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...
Microsatellite markers for mungbean

(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.
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 MgCl2, 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-

| Genotype | Species | Germplasm Bank | 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.

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 alignment1 |
|--------------------------|------------------------------------------|
| 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.
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; Exeter Corporation). The polymorphism information content (PIC) value for each SSR locus was calculated as [PIC = 1 – (Σpi²)], following the method of Anderson et al. (1993). The resolving power (Rp) for each primer was calculated as Rp = ΣIb, where Ib (band informativeness) is calculated as [1 – (2 × (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 (DIav) as MI = DIav × EMR, where DIav = 1 – Σpi² and effective multiplex ratio (EMR) = np × β, 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%).

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 WG37 and PLM562, which depicts their close relatedness. The wild genotypes (TCR, ET58960, 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-
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 ($\geq 0.86$) was observed between the wild salt-tolerant and all the other salt-susceptible genotypes.

### 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  | CAAGCATCCCAAGTTGTTCG       | TTCTCCACTCGAAATATACCA      | (TC)8  | 320-390              | 52      | SOS2                   |
| SSR3233  | AAGGCTAAAGCAGACAGAAGACA    | GAAAGGAGACCAAGACAGATCCA    | (A)11  | 380-450              | 50      | SOS2                   |
| SSR3435  | CATGGAAGGTAGTGAAGAAAAAG    | TTACCTTGAACTTGCTGGAGG      | (TC)6  | 312                  | 60      | SOS2                   |
| SSR3637  | CCCCCTGTCCTCTCTCTCTCA      | AGTTAGAATCTTAATCTGCATG     | (CA)6  | 200-340              | 52      | SOS2                   |
| SSR3839  | TTATGTTTATGAGCAACAGG       | AACTAAAGGGCTAACAGCCTCACTC | (T)11,(CAATT)2 | 320-450 | 51 | SOS3                   |
| SSR4041  | CTCAGGACAGTAAATGGAAGAGGG   | GTCTCCTAAAGAAGCAGTACCC    | (TATT)2 | 280-300              | 60      | SOS2                   |
| SSR4243  | CTGGTTTCAGTATGTATGTATTTT  | CAAAGACAGACATCCTTTAC      | (TATT)2 | 250-410              | 56      | SOS2                   |
| SSR4445  | TAAGGTTGAAGTCAACAGAAGC     | CAGAAGACAGAAGAAGAAGAGCA   | (CTCC)2 | 290-450              | 55      | SOS2                   |
| SSR4647  | GTTTTACAAGTGAATGATGCTG    | AACTAAAGGGCTAGGCTAGG      | (TTTA)2,(GAAT)2 | 300-380 | 52 | SOS2                   |
| SSR4849  | ACACATTGGACAGACACACT      | TGAGAGAGAGAGAACAGAAGAGG   | (TTCA)3,(ATAGGA)2 | 350-475 | 56 | SOS2                   |
| SSR5051  | CATGACTCTGCTCTCTCTCTTG    | AACCTTCTAAGCTACCTCCCTCT   | (CTAT)2,(TGATT)2 | 300-420 | 52 | SOS2                   |
| SSR5253  | AGAAGAAGGAAAAAGCTCTCA    | ATGAGAATGGAGAAAGAAGAGG    | (AGCAT)2 | 340-370              | 52      | SOS2                   |
| SSR5455  | GCGTACATCTTTGGAGTAGGAGA   | ATGAGAGAGAGAGAGAGAGAGA   | (TTGGA)2,(TTTAC)2 | 390-410 | 53 | SOS2                   |
| SSR5657  | TAACCTTCTGACTTCTCTGTGT   | AGAAGACAGACATCCTTTAGG     | (AAAGA)2 | 340-500              | 51      | SOS2                   |
| SSR5859  | ATTACGGGCAATTACCTTCTTTA  | GGGTCTCTGCACCTAATCTTCTT   | (ATACA)2 | 340-450              | 53      | SOS2                   |
| SSR6061  | ACACAGGTTAGTGGACTTCTTGA  | AATGGAACAGTCAAGTGCTGAG    | (AGATG)2 | 380-400              | 52      | SOS2                   |
| SSR6263  | TTCTCTGACATGAGAGACAAA    | CTACCACTACACACAGAAGAAGTA  | (CTTT)2 | 260-410              | 52      | NIXI2                  |
| SSR6465  | GGCAGCATTGGCTCTTGTAGT    | GTAGAGAGAGAGAGAGAGAGAGA  | (GGTTT2,(TTGT)2 | 350-440 | 55 | NIXI2                  |
| SSR6667  | TTCTTCTGACATGACCTTTGGCT | CAGAGGACAGAAGAGGAGTTTA   | (CTTGA)2 | 370-460              | 51      | NIXI2                  |
| SSR6869  | ATCTCTGCTATCTCATATCTCA   | AACATCAAGACAGACCTCCCTC   | (TTGTTA)2,(TATA)4 | 350-400 | 50 | NIXI2                  |
| SSR7071  | CAACCTTCTGTCTTCTGTGGT    | AGTGCAGGACTGCAAGGATGACT   | (GCAAT2,(TTTG)2 | 310-400 | 53 | NIXI2                  |
| SSR7273  | TCTAGGTCTATCTTTTGTGGGC  | AATTGGAAGAGAGAGAGAGAGA   | (CTTTT)2 | 260-410              | 52      | NIXI2                  |
| SSR7475  | GCCAAATGTCACATTTTCTGTG   | AATTTTAAAGAAGACATCCTTCTCT | (CAAT)2,(ATT)2 | 160-190 | 53 | NIXI2                  |
| SSR7677  | GAGGCTTTGGTGAAGATGCTA   | CCTGCTGCTCAAGGATAAGAAGA   | (ATA)4  | 120-260              | 55      | NIXI2                  |
| SSR7879  | ACAGGCTGATTGAGAAGACAA   | AATTTTAAGAGGTAGAAGAGG     | (TGTG)2 | 150-250              | 56      | NIXI2                  |
| SSR8081  | GTGAGGCAGAATCCTCAATAA    | GCCACTGCTAGAAGATGCTAG     | (GCAAT2 | 120-160              | 56      | NIXI2                  |
| SSR8283  | GGCAGCATTGAGTACAGGAC     | AATTTTAAAGAAGACATCCTTCTCT | (CTTT)2 | 110-200              | 52      | NIXI2                  |
| SSR8485  | ACTGGAGTTGGAAAAACATC    | ACCATCAAGACAGAAGAGAGAAGA  | (CTTGTTG2 | 280-325 | 52 | NIXI2                  |
| SSR8687  | TTTTTTTTTTTTTTTTTTTTT   | GGCTTTTCTTCTTAGCTGACTAG    | (CTTTCTC1 | 230-260 | 53 | SOS3                   |
| SSR8889  | CTTCTCTCCTCTGATATCTTT  | GGGTTTGTCTTCTGATCTGACTG   | (TTGGT)2 | 220-310              | 53      | SOS2                   |
| SSR9091  | GTGCTGTTGCTGACAGATTAAA  | ATCTGTTGAGAAAGAAGTCCAGG   | (TTGGA)2 | 120-240              | 57      | NIXI2                  |
| SSR9293  | CTTATGTGTTTGTGCTCTCTGCTG | ATCTTACATTTGAGCTAGCTTTGTTA | (TTTGT)2 | 120-180              | 57 | NIXI2                  |
| SSR9495  | TTCTTAAAGACAGAAGAGCTCA  | GCTATCTTCTCTGTTGCTTAGCAGA | (AAAGTC)2 | 180-250              | 57      | NIXI2                  |
| SSR9697  | CTGGATTTTCTGCTTGCTGAGGA  | GCTGCTGCTGTCAAGTGTGAACTG  | (AAGTC)2 | 280-310              | 57      | NIXI2                  |
| SSR9899  | CCTACTGAAAAATGTGGCTGTCGT | GCAGAGAGTAGGAGGAAAGAGGAGA  | (AGCAT2,(AAGG)2 | 200-240 | 55 | NIXI2                  |
| SSR100101| GCCACACTTCATCTCATACTACA  | GGCACACTTCATCTCATACTACA    | (CAAT)2 | 100-125              | 51 | SOS3                   |
| SSR102103| ATGATGGCAGATGCTCTGTTTCTTC | GCCATCAAGGTTTATATGACA     | (ATGA)2 | 180-220              | 52      | SOS2                   |
| SSR104105| TTTGCTATTGCTCTTGCTCAAGC  | GCCACACTTCATCTCATACTACA   | (ATTA)2 | 290-350              | 53      | SOS3                   |

### 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. Sub-cluster IIA consisted of two wild genotypes TCR86 (V. trilobata) and ET528960 (V. luteola), both of which
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 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             | 0.652     |
| 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 (Mlav) 0.671 (PICav)
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

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**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   | 0.05  | 0.10   | 0.14    | 0.02 | 1.00 |            |

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**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 (Decrooq 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.

**Acknowledgements**

Authors are grateful to the Director, NBPGR, New Delhi, India for providing the valuable seed material and necessary support to carry out the research work successfully and to the Department of Biotechnology for funding the work on genetic improvement of legumes. NS greatly acknowledge the support and facilities provided by the Director, Centre for Biotechnology, Maharshi Dayanand University, Rohtak, India.

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