and mapping population

Five sorghum genotypes were used for assessing the functional diversity of EST-microsatellite markers. Of the five genotypes, B35 is a post-flowering drought resistance stay-green derivative of IS12555 from Ethiopia; TX7000 is a pre-flowering drought resistance variety from the USA. M35-1 is a high yielding popular Indian cultivar. 296B is a moderate drought resistance cultivar with a very good combining ability and seed parent of five commercial sorghum hybrids in India, and IS18551 is a landrace from Ethiopia. A F_7 recombinant inbred line mapping (RIL) population consisting of 168 lines derived from the cross 296B and IS18551 was employed for genetic mapping of EST markers.

Drought-stressed cDNA libraries, marker searches and primer design

Expressed sequence tags developed by Pratt et al. (2005) from two subtracted sorghum drought-stressed cDNA libraries, DSAF1 and DSBF1 libraries, constructed from drought-stressed sorghum leaves were used to develop microsatellite markers in the study. DSAF1 and DSBF1 ESTs were derived from B35, a post-flowering drought resistant genotype and TX7000, a pre-flowering drought resistant genotype, respectively. The ESTs were deposited at the NCBI GeneBank and their accession numbers are CF755710–CF762004 (for DSAF1, 6,295 ESTs) and CF769318–CF772914 (for DSBF1, 3,597 ESTs). The criteria for imposing the two drought stresses used for the development of subtracted ESTs were described by the authors (Pratt et al. 2005). Briefly, for DSBF1, water was withheld after 4 weeks from sowing to impose gradual water deficit and to simulate natural drought stress during pre-flowering period. For DSAF1, final irrigation was applied 3 days after anthesis (about 2 months after sowing) to impose gradual drought stress and to simulate natural post-flowering drought stress. The technical aspects of construction of the drought-stressed cDNA libraries, their subtraction by Poly $(A)^+$-RNA obtained from non-stressed leaves, cloning, amplification and sequencing of the clones can be seen in the author's original paper.

The sequences of ESTs of the two libraries were retrieved by electronically segregating them from sorghum ESTs available at the NCBI dbEST database (http://www.ncbi.nlm.nih.gov/). Initially, for avoiding redundancy, the total retrieved 9,892 drought ESTs (DSAF1—6,295 ESTs and DSBF1—3,597 ESTs) were aligned using the TIGR EST clustering tool (http://tigr.nbn.ac.za/pise/tgicl.html) with default settings specified by the program, which employs CAP3 (Huang and Madan 1999) program for assembling the sequences. Finally single original EST representing each consensus contigs, along with the singleton sequences were mined for SSR motifs using the SSRIT software program (http://www.gramene.org/db/searches/ssrtool) with parameters set to identify repeats up to decamers, with at least six repeats. The software Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), was utilized to design primer pairs flanking SSRs. The major parameters set for primer design were as follows: primer length 18–24 bp with 20 bp as the optimum; PCR product size 100–300 bp; optimum annealing temperature 54°C; GC content 35–60% with 50% as the optimum. The marker nomenclature proposed by De Vicente et al. (2004) was followed for naming the markers developed in this study. The canonical name they proposed consists of [Function][Lab Designator][Species][Type of marker][serial nimber of clone]. Hence, the EST markers developed in this study were named Dsenhsbm1–Dsenhsbm109. The marker name denotes drought stress EST (Dse), developed by NRCS-Hyderabad (nh), in *Sorghum bicolor* (sb), a microsatellite marker (m) and followed by serial number of clone. The primers were synthesized by MWG Biotech Pvt. Ltd. Bangalore, India. The developed drought ESTs containing microsatellite motif were searched against the NCBI non-redundant protein database using the BLASTX algorithm (http://www.ncbi.nlm.nih.gov/BLAST) for assigning of putative functions. In BLASTX analysis, ESTs which could not hit against the NCBI non-redundant protein database were considered as 'unknown proteins', whereas ESTs which identified homologous proteins of unknown function from other crops were classified as 'hypothetical proteins'.

DNA extraction, PCR amplification, electrophoresis

The genomic DNA was extracted using the CTAB method (Saghai-Maroof et al. 1984). Touchdown PCR was used to reduce non-specific amplification. Temperature cycling was carried out for both EST-SSRs and genomic-SSR

markers using the Bio-Rad iCycler version 3.3032. Briefly, a hot start of 94°C for 5 min was followed by 1 cycle of 94°C for 10 s, 61°C for 20 s, and 72°C for 30 s, then 9 cycles during which the annealing temperature dropped by 1°C per cycle, then 30 cycles of 94°C for 10 s, 54°C for 20 s, and 72°C for 30 s, and a 20 min final extension at 72°C (Smith et al. 2000). Reactions were run in 5 μl reaction in 96-well PCR plates (Axygen, PCR-96-HS-C) with each PCR contained 2–4 pmol of primer, 1–4 mM $MgCl_2$, 0.1–0.2 mM dNTP, 0.2 U *Taq* DNA polymerase and 1× PCR buffer (*Invitrogen* S. Giuliano Milanese, Italy) and 50 ng of template genomic DNA. After amplification, the presence of amplification products was tested by agarose gel electrophoresis. The successful PCR products were assayed on a Bio-Rad Sequi-Gen^{TM} sequencing electrophoresis apparatus in 5% polyacrylamide gels containing 8 M urea and 1× TBE buffer at 80 W of constant power. The DNA fragments were visualized by silver staining (Fritz et al. 1999) and scored as either parental (A or B), heterozygous (H), or missing data (–).

Statistical analysis

A binary data matrix was derived using a panel of five sorghum genotypes from scoring the presence or absence of expected fragment size of individual EST markers by 1 or 0, respectively. The binary data were used to compare a pairwise similarity matrix using the Dice similarity index (Dice 1945). The similarity matrix was subjected to cluster analysis using the unweighted pair-group method with arithmetic average (UPGMA) algorithm on the NTSYSSpc, version 2.10e (Rohlf 2000).

Genetic mapping

Altogether, 345 microsatellite primer pairs were considered for screening polymorphism between the parental lines of the RIL mapping population. Among them, 236 markers were genomic DNA derived-SSRs that had been developed by Brown et al. (1996), Taramino et al. (1997), Kong et al. (2000) and Bhattramakki et al. (2000) and the remaining 109 markers were drought EST-microsatellites developed in this study. Polymorphic markers between the parental lines were employed in genotyping of entire mapping population. The genomic-SSRs were selected for the construction of frame work map to linkage with the drought EST-microsatellites. The computer software JOINMAP 3.0 (Van Ooijen and Voorrips 2001) was utilized to calculate Chi-square tests of goodness-of-fit to an expected segregation ratio of 1:1 for all markers segregation and finally used for linkage map construction. The allocation of markers to linkage groups was mostly stable for a wide range of LOD grouping thresholds (from ≤4.0 to ≥7.0). Markers that were attributed to a linkage group at a LOD grouping threshold of <4.0 were only included when it was known from other published maps that they belong to this group. More stringent parameter settings were not applied where the marker location was already known from the earlier published reports. The Kosambi function was used to convert recombination into the genetic distance (centimorgans, cM). A "ripple" was performed after three marker additions/insertions. The "jump" and "triplet" thresholds were set to four and nine, respectively. Linkage groups were named according to the nomenclature proposed by Kim et al. (2005). The goodness-of-fit of the constructed maps reflecting the discrepancy between final recombination frequencies in the map and those apparent from individual marker data pairs, was expressed as a chi-square value and computed according to Stam and van Ooijen (1995).

Results

Marker development and their characterization

Out of 9,892 drought ESTs (DSAF1, 6,295 ESTs; DSBF1, 3,597 ESTs) selected for marker development, 392 ESTs (3.9%) were mined with microsatellites of di-, tri-, tetra-, penta- and hexa-nucleotide repeats. Redundancy analysis of the 392 sequences revealed 237 ESTs belonging to 66 clusters and 155 ESTs being singletons. Hence, a total of 221 unique EST sequences represented candidates for SSR-marker development. Of the 221 microsatellite containing ESTs, primer pairs could be designed only for 116 of them (52.4%). The remaining sequences contained either short stretch of DNA sequence flanking the microsatellite motif or the sequences were inappropriate for primer modeling. Of the 116 primer pairs were tested, 109 of them could result in amplification during PCR with genomic DNA, while seven primer pairs failed to yield amplification and were excluded from the analysis. The most common type of SSR motif in the described 109 drought EST-microsatellites was di-motif (62) followed by tri-motif (36), tetra-motif (4), penta-motif (2), and hexa-motif (2). Of all the SSR assayed, 106 markers had perfect repeats and three markers had compound repeats. Among the final set of 109 drought EST-microsatellites, majority showed a size range similar to the expected size, based on the original EST sequence used for primer design. Seven primer pairs (Dsenhsbm24, Dsenhsbm38, Dsenhsbm40, Dsenhsbm50, Dsenhsbm56, Dsenhsbm61, and Dsenhsbm74) showed higher PCR product size than the expected size, suggesting possibility of introns between the primer binding sites of markers. However, the observed increased size did not interfere with the allelic variation of markers, and these SSRs were also

included in the study. Single informative bands were obtained with only 42 cases, while remaining primer sets produced additional fragments, indicating the PCR amplification at more than one locus. In cases of additional fragments, only fragments with expected size range were considered in both mapping analysis and diversity studies. Nine primer pairs (Dsenhsbm2, Dsenhsbm24, Dsenhsbm29, Dsenhsbm30, Dsenhsbm34, Dsenhsbm47, Dsenhsbm65, Dsenhsbm78, and Dsenhsbm96) were detected to amplify dominant product only in some of the five genotypes studied, which is attributable to presence of some modifications in the genomic DNA at the primer binding sites of other genotypes. Among these nine markers, six markers (Dsenhsbm2, Dsenhsbm29, Dsenhsbm47, Dsenhsbm65, Dsenhsbm78, and Dsenhsbm96) were employed in genotyping as dominant markers in the present RIL mapping population. The primer sequence information of 109 drought EST-microsatellites is provided in Table 1.

Marker diversity analysis

For EST-diversity analysis, two varieties B35 and TX7000 that had been originally used for the development of drought stress ESTs, the parents of the mapping population (296B and IS18551), and a popular high yielding Indian variety M35-1 were included. The 109 drought EST-microsatellites yielded 62 (56.8%) polymorphic markers between the parents of at least one of the five sorghum varieties studied (Table 1). The 62 markers yielded a total of 161 alleles, with an average of 2.59 alleles per primer pair assayed. The remaining 47 EST-microsatellites (43.2%) did not yield polymorphism in the panel of five sorghum varieties.

The usefulness of EST-microsatellites was investigated by assessing genetic similarity (GS) among the five sorghum varieties. The genetic similarity coefficient value observed among these varieties ranged from 0.33 to 0.55, suggesting high degree of genetic homogeneity. However, the dendrogram constructed, based on the genetic similarity coefficient matrix grouped them into three clusters (Fig. 1). Cluster A included varieties 296B and B35, whereas cluster B and C consisted of M35-1 and IS18551, and TX7000, respectively. This categorization is well in agreement with their phenology and staygreen expression as 296B (moderately staygreen) and B35 (highly staygreen) are short varieties; whereas M35-1, IS18551 are tall varieties with moderate leaf senescent expression; and TX7000 is a variety with medium height and highly senescent to post-flowering drought stress but resistance to pre-flowering drought stress. This classification is probably indicative of their functional relatedness irrespective of their geographical origins as IS18551 is from Ethiopia, 296B and M35-1 are from India, B35 is a derivative of line IS12555 from Ethiopia, and TX7000 is from the USA. Significantly, although 296B is not as staygreen as B35, QTL mapping studies with 296B for staygreen trait revealed that it is associated with staygreen trait at three major staygreen QTL regions (data not shown) which were earlier identified in B35 which is used as a staygreen donor in QTL mapping studies, further confirming their functional relatedness irrespective of their difference in the overall trait expression.

Linkage mapping

In this study, a total of 345 microsatellite primer pairs were tried between the parental lines 296B and IS18551 of 168 F_7 RIL population. One hundred and forty-one microsatellites consisting of 108 genomic-microsatellites (45.7%) and 33 EST-microsatellites (30.2%) (27 co-dominant and 6 dominant markers) were found polymorphic between the parental lines. Of the 141 microsatellite loci screened in the mapping population, 21 marker loci (14.8%) highly significantly deviated ($P < 0.01$) from the expected Mendelian segregation ratio (1:1) and were skewed towards either the male or female parent. Of these 21 loci, 5 loci (Xtxp323, Xtxp316, Xtxp340, Xtxp319, and Xtxp61) and 7 loci (Xtxp335, Dsenhsbm50, Xtxp32, Xtxp88, Dsenhsbm66, Xtxp43, and Xtxp329) co-segregated together and were clustered on two genomic regions on SBI-01. Likewise, two (Xtxp336 and Xtxp31), three (Xtxp343, Xtxp12, and Dsenhsbm39), two (Xtxp15 and Xtxp23), and two (Dsenhsbm29 and Xtxp210) deviated marker loci were observed to be clustered on SBI-03, SBI-04, SBI-05, and SBI-08 linkage groups, respectively (Fig. 2). The deviated genomic-SSR markers were retained in linkage analysis since their map positions were consistent in the present map with the earlier reports (Bhattramakki et al. 2000). The EST-microsatellites Dsenhsbm50, Dsenhsbm66, Dsenhsbm39, and Dsenhsbm29 were also retained in the analysis as these markers co-segregated with these deviating genomic-SSR markers. Five EST-microsatellites (Dsenhsbm47, Dsenhsbm60, Dsenhsbm65, Dsenhsbm78, and Dsenhsbm96) were found unlinked in the present study. Eight previously mapped genomic-SSRs (Xtxp83, Xgap1, Xcup16, Xtxp55, Xtxp100, Xtxp213, Xtxp283, and Xtxp164) were removed from the final map because of being mapped at positions different from previous reports (Bhattramakki et al. 2000; Wu and Huang 2006) and some were found largely disturbed during the map construction.

The linkage map we constructed includes 128 marker loci of the 141 polymorphic markers used for genotyping. The map contains 28 drought EST-microsatellites of the 109 drought EST-microsatellites developed in this study, and 7 genomic-SSR markers reported earlier but whose map positions were not known (Fig. 2). The map spans a total genetic distance of 1,074.5 cM, with the average and maximal genetic distance between markers being 8.3 and 42.2 cM, respectively. Linkage analysis produced a total of

Table 1 Primer sequences and putative functions of drought EST-microsatellite markers developed in the study

S. No.	Marker	EST Gen Bank accession no.	Type(s) of SSR and number of repeats	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Ann. temp. (°C)	Expected size (bp)	Putative function	*E* value	Species
1	Dsenhsbm1	CF772844	(GA)17	GTGTGGGAAGGAAGGATGGA	CCCCCTCGAGACTAGTTCTCTCT	55	172	Hypothetical protein	6.00E-33	*Zea mays*
2	**Dsenhsbm2**	CF758376	(TTTTA)6	TCTGCTTGGCTACGTTCTGA	TTCCTCCGCTGAATGATTTC	54	247	**No hit**		
3	**Dsenhsbm3**	CF772773	(GA)7	GAGAGCTGCAGGCGAGTAGT	GGTGTCTCCGTCCTTGTCAT	54	172	**No hit**		
4	**Dsenhsbm4**	CF772733	(TG)7	CCAAGGCTGAGGTCAAGAAG	AGCCGAGCTCAACATACAGG	54	243	**Heat shock protein (16.9 KD low molecular weight protein)**	**4.00E-22**	***Oryza sativa***
5	Dsenhsbm5	CF772691	(GA)15	CCATGTGAGGAGAGGGATGT	AGAAAAGGGATTCCCAGCAT	54	236	No hit		
6	Dsenhsbm6	CF772614	(GT)7	CCGGAGCAGGAGTTCTACAG	TCAGATCATGCATTGCACAC	54	190	Raffinose synthase or seed imbibition protein Sip1	5.00E-17	*Oryza sativa*
7	**Dsenhsbm7**	CF772327	(GTC)8	AGGTAGCAATTCGTCGTTGC	ACGGGTGACCTCAACTGAAC	54	199	**No hit**		
8	**Dsenhsbm8**	CF772264	(TC)8	GTGATGCACGTTCTTGTGGA	CGTGTTCCGTCCAATCTACC	54	224	**No hit**		
9	**Dsenhsbm9**	CF757170	(AT)6	GGGGCAAAAATTGGAAAGAT	CGAACAGAATGAACCAGCAA	54	159	**Zinc finger, C3HC4 type (RING finger)**	**5.00E-48**	***Oryza sativa***
10	**Dsenhsbm10**	CF772123	(AG)10	CACGAGGGTCGCTAAACACT	CACTTTGAAAGGGAGCTTGG	54	197	**Ubiquitin-fold modifier 1**	**2.00E-39**	***Oryza sativa***
11	Dsenhsbm11	CF756489	(GT)6	TTCGCTTCAGGAAATTTTGG	AAACATGGGCCGATTATTCA	54	190	ATP phosphoribosyltransferase	1.00E-43	*Oryza sativa*
12	Dsenhsbm12	CF758865	(TA)6	CCGACCCAATCATGAAAACT	CACCTGCGCCAATTTTATC	54	174	Hypothetical protein		
13	**Dsenhsbm13**	CF771490	(TG)10	TGAACTGAACTCGGGGTTTC	CGTCCTGGAGCAAAGGTTAC	54	247	**CCAAT-box transcription factor complex WHAP3**	**2.00E-10**	***Triticum aestivum***
14	**Dsenhsbm14**	CF771447	(AT)9	GTGGCTTGGAGAAAAGGAAG	TGCCACCTCAGTTCATGACTAT	53	224	**No hit**		
15	**Dsenhsbm15**	CF771344	(AT)8	GGTTCAAGTGGGCAAAGTGT	CGAAGCTAACAGCAGCAAGA	54	172	**Permease (nucleobase-ascorbate transporter)**	**3.00E-23**	***Oryza sativa***
16	**Dsenhsbm16**	CF771342	(TG)20	TCCCCTCCACAGTAAAGCTG	CTTCCCCACGTCTTCTTCTG	54	248	**Hypothetical protein**	**1.00E-22**	***Oryza sativa***
17	**Dsenhsbm17**	CF756551	(TGCTGA)6	TCTGCCACTGGCTTAATCTG	CCAAGGCAAGCGAACTTATC	54	191	**No hit**		
18	Dsenhsbm18	CF756371	(CAC)6	GACACAGCCATGGACATTCA	TCAGTGGCTCCTCAACATCA	54	195	Zinc transporter	9.00E-53	*Oryza sativa*
19	**Dsenhsbm19**	CF771058	(GCA)8	CATGATGCAGCAACAACAGC	GAAACCAGAACCGAACCTGA	54	151	**ETHYLENE-INSENSITIVE3-1 protein**	**6.00E-15**	***Oryza sativa***
20	Dsenhsbm20	CF771015	(TA)7	TGTGTGCTTGATGCTTCTCC	CAAGAGCCACCAAATCACCT	54	232	No hit		
21	**Dsenhsbm21**	CF756943	(TG)6	GCTTGATCATGGTCCTTTGG	CAACTCTCAAGCCCATCCAT	54	235	**TPR Domain containing protein**	**1.00E-18**	***Oryza sativa***
22	Dsenhsbm22	CF770940	(CT)8	GAGGTCGACCAGTACGAGGA	GCAATTGCCAAGAGAGGAAC	54	164	Serine/threonine-protein kinase SAPK5 (Osmotic stress/ abscisic acid-activated protein kinase 5)	6.00E-20	*Oryza sativa*
23	**Dsenhsbm23**	CF770851	(AT)12 + (TG)17	GTGAGTTCTCGCAGCCCTAC	CGAACATCACACACACACACA	54	154	**No hit**		
24	**Dsenhsbm24**	CF770735	(GA)8 + (GA)9	CGTCAATAGCAAACCACCAG	CCCCTCGAGACTAGTTCTCTCT	53	210	**Chaperonin 21 precursor**	**3.00E-49**	***Oryza sativa***
25	**Dsenhsbm25**	CF770657	(AT)9	GGCAGAGAATCTGGCACTGT	TTCGGTCTTCGTTGCTTCAT	54	249	**Trehalose-phosphatase**	**2.00E-22**	***Oryza sativa***
26	Dsenhsbm26	CF770414	(CAG)7	CATCCCTAATGGGTCAGCAC	GGCATCATCCATTCATCCTC	54	235	Oligouridylate binding protein	3.00E-21	*Sorghum bicolor*
27	**Dsenhsbm27**	CF770407	(TG)13	GCGACAGGGTTAATTGGTTC	TACTGTGACGGCATGAATGG	54	210	**No hit**		
28	Dsenhsbm28	CF771876	(GCA)6	CCGGTCCGCTACTTCGTG	GCCTCACACGGTCCTATCAG	55	205	No hit		
29	**Dsenhsbm29**	CF770318	(GT)9	AAACACGGCATTTTTCTTGG	AATAGCAATTCCCAGCGAAC	54	208	**Hypothetical protein**	**2.5**	***Oryza sativa***
30	**Dsenhsbm30**	CF770262	(TGA)8	AGTTTGTGTGTGCGCTCGT	CTCCCCATCACGCATCTAGT	54	194	**Stress related protein**	**4.00E-06**	***Oryza sativa***

Table 1 continued

S. No.	Marker	EST Gen Bank accession no.	Type(s) of SSR and number of repeats	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Ann. temp. (°C)	Expected size (bp)	Putative function	*E* value	Species
31	**Dsenhsbm31**	CF770173	(GT)8	ATCTGGCACATGACGTGAAC	TTTACCGGGACCAATACAGG	54	227	**RING-H2 zinc finger protein RHA2a**	**7.00E-11**	***Arabidopsis thaliana***
32	**Dsenhsbm32**	CF770135	(AC)11	GAATGATCACGAGGCTCCAT	AAAGCATGCCTCCCTTCTCT	54	158	**No hit**		
33	Dsenhsbm33	CF771576	(GT)6	ATGCTAATTGGCGTTGGTGT	CGTTTCATTAATCCGCTGCT	54	203	Actin-depolymerizing factor 5 (ADF-5)	9.00E-39	*Oryza sativa*
34	**Dsenhsbm34**	CF769947	(TA)15	CCTGTGCATGTTTCATCCAT	TGAACAGAGAGATTTTCACATGC	53	240	**Hypothetical protein**	**7.00E-05**	***Oryza sativa***
35	**Dsenhsbm35**	CF769371	(TGC)8	TTGGGATTTGGATTTGGATG	TCGACATGGAAATCGACTGA	54	165	**Harpin induced gene 1 homolog**	**6.00E-39**	***Oryza sativa***
36	**Dsenhsbm36**	CF761941	(CT)10	TACGCAAAATGTTCCCACAC	CCCTCGAGTGGTGTTCTAGC	54	228	**No hit**		
37	**Dsenhsbm37**	CF761886	(TG)12	GCCATGCTCTACCATTTCCT	TCGATTTTTCCCGTTTTGTC	54	229	**Serine carboxypeptidase precursor**	**0.012**	***Oryza sativa***
38	Dsenhsbm38	CF772197	(TAA)6	AGTCGACACGATGACAGACG	CGTATTGCGGTGACGTTTAC	54	179	No hit		
39	**Dsenhsbm39**	CF761862	(AG)14	TCAGTGATACAGCCGTCCAG	ATGCATAAACCACGGCTGTC	54	154	**No hit**		
40	**Dsenhsbm40**	CF758966	(TG)6	CTGGTGATGTCAAACGAACG	CAAGACCTGGTACAAGCAAGC	54	161	**No hit**		
41	Dsenhsbm41	CF757105	(TG)6	CTGTTTGGTGGCAATTGATG	TGCCACAGAGAAATCAACCA	54	165	Nitrilase-associated protein	3.00E-10	*Arabidopsis thaliana*
42	Dsenhsbm42	CF761722	(TG)7	CACAGTGCATTGGGTGGTC	CTCGAAGCTTGACGATGACA	55	212	No hit		
43	Dsenhsbm43	CF761658	(AGC)7	ATGTACCCCTACACGCTTCG	GTCGACGACACGCTCCTC	54	179	Hypothetical protein	6E-20	*Oryza sativa*
44	Dsenhsbm44	CF761225	(CGC)7	TAATCCGTCCCCCTCTTCTC	CCGCCTTAGTGATCATCTCC	54	234	40S ribosomal protein S13	2.00E-72	*Zea mays*
45	**Dsenhsbm45**	CF761081	(AG)10	ATAGGGACACGGCAGCACTA	CCCAGGTGAAGATGATCCAC	54	158	**Hypothetical protein**	**4.00E-08**	***Oryza sativa***
46	**Dsenhsbm46**	CF760991	(TA)8	GGCAGCATCTACCAGACCAT	GAAGGCATCTAGCGATCACC	54	210	**VHS2 protein**	**2.00E-22**	***Oryza sativa***
47	**Dsenhsbm47**	CF760921	(GA)12	CACGAGGGGAAAGCACAAC	TAGATGCGTGGTATGGCTGA	55	187	**No hit**		
48	**Dsenhsbm48**	CF757016	(TCTG)6	TGTTCATCTCCAACGTCTCG	GCAGCATCTCGTCAGAATCA	54	212	**No hit**		
49	Dsenhsbm49	CF760757	(AT)10	CTAGCCCACAAACACTTCAGC	CCTTCTCCGTCGACAGTCTC	54	158	Glutaredoxin-C15	9.00E-35	*Oryza sativa*
50	**Dsenhsbm50**	CF760657	(TA)10	TGATGATCATATCCGTGTACGTC	AACCCCTGGCAGAGAGAGA	54	107	**No hit**		
51	Dsenhsbm51	CF760587	(GGC)8	GGTCCCTCTTCCTCATGTCC	GCGAACCTGCTCAACTCAG	54	175	SWIb domain-containing protein	1.00E-11	*Oryza sativa*
52	**Dsenhsbm52**	CF760116	(TA)22	GCTACGGCGATAACTTGGAC	CGTATACGCCACTGTCGTTG	54	167	**Chitinase**	**1.00E-60**	***Zea diploperennis***
53	Dsenhsbm53	CF760043	(ATT)10	CTCAATGTGATGGCTCGTGT	TACGTAGGCGGTAGGCGT	54	198	No hit		
54	Dsenhsbm54	CF756017	(TGA)6	GCGGCTCCATTACCTGATTA	TACAGACACTGCTGCGAGGT	54	195	No hit		
55	**Dsenhsbm55**	CF759253	(TTC)7	TTCACTCGACCCTCTCATCC	GGAAAAAGAACCCCTCTTGG	54	197	**No hit**		
56	**Dsenhsbm56**	CF759200	(AG)7	ATTATCCCCTGCGCTGAAC	TTAGCCCAAGGAAGAACACG	54	215	**Early nodulin**	**5.00E-36**	***Oryza sativa***
57	**Dsenhsbm57**	CF771652	(CATA)6	CAAGCCTTTCCTTGCAGTTC	CACACCTGGCTCTGTTTTCA	54	194	**Major facilitator super family antiporter**	**7.00E-25**	***Zea mays***
58	Dsenhsbm58	CF759033	(TCC)7	GCGTGACCAAGAAATCAAGA	GGAGGACCAAGATGATCCA	53	238	TB2/DP1, HVA22 family protein (ABA responsive)	7.00E-40	*Oryza sativa*
59	Dsenhsbm59	CF759028	(GA)24	ATCAGGATAGGCAGTGCA	AGGCTCTCTCTCTCTCTCTC	50	235	Mitochondrial glycoprotein family protein/MAM33 family protein	4.00E-29	*Arabidopsis thaliana*
60	**Dsenhsbm60**	CF759410	(GCATC)6	GCGCGTAAGTGGAAGCTATT	TGCCACAGCTATCCATGATT	54	194	**No hit**		
61	Dsenhsbm61	CF771228	(TC)6	TGGCTGGGATGTATAGTGACG	GTGTGTCCTTCATGCTCACC	54	250	Hypothetical protein		
62	Dsenhsbm62	CF757171	(TG)6	GTCCGGTGAGCATAGTAGGG	GGGGTACTACAAGCCACGAG	54	160	No hit		

Table 1 continued

S. No.	Marker	EST Gen Bank accession no.	Type(s) of SSR and number of repeats	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Ann. temp. (°C)	Expected size (bp)	Putative function	*E* value	Species
63	**Dsenhsbm63**	CF758650	(AT)7	CGTATGTTGAAGCAGCATGG	GCAAAACCCTAGCGTCAAAC	54	200	**Nucleoside-triphosphatase**	**4.00E-25**	***Oryza sativa***
64	**Dsenhsbm64**	CF758536	(TC)7	CCACAGCATTGACAAGATGC	GACGCACATCAGGAATCAGA	54	215	**HAD-super family hydrolase, subfamily IA, variant 3**	**9.00E-24**	***Oryza sativa***
65	Dsenhsbm65	CF758446	(AGC)15	GTGCTGTATGAGCAGCAGCA	GGAAGCTGTTGAATCGGTTG	55	164	FCA protein	2.00E-04	*Triticum aestivum*
66	**Dsenhsbm66**	CF758303	(CT)17	GCAAATCCTCAAACGAGAGC	TCATATCATGCGTGCCAGAC	54	223	**Transcription factor ZmMybst1**	**2.00E-47**	***Zea mays***
67	Dsenhsbm67	CF756398	(CCG)6	ACTCCATGCTGAGGGTGAAC	CGACGTTGACGAAGAAGGAT	54	231	GA 2-oxidase 5	3.00E-43	*Hordeum vulgare*
68	Dsenhsbm68	CF758163	(AGC)7	AGAAGCGGCAGTACTCTTCG	CACGTCCTTGAGGAGCTTGT	54	212	Hypothetical protein	6.00E-11	*Oryza sativa*
69	**Dsenhsbm69**	CF758093	(CTG)8	GACAGGATCGTCAACTTCTGC	CGAGCATAACACATCCATGC	54	243	**No hit**		
70	**Dsenhsbm70**	CF757959	(GT)9	GTCGCACTGATTTCAACCAA	AACACACGGTCTCAATTACGC	54	235	**No hit**		
71	Dsenhsbm71	CF760550	(CAT)6	CACAGGACAACACCGAATTG	CAGCAGACAGTGGTTGCTTC	54	195	Hypothetical protein	9.00E-44	*Oryza sativa*
72	**Dsenhsbm72**	CF757844	(GATG)8	GGCTTTAACGGAACCAAACC	CACGGACTTCACTTGCGTTATC	55	229	**No hit**		
73	Dsenhsbm73	CF757811	(TG)8	CAATGGACGACGTGTGAAAC	AGCTCCCTCCACAGTAGCAG	54	212	Zinc finger POZ domain protein	3.00E-39	*Oryza sativa*
74	Dsenhsbm74	CF757780	(TC)9	AGGTGAAGACGGCCAAGG	TCACGGTAGGGAAAGACTCG	55	178	No hit		
75	Dsenhsbm75	CF757721	(GCT)7	AGAGGCAGCAAAGCGAGAC	ACTGGTGGGAGTCCGTGTAG	54	218	Zinc finger A20 and AN1 domain-containing stress-associated protein 9 (OsSAP9)	1.00E-31	*Oryza sativa*
76	**Dsenhsbm76**	CF757642	(GT)22	CAGACATCTCCAGGCTTTCC	GGGTATGAGCAACAACAAGG	53	241	**Aminotransferase, classes I and II family protein**	**4.00E-44**	***Oryza sativa***
77	**Dsenhsbm77**	CF757395	(CT)10	GATCATAGTGCAGCCTTCAGC	CTCCTCCTCCTCCCTTCTTC	54	243	**No hit**		
78	**Dsenhsbm78**	CF757313	(TC)7	GCTAGATTCCGCGATTATGG	GGCCCTTGTATCATCCTTGA	54	182	**Nucleic acid binding**	**4.00E-10**	***Arabidopsis thaliana***
79	**Dsenhsbm79**	CF757218	(GT)10	ACTCCAGCTCCGAGTTTCAG	CTTGCAAGACGACCACAAGA	54	201	**Hypothetical protein**	**9.00E-25**	***Oryza sativa***
80	**Dsenhsbm80**	CF757202	(GCA)7	CAAGGAGCTGTGGAAGAAGG	CCTCCTGCTCCTGTGGTAAC	54	178	**Glutamine amidotransferase class-I family protein**	**9.00E-28**	***Oryza sativa***
81	Dsenhsbm81	CF756977	(CT)9	AGATCATCTTCGCCTTCCTG	CGATTCCACCATGAGATGTG	54	157	Hypothetical protein	1.00E-08	*Oryza sativa*
82	Dsenhsbm82	CF759507	(CG)6	GAATCGTCGTCTTTGGCATC	TGCCTTCCTTCACCTTCTTG	54	232	Hypothetical protein	8.00E-18	*Oryza sativa*
83	Dsenhsbm83	CF758416	(TAT)6	AACTGGAGGAGGCAGTTTCA	AGCACCCAAGTTTGCTCAAT	54	241	CTV.22 protein	6.00E-20	*Oryza sativa*
84	**Dsenhsbm84**	CF756742	(TC)11	CAGCTGGCTCCAGATCTTCT	GCATCAACAAACTGGACTGG	54	241	**No hit**		
85	Dsenhsbm85	CF756484	(GA)11	GGCCAATGTCATCAACCTCT	CCCCTCGAGACTAGTTCTCTCT	53	154	Sucrose synthase 3	8.00E-34	*Zea mays*
86	**Dsenhsbm86**	CF758126	(CTG)6	GTGTATCGCGCACTCAACAT	AAGGAGCTAAAGCATATCTTCCA	53	172	**No hit**		
87	Dsenhsbm87	CF755952	(CA)7	CAACCACTCATGAGCAACAAC	CACAGCAAACAAGACCTGGA	54	248	No hit		
88	**Dsenhsbm88**	CF757466	(CGA)6	ACCTTCTGCCGGTGATTATG	TGTTCTTGTTGTGCCGATCT	54	184	**Hypothetical protein**	**2.00E-08**	***Oryza sativa***
89	**Dsenhsbm89**	CF756119	(GCAACG)6	TAAATCGGAGAGCAGGAGGA	TGAACAAGTTGGAGCTGCTG	54	162	**Glycine-rich RNA-binding protein**	**2.00E-19**	***Nicotiana tobacum***
90	Dsenhsbm90	CF756564	(GAC)6	GCTGCTGAGAGACTTGTCCA	CATGCATGGTAACAGCTTGC	54	202	Hypothetical protein	9.00E-17	*Oryza sativa*
91	Dsenhsbm91	CF758698	(TG)6	CCACCAGCCTCCTACTGATCT	GTGCGCTAGCTTCGTCTGAT	54	192	No hit		
92	**Dsenhsbm92**	CF769451	(CT)5 + (TC)6	ATGCCGTCGTCGCTAGTAGT	GCAGTTTTTCTGGGGTCTCA	54	151	**Cytochrome P450**	**3.00E-60**	***Oryza sativa***

Table 1 continued

S. No.	Marker	EST Gen Bank accession no.	Type(s) of SSR and number of repeats	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Ann. temp. (°C)	Expected size (bp)	Putative function	*E* value	Species
93	Dsenhsbm93	CF758348	(TG)6	TCGAGGAGATGTCAGACACG	CTTCAAGGTTCCAAGCTGGT	54	225	Zwille protein	9.00E-38	*Oryza sativa*
94	**Dsenhsbm94**	CF758308	(CGG)6	GATCCCGTTTCCTTTCCTTC	GTCAGTGCACGAGGTAGGTG	54	213	**No hit**		
95	**Dsenhsbm95**	CF757836	(GC)6	GTGGTTTGTTCCAGCCTTTG	GGGGGAGATGTGTTTCTACG	54	182	**No hit**		
96	**Dsenhsbm96**	CF757395	(GAT)6	CGCGTCTGATCATAGTGCAG	CTCCTCCTCCTCCCTTCTTC	54	250	**No hit**		
97	Dsenhsbm97	CF759349	(GAT)6	AAGAGTTTGGCGGTGATGAG	TTCCCCTTCAGCATTACCTG	54	170	No hit		
98	**Dsenhsbm98**	CF756649	(AAG)6	CCACCACTCCACCTAGCTTC	GCTGTAGTAGAAGCCGGGAAC	54	211	**Zinc-induced protein**	**2.00E-12**	***Oryza sativa***
99	**Dsenhsbm99**	CF755963	(GCA)6	GCCAAGGCAGAGAAGAAGAAG	CGACGACGACTACTTGGTGA	54	245	**Drought-induced hydrophobic protein**	**7.00E-22**	***Oryza sativa***
100	Dsenhsbm100	CF771088	(TA)6	ACGCAAGATTGTGATGCAAG	CCATCAATAATCCACGAGCA	54	172	No hit		
101	Dsenhsbm101	CF759123	(CGG)6	CGTTATTTCGCGTTGCAC	AGGTGAAGGCGTCCCTGTAG	55	226	Chromatin structure remodeling complex protein BSH (SNF5)	2.00E-62	*Arabidopsis thaliana*
102	Dsenhsbm102	CF758281	(GAC)6	GAGCTGCTCGGCTACCAC	GCACGAACCTGAAGTCCTGT	54	178	Cytochrome P450 71A1	5.00E-41	*Oryza sativa*
103	**Dsenhsbm103**	CF770294	(CGTA)6	GGCAAGTTCAGGAAGTGGAA	TGTGTGCAGTGCATGCTTAG	54	155	**No hit**		
104	Dsenhsbm104	CF757743	(GT)6	CAAGAGCATATGGCAGTCCA	CACAGACCAAGCATCAGGAA	54	178	No hit		
105	**Dsenhsbm105**	CF760183	(TCC)6	AGGAGAGCCGAGAGCAGAG	GTACTTCTCGGCGATCTTGC	54	158	**Nuclease I**	**2.00E-40**	***Oryza sativa***
106	Dsenhsbm106	CF761761	(GAT)6	AGGAGCCTGAGGAACAAACA	ACAGCCCAAAATGGTTTCTG	54	172	Hypothetical protein	0.046	*Oryza sativa*
107	Dsenhsbm107	CF761385	(AGC)6	TGGAACTGTAAAGCCCGAGT	AGGTGTGGGAACTCATCAGC	54	240	MATH domain containing protein	1.00E-56	*Oryza sativa*
108	**Dsenhsbm108**	CF761332	(TG)6	ATTCAACGGAACCTCAATCG	CTCCTCGTCCTCCTTCTTGA	54	213	**No hit**		
109	Dsenhsbm109	CF757937	(GAC)6	CCATAAGCAAAGAGGGTGGA	CGCTAAGGGTATCAGGTGGA	54	165	No hit		

Markers in bold are the EST markers that showed polymorphism among the five genotypes studied

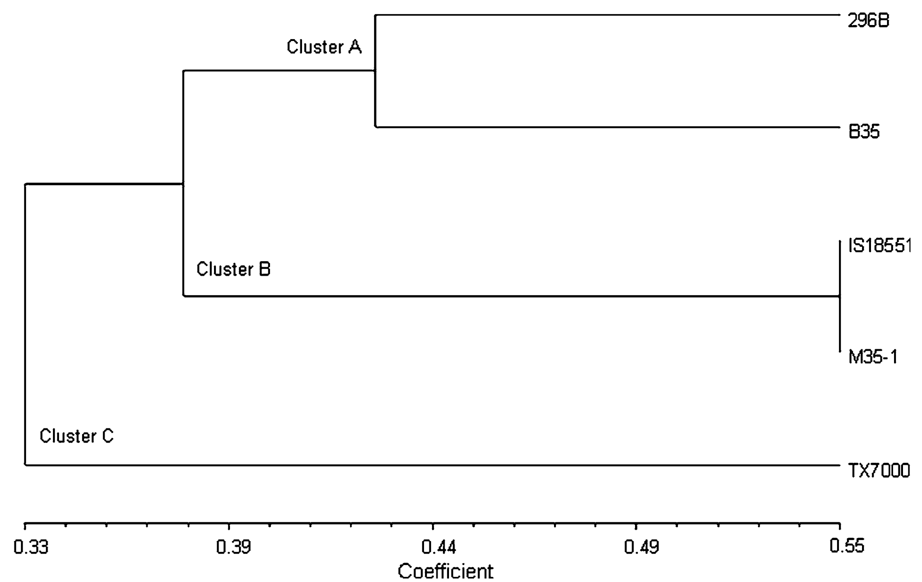

Fig. 1 UPGMA cluster analysis of the five sorghum genotypes used in this study, based on 161 drought EST-microsatellite alleles

15 linkage groups. Their nomenclature was followed according to Kim et al. (2005) by referring to common anchor genomic-SSR markers mapped between this study and that of Bhattramakki et al. (2000). Five chromosomes (SBI-02, SBI-05, SBI-08, SBI-09, and SBI-10) had one linkage group each, whereas five other chromosomes (SBI-01, SBI-03, SBI-04, SBI-06, and SBI-07), were developed with two linkage groups each (Fig. 2). The number of marker loci represented per individual chromosomes ranged from 6 (SBI-10) to 24 (SBI-01). The individual chromosome length ranged from 42.7 cM of SBI-09 to 235 cM of SBI-03 (including SBI-03a and SBI-03b). Except on SBI-05, 35 new mapped microsatellites (28 drought EST-microsatellites and 7 genomic-SSRs) were distributed across all linkage groups. The chromosome SBI-01 contained highest number of new markers (9 markers—7 drought ESTs and 2 genomic-SSRs), followed by SBI-03 (7 markers—drought ESTs), SBI-02 (5 markers—drought ESTs), SBI-04 (3 markers—2 drought ESTs and 1 genomic-SSR), SBI-08 (3 markers—drought ESTs), SBI-09 (3 markers—1 drought EST and 2 genomic-SSRs), SBI-06 (2 markers—drought ESTs), SBI-07 (2 markers—1 drought EST and 1 genomic-SSR), SBI-10 (1 marker—genomic-SSR), respectively. Some of the drought EST-microsatellite markers filled the gaps of earlier SSR scanty regions, and also extended some of the linkage groups.

Assigning functions to the drought EST-microsatellites

BLASTX analysis of the 109 drought EST-microsatellites revealed that 50 ESTs (45.87%) showed significant homology to previously characterized proteins, of which the major proportion (34%) belonging to genes involved in transcription and post-transcriptional regulation. While, 43 ESTs (39.44%) could not hit in the NCBI non-redundant protein database, hence their function is unknown, and 16 ESTs (14.67%) hit against uncharacterized proteins of other crop species (hypothetical proteins). Some EST markers encoding important functional proteins were identified, including ETHYLENE-INSENSITIVE3-1 (EIL-1), heat shock protein, chaperonin, and other proteins that are involved in stress related metabolism (Table 1) (Vierling 1991; Shinozaki and Yamaguchi-Shinozaki 1996, 1997; Seki et al. 2003; Yang et al. 2008).

Discussion

EST-microsatellites frequency, distribution and evaluation

In the present study, we explored microsatellite marker loci in ESTs generated from two subtracted sorghum drought-stressed cDNA libraries DSAF1 and DSBF1 (Pratt et al. 2005). The frequency of SSRs in the 9,892 drought ESTs was 3.9% (392 out of 9,892), in a range reported for EST-SSRs development in a number of Poaceae species, including barley, rice, sorghum, sugarcane, wheat, and maize (1.5–8.5%) (Cordeiro et al. 2001; Hackauf and Wehling 2002; Holton et al. 2002; Kantety et al. 2002; Thiel et al. 2003). This is in contrast to the recent exploration of SSR motifs by Swarup et al. (2006) in redundant EST databases in five species of cereal family and *Arabidopsis* found that the frequency range is from 16.3% in barley to 45.7% in rice, and with sorghum 41.1%. This difference in SSR frequency in ESTs between these studies can be attributed to the SSR search criterion that was used in this study was defined as six repeat units for all types of

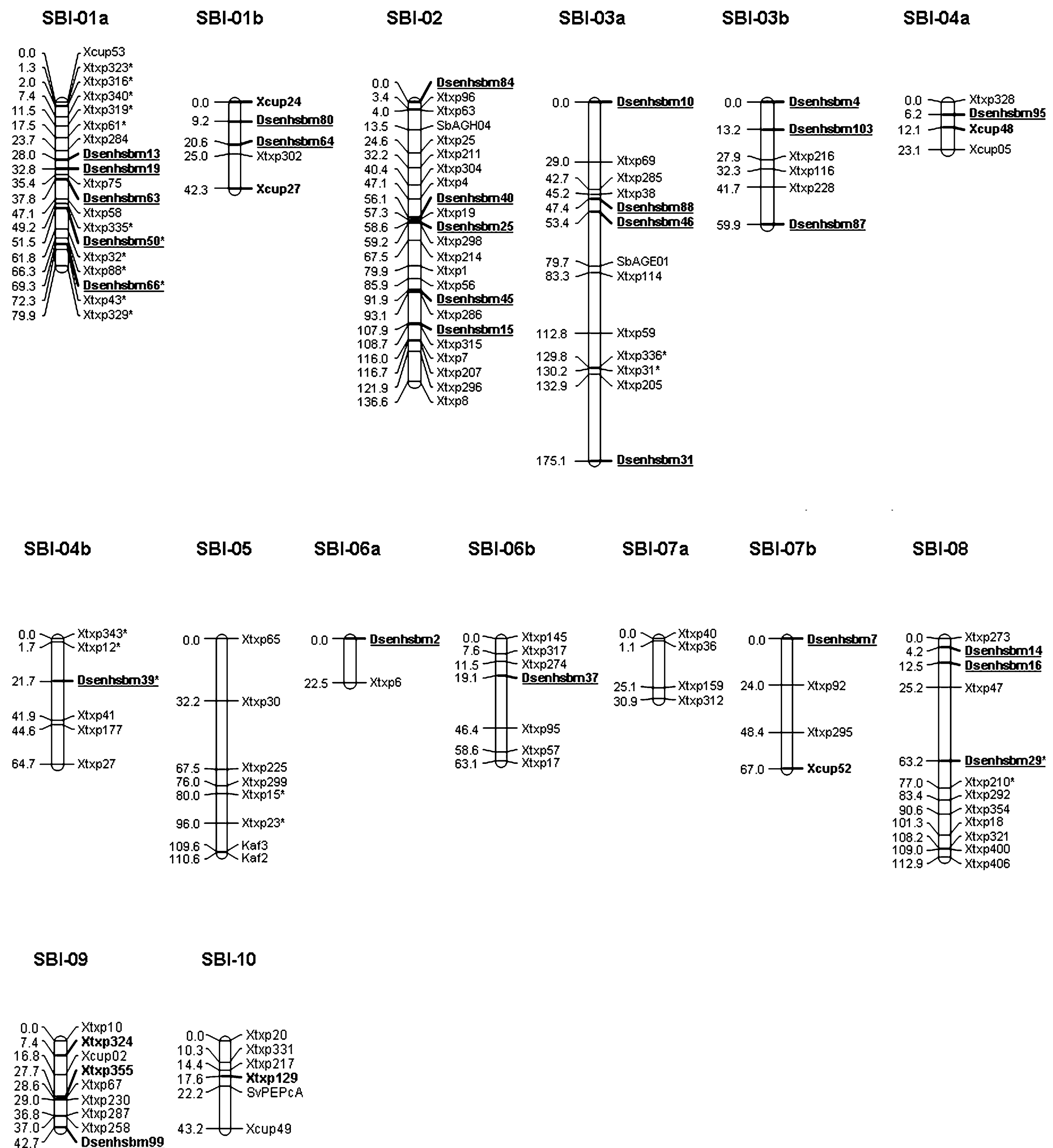

Fig. 2 A genetic linkage map of the *Sorghum bicolor,* based on 128 microsatellite loci using a recombinant inbred line mapping population derived from the parental lines, 296B and IS18551. Linkage groups were named according to the nomenclature of Kim et al. (2005). In cases where two linkage groups were developed for a chromosome, the '*a*' and '*b*' is followed after name of the linkage group. Linkage group name followed by '*a*' refers to the upper portion of the corresponding linkage group of Bhattramakki et al. (2000). Like wise, '*b*' refers to lower portion of the corresponding linkage group. Marker genetic distances are expressed in cM (Kosambi estimates). Thirty-five new microsatellite loci mapped in the study are *bolded*. To distinguish from new genomic-SSRs mapped, the drought EST-microsatellites are *underlined*. Markers highly significantly deviated ($P < 0.01$) from the expected ratio 1:1 were followed by an *asterisk*

SSR motifs, while the higher value observed by Swarup et al. (2006) is likely due to the inclusion of a large number of SSR motifs especially with tri- and tetra-motif types since they adopted a cutoff of $\geq$12 nucleotide sequence length for all types of SSR motifs. Among the 221 non-redundant ESTs containing microsatellite motif identified in this study, the highest proportion motifs comprised di- followed by the tri-motifs. This is in contrast to a majority of the earlier studies where tri-motifs in EST-SSRs were commonly observed (Thiel et al. 2003; Nicot et al. 2004). These apparent differences in the relative abundance of the di- and tri-motifs can again be attributed to the differences in SSR search criteria used for EST database mining in different studies.

Polymorphism of EST-microsatellites

Sixty-two markers (56.8%) of the developed EST markers found to be polymorphic in the set of five sorghum genotypes studied. This level in polymorphism is similar with the results obtained using genomic-SSRs by Bhattramakki et al. (2000), where they identified 165 out of 266 (53%) microsatellite loci to be polymorphic in a set of 18 diverse sorghum lines. In general, EST-SSR markers have been reported to show lower polymorphism than SSR markers derived from genomic libraries (Cho et al. 2000; Eujayl et al. 2002). With the similar level of polymorphism observed with EST-microsatellites in the present study to that of Bhattramakki et al. (2000) using genomic-SSRs suggest that, in sorghum, defining $\geq$6 units for all types of SSR motifs using EST sequences would result in higher polymorphism and the developed EST-microsatellites in the present study are high polymorphic in nature.

Genetic linkage map

We have reported a new genetic map, which includes 28 new microsatellite loci from drought ESTs and 7 genomic-SSRs that were earlier reported but not mapped. Altogether the map of 128 microsatellite loci of this study is the third largest map of microsatellites reported in sorghum only after Bhattramakki et al. (2000) (linkage map composed of 147 SSR loci) and Menz et al. 2002 (linkage map composed of 136 SSR loci). The total map length of the present study is comparable with the reported SSR maps of sorghum (Bhattramakki et al. 2000; Wu and Huang 2006). The positions and colinearity of the anchor microsatellite loci (commonly mapped genomic DNA derived-SSRs) between this study and that of Bhattramakki et al. (2000) are in good agreement. The anchor genomic-SSR loci were distributed over all 15 linkage groups identified in the study and facilitated assigning of ESTs to chromosomes and subsequently to identify their positions on the linkage map. We could observe change in marker order at four positions of consecutive markers. The four positions involved Xtxp41 and Xtxp177 on SBI-04b, Xtxp317 and Xtxp274 on SBI-06b, Xtxp225, Xtxp229, and Xtxp15 on SBI-05, and Xtxp20, Xtxp331, and Xtxp217 on SBI-10. The flips of consecutive markers of closely linked markers are not surprising as these changes are common since alternative order for closely linked loci is possible in the construction of linkage maps. Recently, the flips of consecutive markers were also noted in sorghum by Wu and Huang (2006) while constructing the linkage map with 118 microsatellite loci, at five consecutive primer pairs. However, the changes in marker order of the three anchor genomic-SSR markers involving Xtxp225, Xtxp229, and Xtxp15 on SBI-05, Xtxp317 and Xtxp274 on SBI-07b, and Xtxp20, Xtxp331, and Xtxp217 on SBI-10 are in agreement with corresponding linkage groups of Menz et al. (2002).

In the present study, 21 markers showed highly significant deviation ($P < 0.01$) from the expected 1:1 segregation ration, and together clustered on six chromosomal regions, with two on SBI-01, one each on SBI-03, SBI-04, SBI-05, and SBI-08. Only on SBI-08, alleles were from the parental line 296B whereas for remaining all the genomic regions the marker alleles were from the parental line IS18551. Of the five chromosomes hosting significantly deviated markers identified in the study, the three chromosomes (SBI-01, SBI-04, and SBI-08) were earlier detected to host significantly segregation distorted markers by Menz et al. (2002) in a RIL mapping population of crosses BTx623 $\times$ IS3620C. In comparison with the study of Menz et al. (2002), the distorted marker alleles found on SBI-01 in the present study deviated towards the non-elite male parent (IS18551), whereas they found deviation of markers towards the elite female parental line, BTx623. Menz et al. (2002) suggested that the segregation distortion on SBI-01 could be the result of shattering genes located in this chromosomal region. The phenomenon deviation of markers is also reported in earlier studies in sorghum (Chittenden et al. 1994; Dufour et al. 1997; Wu and Huang 2006). The segregation distortion of markers has also been described in many plant DNA marker-based linkage maps, and has been attributed to causes such as segregation distortion, deleterious recessive alleles, self-incompatibility alleles, structural rearrangements, and differences in DNA content (Taylor and Ingvarsson 2003; Moretzsohn et al. 2005).

Most of the marker loci mapped in the study were evenly distributed throughout the linkage map covering the gaps of earlier map (Bhattramakki et al. 2000), especially, at the lower portion of linkage groups of SBI-01 around the genomic-SSR Xtxp302 and on SBI-03 around the Xtxp205 and Xtxp228; the mapping of five markers (Dsenhsbm66, Xcup24, Dsenhsbm80, Dsenhsbm64, and Xcup27), four

markers (Dsenhsbm31, Dsenhsbm4, Dsenhsbm103, and Dsenhsbm87) on the SBI-01 and SBI-03 covering the gaps of microsatellite scanty regions of about 50 cM, and 40 cM, respectively. Similarly, we mapped three new microsatellite loci (Dsenhsbm95, Xcup48, and Dsenhsbm39) into the gap of about 60 cM at the upper half of the SBI-04. Some of the microsatellite loci placed on the map located to, and extended the ends of linkage groups SBI-02 (Dsenhsbm84), and SBI-03 (Dsenhsbm10).

The Drought ESTs as a source of microsatellite markers

Microsatellite markers identified from economically important genes are highly useful since SSR alleles derived from genes can be associated with a high degree of certainty with trait-influencing mutations in the genes concerned. Mapping of functionally defined genes on linkage map permits evaluation of co-location between genic-markers and QTL of any trait (Aubert et al. 2006) and provides a basis for both understanding the genetic base of a trait (Matthews et al. 2001; Zhang et al. 2004) and marker-assisted selection (MAS) in breeding programs. In the present study, EST Dsenhsbm19 coding for ETHYLENE-INSENSITIVE3-1 (EIL-1) protein, a key transcription regulator of ethylene biosynthesis (Chao et al. 1997; Solano et al. 1998), was mapped on linkage group SBI-01, suggesting its role in drought stress adaptation since ethylene is involved in the regulation of leaf senescence (Yang et al. 2008). Similarly, some functional proteins or proteins that are involved in stress related metabolism were mapped on the linkage map and/or identified as polymorphic markers. For instances, ESTs Dsenhsbm4 (mapped on SBI-03) and Dsenhsbm24 were identified coding for heat shock protein and chaperonin, respectively, which are involved in protecting macromolecules such as enzymes and lipids under severe drought stress (Vierling 1991; Zhu et al. 1997). Likewise, ESTs Dsenhsbm30 and Dsenhsbm99 (mapped on SBI-09) encoding stress related protein and drought induced hydrophobic protein, respectively, appear to be involved in stress related metabolism. In this respect, the markers developed and characterized in this study will not only provide reproducible co-dominant, and easily scorable markers, but also hypothetically include candidate genes that have the potential of being causally linked to the drought tolerance. Therefore, the 28 ESTs mapped on the linkage map in the study may assist to study association of the molecular variability of the genes with if any phenotypic variability of traits related to drought resistance and other agronomic importance in sorghum.

Furthermore, we found that 56.8% of the tested EST–SSR primer pairs were polymorphic among the five sorghum cultivars used, it is expected that 109 EST based primer pairs designed in the study may potentially provide at least about 62 novel gene-based markers for sorghum research. In addition, among the mapped genomic-SSRs, seven genomic-SSRs were identified that had not been mapped on the sorghum linkage map before. Therefore, they provide additional resource for sorghum genetic mapping. With high transferability of EST-SSRs among the cereals (Zhang et al. 2005), the EST markers of the study offer new links for comparative mapping between sorghum and other members of the Poaceae family. Finally, the EST-microsatellite markers, with their ease in applicability over other marker classes such as RFLPs and SNPs, have the potential for assaying the functional diversity and allele mining in germplasm collection or natural population in drought screening programmes. Eventually, the allelic variation observed at those loci among drought resistant versus drought susceptible cultivars, and their contribution to the phenotype should enable the breeder to design superior genotypes to drought tolerance comprising a combination of favorable alleles at all loci by ‘breeding by design’ approach (Peleman and van der Voort 2003).

In conclusion, this study reports the identification, validation, and mapping of new sorghum microsatellite markers derived from drought stress ESTs. The EST-microsatellites developed in the study will have applications in functional diversity studies, association mapping studies, QTL mapping studies for drought and other agronomic traits in sorghum, and synteny studies between sorghum and other members of the Poaceae family.

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