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Salt stress responsive genetic polymorphism analysis among promising rice genotypes using microsatellite markers

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Abstract
Using a panel of six salt stress response related microsatellite primer pairs, the molecular level polymorphism was examined amongst sixteen rice genotypes. Altogether 49 allelic variants including 30 shared and 19 unique alleles were detected with an average of 8.16 alleles per primer pair. Considerably greater polymorphism per cent was recorded for the primer pairs RM 140, RM 10764, RM 10772 and RM 10864. Amongst primer pairs utilized in the present study, RM 493 and RM10772 had relatively greater polymorphism information content. Considering the number of alleles, polymorphism per cent and polymorphism information content along with gene diversity as the basis, RM 10772 appeared to be comparatively most polymorphic and informative primer for the purpose of salt stress response related discrimination of entries. Ample genetic variability revealed by the analysis of amplification profiles based differentiation and divergence pattern conveniently allowed unambiguous discrimination and classification of the tolerant and susceptible entries. Similarity coefficients based hierarchical classification pattern was completely validated by the principal coordinate analysis based spatial distribution of the genetic profiles of sixteen genotypes. Neighbor joining tree based on six salt stress responsive microsatellite primers dependent similarity indices also discriminated the susceptible and tolerant entries. Precise analysis of the genetic polymorphism indicated that the improvement in salt tolerance can be achieved by selecting parental genotypes from different clusters for combining different salt tolerance components into a single genetic background. Presence of ample genetic variability, as revealed by salt stress response related microsatellite primer pairs, signified the prospects for developing highly salt tolerant rice varieties through a combination of superior alleles of genes controlling salt tolerance in different genotypes.

Keywords: Rice, salt tolerance; genetic variability; microsatellite; genetic similarity; genetic polymorphism, cluster analysis

Introduction
Salt stress adversely affects germination, growth and development of the plant, decreases leaf expansion leading to diminished photosynthetic area and dry matter production (Kumari *et al*., 2016; Kumari *et al*., 2018) [19, 21] and consequently yield loss owing to osmotic stress, ionic toxicity, oxidative stress and nutritional imbalance in plants. Being a highly salt-sensitive crop, soil salinity has greater impact for rice productivity. As it is widely emphasized, an excellent approach for the sound management of salt stress is to grow rice genotypes with a high level of salt tolerance. Since tolerance to salt stress varies with rice genotypes, an improvement of salt stress tolerance in commercial varieties would not only increase grain yield of rice under salt-stress condition, but also may extend rice growing area to the region with moderate salt content in soils.

Many research efforts have been made to understand the genetic mechanism of salt tolerance, which suggest that salt tolerance is a complex trait and phenotypic responses of plants to salinity stress are highly affected by the environment (Koyama *et al*., 2001; Flowers, 2004; Bharti *et al*., 2016) [17, 9, 2]. Seedling stage tolerance to salt stress correlates poorly with tolerance during reproductive stage of rice genotypes, suggesting the complexity of salinity tolerance and involvement of different sets of processes and traits at each stage (Rani and Sharma, 2017) [26]. Salinity effects on the growth of rice have been found to be related to different stages of plant development, salt concentration, types of salt, and duration of exposure to salt, water regime, temperature, humidity and solar radiation (Bharti *et al*., 2016) [2].

Differential mechanisms, such as, sodium exclusion, higher tissue tolerance by compartmenting salts into the apoplasts, effective sequestration of toxic salts into older tissues, stomatal responsiveness and up-regulation of the antioxidant system during stress are known to contribute to salt tolerance (Yeo and Flowers 1986) [31]. But, only a few of the mechanisms, which contribute to tolerance, are seemed to be possessed by most of the salt-tolerant cultivars, signifying the prospects for developing highly tolerant rice varieties through combining superior alleles of genes controlling these traits. Several highly tolerant traditional genotypes provide opportunities to improve salinity tolerance of rice through breeding (Gregorio *et al*., 2002, Ganeshan *et al*., 2016) [10, 11] and hence it is imperative to analyze the salt stress mechanism at molecular level for identification of salt stress responsive novel genes and genomic regions (Das *et al*., 2015; Kumar *et al*., 2015) [5, 18] for development of salt tolerance in rice varieties.

Amongst genomic markers, microsatellites are quite popular in the molecular map construction, gene mapping, construction of fingerprints, gene tagging, genetic purity test, assessment of genetic variation and elucidation of genetic relationships within and among species (Jin *et al.* 2010; Matin *et al.* 2012; Kumari *et al*., 2016; Kumari *et al*., 2018) [14, 24, 20, 22] due to their multi-allelic nature, high reproducibility, co-dominant inheritance, simplicity, abundance, stability, extensive genome coverage and simple reproducible assays. Because of all these positive features, microsatellite markers are ideal for fingerprinting and biological individualization and hence, more popular in rice because they are highly informative, co-dominant, easily analyzed, abundant and cost effective (Prabakaran *et al.* 2010) [25]. Keeping all above into consideration in the light of the fact that microsatellite markers based detection and analysis of salt stress response related genetic polymorphism can assist in discrimination of genotypes and marker assisted selection, the present investigation was undertaken to examine the salt stress responsive genetic polymorphism and to determine the nature and extent of differentiation and divergence amongst rice genotypes.

Materials and Methods

Experimental materials comprised sixteen rice genotypes including two tolerant (CSR-36 and Pokkali) and two susceptible (IR-64 and IR-29) reference genotypes and twelve genotypes (Rudra, CSR-2K-219, Rajendra Bhagwati, BPT-5204, Pusa Basmati-1, MTU-7029, CSR-27-192, CSR-2K-242, CSR-2K-262, NDRK-11-1, NDRK-11-3, NDRK-11-5) having variable degree of tolerance to salt stress. Seeds of the sixteen entries were planted in aluminium containers filled with soil and leaves were collected from the two weeks old seedlings of each entry for DNA isolation and amplification. Total genomic DNA was isolated using the standard protocol (Doyle and Doyle, 1990) [8] with slight modifications in the basic protocol. Using the standard protocol of polymerase chain reaction adjusted to laboratory conditions, targeted amplification of extracted genomic DNA was performed with known six pairs (Table 1) of salt stress responsive forward and reverse microsatellite primer pairs in the reaction mixture (15 µl) containing 2.4 µl water (Protease and Nuclease free), 3.0 µl 5X PCR buffer, 1.5 µl 10 mM $MgCl_2$, 3.0 µl 1 mM dNTPs mixture, 1.2 µl Primer F, 1.2 µl Primer R, 0.2 µl Taq Polymerase (1 unit) and 2.5 µl DNA sample. The reaction condition in a thermal cycler was optimized keeping initial denaturation at 95^0 C for 5 min, 35 cycles of denaturation at 94 ^{0}C for 1 min, primer annealing at 54-56 ^{0}C (varied with different primers) for 40 sec and extension at 72 ^{0}C for 1 min followed by final extension at 72^0 C for 5 min and cooling at 4 ^{0}C.

The products of amplification reaction were resolved by agarose (2%) gel electrophoresis at 120 V for 1 and ½ hour and then visualized and documented in gel documentation system. Using the ladder (50 bp; Fermentas), the size of amplified products was determined in relation to the size of markers in ladder with the help of gel reader (Alpha View Gel Reader). The position of the bands on the gel corresponded to the location of the bands along Y-axis (ranging from 0 to 1030). The Rf value for each band was determined assuming the location of well as initial position (Rf=0) and the position of migrated dye as final position (Rf=1) as a frame of reference. All the genotypes under evaluation in the present investigation were scored for the presence and absence of the bands and the data were entered into binary matrix as discrete variables. Suitability of the marker based polymorphism for characterization and differentiation of the entries was evaluated by computing the polymorphism per cent (Kumari *et al*., 2018) [22].

Table 1: List of six primers utilized for amplification of rice genomic DNA extracted from sixteen genotypes used in the present study

S. No	Primer	Chromosome Number	Primer sequence (5'-3')	Repeat motif	Annealing temp.(^{0}C)
1.	RM 140	1	(F)TGCCTCTTCCCTGGCTCCCCTG (R)GGCATGCCGAATGAAATGCATG	$(CT)_{12}$	55
2.	RM 10665	1	(F)CCTGCTGCAATTGATGACAAGC (R)TGGACAGAATGAAGCATCTGTGG	$(ATAC)_6$	54
3.	RM 10772	1	(F)GCACACCATGCAAATCAATGC (R)CAGAAACCTCATCTCCACCTTCC	$(CTT)_{16}$	55
4.	RM 10864	1	(F)GAGGTGAGTGAGACTTGACAGTGC (R)GCTCATCCAACCACAGTCC	$(GT)_{27}$	55
5.	RM 10764	1	(F) AGATGTCGCCTGATCTTGCATCG (R)GATCGACCAGGTTGCATTAACAGC	$(AT)_{28}$	55
6.	RM 493	1	(F) TAGCTCCAACAGGATCGACC (R) GTACGTAAACGCGGAAGGTG	$(CTT)_9$	56

Principal coordinate analysis was conducted to obtain a two-dimensional ordination of the microsatellite primers dependent genetic profiles of the entries subjected to molecular characterization. The binary data matrix based on presence and absence of bands was subjected to further analysis and genetic similarities among entries were calculated using the software NTSYS-pc (Rohlf 2000) [28]. Genetic similarity among entries was analyzed by calculating the similarity coefficient (Dice 1945) [7] for pair-wise comparisons based on the proportions of shared bands produced by the primers as: *Similarity coefficient = 2a/(2a+b+c)*, where, *a*, *b* and *c* represent number of bands

shared between J^{th} and K^{th} genotypes, number of bands present in J^{th} genotype but absent in K^{th} genotype and number of bands absent in J^{th} genotype but present in K^{th} genotype, respectively.

Utilizing the data on similarity coefficients and employing sequential agglomerative hierarchical non-overlapping (SAHN) clustering approach for tree building, cluster analysis was performed and the dendrogram was obtained by un-weighted pair-group method using arithmetic mean (UPGMA). An analysis of the nature and magnitude of differentiation and divergence at molecular level between rice genotypes under evaluation in relation to their salt stress response related genetic polymorphism as revealed by the six salt stress response related microsatellite primer pairs was performed by clustering the genotypes into different groups and identifying the clusters at appropriate phenon level.

Computational analysis for estimating the major allele frequency, gene diversity, polymorphism information content and heterozygosity was performed with the help of Power Marker (Liu and Muse 2005) [23] software. The proportion of heterozygous genotypes in the population at a single marker locus in question was expressed as heterozygosity. Probability that the two randomly chosen alleles from the population are different at the marker locus in question was considered as gene diversity. The comparison of the polymorphism information content (PIC) of the primer pairs at each marker locus (Botstein *et al.*, 1980) [2] reflected allelic diversity.

Results and Discussion

All the genotypes under evaluation in the present study yielded amplified products with all the six primer pairs utilized during primer directed amplification of targeted genomic regions. Only one but polymorphic band was visualized representing the amplified products generated in all the sixteen genotypes in combinations with five out of the six primer pairs utilized during amplification (Fig. 1). Only in the case of amplification reaction carried out with the primer pair RM 493, one band was visualized in five out of sixteen genotypes, whereas two bands were visualized in ten genotypes and three bands in one genotype. Altogether thirteen types of amplified products including nine shared bands and four unique bands (Table 2) were generated by this primer pair. All the primer pairs generated unique alleles. Molecular size of the amplified products varied considerably with the genotype and the primer pair. The Rf value obtained for different bands and the position of the bands along the Y-axis completely corresponded with the molecular size of the bands in all the cases. Since Rf values were calculated on the basis of location of wells and the dye front as a frame of reference, the complete correspondence of Rf values with molecular size of the bands was in accordance with the expectation. Recognizable polymorphism among the genotypes was displayed in respect of the number and position of bands, in addition to presence or absence of bands.

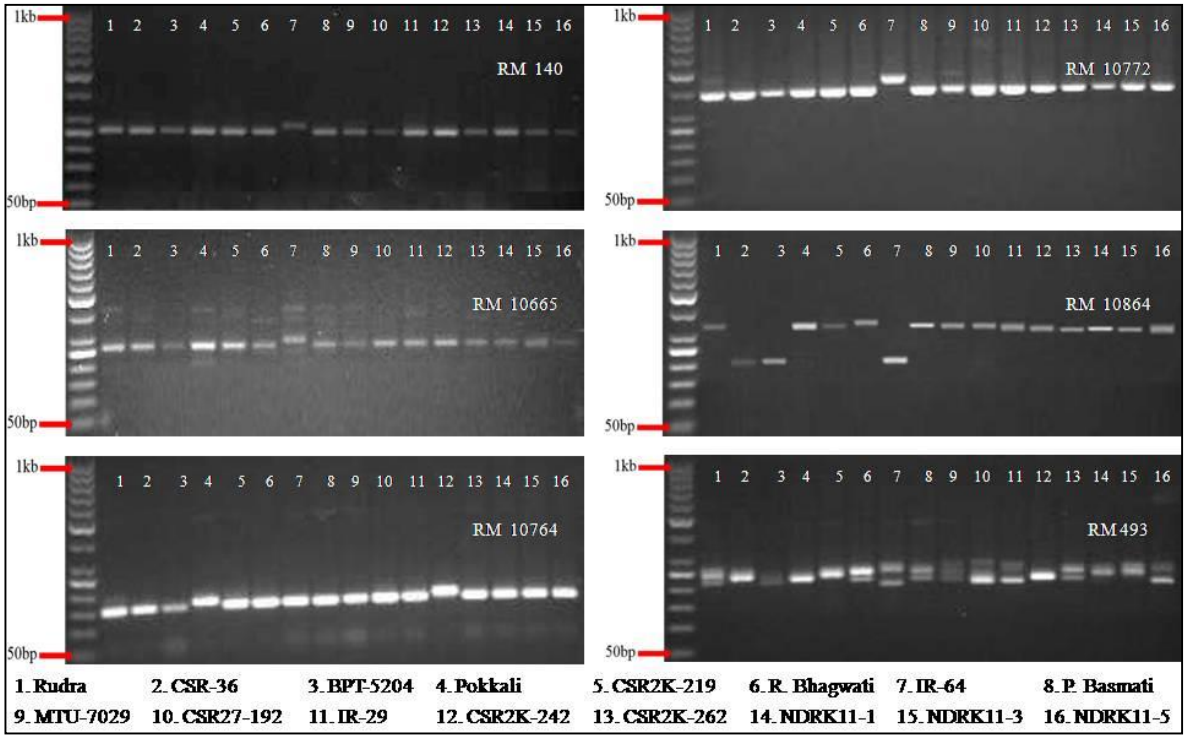

Fig 1: Amplification patterns of targeted genomic regions in sixteen rice genotypes obtained by using a panel of six salt stress response related microsatellite primers

Differential ability of the primer pairs to reveal variability among the genotypes was clearly reflected, since the number and proportion of unique alleles varied considerably among the primer pairs. While some of the primers generated only few allelic variants due to variation in the length of simple sequence repeats among the genotypes, some generated several allelic variants apparently because of existence of different number of repeats of the core unit of a microsatellite. Altogether 49 allelic variants including 30 shared and 19 unique allelic variants were detected with an average of 8.16 alleles per primer pair (Table 2). Considerably greater polymorphism per cent was recorded for the primer pairs RM 140, RM 10764, RM 10772 and RM 10864. A comparative analysis of polymorphism information content of the primer pairs indicated that the level of polymorphism was quite high since the values varied from 0.769 in the case of RM 10864 to 0.883 in the case of RM 493 with an average of 0.806 per microsatellite primer pair (Table 2). Remarkably greater magnitude of value was obtained in the cases of primer pairs RM 493 and RM10772 in descending order of magnitude.

Table 2: Analysis of primer pairs used for the amplification of genomic DNA extracted from twelve varieties of rice

Primer	Allele size difference (bp)	Allele size range (bp)	No. of alleles	No. of unique alleles	Major Allele frequency	Gene Diversity	PP	PIC
RM 140	057	230-287	07	3	0.25	0.81	42.8	0.786
RM 10665	047	278-325	06	1	0.25	0.81	16.6	0.785
RM 10764	052	162-214	07	3	0.31	0.80	42.8	0.778
RM 10772	108	392-500	09	5	0.25	0.85	55.5	0.835
RM 10864	137	220-357	07	3	0.31	0.79	42.8	0.769
RM 493	085	222-307	13	4	0.22	0.89	30.7	0.883

Considering the number of alleles and polymorphism per cent, in conjunction with polymorphism information content, RM 10772 appeared to be the most polymorphic and informative primer pair with greater gene diversity. Allelic variants revealing high degree of genetic polymorphism clearly reflected that the genotypes under evaluation in the present study harboured ample salt stress response related genetic differentiation and divergence. Several earlier studies have also reported remarkable allelic diversity of different salt stress responsive microsatellite loci (Dhar *et al.*, 2012; Karmakar *et. al.*, 2012; Roychowdhury *et al.*, 2013; Krishnamurthy *et al.*, 2014; Ali *et al.*, 2014; Rawal *et al.*, 2014; Islam *et al.*, 2015; Chowdhury *et al.*, 2016; Samal *et al.*, 2016; Hani *et al.*, 2017) [6, 15, 29, 16, 1, 27, 13, 4, 30, 12].

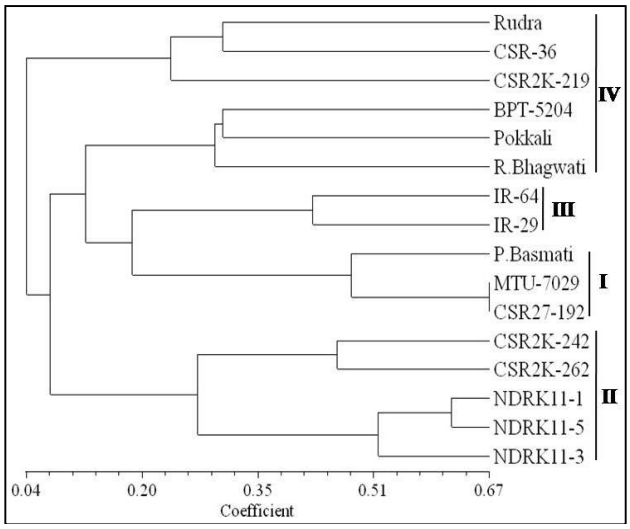

Fig 2: Dendrogram based on six salt stress responsive microsatellite primers dependent similarity indices.

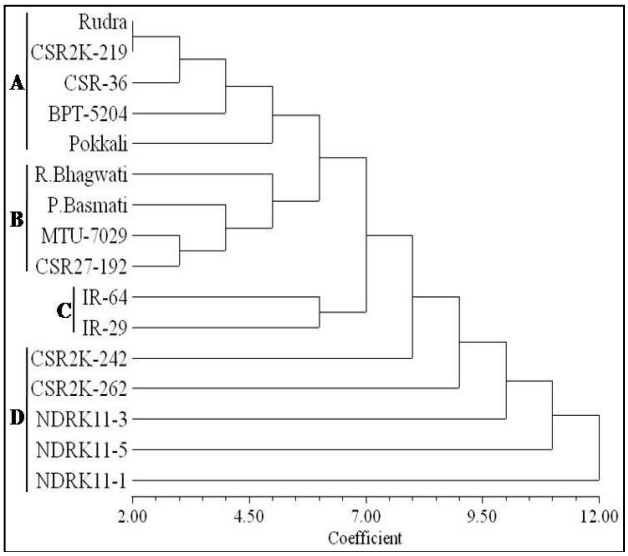

Fig 3: Neighbour joining tree based on six salt stress responsive microsatellite primers dependent similarity indices.

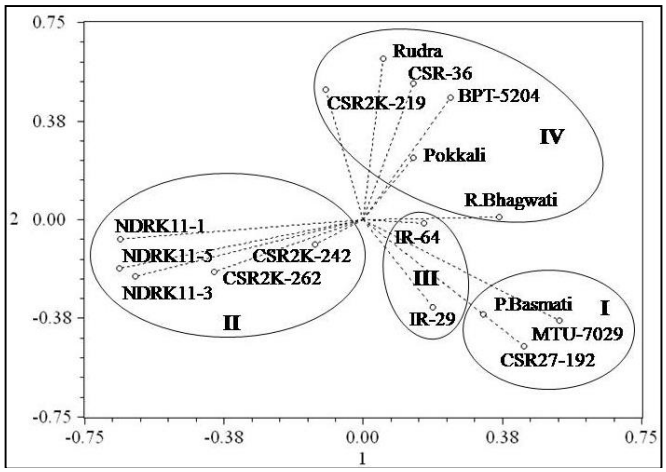

Fig 4: Principal co-ordinate analysis based two dimensional ordinations of six microsatellite primers dependent genetic profiles of sixteen rice genotypes.

Similarity coefficients of the pair-wise combinations of genotypes ranged from 0.133 to 0.667, indicating a considerably greater extent of genetic variation among the rice genotypes evaluated in the present study. Such a large range of similarity coefficients provided greater confidence for the assessment of genetic differentiation and divergence pattern and clustering of genotypes for elucidation of genetic relationship (Dhar *et al.*, 2012; Karmakar *et. al.*, 2012; Roychowdhury *et al.*, 2013; Krishnamurthy *et al.*, 2014; Islam *et al.*, 2015) [6, 15, 29, 16, 13]. Amongst pair-wise genotypic combinations, the similarity coefficient was found to be the maximum between MTU-7029 and CSR27-192 (0.667), followed by relatively higher magnitude of similarity coefficient between NDRK11-1 and NDRK11-5 (0.615), NDRK-11-3 and NDRK-11-5 (0.571), Pusa Basmati and MTU-7029 (0.533). These genotypic combinations appeared to possess a relatively high degree of similarity with respect to the nucleotides sequence composition at the primer binding sites and the molecular size of the genomic regions spanned by the salt stress responsive six primer pairs.

Hierarchical classification pattern of the entries, as indicated by dendrogram, was observed to be related to known genotypic response to salt stress documented in literature. The entries were basically divided into four multi-genotypic groups (Fig. 2) amongst which the first group consisted of three moderately salt tolerant entries, whereas the second multi genotypic group included five moderately tolerant genotypes. Interestingly, the two salt stress susceptible genotypes, namely, IR 64 and IR 29, were accommodated into third group, whereas the fourth group consisted of six tolerant genotypes. Apparently therefore, the salt stress response related genomic regions of the six genotypes belonging to fourth group, which included the two highly tolerant genotypes, were relatively more similar. Genotypic relationships reflected by the dendrogram (Fig. 2) and the neighbor joining tree (Figure 3) were almost in complete

agreement. Neighbor joining method is based on the idea of parsimony and it does yield relatively short estimated evolutionary trees. It does not attempt to obtain the shortest possible tree for a set of data. Rather, it attempts to find a tree that is usually close to the true phylogenetic tree and because of this slight non-correspondence in classification pattern may occur.

Principal coordinate analysis based inferences completely corroborated the results derived from the similarity coefficients based numerical taxonomic approach of hierarchical classification. Spatial distribution pattern of the genotypes along the two principal axes in the two dimensional plot of six salt stress responsive microsatellite primer pairs dependent genetic profiles indicated that the genotypes Rudra, CSR-2K-219, BPT-5204, CSR-36, MTU-7029, CSR-27-192, NDRK-11-1, NDRK-11-3 and NDRK-11-5 were placed far away from the centroid of the clusters and rest of the genotypes were placed more or less around the centroid (Fig. 4). Highly susceptible, moderately tolerant and highly tolerant genotypes were distinctly divided into different genotypic groups without intermixing of genotypes using both the approaches,

Salt stress response related six microsatellite primer pairs based detection and analysis of genetic polymorphism facilitated discrimination and unique genotyping of sixteen genotypes of rice and established relationship between different rice genotypes according to their salt stress responsiveness. Among the four clusters generated, two contained moderately tolerant genotypes and each one of another two clusters consisted of highly tolerant and highly susceptible genotypes. Further, the differentiation and divergence revealed at the molecular level indicated the existence of remarkable extent of genetic variability for the components associated with salinity stress response of the genotypes evaluated because salinity stress response related primer pairs were used during molecular characterization of the genotypes. The possibility of the presence or absence of a particular allele in contrasting genotypes was discarded since null alleles were not detected with any primer pair. Apparently therefore, differential behavioral response of rice genotypes to salt stress may be related to the variation revealed in salt stress response related microsatellite loci. Hence, salt stress response related discrimination of rice genotypes and selection of parental genotypes for genetic improvement in relation to salt tolerance can be effectively and efficiently performed by the use of these microsatellite markers. Parental genetic diversity will undoubtedly increase the probability of identifying desirable recombinants during screening for improvement in salt tolerance, since tolerance to salt stress is a multi-genic phenotype of the plant (Zeng *et. al*., 2004) [32]. Parental selection and inter-crossing of diverse genotypes from microsatellite markers based different clusters can therefore lead to successful pyramiding of different salt tolerance components.

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