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Molecular Characterization of Selected M5 Lines of Rice after TILLING for Salinity Tolerance Using 20 SSR Primers

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Authors' contributions

This work was carried out in collaboration between all authors. Author A. Raihan designed the molecular study, established the protocol and wrote the manuscript drafts. Author ASMN designed the phenotypic study and performed the statistical analysis. Author A. Rahman performed the statistical analysis of the paper, did the referencing and information checking for the manuscript. Author LR provided intellectual guidance during experimentation. All authors read and approved the final manuscript.

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ABSTRACT

About 20% of the net cultivable land is affected by various levels of salinity, which leads to season loss and crop loss. A rice variety BINA 7 was used to develop saline tolerant, high yielding varieties through TILLING. 10 rice lines including 5 from TILLING (Targeting Induced Local Lesions in Genomes) population were studied in Patuakhali, the coastal zone of Bangladesh between July to December, where maximum salinity reached during harvest was close to 8 ds/m^2. Traits like plant height, effective tillers, thousand seed weight on randomly selected plant basis and yield (kg/plot) were recorded. Appropriate statistical analysis was done. This was followed by Genetic Fingerprinting using saline trait specific, 20 SSR primers of the 5 TILLING population along with parent. Data Analysis of band position and creation of dendogram along with genetic distance

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between the materials have been reported. Indications of positive relationship between the molecular charactization and morphological studies gave direction towards possible saline tolerant lines. It was clearly seen from the 20 SSR markers used that the lines developed through the TILLING technique has more possibility of being saline tolerant than the parent,e.g. primer RM 585 bound between 175-650 bp to all except the parent. Such results indicated the TILLING lines to be diverse than the parent.

Keywords: Rice mutant; molecular characterization; Oryza sativa L.; genetic fingerprinting.

1. INTRODUCTION

Soil salinity is one of the most important environmental factors restricting rice (*Oryza sativa* L.) production in Bangladesh since the crop is classified as salt sensitive, especially in the early growth stages- germination, seedling, etc [1]. In fact to be more precise rice is sensitive to salinity at the seedling stage and becomes tolerant at the vegetative stage and very susceptible at the reproductive phase in terms of grain yield [2]. The deterioration in crop growth is mainly due to osmotic stress, ionic toxicity, oxidative stress and nutritional imbalance [3]. Abiotic stress, e.g. salt stress and heat stress can reduce average yield by 50% [4]. Rice is the main staple food in Bangladesh. Currently 2.8 million hectares of rice land in the coastal belt of the country is affected by salinity [5]. The coastal areas are particularly vulnerable to salinity due to saline water intrusion and rise in the sea level as a consequence of global warming.

Sodicity is a condition when there is high exchangeable sodium in the soil resulting in deterioration of soil physical properties. Salt concentration in a soil is measured in terms of its Electrical Conductivity (EC); the SI unit of electrical conductivity is ds/m. The rice crop is within the sensitive division from 0 to 8 ds/m [6]. It is necessary to identify the sensitivity and tolerance level of varieties at early seedling stages for successful crop production in a saline environment. Tolerance to abiotic stresses such as salinity can be increased through the TILLING technique causing induced micro mutations in the genetic makeup of the crop [7]. 92 lines from a TILLING experiment were taken under salt stress study *in vitro* and 5 lines from there was selected for further molecular studies.

TILLING (Targeting Induced Local Lesions in Genomes) is a reverse genetic strategy [8] to make it more suitable for large scale screening of chemically induced mutations in arabidopsis, tetraploid and hexaploid wheat, barley, maize [9] and other crops. Point mutations can be used by many agents but the chemical alkylating agent Ethyl Methane Sulfonate (EMS) has been the mutagen of choice for the creation of TILLING populations in plants. EMS can be used to induce primarily recessive loss of function mutations at frequency several times more than insertions or deletions [10]. TILLING is a powerful tool for reverse genetics, combining traditional chemical mutagenesis with high throughput PCR based mutation detection to discover induced mutations that alter protein functions. TILLING is not only useful for functional genomics but also for crop improvement where heteroduplex mismatch cleavage assay has high sensitivity in pooled samples and is therefore high throughput and low cost.

TILLING in polyploidy is more complicated due to higher number of the requirement of the homoeoallele of specific PCR primers for optimal sensitivity in SNP [11] than the diploids. TILLING is unique in its detection of point mutants.

The most important detection method is based on mismatch cleavage assay using endonuclease Cell. For this method locus specific PCR is essential. Polyploidy is common in plants. Conserved regions of a gene often represent functional domains and have high sequence similarity between homoeologous loci. The advantage are reflected by the successful implementation of TILLING in several plant species such as Arabidopsis, maize, wheat, potato, barley, rice, pea, *Lotus japonicus* and soybean [12]. The TILLING method has further been streamlined through the development of computational tools for assay design and data analysis. The CODDLE (Codon Optimized to Detect Deleterious Lesions) uses the expected spectrum of mutations for a given mutagen to calculate the -1.5kb region of a gene that contains highest number of potential mutations that could adversely affect gene function [13].

It is important to note that almost all literature related to TILLING on different crops as can be found in recent literature are on molecular level identification of the effect of EMS on the specific area and allelic genes available in the plant. After having that identification the gene/s can be used

for future breeding activities or variety having some desirable traits can also be used for cultivation. However, there are no matching studies on identifying the variable phenotypes and then testing the changes that has taken place at the allelic level so as to determine the possible adaptation to the environment and stability as to traits of importance. Such studies can support two important demands one to know whether the stability of the genetic effect already changed for production of the crop products of economic importance and the mutation that has taken place in the gene of the plant system being worked with can remain stable. There is another important field for such TILLING population which can be done through phenotypic assessment of the population under TILLING. Increasing the chances of point mutation is in fact increasing the chances of variation being created by natural mutation with subsequent selection process for adaptation of the species usually achieved through thousands of years of natural selection pressure.

Based on the above information it was planned that whether on TILLING results into point mutants that can be directly selected on its phenotypic performance first followed by molecular level of identification of the effect of EMS on the plant body system.

2. MATERIALS AND METHODS

2.1 Summary of the TILLING Technique

In order to test this concept, experiments were conducted with three mega rice varieties of Bangladesh. These are BRRI Dhan29, BRRI Dhan47 and BINA Dhan7. The technique followed here covers treating the rice seeds with three levels of EMS Viz.; 1.4, 1.6 and 1.8 with a control using normal seeds, dehusked seeds, sprouted seeds and seedlings. All the seedlings treated with different doses of EMS were then planted in during Boro season in the field prepared specifically for this purpose. The crop raising followed all the standard cultivation practices for HYV rice varieties of Bangladesh as recommended by institutes that breed the varieties. The seeds were harvested from 945 surviving plants. The harvested seeds were again processed for sowing and transplanting during the T. Aman season using 100 seeds from each of the plants. Thus a population of 94,500 was raised during this season. The population was then subjected to three tire selection one in the field covering three selection times by the breeder himself and at least one other independently using tagging system. The plants thus selected were again subjected to final selection at harvest mostly based of stature, panicle and duration. The third tire of selection was based on quantitative traits after collection of data. 160 plant lines were selected based on traits considered important for selection of a rice variety from amongst the 945 promising lines. Seeds from these plants were again sown and used for transplanting and final M4 seeds were collected at the end of harvest. Mutant lines were selected for screening in the saline soils of the coastal belt of Bangladesh. 5 mutant lines underwent field trial in Patuakhali during the winter-spring rice season under standard recommended agronomic practices. Soil salinity reached up to 9 ds/m^2, but the yield was still comparable with the highest national average for the region.

2.2 Effect of Salt Stress on Germination of Selected TILLING Population of Rice *in vitro*

Seeds of 92 lines from the TILLING population and 2 check varieties of rice were screened for tolerance to salt stress on germination *in vitro* on petri dishes. Healthy uniform seeds of all varieties were treated with mercuric chloride, 70% ethanol and distilled water for sterilization. Twenty seeds for each line was were allowed to germinate on a filter paper in 9 cm diameter petri dishes. Each filter paper was be moistened with 3 salt solutions (NaCl) and a 0 (distilled water) as control. The 3 solutions had salt concentrations of 6, 9 and 12 ds/m respectively. NaCl was chosen since it would not cause precipitation of other ions. The petri dishes were arranged in Completely Randomized Design (CRD) and kept under temperature of 25±1°C with 8 hrs photo period [14]. Three replications of each treatment were carried out. Germination percentage and growth till seedling stage for each line was measured. Lines were then selected against salt stress along with plant phenotypic data from the field. Molecular analysis was done on 5 of the lines selected. More information is given in the "Results and Discussion" section below.

2.3 Extraction of DNA and the Solutions Used

Molecular characterization was done to compare phenotypic characteristics against salinity tolerance. Around 0.1-0.15 gm of young leaves

were collected from 20 day old seedlings for DNA extraction and crushed thoroughly using liquid nitrogen in a mortar pestle using the modified CTAB method [15] and then DNA was quantified using a double beam spectrophotometer at 260nm and 280 nm wavelengths.

Since "crushing" is one of the most important steps towards proper extraction of DNA, liquid nitrogen was used to break the fibrous tissues. 650 µl extraction buffer was added, the sample ground for the second time and poured into eppendorf tubes. 150 ul of 5% SDS, 10% PVP & 20% CTAB was added and mixed by inversion. The samples were warmed in the water bath at 65ºC for 40 minutes while being inverted 3-4 times during incubation. Equal volume of Chloroform: Isoamyl Alcohol (24:1) solution was added after cooling sample up to room temperature, mixed by inversion and centrifuged at 14000 rpm for 30 minutes. The aqueous upper phase was removed again and equal volume of ice cold isopropanol and 150 µl 5 M NaCl was added and mixed 1-2 times by inversion. Cottony DNA can be observed in this step. The samples were refrigerated at -20°C for an hour to complete precipitation and then centrifuged at 10000 rpm for 20 minutes at room temperature for DNA pellet formation.

The supernatant was discarded and the pellets were washed with 70% ice cold ethanol. The samples were centrifuged for the final time at 10000 rpm for 10 minutes for pellet formation. The supernatant was discarded from the tube and the pellet washed again with 70% ice cold ethanol about 2-3 times by pipetting, each time discarding the alcohol. The remaining pellet in the eppendorf tube was dried thoroughly and dissolved in 50 µl of nuclease free water finally storing at -20°C for future use.

2.4 PCR Analysis of Isolated DNA

20 SSR primers specific towards the loci contributing towards salinity tolerance were used during the PCR method for DNA amplification purpose. Details on the primers are given in Table 1. The PCR protocol followed was 3 minutes in 94°C in stage one followed by 40 cycles in stage 2 at 1 minute in 94°C, 2 minute at annealing temperatures 55°C, then 1 minute 30 seconds at 72°C followed by stage 3 where 7 minute in 72°C then infinitely stored at 4°C [18]. Each 25µl reaction volume contained 2.5 µl MgCl2 mix reaction buffer (10x), 0.5 µl dNTP mixture (2.5 mM), 2.6 µl of primer (10µM), 0.15 µl Taq DNA polymerase (G-bioscience) and 2 µl of DNA (50 ng/µl).

The amplified DNA were then run on 2% ultrapure agarose for 1 hr 25 min at 70 volts and the image was viewed under UV in a Gel Documentation system. Both the horizontal electrophoresis machine and the gel documentation system are made by Biometra. Analysis to measure the resultant bands against the 100 bp DNA Ladder was done using the DNAfrag software [16]. Other analysis such as cluster was done and genetic distances were also done using the "Statistica" software [17].

3. RESULTS AND DISCUSSION

3.1 Phenotypic Data from Salt Stress Test in Comparison to Field Phenotypic Data

The mutant rice lines M_4-16, M_4-58, M_4-59, M_4-62 and M_4-91 were selected for molecular analysis after carrying out salt stress on germination percentage *in vitro* and plant phenotypic data from the field. The germination percentage of line M_4-58 in comparison with the control was found to be the highest at 12 ds/m at 98%. Lines M_4-59 and M_4-16 had a germination percentage of 95%. Lines M_4-62 and M_4-91 had mediocre germination percentage at 12 ds/m of around 60% but high germination percentage of 98% up to 9 ds/m.

The phenotypic plant data for these lines were found to be appropriate for rice production in Bangladesh and is given in Table 1.

3.2 Gel Image Analysis of the Mutant Rice Lines

Majority of the genes responsible for saline tolerance in rice is found in chromosome 1, while few more genes exist in chromosomes 2, 6, 7 and 8. 20 genetic markers for these genes in the form of SSR primers were chosen and Table 2 gives a list of primers along with their sequences, allele number, motifs and expected range of base pair positions. RM 324 has the highest allele number of (6) while RM 121and RM 10694 has the lowest allele number of (2). No bands were found for primer RM 11757.

3.3 Gel Image Analysis

Figs. 1-6 show the image of the gels run for the 5 mutant lines against the parent BINA Dhan 7. Table 3 mentions the difference in base pair

positions within certain SSR primers from the 20 used to test against salinity tolerance. These clear differences in base pairs among the mutants compared to the parent, gives an indication of the mutants having a stronger possibility to be saline tolerant.

3.4 Cluster Analysis to Determine Genetic Relationship

Cluster Analysis is used to group and to construct a dendogram as shown in Fig. 7. Genetic relationships among the varieties at the average distance of linkage distance 2 showed two major clusters present. BINA Dhan 7 and M_4-91 are the two major clusters. From the information derived from the dendogram it can be suggested the mutant M_4-16 is more closely related genetically to the parent BINA Dhan 7, while M_4-91 is the most distantly related. The cluster of M_4-59 and M_4-62 has the least genetic difference. M_4-58 also appears to be more different than the parent Bina Dhan 7 after the mutative effects from the TILLING technique. Further information from Table 3 corroborates the information and suggestion.

Table 1. Important phenotypic data for considering developed rice lines as promising in context of Bangladesh

Line	Plant height (cm)	Effective tiller	Panicle length (cm)	Seed Weight (g)	Duration (days)
M4-16	103	9	25.76	30	155
M4-58	100	11	25.50	30	148
M4-59	93	12	25.46	31	148
M4-62	103	9	25.62	34	155
M4-91	95	13	22.58	22	150

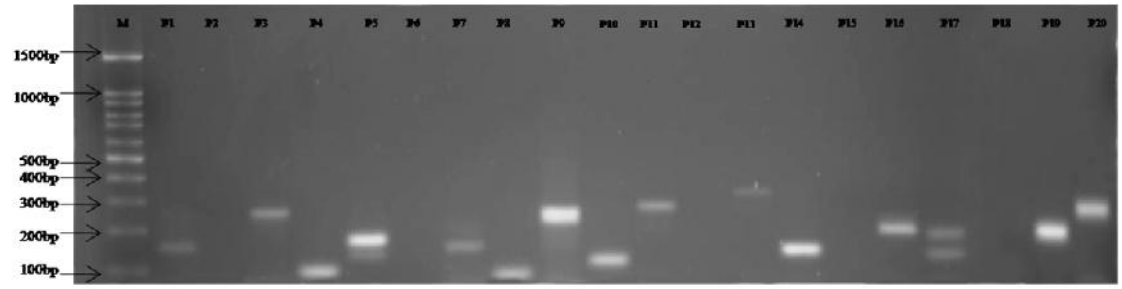

Fig. 1. Gel image of banding profile of the parent Bina Dhan 7 using 20 SSR markers linked with saline tolerance. Lane M is the 100 bp molecular weight marker DNA or DNA ladder. A banding pattern of 15 SSR markers which include primers 1, 3, 4, 5, 7, 8, 9, 10, 11, 13, 14, 16, 17, 19 and 20. The markers have been indicated in the image as P1 to 20

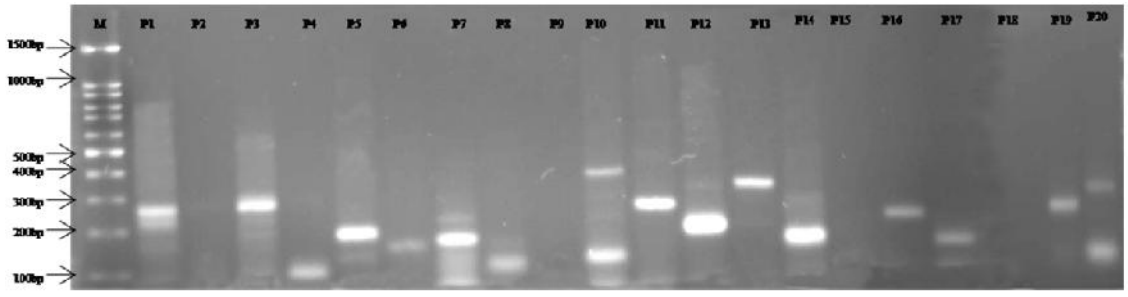

Fig. 2. Gel image showing the DNA binding pattern of the mutant rice line M_4-16 using the same 20 SSR markers as the parent linked with saline tolerance. Lane M is the 100 bp molecular weight marker DNA or DNA ladder. A banding pattern of 16 SSR markers which include primers 1, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 16, 17, 19 and 20. The markers have been indicated in the image as P1 to 20

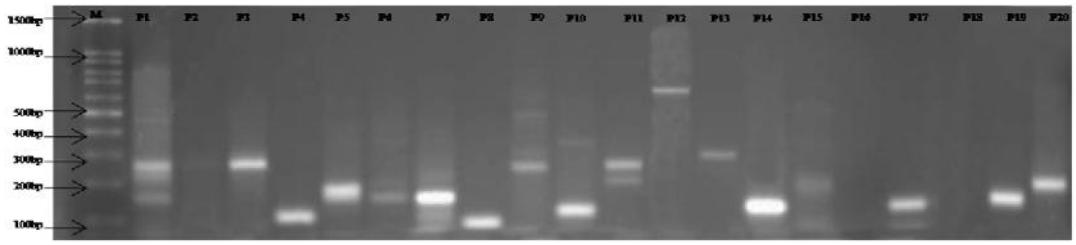

Fig. 3. Gel image showing the DNA binding pattern of the mutant rice line M_4-58 using the same 20 SSR markers as the parent linked with saline tolerance. Lane M is the 100 bp molecular weight marker DNA or DNA ladder. A banding pattern of 17 SSR markers which include primers 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 19 and 20. The markers have been indicated in the image as P1 to 20

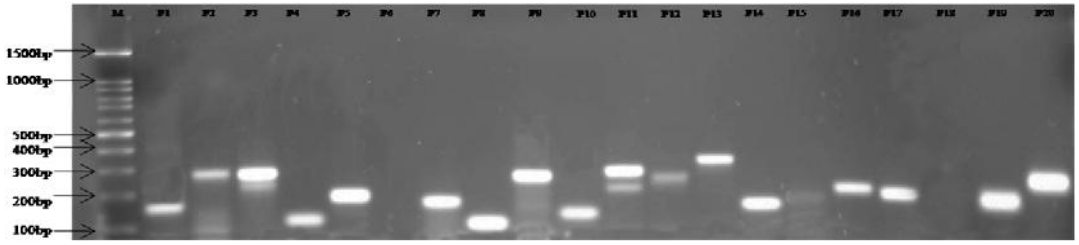

Fig. 4. Gel image showing the DNA binding pattern of the mutant rice line M_4-59 using the same 20 SSR markers as the parent linked with saline tolerance. Lane M is the 100 bp molecular weight marker DNA or DNA ladder. A banding pattern of 18 SSR markers which include primers 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 19 and 20. The markers have been indicated in the image as P1 to 20

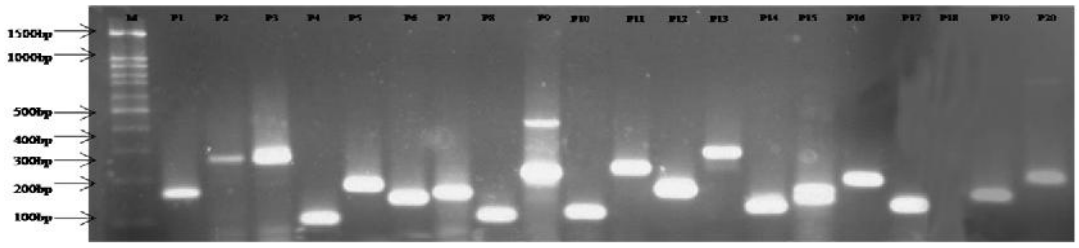

Fig. 5. Gel image showing the DNA binding pattern of the mutant rice line M_4-62 using the same 20 SSR markers as the parent linked with saline tolerance. Lane M is the 100 bp molecular weight marker DNA or DNA ladder. A banding pattern of 19 SSR markers which include primers 1, 2, 3, 4, 5, 6,7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 19 and 20. The markers have been indicated in the image as P1 to 20

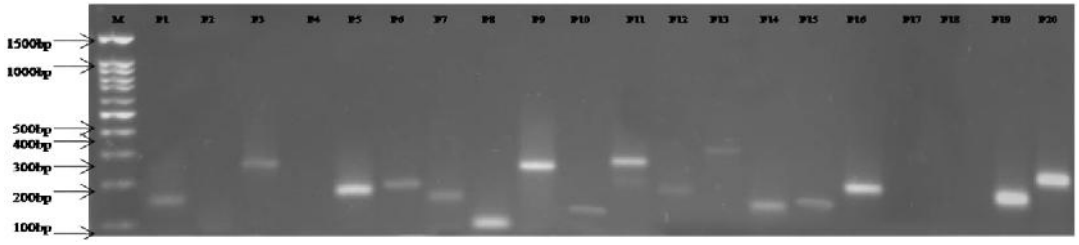

Fig. 6. Gel image showing the DNA binding pattern of the mutant rice line M_4-91 using the same 20 SSR markers as the parent linked with saline tolerance. Lane M is the 100 bp molecular weight marker DNA or DNA ladder. A banding pattern of 16 SSR markers which include primers 1, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 19 and 20. The markers have been indicated in the image as P1 to 20

Table 2. List of SSR saline tolerant markers along with details of their associated loci across BINA 7 and the mutants

Sl. no.	Marker	Chr. no	Motif	Position (cM)	Sequences of primers (5'-3')	Allele no	Size range (bp)	PIC value
1	RM 24	1	(GA)29	53.5	F:GAAGTGTGATCACTGTAACC R:TACAGTGGACGGCGAAGTCG	4	151-257	0.671
2	RM 121	6	(CT)7	53.5	F:ACCGTCGCCTTCCACTTTCCCC R:TTCGGGGTTGCCGGTGATGTTG	2	268-280	0.591
3	RM 140	1	(CT)12	60.1	F:TGCCTCTTCCCTGGCTCCCCTG R:GGCATGCCGAATGAAATGCATG	3	250-307	0.487
4	RM 310	8	(GT)19	24.9	F:CCAAAACATTTAAAATATCATG R:GCTTGTTGGTCATTACCATTC	3	100-133	0.561
5	RM 324	2	(CAT)21	67.5	F:CTGATTCCACACACTTGTGC R:GATTCCACGTCAGGATCTTC	6	129-210	0.778
6	RM 336	7	(CTT)18	80	F:CTTACAGAGAAACGGCATCG R:GCTGGTTTGTTTCAGGTTCG	3	148-217	0.625
7	RM 337	8	(CTT)4-19-(CTT)8	1.1	F:GTAGGAAAGGAAGGGCAGAG R:CGATAGATAGCTAGATGTGGCC	4	161-215	0.625
8	RM 490	1	(CT)13	40.7	F:ATCTGCACACTGCAAACACC R:AGCAAGCAGTGCTTTCAGAG	4	100-127	0.723
9	RM 493	1	(CTT)9	79.7	F:TAGCTCCAACAGGATCGACC R:GTACGTAAACGCGGAAGGTG	5	228-400	0.741
10	RM 510	6	(GA)15	13.4	F:AACCGGATTAGTTTCTCGCC R:TGAGGACGACGAGCAGATTC	3	124-379	0.565
11	RM 562	1	(AAG)13	99.2	F:CACAACCCACAAACAGCAAG R:CTTCCCCCAAAGTTTTAGCC	5	199-306	0.666
12	RM 585	6	(TC)45	12.9	F:CAGTCTTGCTCCGTTTGTTG R:CTGTGACTGACTTGGTCATAGG	5	175-650	0.781
13	RM 594	1	(GA)n	74.4	F:GCCACCAGTAAAAGCAATAC R:TTGATCTGCTAGTGAGACCC	3	307-364	0.668

Sl. no.	Marker	Chr. no	Motif	Position (cM)	Sequences of primers (5'-3')	Allele no	Size range (bp)	PIC value
14	RM 7075	1	(ACAT)13	74.2	F:TATGGACTGGAGCAAACCTC R:GGCACAGCACCAATGTCTC	3	138-189	0.611
15	RM 8094	1	(AT)31	60.6	F:AAGTTTGTACACATCGTATACA R:CGCGACCAGTACTACTACTA	3	162-210	0.594
16	RM 10694	1	(AC)18	10,969,872-10,970,066 bp	F:TTTCCCTGGTTTCAAGCTTACG R:AGTACGGTACCTTGATGGTAGAAAGG	2	218-235	0.481
17	RM 10793	1	(ATAG)7	12,569,890-12,570,013 bp	F: GATTGCCAACTCCTTCAATTCG R:TCGTCGAGTAGCTTCCCTCTCTACC	3	145-210	0.541
18	RM 11757	1	(TTG)48	33,473,203-33,473,801 bp	F:GCTTGTTGCCTGTGAACAGTAGC R:TGTCAGCATGCAACATCAATCC	0	0	-
19	RM 1287	1	(AG)17	58.1	F:GTGAAGAAAGCATGGTAAATG R:CTCAGCTTGCTTGTGGTTAG	4	159-228	0.667
20	RM 3412	1	(CT)17	62.5	F:AAAGCAGGTTTTCCTCCTCC R:CCCATGTGCAATGTGTCTTC	4	207-281	0.667

Information on marker positions has been collected from www.gramene.org

Table 3. Band positions (bp) of 6 SSR primers exhibited the distinct differences between the parents

Primers / Varieties	RM 121	RM 336	RM 510	RM 585	RM 8094	RM 10694
Bina Dhan 7	-	-	132	-	-	228
M_4-16	-	161	379	196	-	235
			129	178		
M_4-58	-	148	130	650	210	-
M_4-59	280	-	151	280	210	232
M_4-62	268	162	138	263	189	218
				175	162	
M_4-91	-	194	155	216	-	-

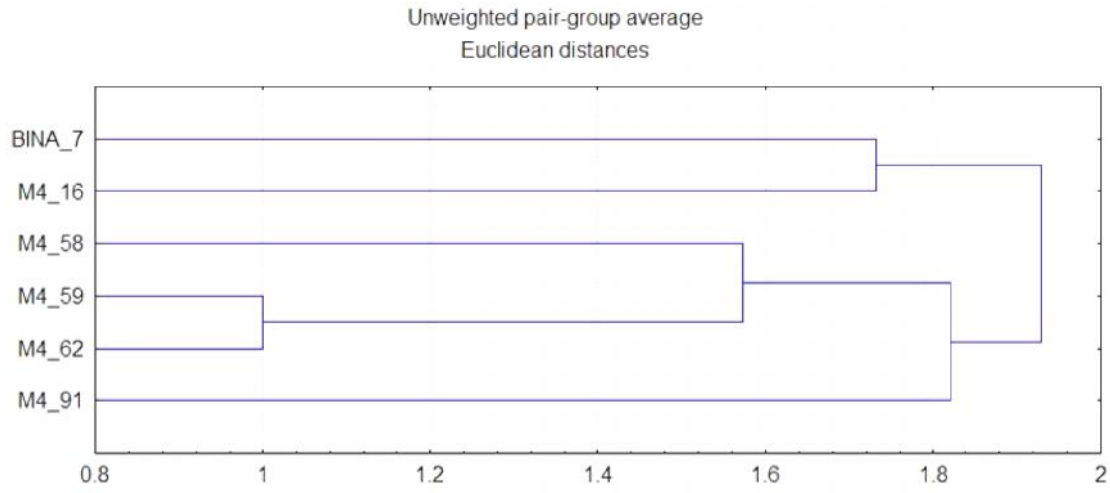

Fig. 7. UPGMA cluster dendrogram showing genetic relationship between BINA 7 and mutants of rice according to 20 microsatellite marker analysis

Table 4. Summary of linkage distances for different pairs of BINA Dhan 7 and 5 Mutant lines for SSR markers

Variable	BINA-7	M4-16	M4-58	M4-59	M4-62	M4-91
BINA-7	0.00	1.73	2.00	1.73	2.00	2.24
M4-16	1.73	0.00	1.73	2.00	1.73	2.00
M4-58	2.00	1.73	0.00	1.73	1.41	1.73
M4-59	1.73	2.00	1.73	0.00	1.00	2.00
M4-62	2.00	1.73	1.41	1.00	0.00	1.73
M4-91	2.24	2.00	1.73	2.00	1.73	0.00

3.5 Genetic Distance Analysis

The values of pair-wise comparisons of genetic distances analyzed by using computer software "Statistica" between varieties were computed from combined data for the 20 primers, ranged from 1.00 to 2.24. Table 4 shows the summary of this analysis. The highest linkage distance (2.24) was recorded in between the parent BINA Dhan 7 and the mutant M_4-91 suggesting the highest difference between parent and mutant. The lowest linkage distance can be seen between the parent BINA Dhan 7 and the mutants M_4-16. The two mutants M_4-62 and M_4-59 are the most similar to each other.

4. CONCLUSION

Molecular characterization of the 5 mutant lines in comparison to the parent BINA Dhan 7 has

shown that the lines have the ability to be more saline tolerant. This correlated with the phenotypic data from the field. Various types of data analysis from the gel image has proven that through the TILLING technique to develop lines which exhibit enough individuality or variation from the parent to be submitted for registration as a parent. Through comparison between phenotypic and simultaneous marker assisted selection through molecular characterization, it is possible to develop a rice variety within a commercially acceptable period of time.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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