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Development of new gene-specific markers associated with salt tolerance for mungbean (*Vigna radiata* L. Wilczek)

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Abstract

Thirty eight novel microsatellite markers (SSRs) specific to candidate genes involved in salt tolerance were developed for detection of genetic variations in 12 mungbean genotypes variably adapted to salt stress. A 100 out of 124 putative alleles were found polymorphic between wild and cultivated genotypes (inter-specific, 80.65%), 65 were within cultivars of mungbean (intra-specific, 52.42%) and 52 were within wild genotypes (inter-specific, 41.94%). The polymorphism varied from 86.84% to 100%, while the number of polymorphic alleles ranged from 1 to 4 with an average value of 2.63 per locus. The polymorphism information content (PIC) values ranged from 0.326 to 0.875 with an average value of 0.671, which shows their effectiveness in genetic analysis. Cluster analysis resulted in the distribution of salt tolerant and susceptible genotypes in separate groups which revealed the presence of inherent variations among mungbean cultivars. These variations were explored effectively for SSR markers studies. The developed SSR markers may help along with already available markers to execute further research on mungbean. The markers may be coupled with specific loci linked with salt tolerance. The developed markers will help to identify the QTLs (quantitative trait loci) or other important genes. These markers can also be utilized for testing the purity of hybrids or diversity assessment of *Vigna* species for important agronomic traits.

Additional key words: polymorphism; salt stress; microsatellite markers; genetic diversity; clustering; QTLs.

Introduction

Mungbean (*Vigna radiata* L. Wilczek) is an important grain legume. It is a rich source of proteins, vitamins, and minerals, especially for the vegetarian Asian population, particularly in South Asia (Tomooka *et al.,* 2002). Mungbean is a self-pollinated diploid plant having 2n = 2x = 22 chromosomes with a genome size of 579 Mb/1C. Its short life span (55-90 days) and a capacity to restore soil fertility (through nitrogen fixation) make it a valuable crop in various cropping systems, particularly wheat-rice cropping system (Somta & Srinives, 2007). Mungbean belongs to the Asian *Vigna* subgenus *Ceratotropis,* having South Asia as its center of diversity (Ajibade *et al.,* 2000; Undal *et al.,* 2011). The genus *Vigna* is composed of more than 150 species originating mainly in Africa and Asia (Polhill & Van der Maesen, 1985; Undal *et al.,* 2011). It is a genetic resource owing to its stress (salt)-resistant genetically diverse germplasm that could be of practical value for salinity-based breeding programs (Win *et al.,* 2011). Intra-specific variations among the close wild relatives of Asian *Vigna* stay on priority in crop improvement programs (Bisht *et al*., 2005). These variations include desired characters including resistance to biotic/abiotic stresses and agronomic traits

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Abbreviations used: EMR (effective multiplex ratio); MI (marker index); PCR (polymerase chain reaction); PIC (polymorphism information content); QTL (quantitative trait locus); RAPD (random amplified polymorphic DNA); Rp (resolving power); SOS (salt overlay sensitive); SNP (single nucleotide polymorphism); SSR (simple sequence repeat); STMS (sequence tagged microsatellite site); UPGMA (unweighted pair group method with arithmetic mean).

(Bisht *et al*., 2005). Immediate focus on favorable agronomic traits is required for breeding-assisted improvements in mungbean. Worldwide, a total of 43,027 mungbean accessions are available *ex situ* at different Institutes (AVGRIS, 2012; WIEWS, 2012).

India is the largest producer of pulses, contributing 35.7% to the world's total pulse production (FAOSTAT, http://faostat.fao.org). India contributes about 54% to the total production of mungbean (Lambrides & Godwin, 2007). An area of 3.55 million hectares was under cultivation for this crop in 2010-11, and produced 1.82 million tons with an average yield of 512 kg ha^{-1} (MULLaRP, www.aicrpmullarp.res.in). In 2011-12, the production was reduced to 1.27 million tons (INDIASTAT, http://indiastat.com). Mungbean production is decreasing mainly due to increasing soil salinity in irrigated land agriculture (Saha *et al.,* 2010). The crop is sensitive to salt stress and at 50 mM NaCl concentration more than 50% reduction in yield has been observed (Salim & Pitman, 1988). The low productivity of mungbean makes it insufficient for internal consumption. However, sufficient research has not been performed in *Vigna* for identification and development of breed cultivars that are adapted to salt stress conditions (Singh & Singh, 2011).

Response to salinity is a polygenic trait. Plants maintain their ionic and osmotic homeostasis by preventing accumulation of Na^+ ions inside the cells. This mechanism involves either restriction of Na^+ influx or activation of Na^+ efflux and sequestering of Na^+ inside the vacuole (Agarwal *et al.,* 2013). The salt overlay sensitive (SOS) pathway regulates the Na^+ flux. This pathway is governed by three genes, namely, *SOS1*, *SOS2*, and *SOS3*. Change in either intra- or extra-cellular sodium (primary signal) is perceived by SOS3, which further activates SOS2, a serine/threonine kinase. The SOS2 in turn activates SOS1, probably via phosphorylation. The SOS1 is a plasma membrane Na^+/H^+ antiporter that promotes Na^+ efflux in the external medium. The SOS pathway also regulates Na^+/H^+ antiport (NXH1) located in vacuolar membrane and compartmentalizes Na^+ in vacuoles to reduce cytosolic Na^+ that is important for salt tolerance in plants.

The inherent variations in physiological aspects for salt tolerance are independent of the growth stage and remain unaffected by the environment. The reliable evaluation of these inherent variations, based on DNA polymorphisms, provides indirect selection of resistant germplasm in salt-free environments. Identification of DNA markers linked to salt tolerance can facilitate marker-assisted selection. A few PCR-based DNA markers, including random-amplified polymorphic DNA (RAPD), sequence-tagged microsatellite site (STMS), single nucleotide polymorphism (SNP), and simple sequence repeat (SSR) have been developed in mungbean (Kumar *et al.,* 2011; Van *et al.,* 2013). Gupta & Gopalakrishna (2013) also reviewed genetic linkage maps, comparative genome mapping, and gene/quantitative trait loci (QTLs) mapping for agronomically important traits of the genus *Vigna*. SSR markers are important owing to their co-dominant inheritance, relative abundance, high reproducibility, polymorphism, and simplicity of genotyping (Tautz & Renz, 1984; Varshney *et al.,* 2005). The number of genome-wide polymorphic SSR markers is limited for mungbean (Kumar *et al.,* 2004; Somta *et al.,* 2008; Tangphatsornruang *et al.,* 2009). Development of SSRs based on SSR-enriched libraries, cloning, and sequencing is expensive and time-consuming (Yu *et al.,* 2009). Searching for SSRs in the conserved regions of *SOS* and *NHX1* genes is a cost-effective approach to discover new DNA markers for mungbean and the related *Vigna* species.

In the present study, gene-specific microsatellite markers (SSRs) for mungbean were identified. In addition, the markers were characterized to identify inherent polymorphisms between salt-tolerant and salt-susceptible genotypes. These genetically diverse genotypes may be helpful in executing further research on salt-stressed mungbean.

Material and methods

Plant materials

Twelve cultivated and wild relatives of mungbean with genetically diverse backgrounds and different adaptations to salinity stress were used in this study (Table 1). In a separate experiment, these genotypes were screened for salt tolerance (data not shown). On the basis of higher root:shoot ratio, K^+/Na^+ ratio, chlorophyll content, photosynthetic rate, seed yield, and membrane permeability, two wild genotypes, ET-528960 (*V. luteola*) and TCR-86 (*V. trilobata*) and five cultivated genotypes, PLM-380, PLM-562, PLM-891, IC-615, and WGG-37, were identified as salt-tolerant variants. A wild genotype, BB-9-2R (*V. sublobata*), and four cultivated genotypes, IC-10492, IC-2056, PLM-32, and K-851, were identified as salt-susceptible variants.

Table 1. Selected Indian mungbean genotypes used in this study

Genotype[1]	Species	Germplasm Bank[2]	Response towards salt stress
IC-10492 (C)	*Vigna radiata*	NBPGR	Salt susceptible
PLM-32 (C)	*Vigna radiata*	NBPGR	Salt susceptible
IC-2056 (C)	*Vigna radiata*	NBPGR	Salt susceptible
PLM-562 (C)	*Vigna radiata*	NBPGR	Salt tolerant
PLM-380 (C)	*Vigna radiata*	NBPGR	Salt tolerant
WGG-37 (C)	*Vigna radiata*	NBPGR	Salt tolerant
PLM-891 (C)	*Vigna radiata*	NBPGR	Salt tolerant
IC-615 (C)	*Vigna radiata*	NBPGR	Salt tolerant
K-851 (C)	*Vigna radiata*	IARI	Salt susceptible
BB-9-2R (W)	*Vigna sublobata*	NBPGR	Salt susceptible
TCR-86 (W)	*Vigna trilobata*	NBPGR	Salt tolerant
ET-528960 (W)	*Vigna luteola*	NBPGR	Salt tolerant

[1] C: cultivated genotypes; W: wild genotypes. [2] NBPGR: National Bureau of Plant Genetic Resources, New Delhi; IARI: Indian Agricultural Research Institute, Div. of Genetics, New Delhi.

Development of microsatellite markers (gene-specific SSRs)

The sequence of the genes involved in the SOS pathway and vacuolar Na^+/K^+ antiport that were available for model plants and related legumes was searched in the GenBank (NCBI) database. The sequences obtained for the *SOS* genes were further searched for homology by BLAST at NCBI. Highly homologous sequences (Table 2) were aligned to find out the conserved regions for the genes (*SOS1, SOS2, SOS3,* and *NHX1*). The conserved regions from *Glycine max* sequences were searched for the presence of SSRs and SSR primers were designed using the WebSAT software (Martins *et al.,* 2009).

DNA extraction, quantification, and PCR analysis with SSR primer pairs

Genomic DNA was extracted from young leaves (18-day-old) of the 12 genotypes of mung bean (Table 1) by using the Gene Elute Plant Genomic DNA Extraction Kit (Sigma) according to the manufacturer's instructions. The isolated DNA was quantified by 0.8% agarose gel electrophoresis in 1X Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer using a ladder of known concentration (100 ng μL^{-1}) of phage lambda DNA (Fermentas). Ethidium bromide-stained gel was visualized and documented by using the Gel Documentation System (CFW-1312M; BioRad). The reaction mixture was prepared in 20 µL volume containing 30 ng of template DNA, 1X final concentration of *Taq* buffer B, 25 mM $MgCl_2$, 5.0 µM of each forward and reverse primer, 2.5 mM of deoxynucleotide triphosphates (dNTPs) and 0.03 U of Taq, DNA polymerase (5 U μL^{-1}) and run on a thermo cycler (Gene AMP PCR System 9700; Applied Biosystems, USA). The PCR conditions used for amplification of SSRs consisted of initial denaturation at 94°C for 45 s, followed by 38 cycles, each consisting of denaturation at 94°C for 30 s, annealing at 50-60°C for 1 min, and elongation at 72°C for 45 s, with a final extension at 72°C for 10 min. The PCR amplified products were resolved on 3.0% agarose gel electrophoresis in 1X TAE buffer at a constant power supply with known concen-

Table 2. Plant sources used for genetic alignment to find out the conserved sequences in the candidate genes of SOS pathway

Candidate genes involved	Plant sequences used for genetic alignment[1]
SOS1 (sodium proton plasma-membrane antiporter)	XM_003532633, GLYMA08G09730, EF219135
SOS2 (protein kinase)	AK285983, AB303675, GLYMA02G44380
SOS3 (calcium sensor)	BT098815, GLYMA06G13420, XM_003602196
NHX1 (sodium proton vacuolar antiporter)	JN656211, JN641304, AY972078, GLYMA20G37370

[1] NCBI accessions.

tration of DNA ladder (50 ng μL^{-1}; Fermentas) as a molecular weight marker. Ethidium bromide-stained gels were visualized and photographed by using the Gel Documentation System.

Statistical analysis

The reproducible DNA bands specific to each primer set were scored manually in all genotypes based on the positions of the bands relative to the known molecular weight ladder. The bands were recorded as 0 or 1 depending on their absence and presence, respectively, in the data matrix. Null allele was assigned to the genotype for the SSR locus whenever the amplification product was difficult to detect for a particular genotype/marker combination. The binary data matrix was subjected to an unweighted pair group method with an arithmetic mean (UPGMA) cluster analysis by NTSYS-pc software (version 2.1.1.2; Exeter Corporation). The polymorphism information content (PIC) value for each SSR locus was calculated as [$PIC = 1 - (\Sigma p_i^2)$], following the method of Anderson *et al.* (1993). The resolving power (Rp) for each primer was calculated as $Rp = \Sigma Ib$, where Ib (band informativeness) is calculated as $[1 - (2 \times (0.5 - p)]$, with p being the proportion of the genotype of different *Vigna* species containing that band (Prevost & Wilkinson, 1999). Marker index of each SSR primer was calculated using average diversity index (DI_{av}) as $MI_{DI} = DI_{av} \times EMR$, where $DI_{av} = 1 - \Sigma p_i^2$ and effective multiplex ratio (EMR) = $np \times \beta$, which is the product of number of polymorphic alleles (np) and fraction of the polymorphic markers (β).

Results

Amplification of SSR markers and polymorphism in mungbean genotypes

The developed microsatellite markers (Table 3) showed significant amplification in the investigated genotypes and produced 124 reproducible putative alleles with an average value of 3.26 alleles/locus, indicating their efficient transferability and presence of greater magnitude of diversity among the plant materials. All SSR primers produced greater number of alleles in the salt-tolerant genotypes as compared with the salt-susceptible genotypes. The SSR9293 produced one unique allele (180 bp) in all salt-tolerant genotypes; the SSR3435 produced one unique allele (312 bp) in all salt-tolerant cultivated and wild genotypes (except for PLM562); and the SSR6263 produced two unique alleles (320 and 410 bp) only in the cultivated salt-tolerant genotypes.

The SSR primers exhibited significant polymorphism between wild and cultivated genotypes and also within the salt-tolerant and -susceptible cultivars. The five primers SSR4647, SSR5455, SSR6465, SSR7879, and SSR9899 did not produce any polymorphic allele within the wild genotypes, where polymorphism obtained by 33 SSR primers was 86.84%. Out of 124 putative alleles, 100 were polymorphic between the wild and cultivated genotypes (inter-specific polymorphism 80.65%), 65 were polymorphic between the salt-tolerant and salt-susceptible cultivated genotypes (intra-specific polymorphism 52.42%), and 52 were polymorphic between the salt-tolerant and salt-susceptible wild genotypes (inter-specific polymorphism 41.94%). The number of polymorphic alleles ranged from 1 to 4, with an average value of 2.63/SSR loci. The PIC value ranged from 0.326 to 0.875, with an average value of 0.671, confirming the potential of the developed microsatellite markers in the genetic analysis study for the selection of most divergent parental lines for the genetic improvement of mungbean for saline soils. The power of resolution determines the information content of any primer. High resolving power (Rp = 3.76) was obtained for SSR6263, whereas a high value for marker index (MI = 3.00) was obtained for SSR4445. The Rp, MI, and PIC values obtained for the developed SSRs markers are given in Table 4.

Genetic similarity and distances among mungbean genotypes

The genetic similarity values were obtained by subjecting the marker binary data to cluster analysis (Table 5). The maximum similarity value of 0.56 was found between two salt-tolerant cultivated genotypes (PLM562 and PLM380) of mungbean, followed by 0.44 between WGG37 and PLM562, which depicts their close relatedness. The wild genotypes (TCR86, ET528960, and BB 9 2R) showed less genetic similarity with the cultivated accessions, and the order of similarity obtained was as follows: *V. trilobata* (0.02-0.27) > *V. luteola* (0.03-0.16) > *V. sublobata* (0.00-

Table 3. List of the gene-specific simple sequence repeats markers (SSRs) developed for mungbean from conserved regions of the genes (*Glycine max*) involved in salt overlay sensitive (SOS) signaling pathway

Primer	FW-Primer Sequence (5'-3')	RW-Primer Sequence (5'-3')	Motifs	Size range (min-max)	Tm (°C)	Candidate gene involved
SSR3031	CAAGCATCCAAATGTTGTTCG	TTCCTCCCATCGAAATATACCA	(TC)8	320-390	52	*SOS2*
SSR3233	AAGGCTAAACGAGACAGAAGACA	GAAAGGAAGCCAACAAGATTCA	(A)11	380-450	50	*SOS2*
SSR3435	CATGGAAGGATGAGTGAAAATG	TTACCCTAACTTGCTGGGAGAG	(TC)6	312	60	*SOS2*
SSR3637	CCCTTTTCGTCTCTCTCTCTCA	ATGATTACCCTAACTTGCTGGG	(CA)6	200-340	52	*SOS2*
SSR3839	TTAGTTTCATTCAGCACAAGCC	AACTTAAAAGCCTAACTCACCCTG	(T)11,(CAATT)2	320-450	51	*SOS3*
SSR4041	CTAAGCACGATAATTGAAGGGG	GTCTCTCCAAGAACCATCCG	(TATTT)2	280-300	60	*SOS1*
SSR4243	CTGTTTCTGCATGTGATGGTTT	CAAAAGCAAGACCCATTCCTAC	(TATTT)2	250-410	56	*SOS1*
SSR4445	TAAGGGTGAAAGTCAACAAAGC	CAGACAGGACAAACAGACATGA	(CTCCC)2	290-450	55	*SOS1*
SSR4647	TGTTTACAAGTGAGATAGGTGCTGT	AACTAAGGTGGGCTAGGGTGA	(TTTTA)2,(GGAATA)2	300-380	52	*SOS1*
SSR4849	ACACATTTGCAGACAACCAATC	TGAGAGAGAGAGACGAAAAGGG	(TTCA)3,(ATAGGA)2	350-475	56	*SOS2*
SSR5051	CATTGCCTTCTGACTTTTCCTT	ACCTCCTAACTCATCCATCCCT	(CTTAT)2,(TGATT)2	300-420	52	*SOS2*
SSR5253	AGAGAAGTGGGGAAAATGCTTA	ATTGATGGCACAGGATAACTGA	(AGCAT)2	340-370	52	*SOS2*
SSR5455	GCGTGACATTATTGAAGTTGGA	ATCGACATTTTGGGAAGAGAGA	(TTGGA)2,(TTTAC)2	390-410	53	*SOS2*
SSR5657	TAACCTTCTGCATTTCCTTGGT	AGACAGCTTCAACATCATCGAG	(AAAGA)2	350-400	51	*SOS2*
SSR5859	ATTACGGGCATTATTCTCCCTT	GGGGTGCGTGTACCTATCTTTA	(ATACA)2	340-450	53	*SOS3*
SSR6061	CACAGGGTGAGTTAGGCTTTTA	AATGAAACAGTACGAGTGCCAG	(AGATG)2	380-400	52	*SOS3*
SSR6263	TTTCCCATGTAGGGACCAAA	CCACCACTAAACAGCAAAATGA	(CTTTT)2	260-410	52	*NHX1*
SSR6465	GGCAGCATTTCTTTGTGTAGTG	GTGAACAGAGTGCCCATATCAG	(GGGTT)2,(TGTTT)2	350-440	55	*NHX1*
SSR6667	TTCTTTGCAGACATCCTTTGTG	CAGAAGGCAGAACAGGGTTTTA	(CTTGTA)2	370-460	51	*NHX1*
SSR6869	ATTCCTGCTTCATCCAATCTCA	AACATCATAAGAGCAACCTCGC	(TTGTTA)2,(TTA)4	350-400	50	*NHX1*
SSR7071	CAACCTTGTCTTTTGTTGCTGA	AGTTGCTACAGATGTTCCAGGG	(TCTTTC)2	380-425	53	*NHX1*
SSR7273	TCTAGGTTATCATTTGGTGGGC	AATGGACACTGGCTAAGGATGT	(GCAAT)2,(TTTGT)2	310-400	53	*NHX1*
SSR7475	GCAAAAGCTCGCATATTCTTGT	AAAACAAAACACCTCCCCTTCT	(CAATT)2,(ATTTT)2	160-190	53	*NHX1*
SSR7677	GAGGGTGTTGTGAATGATGCTA	CCTGTCTGCAAGGTAATGAAGA	(ATA)4	280-325	55	*NHX1*
SSR7879	ACATTGGCAGGTATCTTTGCAT	AATTGAGATTGGATGAAGCAGG	(TGTTT)2	150-250	56	*NHX1*
SSR8081	GTCGGGGCACACTTCACTAA	GGACACTGGCTAAGGATGTCAG	(GCAAT)2	120-160	56	*NHX1*
SSR8283	GGCACACTTCACTAAGAAGCAA	AATGGACACTGGCTAAGGATGT	(TTTGT)2	110-200	52	*NHX1*
SSR8485	ACTGGAGTTGGAAAACATCACT	ACCATCACATGCAGAAACAGTA	(CTGTTG)2	280-325	52	*SOS1*
SSR8687	TATATTTTATTGGCTCCCCTCC	GGCTTTTGGCTTAGAGTCAGAT	(CTCCCT)1	230-260	53	*SOS1*
SSR8889	CTCCCTCTCCCTGGATATTCTT	GGCTTTTGGCTTAGAGTCAGAT	(TGTTT)2	220-310	53	*SOS1*
SSR9091	GTGCATTGTAGTCGGAGCATTA	ACTGATAAGGAAAGTGCCAAGG	(TTGGA)2	120-240	57	*SOS1*
SSR9293	CTTATATTTGGTCTGGCCCTTG	CTCATCATCTCCAAGTTCACCA	(TGTTT)2	120-180	57	*SOS1*
SSR9495	TTCCAAAACCAGACAAAGCCTA	GCTACTTCTCTCCTGATCTGCAA	(AAGTC)2	180-250	57	*SOS2*
SSR9697	CTGGTATATTTCGATGGGAGGA	TCTGTCTCGTTTAGCCTTGGAT	(TAAAAA)2	280-310	57	*SOS2*
SSR9899	CCTACATGAAAATATGTGGCTG	GCAGAGAGTGAAGCAAACAAT	(AGCAT)2,(AAAGT)2	200-240	55	*SOS2*
SSR100101	GGCCTTGCATGAACTCTACA	GGACATCTCATCTAAACTGGAAAAC	(CAATT)2	100-125	51	*SOS3*
SSR102103	ATGAGTCGGATCTTGAGCTTTC	TCCATCACCGTTTATATCAGCA	(ATGAT)2	180-220	52	*SOS3*
SSR104105	TTTGCATTTTGGTCTCTACAGC	CACCCACTATTGCTCAAACAAA	(TTTAA)2	290-350	52	*SOS3*

0.07). The lowest similarity value obtained between the salt-tolerant and salt-susceptible genotypes depicted the presence of high genetic variations among the investigated genotypes, which is a key requirement for the genetic augmentation of any crop through breeding. The genetic dissimilarity was calculated from the Jaccard similarity values; the highest genetic distance (≥ 0.86) was observed between the wild salt-tolerant and all the other salt-susceptible genotypes.

Clustering

Cluster analysis grouped the 12 mungbean genotypes into two clusters (Fig. 1): Cluster I consisted of single wild genotype BB 9 2R (*V. sublobata*), which is a highly salt-susceptible wild relative of mungbean; Cluster II consisted of the remaining 11 wild and cultivated genotypes in three different sub-clusters. Subcluster IIA consisted of two wild genotypes TCR86 (*V. trilobata*) and ET528960 (*V. luteola*), both of which

Table 4. Details of the observations recorded (resolving power, marker index, and PIC values) among 12 mungbean genotypes using 38 SSR markers

Marker code	Resolving power (Rp)	Marker index (MI)	PIC values
SSR3031	1.00	1.75	0.875
SSR3233	1.33	1.53	0.764
SSR3435	1.00	0.75	0.750
SSR3637	1.17	2.60	0.868
SSR3839	1.83	2.15	0.715
SSR4041	1.67	1.06	0.528
SSR4243	2.00	2.29	0.764
SSR4445	2.33	3.00	0.750
SSR4647	1.67	1.56	0.778
SSR4849	1.50	2.48	0.826
SSR5051	1.50	2.31	0.771
SSR5253	1.17	1.60	0.799
SSR5455	1.67	1.31	0.653
SSR5657	1.00	1.75	0.875
SSR5859	2.33	2.13	0.708
SSR6061	1.00	1.75	0.875
SSR6263	3.76	1.94	0.486
SSR6465	3.50	1.31	0.438
SSR6667	2.17	2.10	0.701
SSR6869	2.33	1.25	0.625
SSR7071	2.33	1.63	0.542
SSR7273	3.50	0.65	0.326
SSR7475	1.33	2.38	0.792
SSR7677	2.33	1.03	0.514
SSR7879	2.17	2.23	0.743
SSR8081	1.67	1.47	0.736
SSR8283	3.33	1.92	0.639
SSR8485	2.17	1.94	0.646
SSR8687	2.67	1.06	0.528
SSR8889	2.67	1.50	0.500
SSR9091	2.33	2.83	0.708
SSR9293	1.67	1.19	0.597
SSR9495	2.33	1.94	0.486
SSR9697	2.17	1.81	0.604
SSR9899	2.00	1.63	0.542
SSR100101	1.33	1.44	0.722
SSR102103	1.83	1.43	0.715
SSR104105	2.17	1.81	0.604
Total alleles	1.998 (Rpav)	1.750 (MIav)	0.671 (PICav)

showed high adaptability under salt stress in our earlier study (data not shown). Sub-cluster IIB consisted of 5 cultivated genotypes (PLM562, PLM380, WGG37, IC615, and PLM891) in one group, all being salt-tolerant, whereas 4 cultivated genotypes (IC10492, PLM32, IC2056, and K851) were clustered in another group and were all salt-susceptible. The SSR analysis differentiated the wild and cultivated genotypes into separate clusters, indicating the existence of diversity at molecular level among different *Vigna* species. The dendrogram exposed the allelic richness of all the clusters of different sizes for purpose of grouping different mungbean genotypes.

Discussion

Assessment of inherent variability is the foremost requirement for the development of salt-resistant and high-yield varieties of mungbean. All microsatellite markers used in this study showed considerable amplification and highly significant polymorphism (86.84-100%) within the analyzed genotypes from different *Vigna* species. The obtained polymorphism is significantly higher as compared to that reported earlier (Chaitieng, 2006; Somta *et al.,* 2009; Sudha *et al.,* 2012). The SSR markers were developed from the candidate gene *Glycine max*, which is involved in the SOS pathway. Amplification of a particular locus of a genome using primers designed from other related species depends on the evolutionary distance between the two species and also on the evolution rate of the genomic sequence where the primer sequence is located (Souframanien & Gopalkrishna, 2009; Dikshit *et al.,* 2012). High allelic frequency obtained in the salt-tolerant genotypes as compared with the salt-susceptible genotypes indicates that the SSRs used produced more specific bands in the tolerant genotypes. The average number of polymorphic alleles (2.63) suggests considerable differences in the allelic diversity among all loci, validating the usefulness of these primers in the genetic studies of mungbean or other related legumes or crops. The PIC value (0.326-0.875) obtained in this study was significantly higher than that reported earlier for SSR markers developed for mungbean (Kumar *et al.,* 2002a,b; Gwag *et al.,* 2006; Somta *et al.,* 2009).

The differences in number of alleles between the salt-tolerant and salt-susceptible genotypes reported in the present study may be due to their different species origin. Wild relatives are considered as natural sources of genetic variations that can be used to create variability in the mungbean cultivars with a narrow genetic base through hybridization (Kumar *et al.,* 2004; Pandiyan *et al.,* 2012a,b). Different *Vigna* species also showed different adaptations toward physiographic, edaphic, and environmental factors that affect both plant growth and economic yield.

Table 5. Jaccard similarity coefficient values based on 38 SSRs primers sequence data among 12 mungbean genotypes

Genotype	*V. luteola*	*V. sublobata*	PLM380	WGG37	PLM562	IC2056	PLM32	PLM891	IC10492	K851	IC615	*V. trilobata*
ET-528960	1.00											
BB-9-2R	0.03	1.00										
PLM-380	0.12	0.05	1.00									
WGG-37	0.09	0.02	0.37	1.00								
PLM-562	0.16	0.03	0.56	0.44	1.00							
IC-2056	0.07	0.03	0.16	0.23	0.14	1.00						
PLM-32	0.06	0.00	0.17	0.28	0.18	0.27	1.00					
PLM-891	0.09	0.03	0.26	0.15	0.25	0.09	0.11	1.00				
IC-10492	0.04	0.03	0.16	0.27	0.17	0.22	0.32	0.02	1.00			
K-851	0.07	0.06	0.14	0.07	0.19	0.16	0.05	0.18	0.14	1.00		
IC-615	0.10	0.06	0.22	0.16	0.19	0.23	0.14	0.24	0.12	0.13	1.00	
TCR-86	0.14	0.07	0.21	0.13	0.27	0.04	0.06	0.05	0.10	0.14	0.02	1.00

The repetition of the primer-binding sites in multiple copies of genes in wild accessions may account for multiple bands. Various genomic SSR markers also showed multiple bands (not stutter bands) in wild accessions with primer-binding sites of different sequences (Decroocq *et al.,* 2003). The markers with PIC values of ≥0.5 are highly informative and widely useful in discriminating the polymorphism rate of a marker at a particular locus (De Woody *et al.,* 1995). The highest genetic distance (≥0.86) observed between the wild salt-tolerant and all other salt-susceptible genotypes revealed that the analyzed genotypes harbored sufficient genetic variations due to differences in their origin, ecotype, and speciation (Ram *et al.,* 2007; Senguttuvel *et al.,* 2010). Genotypes showing genetic distance values of ≥0.70 can be considered as highly genetic divergent for the trait of interest that can facilitate the selection of parental lines for breeding salt-tolerant variants.

Cluster analysis grouped the salt-tolerant and salt-susceptible wild and cultivated genotypes of mungbean in separate clusters with respect to their similarity values. The low genetic similarity obtained in this study among different *Vigna* species (Table 5) and the

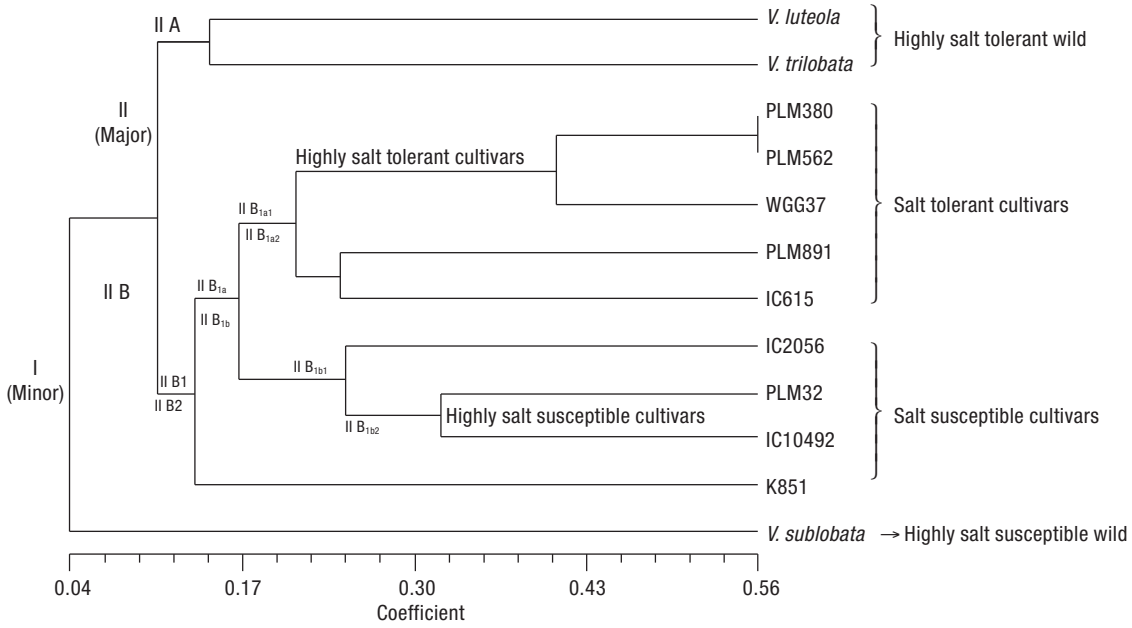

Figure 1. Dendrogram constructed using unweighted pair group method with arithmetic average (UPGMA; Jaccard similarity). Cluster analysis based on 38 SSR markers.

formation of more clusters through cluster analysis of the SSRs data also confirmed the presence of high diversity among the selected genotypes at the molecular level, which is the key requirement for the genetic improvement of valuable agronomic traits in vulnerable crops through breeding (Vijayanand *et al.,* 2009; Pandiyan *et al.,* 2010, 2012a,b). This observation confirmed the effectiveness of the developed SSR markers to explore the genetic variations within mungbean cultivars possessing a narrow genetic base as well as within related legumes or crop species (Kumar *et al.,* 2004). High resolution power (Rp) and marker index (MI) further confirmed that these SSR markers were highly informative. Therefore, the wild relatives of mungbean can be used for the improvement of the cultivated genotypes for salt tolerance by broadening their genetic base. Assessment of the diversity among the investigated genotypes would be of huge importance for designing breeding strategies for the improvement of quantitative trait regulating salt tolerance (Pandiyan *et al.,* 2012a,b). The markers used in the present study were developed from the conserved regions of the genes involved in cellular signaling pathway conferring ionic homeostasis under salt stress. Clustering of involved genotypes in distinct groups clearly indicate their potential for the selection of highly divergent parental lines for breeding salt-tolerant variants. The sequence conservation during evolution is the basis of cross-species utilization of SSR primers in assessing phylogenetic relationships across species and genera (Decroocq *et al.,* 2003). High polymorphism obtained for these SSR markers may reveal variations for the candidate genes involved at the sequence level among mungbean cultivars and other *Vigna* species.

Soil salinity is a major constraint of mungbean production in Southeast Asia. The SSR markers developed in this study are highly informative and can be used effectively to explore the inherent variations for salt tolerance among mungbean cultivars possessing a narrow genetic base. The conserved regions may be promising and useful for the development of cost-effective microsatellite markers (SSRs) for mungbean. As the SSRs are candidate genes, variations may be present at the gene sequence level. Based on the analysis, the genotypes PLM380, PLM562, PLM891, IC615, and WGG37 can be used as male parents (♂) and the genotypes IC10492, PLM32, K851, IC2056, and BB 9 2R (*V. sublobata*) can be used as female parents (♀) in breeding for salt-tolerant variant. The wild genotypes (*V. luteola* and *V. trilobata*) can also be taken as male parents (♂) as they can be the source of major genes responsible for salt tolerance to be transferred through breeding. The findings of the present study will help implement further research and aid in the molecular identification of mungbean cultivars and marker-assisted breeding in mungbean. The microsatellite markers used in this study can be used for assessment of genetic diversity, construction of linkage maps, identification of true hybrids in mungbean or other related legumes as well as add to the available mungbean markers. These markers can also be used in genetic analysis of large number of mungbean genotypes or related *Vigna* species for their further validation.

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References

Agarwal PK, Shukla PS, Gupta K, Jha B, 2013. Bioengineering for salinity tolerance in plants: state of the art. Mol Biotechnol 54: 102-123.

Ajibade SR, Weeden NF, Chite SM, 2000. Inter simple sequence repeat analysis of genetic relationships in the genus *Vigna*. Euphytica 111: 47-55.

Anderson JA, Churchill GA, Autrique JE, Tanksley SD, Sorrells ME, 1993. Optimizing parental selection for genetic linkage maps. Genome 36: 181-186.

AVGRIS, 2012. AVRDC Vegetable Genetic Resources Information System. Available at http://203.64.245.173/search_result.asp?VINO=&ACCNO=&TEMPNO=&SPECIE=VIGNARADIATA&PEDCUL=&SUBTAX=&COUNTR=&NOTES= [accessed on 25 May 2012].

Bisht IS, Bhat KV, Lakhanpaul S, Latha M, Jayan PK, Biswas BK, Singh AK, 2005. Diversity and genetic resources of wild *Vigna* species in India. Genet Resour Crop Evol 52: 53-68.

Chaitieng B, Kaga A, Tomooka N, Isemura T, Kuroda Y, Vaughan DA, 2006. Development of a black gram [*Vigna mungo* (L.) Hepper] linkage map and its comparison with an azuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi] linkage map. Theor Appl Genet 113: 1261-1269.

De Woody JA, Honeycutt RL, Skow LC, 1995. Microsatellite markers in white tailed deer. J Hered 86: 317-319.

Decroocq V, Favé MG, Bordenave L, Decroocq S, 2003. Development and transferability of apricot and grape EST microsatellite markers across taxa. Theor Appl Genet 106: 912-922.

Dikshit HK, Singh D, Singh A, Jain N, Kumari J, Sharma TR, 2012. Utility of adzuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi] simple sequence repeat (SSR) markers in genetic analysis of mungbean and related *Vigna* spp. Afr J Biotechnol 11: 13261-13268.

Gupta SK, Gopalakrishna T, 2013. Advances in genome mapping in orphan grain legumes of genus *Vigna*. Indian J Genet Pl Br 73: 1-13.

Gwag JG, Chung JW, Chung HK, Lee JH, Ma KH, Dixit A, Park YJ, Cho EG, Kim TS, Lee SH, 2006. Characterization of new microsatellite markers in mung bean, *Vigna radiata* (L.). Mol Ecol Notes 6: 1132-1134.

Kumar SV, Tan SG, Quah SC, Yusoff K, 2002a. Isolation of microsatellite markers in mungbean, *Vigna radiata*. Mol Ecol Notes 2: 96-98.

Kumar SV, Tan SG, Quah SC, Yusoff K, 2002b. Isolation and characterization of seven tetranucleotide microsatellite loci in mungbean, *Vigna radiata*. Mol Ecol Notes 2: 293-295.

Kumar S, Gupta S, Chandra S, Singh BB, 2004. How wide is the genetic base of pulse crops? In: Pulses in new perspectives (Masood A, Singh BB, Kumar S, Vishwa D, eds). Indian Institute of Pulses Research, Kanpur, India. pp: 211-221.

Kumar J, Choudhary AK, Solanki RK, Pratap A, 2011. Towards marker-assisted selection in pulses: a review. Plant Breeding 130: 297-313.

Lambrides CJ, Godwin ID, 2007. Mungbean. In: Pulses, sugar and tuber crops. Series: Genome mapping and molecular breeding in plants (Kole C, ed). Springer Berlin Heidelberg. Volume 3. pp: 69-90.

Martins WS, Lucas DCS, de Souza Neves KF, Bertioli DJ, 2009. WebSat – A web software for microsatellite marker development. Bioinformation 3: 282-283.

Pandiyan M, Senthil N, Sivakumar P, Muthiah AR, Ramamoorthi N, 2010. Genetic diversity analysis among greengram genotypes using RAPD markers. Elect J Plant Breed 1: 466-473.

Pandiyan M, Senthil N, Packiaraj D, Gupta S, Nadarajan N, Pandian RT, Suresh R, Jagadeesh S, 2012a. Characterisation and evaluation of 646 greengram (*Vigna radiata*) genotypes for constituting core collection. Wudpecker J Agric Res 1: 294-301.

Pandiyan M, Senthil N, Anitha M, Raveendran M, Sudha M, Latha M, Nagarajan P, Toomoka N, Balasubramanian P, 2012b. Diversity analysis of *Vigna* sp through morphological markers. Wudpecker J Agric Res 1: 335-340.

Polhill RM, Van der Maesen LJG, 1985. Taxonomy of grain legumes. In: Grain legume crops (Summerfield RJ, Roberts EH, eds). Collins, London, UK. pp: 3-36.

Prevost A, Wilkinson MJ, 1999. A new system of comparing PCR primers applied to ISSR finger printing of potato cultivars. Theor Appl Genet 102: 440-449.

Ram SG, Thiruvengadam V, Vinod KK, 2007. Genetic diversity among cultivars, landraces and wild relatives of rice as revealed by microsatellite markers. J Appl Genet 48: 337-345.

Saha P, Chatterjee P, Biswas AK, 2010. NaCl pretreatment alleviates salt stress by enhancement of antioxidant defense and osmolyte accumulation in mungbean (*Vigna radiata* L. Wilczek). Indian J Exp Biol 48: 593-600.

Salim M, Pitman MG, 1988. Salinity tolerance of mungbean (*Vigna radiata* L.): seed production. Biol Plantarum 30: 53-57.

Senguttuvel P, Raveendran M, Vijayalakshmi C, Thiyagarajan K, Kannan Bapu JR, Viraktamath BC, 2010. Molecular mechanism of salt tolerance for genetic diversity analysed in association with Na^+/K^+ ratio through SSR markers in rice (*Oryza sativa* L.). Int J Agric Res 5: 708-719.

Singh DP, Singh BB, 2011. Breeding for tolerance to abiotic stresses in mungbean. J Food Legumes 24: 83-90.

Somta P, Srinives P, 2007. Genome research in mungbean [*Vigna radiata* (L.) Wilczek] and blackgram [*V. mungo* (L.) Hepper]. ScienceAsia 33: 69-74.

Somta P, Musch W, Kongsamai B, Chanprame S, Nakasathien S, Toojinda T, Sorajjapinun W, Seehalak W, Tragoonrung S, Srinives P, 2008. New microsatellite markers isolated from mungbean [*Vigna radiata* (L.) Wilczek]. Mol Ecol Resour 8:1155-1157.

Somta P, Seehalak W, Srinives P, 2009. Development, characterization and cross-species amplification of mungbean (*Vigna radiata*) genic microsatellite markers. Conserv Genet 10: 1939-1943.

Souframanien J, Gopalkrishna T, 2009. Cross-species amplification of microsatellite loci and diversity analysis in blackgram. J Food Legumes 22: 11-17.

Sudha M, Anusuya P, Mahadev NG, Karthikeyan A, Nagarajan P, Raveendran M, Senthil N, Pandiyan M, Angappan K, Balasubramanian P, 2012. Molecular studies on mungbean [*Vigna radiata* (L.) Wilczek] and ricebean [*Vigna umbellata* (Thunb.)] interspecific hybridisation for *Mungbean yellow mosaic virus* resistance and development of species-specific SCAR marker for ricebean. Arch Phytopathol Plant Prot 46: 503-517.

Tangphatsornruang S, Somta P, Uthaipaisanwong P, Chanprasert J, 2009. Characterization of microsatellites and gene contents from genome shotgun sequences of mungbean [*Vigna radiata* (L.) Wilczek]. BMC Plant Biol 9: 137-148.

Tautz, D, Renz P, 1984. Simple sequences are ubiquitous repetitive components of eukaryotic genome. Nucleic Acids Res 12: 4127-4138.

Tomooka N, Yoon MS, Doi K, Kaga A, Vaughan D, 2002. AFLP analysis in diploid species in the genus *Vigna* subgenus *Ceratotropis.* Genet Resour Crop Evol 49: 521-530.

Undal VS, Thakare PV, Chaudhari US, Deshmukh VP, Gawande PA, 2011. Estimation of genetic diversity among wild *Vigna s*pecies revealed by RAPD markers. Ann Biol Res 2: 348-354.

Van K, Kang YJ, Han KS, Lee YH, Gwag JG, Moon JK, Lee SH, 2013. Genome-wide SNP discovery in mungbean by Illumina HiSeq. Theor Appl Genet 126: 2017-2027.

Varshney RK, Graner A, Sorells ME, 2005. Genic microsatellite markers in plants: features and applications. Trends Biotechnol 23: 48-55.

Vijayanand V, Senthil N, Vellaikumar S, Paramathma M, 2009. Genetic diversity of Indian Jatropha species as revealed by morphological and ISSR markers. J Crop Sci Biotech 12: 115-120.

WIEWS, 2012. Germplasm report. World Information and Early Warning System on PGRFA. http://apps3.fao.org/wiews/germplasm_report.jsp?i_STID=&i_RC=&i_VINST=&i_LT=N&i_d=false&i_j=&i_r=0&i_a=Navigate&i_t=&i_m=true&i_f=&i_op=&i_np1=&i_np2=&i_FC=&i_FG=&i_FP=&i_s=N&i_UP=N&i_TI=Y&i_TC=Y&i_TR=Y&i_SO=N&i_DA=&i_CHLE=&i_SELE=&i_CHGP=&i_SEGP=&i_CHPG=&i_SEPG=&i_CHGE=&i_SEGE=&i_CHOT=&i_SEOT=&i_All=&i_l=EN [accessed on 25 May 2012].

Win T, Oo AZ, Hirasawa T, Ookawa T, Yutaka H, 2011. Genetic analysis of Myanmar *Vigna* species in responses to salt stress at the seedling stage. Afr J Biotechnol 10: 1615-1624.

Yu JW, Dixit A, Ma KH, Chung JW, Park YJ, 2009. A study on relative abundance, composition and length variation of microsatellites in 18 underutilized crop species. Genet Resour Crop Evol 56: 237- 246.