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Development and utilization of novel intron length polymorphic markers in foxtail millet (*Setaria italica* (L.) P. Beauv.)

Sarika Gupta, Kajal Kumari, Jyotirmoy Das, Charu Lata, Swati Puranik, and Manoj Prasad

Abstract: Introns are noncoding sequences in a gene that are transcribed to precursor mRNA but spliced out during mRNA maturation and are abundant in eukaryotic genomes. The availability of codominant molecular markers and saturated genetic linkage maps have been limited in foxtail millet (*Setaria italica* (L.) P. Beauv.). Here, we describe the development of 98 novel intron length polymorphic (ILP) markers in foxtail millet using sequence information of the model plant rice. A total of 575 nonredundant expressed sequence tag (EST) sequences were obtained, of which 327 and 248 unique sequences were from dehydration- and salinity-stressed suppression subtractive hybridization libraries, respectively. The BLAST analysis of 98 EST sequences suggests a nearly defined function for about 64% of them, and they were grouped into 11 different functional categories. All 98 ILP primer pairs showed a high level of cross-species amplification in two millets and two nonmillets species ranging from 90% to 100%, with a mean of ~97%. The mean observed heterozygosity and Nei's average gene diversity 0.016 and 0.171, respectively, established the efficiency of the ILP markers for distinguishing the foxtail millet accessions. Based on 26 ILP markers, a reasonable dendrogram of 45 foxtail millet accessions was constructed, demonstrating the utility of ILP markers in germplasm characterizations and genomic relationships in millets and nonmillets species.

Key words: Foxtail millet (*Setaria italica* L.), intron length polymorphism, molecular markers, transferability, genetic diversity.

Résumé : Les introns sont des séquences non-codantes au sein d'un gène. Ils sont transcrits mais épissés hors de l'ARNm précurseur et sont abondants dans les génomes eucaryotes. Le peu de marqueurs codominants et l'absence d'une carte génétique saturée constituent des contraintes chez le millet des oiseaux *(Setaria italica* (L.) P. Beauv.). Les auteurs décrivent ici le développement de 98 nouveaux marqueurs exploitant le polymorphisme de longueur des introns (ILP) chez le millet des oiseaux et l'information de séquence de la plante-modèle qu'est le riz. Au total, 575 séquences non-redondantes d'EST ont été obtenues, dont 327 et 248 séquences provenaient de banques d'hybridation suppressive soustractive préparées à partir de plantes ayant subi, respectivement, un stress de sécheresse ou de salinité. Des analyses BLAST des 98 séquences d'EST suggèrent une fonction vraisemblablement définie pour environ 64 % d'entre elles et elles ont été classées au sein de 11 catégories fonctionnelles. Les 98 paires d'amorces pour ces marqueurs ILP ont présenté une très grande transportabilité interspécifique puisqu'elles ont produit un amplicon chez deux espèces de millets ainsi que deux espèces autres à une fréquence qui variait entre 90 et 100 %, pour une moyenne de 97 %. L'hétérozygotie observée moyenne et la diversité génétique de Nei moyenne étaient respectivement de 0,016 et 0,171, ce qui démontre l'efficience des marqueurs ILP pour la différenciation des accessions de millet des oiseaux. À l'aide de 26 marqueurs ILP, un dendrogramme raisonnable a été produit pour 45 accessions du millet des oiseaux, démontrant ainsi l'utilité des marqueurs ILP pour des fins de caractérisation des ressources génétiques et des relations génomiques au sein des millets ou d'autres espèces.

Mots-clés : millet des oiseaux (*Setaria italica* L.), polymorphisme de longueur des introns, marqueurs moléculaires, transportabilité, diversité génétique.

[Traduit par la Rédaction]

Introduction

Foxtail millet (*Setaria italica* (L.) P. Beauv.) is an important food grain crop in temperate, subtropical, and tropical Asia and in parts of Southern Europe and is grown for forage in North America, South America, Australia, and North Africa. The foxtail millet genome is being sequenced by the US Department of Energy Joint Genomic Institute and BGI (formerly the Beijing Genomics Institute), China. Foxtail millet together with proso millet (*Panicum miliaceum* L.) ranks second in total world production of millets after pearl millet (*Pennisetum glaucum* L.) (FAO STAT data 2005;

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http://faostat.fao.org/). Foxtail millet is a diploid ($2n = 2x = 18$), self pollinating, C_4 panicoid crop with a small genome of ~490 Mb and a low repetitive DNA content (30%). The genome structure of foxtail millet is highly conserved, relative to the ancestral grass lineage, making it a suitable model species for genetic and molecular studies (Devos et al. 1998; Jayaraman et al. 2008). Furthermore, foxtail millet is a close relative to several important biofuel crops such as switchgrass (*Panicum virgatum* L.), Napier grass (*Pennisetum purpureum* Schumach.), and pearl millet and hence has been suggested to represent an appropriate model for this class of crop species (Doust et al. 2009).

Recent advances in molecular biology provide novel tools for addressing evolutionary, ecological, and taxonomic research questions. DNA variation is frequently exploited in molecular genetic marker systems, and the application of molecular markers to advance research and commercial activities is now well established. Molecular markers are, therefore, powerful devices for plant genetic research and breeding. Various types of molecular markers have been developed since 1980 such as restriction fragment length polymorphism (RFLP; Botstein et al. 1980), random amplified polymorphic DNA (RAPD; Williams et al. 1990), amplified fragment length polymorphism (AFLP; Vos et al. 1995), simple sequence repeat polymorphism (SSR; Becker and Heun 1995), single-nucleotide polymorphism (SNP; Kruglyak 1997), and intron length polymorphism (ILP; Choi et al. 2004). Introns are the noncoding sequences present in almost all eukaryotes and are not innocent sequences (Hawkin 1988; Deutsch and Long 1999). Recent research has proved that they actively participate to control the gene expression (Braglia et al. 2010). ILP is a new type of molecular marker, which has not been reported extensively. Wang et al. (2006) reported that ILPs have significant subspecies specificity in rice. This characteristic could be useful for genetic study and breeding in rice. It might be useful for the studies of genome evolution and inter-subspecies heterosis and for cross-subspecies marker-assisted breeding. There are a number of advantages for using ILPs over the other so far reported DNA markers. First, ILPs are specific, codominant, neutral, convenient, and reliable. They are specific for being sequence-tagged sites markers, providing complete information about genotypes, showing no particular phenotype, detectedable by PCR, and give stable results (Braglia et al. 2010). Second, ILP markers have high transferability rates among the plant species. This characteristic would make ILP markers very useful for the construction of molecular marker maps in related plant species for which less genomic data is available and for investigations of phylogenetic relationships.

To our knowledge, there is no report on the development and use of ILP markers in foxtail millet. Hence, the present study was undertaken with the following three objectives: (1) to develop and characterize ILP markers for foxtail millet, (2) to assess their potential for cross-species transferability, and (3) to analyze genetic diversity in a set of 45 foxtail millet accessions. We performed a genome-wide search for ILPs and a large-scale exploitation of candidate ILP markers via electronic-PCR, based on the 575 nonredundant EST sequences of foxtail millet derived from two suppression subtractive hybridization (SSH) libraries. We developed a set of 98 ILP markers selected from the candidate genes and validated them as functional markers by experiment.

Materials and methods

Plant materials, stress treatment and DNA isolation

Seeds of two foxtail millet cultivars, namely 'Prasad' (dehydration and salt tolerant) and 'Lepakshi' (dehydration and salt susceptible), were germinated, sown in composite soil (peat compost to vermiculite, 3:1), and grown under optimal conditions (Zhang et al. 2007). Dehydration and salinity stress treatments were given with 20% PEG and 250 mmol/L NaCl, respectively (Lata et al. 2010; Puranik et al. 2011). For the diversity study, a total of 45 foxtail millet accessions (40, *S. italica*; 2, *Setaria sphacelata* (Schumach.) Stapf & C.E. Hubb. ex M.B. Moss; and 3, *Setaria verticillata* (L.) P. Beauv.) were used. For the testing of transferability, two grass species, namely rice (*Oryza sativa* L. 'IR64') and wheat (*Triticum aestivum* L. 'PH132'), and three millet species, namely foxtail millet 'Prasad', pearl millet 'T1', and guinea grass 'SPM92', were used (Table 1). Genomic DNA was extracted from fresh leaf material of field-grown plants following a modified CTAB method (Saghai-Maroof et al. 1994), and the concentration of isolated DNA was determined on agarose gels using known concentrations of lambda DNA marker (New England Biolabs Inc., Ipswich, Massachusetts).

Construction of subtracted cDNA libraries, sequencing and data analysis of ESTs

Total RNA was isolated from whole seedlings by a modified hot phenol method using lithium chloride (Logemann et al. 1987). polyA$^+$ RNA was purified by a mRNA isolation kit (Roche Applied Science, Indianapolis, Indiana). Subtracted cDNA libraries were constructed by using the PCR-Select cDNA subtraction kit (Clontech Laboratories Inc., Palo Alto, California) following the manufacturer's protocol. For dehydration stress, total RNA extracted from whole seedlings of 'Prasad' induced by 0.5 or 6 h dehydration stress was used as tester for the early- and late-SSH libraries, respectively. Total RNA from unstressed seedlings was used as driver for both the libraries. For salinity stress, both forward (6 h salt-treated 'Prasad' as tester and 6 h salt-treated 'Lepakshi' as driver) and reverse (6 h salt-treated 'Lepakshi' as tester and 6 h salt-treated 'Prasad' as driver) SSH cDNA libraries were constructed. The subtracted and enriched DNA fragments were directly cloned into a T/A cloning vector (Promega Corp., Madison, Wisconsin). The recombinant (white) colonies were randomly picked up and cultured overnight at 37 °C in 96-well plates with 150 µL Luria–Bertani medium plus ampicillin. Sequences of the recombinant plasmids were determined with the ABI Sequencer, version No.3770 using M13 forward primer. Adaptor and vector sequences were removed using the VecScreen system provided by the National Center for Biotechnology Information (NCBI). The putative functions of the ESTs were assigned by comparison with the nonredundant database at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) using the BLASTx and BLASTn algorithms (Altschul et al. 1997). The optimal parameters set for ESTs have E value less than 1×10^{-10} and are more than 100 nucleotides long.

Table 1. Description of plant material used in the present study.

Accession No.	Name of species	Source*
IC257885	*Setaria italica*	NBPGR, Hyderabad, India
IC257890	*S. italica*	NBPGR, Hyderabad, India
IC257893	*S. italica*	NBPGR, Hyderabad, India
IC257894	*S. italica*	NBPGR, Hyderabad, India
IC283695	*S. italica*	NBPGR, Hyderabad, India
IC283694	*S. italica*	NBPGR, Hyderabad, India
IC283708	*S. italica*	NBPGR, Hyderabad, India
IC306468	*S. italica*	NBPGR, Hyderabad, India
IC308933	*S. italica*	NBPGR, Hyderabad, India
IC308934	*S. italica*	NBPGR, Hyderabad, India
IC308941	*S. italica*	NBPGR, Hyderabad, India
IC308944	*S. italica*	NBPGR, Hyderabad, India
IC308946	*S. italica*	NBPGR, Hyderabad, India
IC308955	*S. italica*	NBPGR, Hyderabad, India
IC308956	*S. italica*	NBPGR, Hyderabad, India
IC308959	*S. italica*	NBPGR, Hyderabad, India
IC308960	*S. italica*	NBPGR, Hyderabad, India
IC308966	*S. italica*	NBPGR, Hyderabad, India
IC308967	*S. italica*	NBPGR, Hyderabad, India
IC308972	*S. italica*	NBPGR, Hyderabad, India
IC308976	*S. italica*	NBPGR, Hyderabad, India
IC308978	*S. italica*	NBPGR, Hyderabad, India
IC343853	*S. italica*	NBPGR, Hyderabad, India
IC344033	*S. italica*	NBPGR, Hyderabad, India
IC345121	*S. italica*	NBPGR, Hyderabad, India
IC345123	*S. italica*	NBPGR, Hyderabad, India
IC345140	*S. italica*	NBPGR, Hyderabad, India
IC369718	*S. italica*	NBPGR, Hyderabad, India
IC382958	*S. italica*	NBPGR, Hyderabad, India
IC384077	*S. italica*	NBPGR, Hyderabad, India
IC384080	*S. italica*	NBPGR, Hyderabad, India
IC413272	*S. italica*	NBPGR, Hyderabad, India
IC426717	*S. italica*	NBPGR, Hyderabad, India
IC426728	*S. italica*	NBPGR, Hyderabad, India
IC430608	*S. italica*	NBPGR, Hyderabad, India
IC436885	*S. italica*	NBPGR, Hyderabad, India
IC438725	*S. italica*	NBPGR, Hyderabad, India
EC539248	*S. italica*	NBPGR, Hyderabad, India
EC539251	*S. italica*	NBPGR, Hyderabad, India
'Krishnadeva Raya'	*S. italica*	UAS, Bangalore, India
EC539290	*S. sphacelata*	NBPGR, Hyderabad, India
EC539291	*S. sphacelata*	UAS, Bangalore, India
EC539293	*S. verticillata*	UAS, Bangalore, India
EC539297	*S. verticillata*	UAS, Bangalore, India
EC539300	*S. verticillata*	UAS, Bangalore, India
'Prasad'	*S. italica*	NBPGR, Hyderabad, India
'Lepakshi'	*S. italica*	NBPGR, Hyderabad, India
'T1'	*Pennisetum glaucum*	IGFRI, Jhansi, India
'SPM92'	*Panicum maximum*	IGFRI, Jhansi, India
'IR64'	*Oryza sativa*	IARI, New Delhi, India
'PH132'	*Triticum aestivum*	CCSU, Meerut, India

*NBPGR, National Bureau of Plant Genetic Resources; UAS, University of Agricultural Sciences; IGFRI, Indian Grassland and Fodder Research Institute; IARI, Indian Agricultural Research Institute; CCSU, Chaudhury Charan Singh, University.

Development of putative intron length polymorphic (PIP) markers

The monocot model plant rice was used as the subject species; whereas the available nonredundant EST sequences of foxtail millet obtained from the two SSH libraries of dehydration- and salinity-stressed conditions were used as query species (Lata et al. 2010; Puranik et al. 2011). For the development of specific intron-based markers in foxtail millet, we

used model plant rice sequences as a reference to predict intron positions in its EST sequences and then designed a pair of primers on both sides of each intron position using the PIP database (http://ibi.zju.edu.cn/pgl/pip/) (Yang et al. 2007). A query EST was thought to be homologous to a subject coding sequence only if there were at least 100 bp overlapping and 80% similarity between them. The corresponding position and length of identified introns from the subject species were obtained from the PIP database (Yang et al. 2007). Primer pairs were designed to amplify fragments in a 100- to 1000-bp range; the optimal melting temperature and size were set at 55 °C and 20 bases, respectively; and the GC components ranged from 45% to 65%, with 50% as the optimum. The designed primers were tested by electronic PCR (e-PCR) on the EST sequences of foxtail millet. We took a putative ILP locus as a candidate ILP marker when it was successfully and uniquely detected by the e-PCR.

PCR amplification and sequence analysis

ILP amplification was carried out in a 25 μL volume containing 1 U *Taq* DNA polymerase (Invitrogen Inc., Carlsbad, California), 50 ng of genomic DNA, 10 μmol/L of each primer, 0.5 mmol/L of each dNTP, and 2.5 μL of 10× PCR buffer. DNA amplification was carried out in the iCycler thermal controller (Bio-Rad, Hercules, California) using the following PCR profile: an initiation denaturation of 3 min at 94 °C; followed by 35 cycles of 30 s at 94 °C, 30 s at 50–60 °C, and 1 min at 72 °C; and a final step of 10 min at 72 °C. The amplification products were resolved either on agarose or a microchip-based electrophoresis system (MultiNA; Shimadzu Corporation, Kyoto, Japan). In brief, the technique (microchip based) used four microchips in parallel for the electrophoresis of DNA samples in an 8-strip or 96-well plate format. The SYBRGold dye was used for detection of DNA fragment and results are displayed as a digital gel and electropherogram. An internal marker consisting of a lower and upper marker served for inter-chip normalization and a 25-bp ladder was used for fragments sizing. The 2.0% agarose gels were run in Tris-borate EDTA (TBE) buffer (pH 8.0), stained with ethidium bromide, and analyzed using the GelDoc-It imaging system (UVP, Cambridge, UK). Standard size markers were used to determine the fragment sizes for each locus (New England Biolabs Inc.). Results were confirmed by three replicate assays. The amplified products were eluted from the gel (QIAGEN GmbH, Hilden, Germany) and cloned into the pGEM-T Easy vector (Promega Corp.) following the manufacturer's instructions. The recombinant plasmids were purified using the QIAprep Spin Miniprep Kit (QIAGEN), following the manufacturer's protocol, and sequenced with the M13 universal primers using an automated sequencer (3730xl DNA Analyzer; Applied Biosystems, Foster City, California). The obtained sequences were aligned using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Determination of genetic relationships and diversity

To determine the genetic relationships among the different species, the ILP marker profiles were scored manually: each allele was scored as present (1) or absent (0) for each of the ILP loci. Polymorphic informative content (PIC) was used to measure allele diversity at each locus. PIC value uses both the number of alleles at a locus and the relative frequencies of those alleles in a population and was calculated (Roldán-Ruiz et al. 2001) as: $PIC_i = 2f_i\ (1 - f_i)$, where f_i is the frequency of the amplified allele (band present) and $(1 - f_i)$ is the frequency of the null allele (band absent) of marker *i*. The genetic similarity among the accessions was calculated according to the Jaccard coefficient (Jaccard 1908) using the NTSYS-pc software package version 2.02e (Rohlf 1997). The pairwise similarity matrix of Jaccard coefficients was used to construct a phylogenetic tree using the unweighted pair-group method of arithmetic average (UPGMA) and the neighbor-joining (NJoin) module of the NTSYS-pc. Observed heterozygosity (H_O), Nei's average gene diversity (Nei 1973), fixation index (F_{IS}), and Shannon's informative index (I) were carried out using POPGENE 1.32 (Yeh and Boyle 1997) based on codominant markers with diploid individuals.

Results and discussion

Development of ILP markers

Two SSH forward libraries (0.5 and 6 h PEG induced) were constructed for dehydration stress from tolerant 'Prasad', whereas another two SSH libraries (forward and reverse) were constructed from salt-tolerant 'Prasad' and salt-sensitive 'Lepakshi' (Lata et al. 2010; Puranik et al. 2011). A total of 575 nonredundant EST sequences (~24%) were found after sequencing and screening of the above mentioned libraries, among which 327 (56.9%) and 248 (43.1%) sequences were obtained from dehydration- and salt-stressed libraries, respectively. These sequences were searched for ILPs against rice genome sequences using the PIP database (Yang et al. 2007). A total of 98 (~17%) primer pairs were developed from these 575 nonredundant EST sequences, of which 42 and 56 primer pairs were from dehydration- and salt-stressed libraries, respectively (Table 2). The remaining sequences either showed no Blast hit or the sequences were inappropriate for designing primers. The characteristics of the 98 primer pairs are shown in Table 2.

Functional classification of ESTs

The BLAST analyses of the 98 EST sequences suggested a nearly defined function for about 64% of the sequences, and these sequences were grouped into 11 different categories on the basis of their putative functions (Table 2; Fig. 1A). The largest category (~36%) contained EST sequences with hypothetical or unknown functions or with no similarities to previously sequenced genes, which indicates the uniqueness and presence of putative novel genes that are reported here for the first time and may be specific to foxtail millet. Also, these transcripts may represent an untapped gene source from foxtail millet showing specific responses to dehydration and salt stress in an orphan crop known to possess natural adaptation capacity to abiotic stresses. The second largest category (17%) comprised of photosynthesis or energy related transcripts followed by metabolism (15%) related transcripts. Other transcripts present were few in numbers in both the libraries. It has been observed that two stress libraries contained five distinct types of transcripts (Fig. 1B). Salinity stress transcripts related to cell cycle and DNA processing and translation/post translation were found. Relatively higher levels of these transcripts in the tolerant cultivar under salin-

Table 2. Characteristics and putative function of the 98 ILP markers developed in the plant species foxtail millet (*Setaria italica*).

Marker	Clone name	Corresponding rice locus	GenBank accession No.	Primer sequences (5′–3′)	*T*m (°C)	Expected size (bp)	*E* value	Putative function
FM_ILP001	D_115	LOC_Os04g38870	GT090829	F: CCCGAGTCCAAGGTCTTCTA	53	182	5×10^{-42}	14-3-3-like protein
				R: GTATTCTCGGCAGCGTCCT	54			
FM_ILP002	D_117	LOC_Os03g57290	GT090831	F: CCCAAGCAGCAACCTCTTAG	54	586	1×10^{-123}	Cullin-4B, putative
				R: CTGGAGGGTATCCTCGACAA	54			
FM_ILP003	D_121	LOC_Os02g50760	GT090835	F: GGCTTCCAACCATGTGAAGT	54	213	3×10^{-91}	Heat shock protein
				R: GGGTTCAGCACATACAACCTC	53			
FM_ILP004	D_136	LOC_Os09g19734	GT090850	F: GGAACAGCAAAGCTCAAGATG	54	384	3×10^{-24}	Isochorismate synthase
				R: CCGTAGATGTGAAAGCGAACT	53			
FM_ILP005	D_147	LOC_Os09g23280	GT090861	F: GCTATTGCAGACAGAGAGAACG	53	635	7×10^{-87}	Pyrrolidone carboxyl peptidase
				R: CTTGGTTCTCAAGAGCAAACCT	54			
FM_ILP006	D_174	LOC_Os03g18570	GT090888	F: GGAGAACAGCAACCAGGAGA	54	874	3×10^{-95}	40S ribosomal protein S7
				R: GTATGGGACGTGGATCACAA	53			
FM_ILP007	D_179	LOC_Os10g27230	GT090893	F: AATTATTCCCGCTGCTCAGA	54	190	5×10^{-140}	Endonuclease exonuclease / phosphatase
				R: TCAGCTCCATGGTTACCAAA	53			
FM_ILP008	D_181	LOC_Os01g09320	GT090895	F: TTGGTCTGATTATGGCTGGAG	54	208	1×10^{-34}	NADP-malic enzyme
				R: GGAAGATCGATCCCTTCTCA	53			
FM_ILP009	D_186	LOC_Os02g54254	GT090900	F: TGCAACAGCTACTCAGCTTG	51	218	2×10^{-114}	Lysine-ketoglutarate reductase
				R: ATACTCTTGCAACGGCAGCA	56			
FM_ILP011	D_203	LOC_Os03g18130	GT090917	F: AGGCCCCCAACAAAGAAGAG	57	180	3×10^{-59}	Glutamine-dependent asparagine synthetase
				R: CAAGCAGAGGTTGCCTTGTT	54			
FM_ILP012	D_204	LOC_Os04g49510	GT090918	F: AGTGTTCACATCCGCACAAG	53	367	1×10^{-125}	Respiratory burst oxidase-like protein
				R: GTCAGCTCGAAGGAGTCCAC	53			
FM_ILP013	D_212	LOC_Os01g25820	GT090926	F: GGATAGCCCAGGCAATCACT	55	180	6×10^{-65}	Unknown
				R: GTCCCGAAGAAGGAAAGGTT	54			
FM_ILP014	D_228	LOC_Os07g04690	GT090942	F: CATGTGCTACCTACGGAGAGC	54	182	2×10^{-67}	UDP-glucose 4-epimerase
				R: GTCCTCTGCCATCTTCTTGG	53			
FM_ILP015	D_230	LOC_Os05g33570	GT090944	F: GGAATCCTAGCACTGGAGAGAA	54	336	6×10^{-58}	Unknown
				R: TTCATGGCATCAAGATCCTC	52			
FM_ILP016	D_232	LOC_Os01g45990	GT090946	F: TCAACTTCTGAAGCGTGGTTT	54	192	2×10^{-84}	Potassium uptake channel
				R: AAGAAGCTTGACGCATTGCT	54			
FM_ILP017	D_234	LOC_Os06g45820	GT090948	F: AGAAAGAATTGCCCGGAACT	54	123	3×10^{-112}	Filamentation temperature-sensitive
				R: AGGCCTATGAGATTGCTTTGA	53			
FM_ILP018	D_236	LOC_Os10g26520	GT090950	F: TTTACTGAGAGGCGACCTGTT	53	630	1×10^{-76}	Serine / threonine kinase
				R: CAGGTCCAACTGGGGATAGA	54			
FM_ILP019	D_250	LOC_Os08g07960	GT090964	F: AAAGACACAGATCCCGCAAG	54	147	1×10^{-119}	Rae1-like protein
				R: CAGCTGCAAAGACATCCATC	53			
FM_ILP020	D_252	LOC_Os02g28830	GT090966	F: GAGAAGAACCCACGTCGAAC	53	216	2×10^{-128}	Unknown
				R: TATGGCAAGAGCCTCCTCAT	54			
FM_ILP021	D_254	LOC_Os04g52500	GT090968	F: TTTGTCGAAGGATGGGAGTC	54	180	1×10^{-53}	Hypothetical protein
				R: TAGGGTTTGCCAACAACTCA	53			
FM_ILP022	D_273	LOC_Os05g33810	GT090987	F: AACCAGGGCGAATGTAACC	54	184	3×10^{-74}	Hypothetical protein
				R: ATGACTGGAATCCTGCTGAA	52			
FM_ILP023	D_277	LOC_Os05g50970	GT090991	F: AGGCAGAAGGAGAGGGAGAG	54	210	1×10^{-93}	Hypothetical protein
				R: TGCTTGCCCTTTTTCCTCTA	54			
FM_ILP024	D_283	LOC_Os12g08260	GT090997	F: CAAAAAGGACGCATGTGCTA	54	190	3×10^{-99}	Hypothetical protein
				R: ATCACCGGTGCTTCCATAAC	54			
FM_ILP025	D_286	LOC_Os09g29070	GT091000	F: GTGGGAGAACTTCGGAAATG	53	206	2×10^{-47}	Hypothetical protein / g-6-p isomerase-like prot
				R: GTTCCTGGTTTCCTCATCCA	53			
FM_ILP026	D_289	LOC_Os09g39390	GT091003	F: CAGATCTCGGAAGAGGATGG	53	630	1×10^{-83}	Aldo-keto reductase / oxidoreductase

Table 2 (*continued*).

Marker	Clone name	Corresponding rice locus	GenBank accession No.	Primer sequences (5′–3′)	*T*m (°C)	Expected size (bp)	*E* value	Putative function
				R: CCTTCAGGTCTGTGATGTGC	52			
FM_ILP027	D_298	LOC_Os05g51670	GT091012	F: GCACATAGCAGCTCTGAGGA	53	202	3×10^{-120}	UDP-glucose-4-epimerase
				R: CCATTTCCAGCACTGACGTA	53			
FM_ILP028	D_304	LOC_Os11g11000	GT091018	F: GCGATGGTCGTATTGCTTAC	52	332	1×10^{-43}	ABC1 family protein
				R: TCATTAACAGCATGAACAACAGC	54			
FM_ILP029	D_310	LOC_Os08g25624	GT091024	F: CAAGGCTCATGTTGGTGAAA	53	203	7×10^{-54}	Triose phosphate / phosphate translocator
				R: TGTGGATCACTGGTATCATTAAGAG	54			
FM_ILP030	D_315	LOC_Os05g04630	GT091029	F: GATAGGATATCTTTTGTGAAGAGCA	53	180	4×10^{-18}	Aspartic proteinase
				R: AGCTTACCACACGGTGTTCG	55			
FM_ILP031	D_319	LOC_Os06g37180	GT091033	F: CTGGAAAGCCTATTGACAACG	54	214	1×10^{-113}	Vacuolar ATP synthase
				R: TCCCAGTTTGGATCATCTCC	53			
FM_ILP032	D_362	LOC_Os02g35950	GT091076	F: TGCCTCTGTCAAAATTCCTG	52	201	2×10^{-78}	Hypothetical protein
				R: GCTTCCCAATGGTTGATCC	54			
FM_ILP033	D_374	LOC_Os01g16900	GT091088	F: TCTATGTGCTGGTTTGATTGG	52	182	6×10^{-61}	Hypothetical protein
				R: CAGGAGCAGAACCATGAACA	53			
FM_ILP034	D_375	LOC_Os06g08530	GT091089	F: ATGACAAAAGCTTAGTTCACAAAA	52	194	1×10^{-70}	Hypothetical protein
				R: TGAGATCCAAGAAAGGATCAAA	53			
FM_ILP035	D_388	LOC_Os12g42884	GT091102	F: AATGCCACTGTCCCTGCTAT	53	200	5×10^{-117}	Methionine synthase
				R: GCCTTGTGGGAAGAGTAGGT	52			
FM_ILP036	D_393	LOC_Os01g31980	GT091107	F: AGAATGGTGCCATTGCTCTC	54	201	8×10^{-73}	Hypothetical protein
				R: CCAGGTTAACATAGGCACCTAAA	54			
FM_ILP037	D_399	LOC_Os09g28570	GT091113	F: TACTGCATCTGGCACCGAAG	56	298	4×10^{-105}	Putative NADPH oxidoreductase
				R: TCATCTTGACAAACTTTTTGACC	53			
FM_ILP038	D_418	LOC_Os07g42600	GT091132	F: TGGTAGAGAGGGATGGCATA	52	281	4×10^{-82}	Putative alanine aminotransferase
				R: CCTCAGCTTTGTTGCATGTG	54			
FM_ILP039	D_421	LOC_Os05g47980	GT091135	F: TTTTGTTGAGCAAGCCACTG	54	196	9×10^{-33}	ATP synthase
				R: GCACCACCAAAAAGACCAAT	54			
FM_ILP040	S_0434	LOC_Os06g15420	GT228092	F: CCGTAATGCATTCGATGATG	54	272	3×10^{-71}	Asparagine synthetase
				R: TCAATCCAACTATACCCAACACC	54			
FM_ILP041	S_0435	LOC_Os12g41250	GT228093	F: TTTTCGTGTAGATGCTGCTG	51	218	5×10^{-91}	Methylcrotonoyl-CoA carboxylase
				R: GTGATGATGCTTCCCATGC	53			
FM_ILP042	S_0437	LOC_Os05g39770	GT228095	F: CCACTTCTGGGGATTTGAAG	53	300	9×10^{-115}	Aminotransferase
				R: AATCTCCGGCGTTGTCAC	53			
FM_ILP043	S_0440	LOC_Os12g42884	GT228098	F: AAGGTGGTTGAGGTTGATGC	54	704	2×10^{-82}	Methionine synthase
				R: GTCACACGGGGTGATGACTT	54			
FM_ILP044	S_0445	LOC_Os06g07090	GT228103	F: TCTCCCTTTTGAAGCTTTCC	53	480	1×10^{-100}	AP-1 complex subunit gamma-1
				R:TGCTGCAATTCAAGCACAGT	54			
FM_ILP045	S_0446	LOC_Os05g48020	GT228104	F: AGCCAATGCAAATTTGAAGAA	54	177	1×10^{-47}	Syntaxin
				R: ACATAGAAGGATGATTTCGATGC	54			
FM_ILP046	S_0455	LOC_Os01g53520	GT228113	F: CGCTACCTTGAGGGAAATTG	54	182	3×10^{-29}	Seed maturation protein
				R: AATTGCAGCAGCTACCAAGG	54			
FM_ILP047	S_0457	LOC_Os03g27310	GT228115	F: CTCACGGAGGGCAACAGT	53	185	3×10^{-21}	Histone H3
				R: CAGGAAGCAGCTTGCAACTAA	55			
FM_ILP048	S_0458	LOC_Os09g26880	GT228116	F: CTTCTCGTCCACCACCAGTT	54	194	3×10^{-42}	Aldehyde dehydrogenase
				R: GCCCCATGGTAGTGACTGTG	55			
FM_ILP049	S_0459	LOC_Os04g39020	GT228117	F: TGGTTTAGCTGGTGCAGTGA	54	285	9×10^{-51}	Betaine aldehyde dehydrogenase
				R: GGAGCTTGGACTAAGGTTGG	53			
FM_ILP050	S_0460	LOC_Os03g18220	GT228118	F: TGACACATCCTGTGCTGTGA	53	244	5×10^{-52}	Pyruvate decarboxylase
				R: GTCAGTCGGTGCATTGCTT	53			

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Table 2 (*continued*).

Marker	Clone name	Corresponding rice locus	GenBank accession No.	Primer sequences (5′–3′)	*T*m (°C)	Expected size (bp)	*E* value	Putative function
FM_ILP051	S_0461	LOC_Os05g33570	GT228119	F: CCAACCAGGGTGTTCAAGTT	54	350	1×10^{-108}	Cytosolic orthophosphate dikinase
				R: CAGAGCAGCGAAAACTTTGTT	54			
FM_ILP052	S_0462	LOC_Os10g08550	GT228120	F: GGAGGCTATGAAAATGGGTGT	54	186	5×10^{-103}	Enolase2
				R: AAAACCACCCTCATCACCAA	54			
FM_ILP053	S_0466	LOC_Os03g04000	GT228124	F: GGCAGAGTTGGAAGCTCTTTT	54	179	9×10^{-72}	Unknown / Acyl-CoA synthetase
				R: ACGTAGGCCATAGGGAACTG	53			
FM_ILP054	S_0480	LOC_Os04g55180	GT228138	F: CTTCATGGGGCTCCTACTCA	54	515	2×10^{-55}	Unknown / esterase_lipase
				R: CACTGAACCCAAATCCAAGC	54			
FM_ILP055	S_0481	LOC_Os01g12710	GT228139	F: AACACTGCAAAGCTGGACCT	54	485	3×10^{-13}	Hypothetical protein
				R: CAACATGGTCTCCAGAAAGAAG	53			
FM_ILP056	S_0485	LOC_Os10g37210	GT228143	F: CCAGATGGACAGATAACCTTCG	55	551	3×10^{-101}	Unknown ferredoxin-like protein
				R: TAGGATCCCTCAAGCGGAGA	56			
FM_ILP057	S_0488	LOC_Os04g55960	GT228146	F: ATCCAGCTGAAGCTGAACGA	55	177	8×10^{-34}	Unknown NADPH cytochrome p450 reductase
				R: ACTTCAAGAAGGCTCCTCTGACT	54			
FM_ILP058	S_0520	LOC_Os02g03230	GT228178	F: CCGAACAAAGTTGTCACCAA	53	113	3×10^{-82}	Hypothetical protein
				R: CTGGACTCCGGGCCTATC	54			
FM_ILP059	S_0524	LOC_Os02g03040	GT228182	F: CCATATTTATCAAAGTGCCTCTCC	55	130	2×10^{-57}	Arginine / serine-rich splicing factor
				R: GATCTCCAACCAGTGCAAGG	54			
FM_ILP060	S_0531	LOC_Os07g14150	GT228189	F: AACCATCTCGGTTGCTTCTG	54	190	4×10^{-82}	Putative cytidine deaminase
				R: CTCCCCGGTAGGCTTCTTC	55			
FM_ILP061	S_0534	LOC_Os01g70170	GT228192	F: TCCTTCTATCCGGGAAGTCA	53	190	3×10^{-114}	Transaldolase
				R: AGCCCATCAAGGTAAGCATC	53			
FM_ILP062	S_0537	LOC_Os05g04630	GT228195	F: GAATGCAAAGAAGTGGTGAGC	54	219	2×10^{-44}	Aspartic proteinase
				R: AGAGTGAGTGCCGTCAAACAT	53			
FM_ILP063	S_0538	LOC_Os02g57410	GT228196	F: TGGAGGAGGAAGTAGGGAAGA	54	390	6×10^{-98}	Hypothetical protein
				R: CATGGTCCATAGTGGCTTCA	53			
FM_ILP064	S_0541	LOC_Os03g14280	GT228199	F: GGGCGAGTAATCTGGCTTTC	55	105	6×10^{-66}	ATP-dependent Clp protease
				R: TAACTGCCAACCATTTGCTG	53			
FM_ILP065	S_0546	LOC_Os02g37930	GT228204	F: TGACGCTAGGCTTCATATTGG	54	337	1×10^{-7}	Hypoxia induced protein
				R: GAGGAAGTGGGTCGTCGAG	54			
FM_ILP066	S_0550	LOC_Os07g32380	GT228208	F: AGGGCTTTCAGGAAGGAGAC	54	926	1×10^{-101}	Hypothetical protein
				R: TTTGTCTTGCCTTTCCCTGT	54			
FM_ILP067	S_0556	LOC_Os07g32380	GT228214	F: TCGTATTGCACGCATAGAGC	54	189	9×10^{-73}	Hypothetical protein
				R: TGAAGACAAGGTCAAGCAATG	53			
FM_ILP068	S_0562	LOC_Os03g22460	GT228220	F: CATGGAGAACGAGGAGGGTA	54	189	6×10^{-37}	Hypothetical protein
				R: GATCTGCACAGAGGCATGGT	55			
FM_ILP069	S_0565	LOC_Os09g17600	GT228223	F: TTATATTCCCTGACTGGCTGGT	54	178	2×10^{-19}	Hypothetical protein
				R: TCTTTCTTCCATGTTTCAATGC	53			
FM_ILP070	S_0569	LOC_Os06g02380	GT228227	F: GCACCACCCAGGAAGAAGT	54	210	4×10^{-94}	Hypothetical protein
				R: CCAGCAAGCTTTGCTATCCT	54			
FM_ILP071	S_0576	LOC_Os04g37690	GT228234	F: TCTTGCCTCTGTAAAAATTCCTC	53	217	6×10^{-88}	Hypothetical protein
				R: CTGCCTGCTTTCCAATTACTG	54			
FM_ILP072	S_0579	LOC_Os01g70270	GT228237	F: CAAAGTTCAAAAGATATTCAAAGCA	54	539	5×10^{-30}	Auxin response factor
				R: GGTCCACAGATCTGCCAAGT	54			
FM_ILP073	S_0583	LOC_Os08g38880	GT228241	F: TTGCAGCAGTATGCCAACTT	53	206	1×10^{-51}	Hypothetical protein
				R: TCTCGTGTTATGCTGAAACTCA	52			
FM_ILP074	S_0586	LOC_Os03g22730	GT228244	F: GAACCAGCAGATTTCCCAAA	54	188	1×10^{-47}	Hypothetical protein
				R: CTTGGTGATGGTGAGGACAA	53			
FM_ILP075	S_0591	LOC_Os04g55960	GT228249	F: TAGCTCCTCGTCTGCAACCT	54	305	2×10^{-111}	Hypothetical protein

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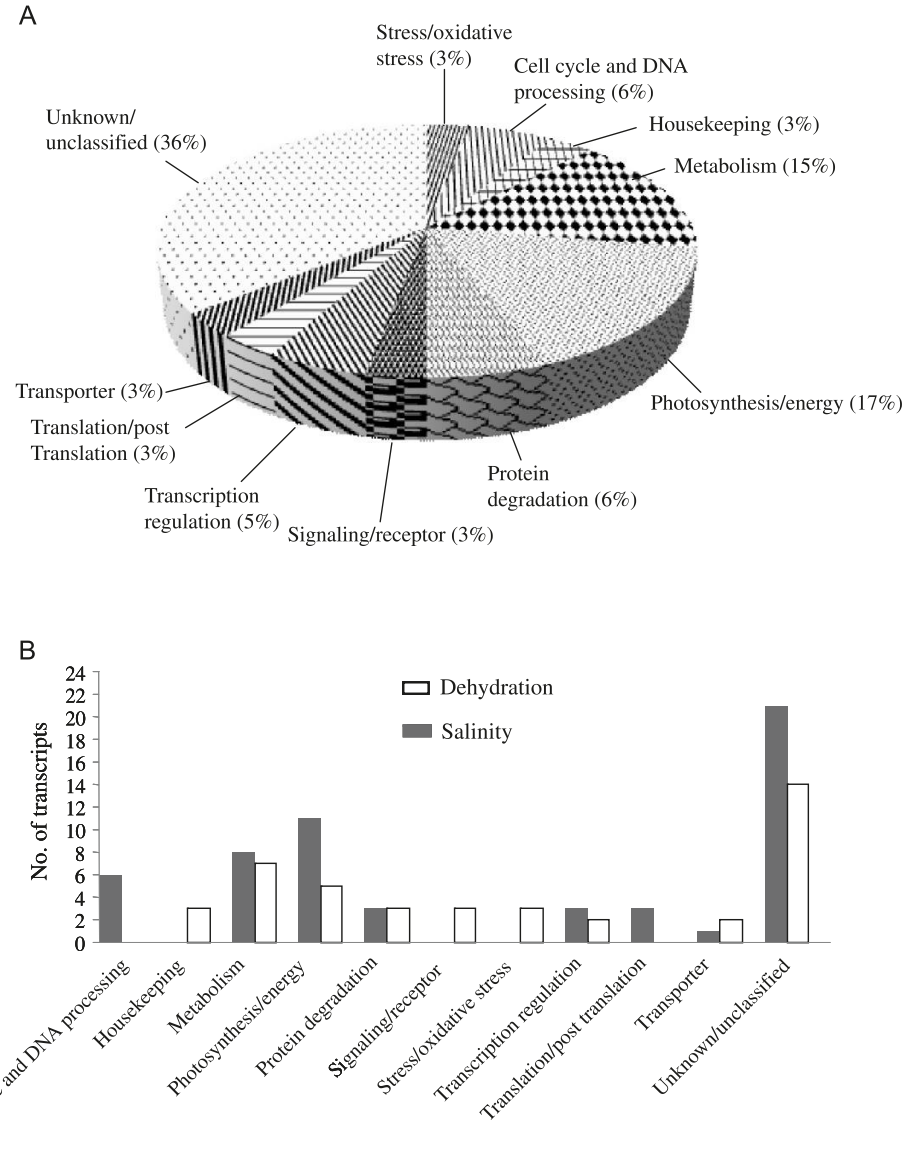
Table 2 (*concluded*).

Marker	Clone name	Corresponding rice locus	GenBank accession No.	Primer sequences (5′–3′)	*T*m (°C)	Expected size (bp)	*E* value	Putative function
				R: CCTGTGGGTGAAGGACCATA	54			
FM_ILP076	S_0593	LOC_Os04g39440	GT228251	F: CGATCTTCATGGAGGCATCT	54	120	2×10^{-59}	GTP binding protein
				R: CATCGAAAACACCATCTTGG	53			
FM_ILP077	S_0594	LOC_Os03g25400	GT228252	R: CATCAATTCCTTGATCGCTTC	53	705	2×10^{-84}	Hypothetical protein
				F: TGAGGAAAACTGGAGAAATGG	54			
FM_ILP078	S_0598	LOC_Os12g12360	GT228256	F: TGAGGAAAACTGGAGAAATGG	53	305	3×10^{-37}	Hypothetical protein
				R: TTTCCATCAGTTCCGTGTGT	52			
FM_ILP079	S_0600	LOC_Os02g32520	GT228258	F: TGCCCTTCACTTCCTTCAG	52	350	9×10^{-78}	Unknown / ATP-dependent
				R: TTTTTCCGACCAGAGTTGCT	54			
FM_ILP080	S_0601	LOC_Os03g44620	GT228259	F: TCAGTCAGAGACAATGTGTGC	50	213	1×10^{-83}	DnaJ protein
				R: TGAAGCGCCTGATACTACCAC	54			
FM_ILP081	S_0611	LOC_Os01g09320	GT228269	F: ACTGATGGTGGGCGAATCT	54	180	2×10^{-75}	Hypothetical protein
				R: GAACTCCTCCAAGGGCAGTG	56			
FM_ILP082	S_0612	LOC_Os01g21160	GT228270	F: TGAACAGCTCTTTCATTGAGGA	54	600	3×10^{-116}	Dihydrolipoyllysine-residue acetyltransferase
				R: TGTTTGGCACGACTAAACCA	54			
FM_ILP083	S_0613	LOC_Os08g45010	GT228271	F: TTAGTTGTGATGAAGAAAGAGTCCA	54	500	1×10^{-110}	Hypothetical protein
				R: ACGCTCATACACACGATCCA	53			
FM_ILP084	S_0620	LOC_Os06g43660	GT228278	F: GGGCTCCCATATACATTCACA	54	188	8×10^{-69}	Hypothetical protein
				R: ACTAGTCCAGCCCACAGACC	53			
FM_ILP085	S_0621	LOC_Os01g45750	GT228279	F: GCACAGTATTGCGTCAAACC	53	819	4×10^{-95}	Na(+) dependent transporter-like
				R: GGGCAACATGAGACCAAAAT	54			
FM_ILP086	S_0625	LOC_Os01g65900	GT228283	F: TAGACCTCACGGAACCTTGC	54	180	2×10^{-54}	Chitin-inducible gibberellin-responsive protein
				R: TCCGTATGGTGAAGGGTCTC	54			
FM_ILP087	S_0626	LOC_Os11g43900	GT228284	F: TGAGGGTGTTGATGATCAGG	53	115	9×10^{-67}	Translationally controlled tumor protein
				R: TTCATGAAGGTCACAAACTGCT	54			
FM_ILP089	S_0646	LOC_Os06g22870	GT228304	F: GAACATTCTCCACGGGACAT	53	110	5×10^{-9}	Hypothetical protein
				R: TTATCTGGTTGCCGACTGAA	53			
FM_ILP090	S_0653	LOC_Os08g42580	GT228311	F:AAGAAGCTATTGTGAGATCAACTGAG	54	633	8×10^{-71}	Hypothetical protein
				R: ATCCTCTGAAGGCCCTCCT	54			
FM_ILP091	S_0662	LOC_Os05g45050	GT228320	F: CGCTACCTTGAGGGAAATTG	54	182	5×10^{-23}	Seed maturation protein (*Zea mays*)
				R: AATTGCAGCAGCTACCAAGG	54			
FM_ILP092	S_0669	LOC_Os02g32520	GT228327	F: TTTTCAACATCCTTCTCCAAATC	54	800	8×10^{-79}	Unknown / ATP-dependent Clp protease
				R: ACCAACATTGGACGTCATCA	53			
FM_ILP093	S_0671	LOC_Os03g08280	GT228329	F: GGTGTGACTGACACGTCCAA	54	450	3×10^{-90}	Proteasome subunit
				R: AAGAAGACCGTGTTTTCAGAGC	54			
FM_ILP094	S_0676	LOC_Os07g10720	GT228334	F: GTCATGATCCGGCCCTCT	54	186	7×10^{-56}	Putative ribosomal protein
				R: CGCACGATGGTCATCAAC	52			
FM_ILP095	S_0678	LOC_Os08g27850	GT228336	F: AGTGGCACATCCCTCAACAC	54	700	1×10^{-44}	Hypothetical protein
				R: TCTTATCCAGCCTTGCTTGC	54			
FM_ILP096	S_0682	LOC_Os04g56530	GT228340	F: GACGAGGATGGTGCTCATAGA	54	266	2×10^{-85}	Hypothetical protein
				R: CCACCAATGGTTTGACAACA	54			
FM_ILP097	D_172	LOC_Os01g48910	GT090886	F: GCGGTTGAGAATCTGGAGAA	60	186	5×10^{-80}	Putative acyl-CoA synthetase
				R: GTTCACCACCGCAACGAG	60			
FM_ILP098	D_225	LOC_Os08g41460	GT090939	F: CGCATTAAACAATTCTCTGCAA	60	425	3×10^{-112}	Calcium-dependent protein kinase
				R: ACATTTCCTTCAACCCAGCA	60			
FM_ILP099	D_110	LOC_Os01g15020	GT090824	F: TTTACCTCATCTACCCTGACATTG	59	188	2×10^{-83}	Putative CTV
				R: TCAACATTTTTAGCGTTTCACC	59			
FM_ILP100	D_422	LOC_Os03g63770	GT091136	F: TTCCACAAAAGTGCCTCGTT	60	217	5×10^{-49}	RCD1 protein
				R: GGTCACCAACTATTTGCCTCA	60			

Fig. 1. Functional classification of 98 differentially expressed transcripts under dehydration and salinity stress in foxtail millet (*Seteria italica.*). (A) Unique transcripts were grouped into 11 functional groups. (B) Comparison of functional classification between unique transcripts identified from dehydration and salinity stress libraries.



ity stress suggests their role in providing better adaptation than the sensitive cultivar. Whereas in dehydration stress three unique types, namely housekeeping, signaling/receptor, and stress/oxidative stress related transcripts, were exclusively identified. The presence of these transcripts only under dehydration stress suggests that the tolerant foxtail millet cultivar seems to hold the efficient mechanisms of signal perception and transduction in adjusting the metabolism to dehydration shock. In summary, the identified transcripts that are preferentially expressed in tolerant cultivar during early abiotic stress responses being specific to salt and osmotic responses would also be an important source of future targets for genetic manipulations.

Evaluation of foxtail millet ILP markers

For validation purpose, the 98 developed ILP primer pairs were evaluated for their ability to detect polymorphisms in the parent DNA of a F_2 mapping population (dehydration- and salt- tolerant 'Prasad' × dehydration- and salt-sensitive 'Lepakshi'), and they produced clear amplification profiles. The result revealed that 29 (~30%) primer pairs were polymorphic, whereas the remaining 69 (~70%) were monomorphic in nature (Figs. 2A–2D). In 'Prasad', 90 (91.8%) of the 98 ILP markers amplified a single locus, whereas 8 (8.2%) markers (FM_ILP006, FM_ILP008, FM_ILP009, FM_ILP014, FM_ILP018, FM_ILP033, FM_ILP063, and FM_ILP089) amplified more than one locus (Table 3). A total of 109 loci were scored using 98 ILP markers, and 1 to 3 alleles were detected at each locus, producing a total of 109 alleles in foxtail millet 'Prasad' (Table 3).

Transferability of foxtail millet ILP markers

The 98 primer pairs assessed for cross-species transferability produced clear amplification profiles and could be validated as useful markers. Details of their cross-species amplification status are shown in Table 3. Of the 98 ILPs as-

Fig. 2. Electropherogram of parent DNA (P_1 and P_2) amplified with ILP markers (A) FM_ILP021; (B) FM_ILP023; (C) FM_ILP041; and (D) FM_ILP081 using MultiNA. M, 25-bp DNA ladder; P_1, 'Prasad'; and P_2, 'Lepakshi'.

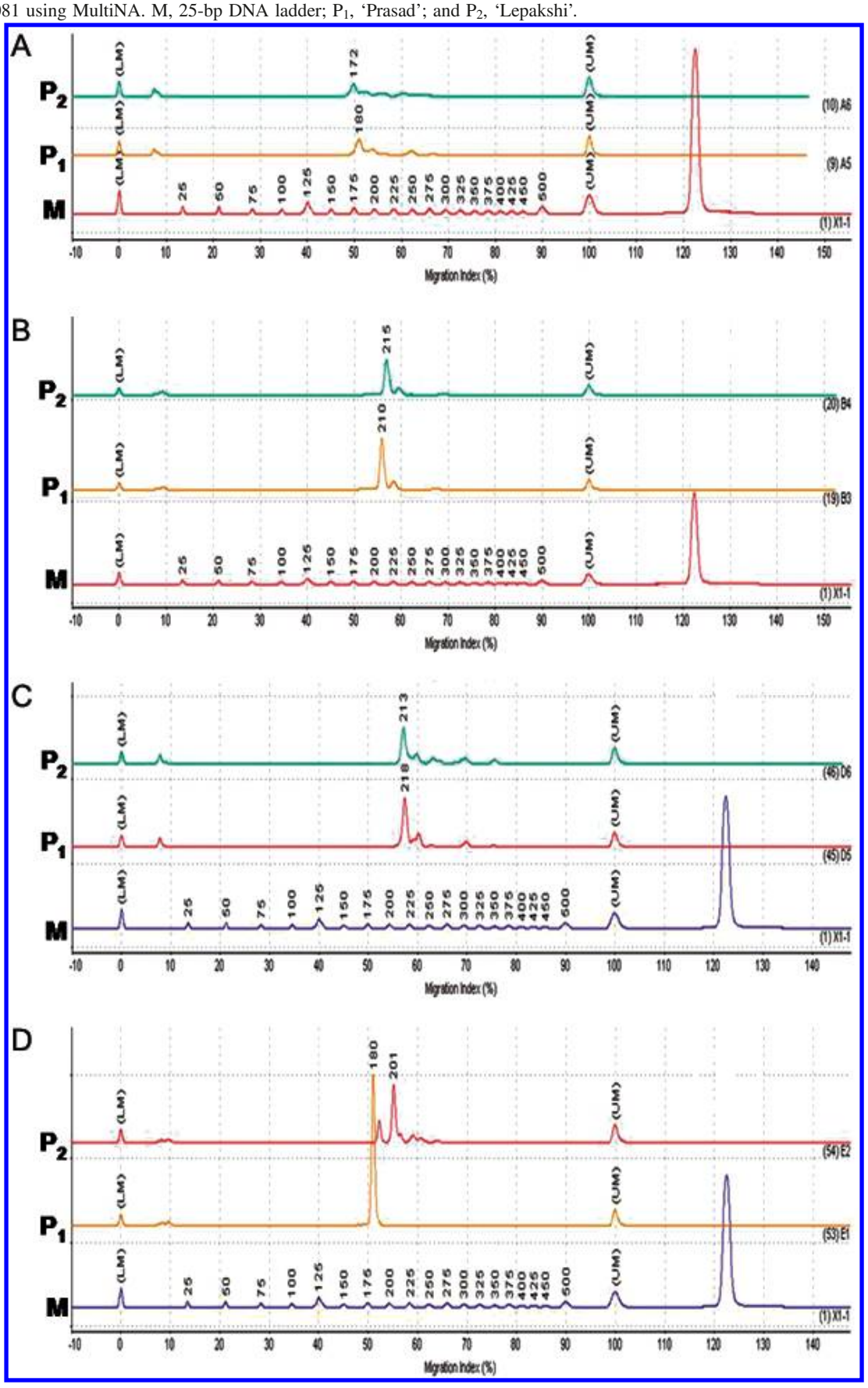


Table 3. Cross-species transferability of 98 ILP markers in millets and nonmillet species.

			Obtained allele size (bp)					
Marker	No. of alleles	Size range of alleles (bp)	Foxtail millet	Rice	Wheat	Pearl millet	Guinea grass	% transferability (each marker)
FM_ILP001	1	182	182	182	182	NA	182	80
FM_ILP002	3	410–586	586	586	465	410	410	100
FM_ILP003	1	213	213	213	213	213	213	100
FM_ILP004	3	141–384	326	384	141	326	326	100
FM_ILP005	4	548–1157	548	635	679	1157, 635	548	100
FM_ILP006	6	141–1341	552, 152	874, 152	1341, 141	965, 874, 152	1341, 152, 874	100
FM_ILP007	3	185–1445	185	190	185, 1445	185	185	100
FM_ILP008	4	208–600	418, 208	208	600, 437	208	418, 208	100
FM_ILP009	7	218–776	218, 400, 776	218, 790	776, 415, 230	337	400	100
FM_ILP011	3	95–180	95	180	150	95	95	100
FM_ILP012	5	180–410	384	367	180, 410	384	326	100
FM_ILP013	2	180–800	180	180	800	180	180	100
FM_ILP014	3	141–271	182, 271	141, 182	182	182	182, 271	100
FM_ILP015	3	250–478	250	336	478, 336	250	250	100
FM_ILP016	4	180–1050	180	192	380, 1050	180	180	100
FM_ILP017	3	123–900	123	123	900	132	132	100
FM_ILP018	7	240–1419	824, 240, 531	630	824, 464	1419	1459	100
FM_ILP019	4	108–283	147	147, 108, 283	283	283, 185	185	100
FM_ILP020	2	216–267	216	216	267, 216	216	216	100
FM_ILP021	2	180–410	180	180	410	NA	180	80
FM_ILP022	2	184–1500	184	184	1500	184	184	100
FM_ILP023	5	90–615	90	210	120	280	615	100
FM_ILP024	2	190–750	190	190	190, 750	190	190	100
FM_ILP025	3	136–206	136	206	180	136	136	100
FM_ILP026	5	387–780	630	630, 387	700, 780	630	582	100
FM_ILP027	2	202–700	202	202	202, 700	202	202	100
FM_ILP028	2	332–400	332	332	400	332	332	100
FM_ILP029	1	203	203	203	203	203	203	100
FM_ILP030	2	180–350	180	180	180	180	350	100
FM_ILP031	2	214–234	214	214	NA	214	234	80
FM_ILP032	2	201–245	245	201	201	201	201	100
FM_ILP033	2	182–220	182, 220	182	182	182	182	100
FM_ILP034	1	194	194	194	194	194	194	100
FM_ILP035	2	180–200	200	200	200	200	200, 180	100
FM_ILP036	3	190–1050	201	201	1050	201	190	100
FM_ILP037	3	200–298	224	298	NA	224	200	80
FM_ILP038	6	90–510	350	281, 510	510, 90	140	150, 350	100
FM_ILP039	3	150–196	196	196	196	175	150	100
FM_ILP040	1	272	272	272	272	272	272	100
FM_ILP041	3	218–350	218	218	350, 218	262	262	100
FM_ILP042	3	274–400	300	300	300, 400, 274	300	300	100
FM_ILP043	4	675–750	704	704	750	675	680	100
FM_ILP044	5	480–1200	648	480	750, 1000, 1200	648	648	100
FM_ILP045	1	177	177	177	NA	177	NA	60
FM_ILP046	1	182	182	182	182	182	182	100
FM_ILP047	1	185	185	185	285	200	215	100
FM_ILP048	1	194	194	194	194	194	194	100
FM_ILP049	4	285–850	285	285	700, 850	310	310	100
FM_ILP050	2	244–405	244	244, 405	244	244	244	100
FM_ILP051	2	350–510	350	350	510	350	350	100
FM_ILP052	1	186	186	186	186	186	186	100
FM_ILP053	1	179	179	179	179	179	179	100
FM_ILP054	4	431–573	515	515	515	476, 431	573	100
FM_ILP055	2	485–525	485	485	525	485	485	100
FM_ILP056	1	551	551	551	NA	551	551	80
FM_ILP057	4	205–310	225	177, 310	225	205	205	100
FM_ILP058	3	113–850	113	113	113, 850	113	655	100
FM_ILP059	2	130–175	130	130	175	130	130	100
FM_ILP060	3	190–1085	305	190	1085	190	305	100
FM_ILP061	3	190–270	220	190	270	220	220	100

Table 3 (*concluded*).

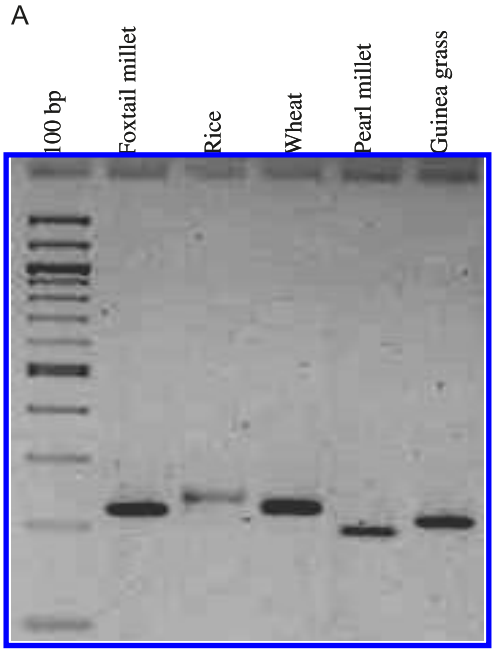
Marker	No. of alleles	Size range of alleles (bp)	Obtained allele size (bp)					% transferability (each marker)
			Foxtail millet	Rice	Wheat	Pearl millet	Guinea grass	
FM_ILP062	3	210–900	210	219	900	219	210	100
FM_ILP063	6	204–900	724, 300, 204	900, 390	900, 390	724, 500	724	100
FM_ILP064	2	105–1050	105	105	105, 1050	105	105	100
FM_ILP065	3	337–624	337	337, 624	400	337	337	100
FM_ILP066	2	926–1125	1125	926	NA	1125	NA	60
FM_ILP067	1	189	189	189	189	189	189	100
FM_ILP068	3	95–600	189	189	600, 95	189	189	100
FM_ILP069	3	178–198	178	178	178	187	198	100
FM_ILP070	3	187–405	187	210	405	187	187	100
FM_ILP071	1	217	217	217	217	217	217	100
FM_ILP072	2	539–565	565	539	565	565	565	100
FM_ILP073	4	200–1200	200	206, 1200	NA	217	217	80
FM_ILP074	2	188–200	200	188	NA	200	200	80
FM_ILP075	2	305–337	305	305	305	337	337	100
FM_ILP076	2	100–120	120	120	120	100	120	100
FM_ILP077	3	648–865	648	705	865	648	648	100
FM_ILP078	3	250–1100	305	305	1100	250	250	100
FM_ILP079	2	322–350	322	350	NA	322	322	80
FM_ILP080	3	115–482	115	213, 115	482	213	482	100
FM_ILP081	3	152–874	152	180	874	152	152	100
FM_ILP082	2	500–600	600	600	NA	500	600	80
FM_ILP083	5	182–1400	182	500	1400	200	330	100
FM_ILP084	1	188	188	188	188	188	188	100
FM_ILP085	2	819–1150	819	819	1150, 819	819	819	100
FM_ILP086	5	180–1773	194	180	194	1773, 1081	620	100
FM_ILP087	2	115–160	115	115	160	150	150	100
FM_ILP089	4	85–220	85, 200	110	85, 220	85	NA	80
FM_ILP090	3	633–800	800	633	NA	800	705	80
FM_ILP091	1	182	182	182	182	182	182	100
FM_ILP092	4	460–980	520	800	980	460, 800	460	100
FM_ILP093	6	95–1500	95	450, 750	1000, 1500	95, 680	95	100
FM_ILP094	4	124–372	124	186	360	124	372	100
FM_ILP095	5	460–1370	790	700, 1370	460	NA	600, 1370	80
FM_ILP096	4	226–266	246	266	246	226	232	100
FM_ILP097	2	186–447	186	447, 186	447, 186	186	186	100
FM_ILP098	3	372–500	372	425	500	372	372	100
FM_ILP099	1	188	188	188	188	188	188	100
FM_ILP100	2	217–250	217	217, 250	217	217	217	100
% transferability (each species)			100	100	90	97	97	

Note: NA, no amplification.

sayed, 98 (100%) gave consistent amplification in foxtail millet, 98 (100%) in rice, 89 (~90%) in wheat, 95 (~97%) in pearl millet and 95 (~97%) in guinea grass, with a mean of ~97% (Table 3). The results suggest that a high proportion of foxtail millet ILP markers are transferable to other plants. Eighty four (85.7%) markers were transferable to other related species. Of the 98 ILP markers, only 60 (~61%) gave amplified fragments of the expected length. All markers that gave consistent amplification in other species were scored as being transferable.

To examine whether the PCR products were really amplified or homologous to the target genes in two millet (pearl millet and guinea grass) and two nonmillet (rice and wheat) species, we randomly picked up a primer pair, FM_ILP096, which amplified variant alleles from 226 to 266 bp (Fig. 3A). As expected, the sequences of cloned PCR products of the investigated species revealed indels and several point mutations, such as single-base insertions, deletions, or translocations; in addition, a polymorphism in intron length was observed (Fig. 3B). Overall, multiple sequence alignment has shown that they were homologous to each other and were composed of conserved exon regions at two end positions and a nonconserved or variable intron region in the middle position. A similar kind of observation was also reported in rice and a medicinal plant, St. John's wort (Wang et al. 2006; Ferreira et al. 2009). Further, higher levels of transferability of ILPs compared with previously identified markers reflect the conserved nature of exon positions in gene sequences and variability in the noncoding sequences (Wang et al. 2006). Hence, in our study the high levels of cross-species amplification indicate that the foxtail millet ILP markers could be successfully useful for comparative mapping in millet and nonmillet species. Similarly, it was shown that EST–SSR has higher cross-species transferability than

Fig. 3. Gel electrophoresis and multiple sequence alignment of size-variant alleles. (A) PCR amplified products of ILP marker FM_ILP096 in foxtail millet 'Prasad', rice 'IR64', wheat 'PH132', pearl millet 'T1', and guinea grass 'SPM92' separated on 2.0% agarose gel. (B) ClustalW sequence alignment of the size-variant alleles amplified using FM_ILP096 primers across five plant species (foxtail millet 'Prasad', rice 'IR64', wheat 'PH132', pearl millet 'T1', and guinea grass 'SPM92'). Asterisks denote similar sequences, dashes represent deletions, and arrows represent primer binding sites. Repeat regions are in bold, point mutations are underlined, and exonic regions are darkly shaded.



B

Forward primer

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Pearl millet    GACGAGGATGGTGCTCATAGACAACCTGGA---AGTTACATACA-------------CCA 44
Guinea grass    GACGAGGATGGTGCTCATAGACAACCTGGAAA-AGTTACATACA-------------CCA 46
Foxtail millet  GACGAGGATGGTGCTCATAGACAACCTGGAAA-AGTTCCATACA-------------CCA 46
Rice            GACGAGGATGGTGCTCATAGACAACCTGAAAGCAATTCCAAACAATCGAACGTTAAATCA 60
Wheat           GACGAGGATGGTGCTCATAGACAACCTGGA---AATTTGTAACA-----TAGTCATATAT 52
                ***************************  *   *  **    ***

Pearl millet    CAAA--TCAGATTCTA----ACTACAAGATCAAATTCATTGA-------------AATGC 85
Guinea grass    CAAAGTTCAGATTCTA----ACTACAAGATCAAATTCATTGA-------------AATGC 89
Foxtail millet  AAAAGTTCAGATTCTACATAGCTACAAGAATCATTTCATTGACAGTGACCTAA--AATGC 104
Rice            TGGAGTTCAAACTTTACAAAGGTACATTAAACATATCGTTGATATTGAGTAAAATGAAAT 120
Wheat           TATTTCTCACGTTGTACATAACAGCATGGAACATGTTTTACTTGAC----------ATGT 102
                   ***   * **      **     *  * *      *

Pearl millet    AGGACCATTTAACCCAG---GCAA--CAAGAATGCCAAACCTTTATGAGTTTCAGCAGAG 140
Guinea grass    AGGACCATTTAACCCAG---GCAAAACAAGAATGCCAAACCTTTATGAGTTTCAGCAGAG 146
Foxtail millet  AGGACCATTTGATCAAG---CTAAGACTAGA-TGTCAAACCTTTATGAGTTTCAGCAGAG 160
Rice            AAAACCATGTAACCAGAAATTTAAAGCGAGGAATGCAAACCTTTATTAATTTTAGCAGTG 180
Wheat           AGAATCAGTGAATCATGA--GAAAAGAGTGAACAGCAAACCTTTATTAGTTTCAGCAGAG 160
                *  * **    * *        **     *     *********** * *** ***** *

Pearl millet    TCAAGCGAGCAATAGCTTCTCGATGGTCAAGTCTTGCAATAAGCAATGTTGTCAAACCAT 200
Guinea grass    TCAAGCGAGCAATAGCTTCTCGATGGTCAAGTCTTGCAATAAGCAATGTTGTCAAACCAT 206
Foxtail millet  TCAATCGAGCAATAGCTTCTCGATGGTCAAGTCTAGCAATTAGCAATGTTGTCAAACCAT 220
Rice            TCAAACGGGCAATAGCTTCTCGGTGGTCAAGCCTTGCAATAAGCAGTGTTGTCAAACCAT 240
Wheat           TCAAACGAGCAATAGCTTCTCGATGGTCAAGTCTTGCAATAAGCAATGTTGTCAAACCAT 220
                **** ** ************** ******** ** ***** **** **************

Pearl millet    TGGTGGTGTTGTCAAACCATTGGTGG 226
Guinea grass    TGGTGGTGTTGTCAAACCATTGGTGG 232
Foxtail millet  TGGTGGTGTTGTCAAACCATTGGTGG 246
Rice            TGGTGGTGTTGTCAAACCATTGGTGG 266
Wheat           TGGTGGTGTTGTCAAACCATTGGTGG 246
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Reverse primer

Fig. 4. Genetic relationship among millets (foxtail millet, pearl millet, and guinea grass) and nonmillet (rice and wheat) species as revealed by 98 ILP markers based on NJoin clustering.

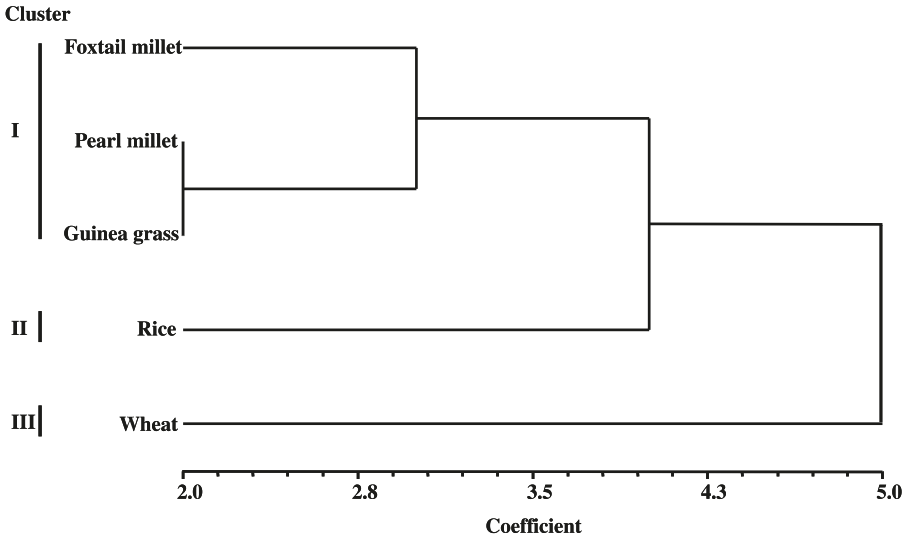
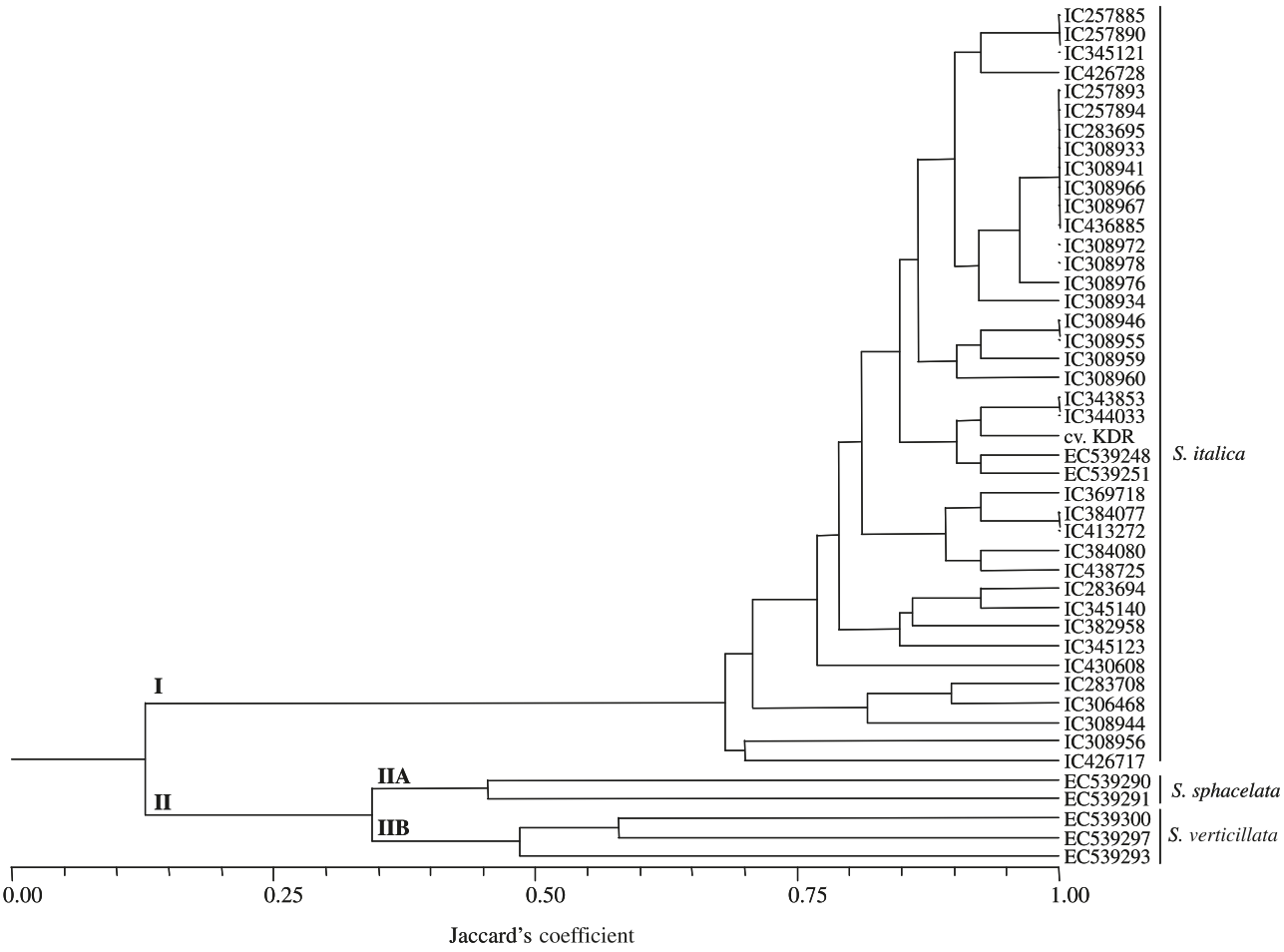


Table 4. Genetic diversity analysis of the 45 foxtail millet (*Setaria italica*) accessions with 26 ILP markers.

Marker	No. of alleles	Size range of alleles (bp)	H_O	Nei	F_{IS}	*I*	PIC
FM_ILP002	4	569–750	0.000	0.392	1.000	0.703	0.20
FM_ILP005	2	548–800	0.000	0.089	1.000	0.188	0.12
FM_ILP007	1	185	0.000	0.000	0.000	0.00	0.0
FM_ILP011	4	95–618	0.046	0.046	–0.500	0.110	0.10
FM_ILP016	2	180–210	0.000	0.324	1.000	0.500	0.32
FM_ILP019	4	82–240	0.088	0.297	0.558	0.612	0.19
FM_ILP021	1	180	0.000	0.000	0.000	0.000	0.20
FM_ILP025	3	136–400	0.000	0.239	1.000	0.453	0.13
FM_ILP026	2	564–630	0.000	0.047	1.000	0.112	0.10
FM_ILP028	4	170–350	0.000	0.444	1.000	0.848	0.22
FM_ILP031	3	214–271	0.000	0.431	1.000	0.765	0.28
FM_ILP032	4	245–400	0.066	0.320	0.508	0.573	0.17
FM_ILP034	2	194–221	0.000	0.088	1.000	0.185	0.11
FM_ILP037	2	224–330	0.000	0.164	1.000	0.300	0.16
FM_ILP054	1	515	0.000	0.000	0.000	0.000	0.16
FM_ILP055	2	300–485	0.000	0.049	1.000	0.117	0.14
FM_ILP056	2	443–551	0.044	0.044	–0.250	0.107	0.04
FM_ILP060	6	130–305	0.044	0.386	0.417	0.848	0.15
FM_ILP061	2	190–220	0.022	0.182	–0.111	0.325	0.18
FM_ILP067	3	189–230	0.000	0.239	1.000	0.453	0.18
FM_ILP068	4	100–240	0.044	0.332	0.528	0.570	0.18
FM_ILP070	1	187	0.000	0.000	0.000	0.000	0.00
FM_ILP071	2	217–236	0.000	0.086	1.000	0.182	0.08
FM_ILP075	3	305–440	0.068	0.131	–0.200	0.292	0.11
FM_ILP096	1	246	0.000	0.000	0.000	0.000	0.20
FM_ILP098	2	372–425	0.000	0.126	1.000	0.245	0.12
Mean	2.6	-	0.016	0.171	0.748	0.326	0.15
SD			0.027	0.150		0.278	

Note: H_O, observed heterozygosity; Nei, Nei's average gene diversity (Nei 1973); F_{IS}, fixation index; *I*, Shannon's informative index; *PIC*, polymorphic information content.

Fig. 5. Dendrodram of 45 foxtail millet (*Setaria italica*) accessions constructed using UPGMA based on 26 ILP markers. The distributions of accessions along with species name are indicated right of the dendrogram.



genomic SSR markers (Choudhary et al. 2009; Gupta and Prasad 2009).

Genetic relationships among the species

The genetic relationships among the investigated species are shown in Fig. 4. The 98 ILP markers showed the ability to clearly separate the millet and nonmillet species into three distinct clusters (clusters I–III). All three millet species, namely foxtail millet, pearl millet, and guinea grass, were grouped together (cluster I) separately from the two nonmillet species, namely rice (cluster II) and wheat (cluster III) (Fig. 4). All the millet species belong to the tribe Paniceae and that may be the reason for their grouping in the same cluster (cluster I). Similar observations have also been reported in previous studies (Wang et al. 2006; Zhao et al. 2009; Gupta and Prasad 2009).

Assessment of genetic diversity

To verify the utility of developed ILP markers, 26 markers were randomly selected from the list of 98 markers to estimate the genetic diversity of 40 cultivated (*S. italica*) and 5 wild (*S. sphacelata* and *S. verticillata*) accessions of foxtail millet. Details of their diversity statistics are shown in Table 4. A total of 67 alleles were detected, ranging from 1 to 6 alleles per locus and a mean of 2.6. Substantial differences in allelic polymorphisms were also detected by ILP markers and allele size variation ranged from 82 to 800 bp. The PIC value was estimated and ranged from 0.0 to 0.32, with a mean of 0.15. The observed heterozygosity for individual loci ranged from 0.000 to 0.088, with a mean of 0.016. The expected heterozygosity ranged from 0.000 to 0.444, with a mean of 0.171. All the loci were tested with the fixation index (F_{IS}), which was positive for 22 loci, indicating an excess of observed homozygotes, and negative for 4 loci, indicating an excess number of observed heterozygotes, with a mean of 0.748 per locus. The Shannon's informative index (I) of the loci ranged from 0.000 to 0.848, with a mean of 0.326 per locus (Table 4).

Genotyping data obtained for all the polymorphic alleles were used to estimate pairwise similarity comparisons among these accessions. The similarity coefficient values of the dendrogram ranged from 0.13 to 1.00 (Fig. 5). The 45 foxtail millet accessions clustered into two major groups: cluster I (with 40 accessions of *S. italica*) and cluster II (with five wild accessions). Cluster II was further divided into two subclusters: cluster IIA (with 2 accessions of *S. sphacelata*) and cluster IIB (with 3 accessions of *S. verticillata*) as shown in Fig. 5. The results suggested that all the *S. italica* accessions grouping was largely consistent, while the other *Setaria* species tended to be grouped together. A comparison of the results indicated that the mean number of alleles per locus (2.6)

in the present study was comparable (2.3) to a recent study in rice using ILP markers (Huang et al. 2010). F_{IS} is a measure of how populations differ genetically. In the present study, the mean F_{IS} was ~1.0 (0.748), indicating that the accessions were fixed for the homozygous allele. The PIC value was calculated to examine the extent of information on diversity that these markers can provide and compare these results with previous published studies. In this study, the mean PIC value was 0.15, lower than that reported in rice (0.45 and 0.44) using ILP markers (Wang et al. 2006; Huang et al. 2010). The probable reason for the difference in results might be attributed to the difference in the number of genotypes and their genetic background and the number of markers used.

Conclusions

In most of the millets and forage grass species either a very limited number of genomic SSRs markers and EST sequences are reported or do not exist at all. To the best of our knowledge, the ILP markers developed in this study are the novel set of publicly available markers in foxtail millet, in addition to 114 that where earlier reported by Jia et al. (2007 and 2009). The present study demonstrates that the foxtail millet ILP markers developed and characterized here are useful in germplasm characterization and genome relationships in both millet and nonmillet species. As well, the ILP markers would be helpful for comparative mapping and construction of a genetic linkage map for QTL discovery in foxtail millet including other millets and forage grass species. Further, the recent completion of foxtail millet whole genome sequencing by the Department of Energy Joint Genome Institute, USA, and BGI, China will provide more useful information regarding the foxtail millet genome that will bring to the research community a huge wealth of information for rapid and inexpensive development of gene-derived molecular markers for effective use in crop improvement programs.

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