Integrating molecular markers forscreening of salinity tolerance in rice genotypes

Abstract

This study assessed the genetic diversity among fifteen rice germplasms, including selected landraces, wild species, released cultivars, improved lines and standard tolerant/susceptible check varieties, using the *Saltol* QTL and other salt stress-associated SSR markers. The analysis revealed a high level of polymorphism and genetic diversity, with an average of 6.19 alleles per locus and a mean gene diversity of 0.73. The polymorphism information content (PIC) values ranged from 0.53 to 0.78, averaging 0.70, indicating the SSR markers' effectiveness in distinguishing the genotypes. Notably, markers RM490, RM7158, RM562, RM3412, RM493, RM7075, RM8094, RM6811 and RM10825 out of total markers were particularly proficient in screening for salinity tolerance.The genetic relationships among the genotypes were visualized through a dendrogram, forming several distinct clusters. Cluster 1, containing Lalkada gold, *Oryza nivara* and Purna, showed strong genetic similarity despite varying salt tolerance levels. Other clusters also displayed genetic groupings with phenotypic variances, highlighting the complexity of salt tolerance as a trait. The discrepancies between phenotypic classifications and genetic clustering were attributed to factors such as genetic recombination, multiple mechanisms of salt tolerance, incomplete marker coverage, gene interactions and environmental influences.The dissimilarity matrix further underscored significant genetic relationships, with highly similar genotypes (e.g., GR 17 and IR 28) and completely dissimilar ones (e.g., GR 17 and IR55179-3B-11-3). These findings demonstrate the utility of SSR markers in genetic diversity assessment and marker-assisted selection. The study emphasizes the importance of considering genetic nuances in breeding programs to develop robust, salt-tolerant rice varieties.

Introduction

Rice (*Oryza sativa* L.) is a self-pollinated cereal crop within the Gramineae family, with a chromosome number of 2n=24, as noted by Hooker in 1885. Typically grown as an annual plant, rice is a monocot and serves as a staple food for over 3.5 billion people worldwide (Xu *et al.,* 2016). It constitutes 50% of agricultural income in Asia and provides nearly 80% of the world's nutrition. In some countries, rice accounts for 75% of daily caloric intake and 55% of protein consumption (Bhuiyan *et al.,* 2002). In most of the countries, crops are mainly raised under field conditions or open environments which are often exposed to biotic as well as abiotic stress. Abiotic stresses like climatic catastrophes like fluctuation of temperature, rainfall, drought, flood, sodicity, salinity, acidity in tropics, temperate, arid, or semi-arid regions which influence plant metabolism directly or indirectly, thereby affecting plant growth development and finally their production(Upadhay and kumar, 2022).In recent times, rice cultivation has expanded into marginal lands where soil salinity levels exceed the thresholds that affect growth and yield. These developments, along with the use of marginal quality water for irrigation, highlight the urgent need for genetic improvement of salt tolerance in rice. However, rice is among the most salt-sensitive cereal crops. The ingress of salinity has deteriorated environmental conditions in coastal areas, negatively impacting agriculture (Zeng *et al.,* 2004). Breeding for salinity tolerance has been slow due to several challenges, including limited understanding of the genetics involved, the complexity of multiple tolerance mechanisms, inadequate screening methods, low selection efficiency, and a

poor grasp of the interactions between salinity and environmental factors (Bhowmik *et al.,* 2009). A significant breakthrough occurred with the identification of major chromosomal regions, known as quantitative trait loci (QTLs), responsible for salinity tolerance, specifically Saltol. The development and use of molecular markers have facilitated the rapid incorporation of these traits into high-yielding and popular rice varieties through marker-assisted backcrossing (Huyen *et al.,* 2012). Recent advances in molecular marker analysis now enable the examination of both simple inherited and quantitative traits, helping to identify individual genes responsible for salinity tolerance. This advancement aids in the selection of rice for this trait, which has traditionally been difficult to breed for due to its low heritability (Aliyu *et al.,* 2011).

DNA markers offer a precise, convenient and reliable means of assessing genetic variability. They are particularly advantageous because they are technically straightforward, time-efficient, highly informative, require only small DNA samples and are unaffected by environmental conditions or the plant's physiological stage. Among the PCR-based markers, SSR markers have proven to be highly effective tools for studying genetic diversity and relationships within and among species. SSR markers are known for being highly polymorphic, easily transferable, abundant in eukaryotic organisms and well-distributed across the genome. They can be easily amplified in PCR reactions using DNA nucleotide primers specific to the unique sequences flanking the repeat motifs (Salgotra*et al.,* 2015). SSR markers have been extensively used in genetic diversity analysis, genotype identification and population structure estimation in numerous rice genetic studies (Islam *et al.,* 2018). Even a small number of SSR markers can provide a comprehensive view of genetic diversity due to their multi-allelic and highly polymorphic nature (McCouch *et al.,* 1997). Assessing genetic diversity in crops is crucial for selecting heterotic germplasm for breeding improvements. Local rice varieties have evolved from their wild progenitors under both natural and human selection, resulting in significant genetic diversity (Huang *et al.,* 2010). Therefore, research has focused on assessing genetic diversity using microsatellite DNA markers in rice germplasm. The main objectives of this research were to evaluate the polymorphism and molecular diversity of 15 rice genotypes using SSR markers, to establish a dendrogram for classifying genotypes into different groups based on genetic distances and to determine the genetic relationships among the rice germplasm.

Material and Method

In the present experiment, total 15 genotypes comprising one wild species (*Oryza nivara)*, two landraces, one derived line and eleven released cultivars were evaluated during *Kharif* 2023. The details of experimental materials under evaluation are given in Table 1. The evaluation of experimental materials was carried out along-with known salt-tolerant and salt-sensitive checks at Rainout Shelter Facility, Regional Rice Research Station, Navsari Agricultural University, Vyara, which is situated at latitude 21° 7' 12.0036" N and longitude 73° 40.0002" E in South Gujarat.

1. Molecular marker analysis

Total 16 SSR makers (Table 3), among which 10 linked to *Saltol* (Waziri *et al.,* 2016) and 6 markers (Satasiya, 2022) linked to other salinity tolerant trait QTLs which are used to diversify the genotypes.

2. DNA extraction, PCR amplification and gel electrophoresis

DNA isolation was performed using fresh leaf tissues (200-250 mg) obtained from 15-day-old seedlings, following the modified CTAB method protocol as described by Doyle and doyle, 1990. Subsequently, the DNA concentration of all samples was adjusted to 100 ng/L using a Nano-Drop spectrophotometer (Thermo Fisher Scientific, America). PCR reactions were conducted using an Eppendorf thermocycler Germany, with a total volume of 10 µL comprising 5 µl of PCR master mix for one genotype (*SafeAmp* 2X PCR Master Mix Kit, TAKARA), 2 µL of primer, 2 µL of H_2O, and 1 µl of template DNA. The thermocycler program was initiated with 5 minutes of initial denaturation at 95 ºC, followed by 35 cycles of denaturation at 95 ºC for 30 s, annealing at 55-58 ºC for 30 s, and extension at 72 ºC for 45 s. A final extension cycle at 72 ºC for 10 min was included (Eppendorf, Mastercycler nexus-gradient PCR). The PCR amplified products, along with the 100 bp DNA ladder, were visualized on a 3.5% agarose gel stained with ethidium bromide and documented using a gel documentation system (Bio-Rad, U.S.A.).

3. Allele scoring

The size (in nucleotide base pairs) of the amplified band for each microsatellite marker was determined based on its migration relative to a molecular weight of size marker (100 bp DNA Ladder). Allele molecular weight data was used to determine major allele frequency, gene diversity, heterozygosity and polymorphism information content (PIC).

4. **Summary of the statistics used in analysis of SSR marker**

Summary of the statistics for all the markers was derived using Power Marker v 3.25 software (Liu and Muse, 2005). This software uses the following formulas to calculate different parameters:

5. **Major allele frequency**

$$\text{Major allele frequency} = \frac{\text{Number of genotypes having major allele}}{\text{Total number of genotypes}} \times 100$$

6. **Gene diversity**

Gene diversity, often referred to as expected heterozygosity, is defined as the probability that two randomly chosen alleles from the population are different. An unbiased estimator of gene diversity at the l^{th} locus was estimated by using the formula,

$$H_e = \left[1 - \sum_{i=1}^{n} pil^2\right] / \left[1 - \frac{1+f}{n}\right]$$

Where,

Pi = i^{th} allele frequency
f = inbreeding coefficient
n = number of individuals

7. Polymorphism information content

As per Botstein *et al.* (1980), PIC was estimated as

$$PIC = \left[1 - \sum_{i=1}^{n} pi^2\right] - \left[\sum_{i=0}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2\right]$$

Where,

P_i and P_j are the frequencies of i^{th} and j^{th} alleles

8. Dissimilarity matrix

Bootstrapping of the Neighbor-Joining (NJ) tree was performed using DARwin 6.0 with 1000 iterations. Processed data was directly used for calculating the dissimilarity matrix using DARwin 6.0 software (Perrier *et al.* 2003). Dissimilarity was calculated by pairwise simple matching using the following formula.

$$d_{ij} = 1 - \frac{1}{L}\sum_{i=1}^{l} \frac{m_i}{\pi}$$

Where,

d_{ij} = Dissimilarity between units i and j
L = Number ofloci
π =Ploidy
m_i = Number of matchingalleles

Construction of dendrogram

Pairwise dissimilarity (simple matching coefficient) matrix was used for constructing a dendrogram using Neighbor-Joining method, proposed by Saitou and Nei (1987) as implemented in DARwin 6.0. This method uses the criterion of relative neighborhood, weighted average for dissimilarity updating, and adjustment to an additive tree distance.

Table1 : Details of genotype used for seedling stage salinity screening

Genotype	Pedigree	DM	PH	PTP	GYPH	GT	STS
GR 17 (Sardar)	Gurjari × Jaya	115-120	120-125	10-12	5500-6000	LB	5
IR55179-3B-11-3	IR4630-22-2-5-1-3 × Nona bokra	125-130	110-115	12-14	5500-5800	SB	3
IR 28	IR8333-6-2-2-1 × IR2040	105-110	100-105	8-10	5000-5500	LS	7
Dandi	PNL-2 × IET-8320	130-135	115-125	8-9	4500-5000	SB	5
FL478	IR29 × Pokkali	115-120	100-105	12-14	4500-4700	MS	3
Pokkali	Landrace	145-150	150-155	10-12	3000-3500	LS	3
NVSR 2272	Jaya × Purna	85-90	125-130	6-7	2800-3000	LS	9
Nona bokra	Landraces	135-140	130-135	6-7	-	LS	3
GR 19	Dandi × IET15429	125-130	115-150	11-13	5000-5500	SB	3
GNR 5	Jaya × GR6	125-130	120-125	10-12	5500-6000	LS	5
GR 25 (Mahatma)	GR7 × Jaya	125-130	125-135	10-12	6200-6500	LB	5
Devlikolam	GR13 × JGL3828	110-115	125-130	10-12	5400-5800	MS	9
Oryza nivara	Wild spp	90	90-100	6-8	-	MS	9
Purna	Annada × RR151-3	95-98	125-130	6-8	2500-2800	SB	9
Lalkada gold	IR 28 × Lalkada	100-105	105-115	8-10	4000-4500	LS	7

DM: Days to maturity**, PH:** Plant height (cm), **PTP:** Productive tillers per plant**, GYPH,** Grain yield per ha**,**
GT: Grain type**, STS:** Salt tolerance score, **LS**: long slander, **MS**: medium slender, **LB**: long bold and **SB**: short bold

Table 2: Standard tolerance score (STS) of visual salt injury at seedling stage (IRRI,

Gregorio *et al.*, 1997)

Score	Observation	Tolerance
1	Normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips or few leaves whitish and rolled	Tolerant
5	Growth severely retarded, most leaves are rolled and only a few are elongating	Moderately tolerant
7	Complete cessation of growth, most leaves dry and some plants dying	Susceptible
9	Almost all plants are dead or dying	Highly susceptible

Table 3. SSR marker used for molecular diversity analysis of 15 rice genotype

Sr. No.	Chr. No.	Primer Name	Genomic location (Mb)	Forword sequence	Reverse sequence
1	1	RM1287	10.83	GTGAAGAAAGCATGGTAAATG	CTCAGCTTGCTTGTGGTTAG
2	1	RM8094	11.23	AAGTTTGTACACATCGTATACA	CGCGACCAGTACTACTACTA
3	1	RM3412	11.23	AAAGCAGGTTTTCCTCCTCC	CCCATGTGCAATGTGTCTTC
4	1	RM493	12.28	TAGCTCCAACAGGATCGACC	GTACGTAAACGCGGAAGGTG
5	1	RM10852	13.97	GAATTTCTAGGCCATGAGAGC	AACGGAGGGAGTATATGTTAGCC
6	1	RM10864	14.25	GAGGTGAGTGAGACTTGACAGTGC	GCTCATCATCCAACCACAGTCC
7	1	RM562	14.62	CACAACCCACAAACAGCAAG	CTTCCCCCAAAGTTTTAGCC
8	1	RM7075	15.11	TATGGACTGGAGCAAACCTC	GGCACAGCACCAATGTCTC
9	1	RM6711	16.11	TAGTGATAGGGGTGGTGTGG	TTACAAGCATGGGAGTTGGG
10	1	RM490	3.05	ATCTGCACACTGCAAACACC	AGCAAGCAGTGCTTTCAGAG
11	1	RM495	0.21	AATCCAAGGTGCAGAGATGG	CAACGATGACGAACACAACC
12	1	RM8115	12.68	TATATAGTAAATTTGTTTGGTGTAGG	ACAGATGGATATTATAAGAAGTAACA
13	6	RM6811	29.22	GGTGATCACCAGCAACACAC	AGCGTGTGACTTCATTGCAC
14	8	RM337	0.15	GTAGGAAAGGAAGGGCAGAG	CGATAGATAGCTAGATGTGGCC
15	2	RM6	29.57	GTCCCCTCCACCCAATTC	TCGTCTACTGTTGGCTGCAC
16	6	RM7158	0.21	GTTAGCAGCGTACCTGGAGC	ATCCTCCCCTCGATTTCATC

Result and discussion

Development of salt tolerant rice genotypes through marker-assisted breeding programs has added advantage over conventional breeding methods (Collard and Mackill, 2008). Molecular markers such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR) and Amplified Fragment Length Polymorphism (AFLP) used for screening genotypes are more reliable than the morphological or physiological markers. Data obtained by molecular markers helps to overcome the limitations associated with G × T interaction of morphological/biochemical markers. Hence, molecular markers are very powerful for genetic diversity estimation as they are environment independent. Among the molecular markers, microsatellite markers have been found to be effective in the identification of genetic variability. Microsatellites or simple sequence repeat (SSR) markers have been more effective due to their dense distribution throughout the genome, high reproducibility, co-dominant alleles and high variability.

In rice, a number of mapping studies have identified quantitative trait loci (QTL) that are associated with salinity tolerance. One such major salinity related QTL in rice is ‘‘*Saltol*’’, associated with seedling stage salt tolerance which maintain Na^+/K^+ ratio in plant. A number of salt stress-related microsatellite markers are mapped to the *Saltol* QTL, which can be used to effectively screen germplasm and landraces. Apart from the *Saltol* region, SSR markers responsive to salt parameters have also been mapped in other rice chromosomal regions (Molla *et al.,* 2015; Thomson *et al.,* 2010). In rice, salinity tolerance naturally exists in landraces/wild germplasms (Majee*et al.,* 2004). The genes/proteins responsible for salinity resistance were used to enhance salinity tolerance ability of modern variety. Hence it is crucial to search for new salt tolerant rice genotypes through screening of available genomic resources. In the present study, however, we have used selected rice landraces, wild species, released cultivar, improved lines and standard tolerant/susceptible check varieties, aims of

study to assess the diversity and different salt tolerance mechanism present in these fifteen rice germplasms.

1. Qualitative and quantitative analysis of genomic DNA

Genomic DNA was extracted from 300 mg of 15-day-old seedlings using the modified CTAB method (Doyle and Doyle, 1990). Quantification of genomic DNA was achieved using a NanoDrop microvolume spectrophotometer. The quality of the DNA was determined by the A260/A280 ratio, which ranged from 2.00 (IR 28) to 2.19 (*Oryza nivara* and Devil Kolam). PCR reactions were carried out in a total volume of 10 µL. The PCR conditions included an initial denaturation for 5 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at temperatures ranging from 54°C to 60°C for 30 seconds, extension at 72°C for 45 seconds and a final extension at 72°C for 10 minutes. A 3.5% agarose gel was used to resolve the bands depending on their molecular size. Images of the band patterns in the gel were taken using a gel documentation system (Bio-Rad).

Table 4:Qualitative and quantitative analysis of genomic DNA

Sr. No.	Genotypes	Concentration of stock solution (ng/µl)	260/280 Ratio	Preparation of working solution (30ng/µl,100µl)	
				Stock solution taken (µl)	Water (nuclease free) added (µl)
1	GR 17	1678.3	2.06	6.0	94.0
2	IR55179-3B-11-3	1885.3	2.16	5.3	94.7
3	IR 28	315.3	2.00	31.7	68.3
4	Dandi	2162.7	2.17	4.6	95.4
5	FL478	2568	2.11	3.9	96.1
6	Pokkali	1735.5	2.12	5.8	94.2
7	NVSR 2272	2143.8	2.12	4.7	95.3
8	Nona bokra	1956.1	2.17	5.1	94.9
9	GR 19	2456.7	2.18	4.1	95.9
10	GNR 5	3893.5	2.11	2.6	97.4
11	GR 25	2276.6	2.13	4.4	95.6
12	Devlikolam	1969.8	2.19	5.1	94.9
13	*Orvzanivara*	1269.9	2.19	7.9	92.1
14	Purna	2733.8	2.15	3.7	96.3
15	Lalkada gold	2197.8	2.12	4.6	95.4
	Average	**2082.87**	-	-	-

In present investigation, the average concentration of DNA extracted from rice leaves was 2082.87 ng/µl, quantified on NanoDrop microvolume spectrophotometer (Table 4). The genotypes GNR 5 and purna showed the highest concentration of 3893.5 ng/µl and 2733.8 ng/µl, respectively. Genotype IR 28 showed the lowest concentration of DNA (315.5 ng/µl).

2. Genotyping by SSR marker

A total of sixteen SSR markers, including markers for the *Saltol* QTL and salt-responsive candidate genes at different loci of rice chromosomes, were selected for the study. The primer sequences of each marker are presented in Table 3. Total 10 SSR markers (RM1287, RM3412, RM8094, RM493, RM8115, RM10825, RM10864, RM562, RM7075, RM6711) are closely linked to the *Saltol* QTL and all these markers falling within the region

between 10.8 mb and 15.8 mb located on chromosome 1. Additionally, other markers (RM495, RM490, RM6, RM7158, RM6811 and RM337) which are not linked to the *Saltol* QTL but associated with salt-responsive genes present on chromosomes 1, 2, 6 and 8 were selected. Primers were chosen from the Anonymous, 2022 and got information from Gramene website (https://archive.gramene.org/markers/microsat). Molecular Profiling of SSR Markers in present in gel image (Photo 1 to 3)

Various biostatistical parameters were calculated which are efficient for diversity analysis. These included amplicon size, number of alleles, major allele frequency, gene diversity, heterozygosity and PIC values (Table 5). The molecular weight of the amplified PCR products ranged from 96 (RM490) to 412 bp (RM337) which reflected remarkable difference in the number of repeats between the different alleles (Adak *et al.*, 2020). A total of 99 alleles were detected among 15 genotypes of rice. The average number of alleles per locus was 6.19 with a range of 4 (RM6811 and RM337) to 8 (RM7075).

Ali *et al.* (2014) reported an average number of alleles per locus of 5.3, which is lower than the current investigation. Similarly, Anyomi*et al.* (2018) found an average of 4.5 alleles per locus and Adak *et al.* (2020) observed an average of 3.90 alleles per locus, both of which are also lower than the current study's findings. In contrast, Darshna *et al.* (2018) identified an average of 8.16 alleles per locus, which is higher than the current investigation.

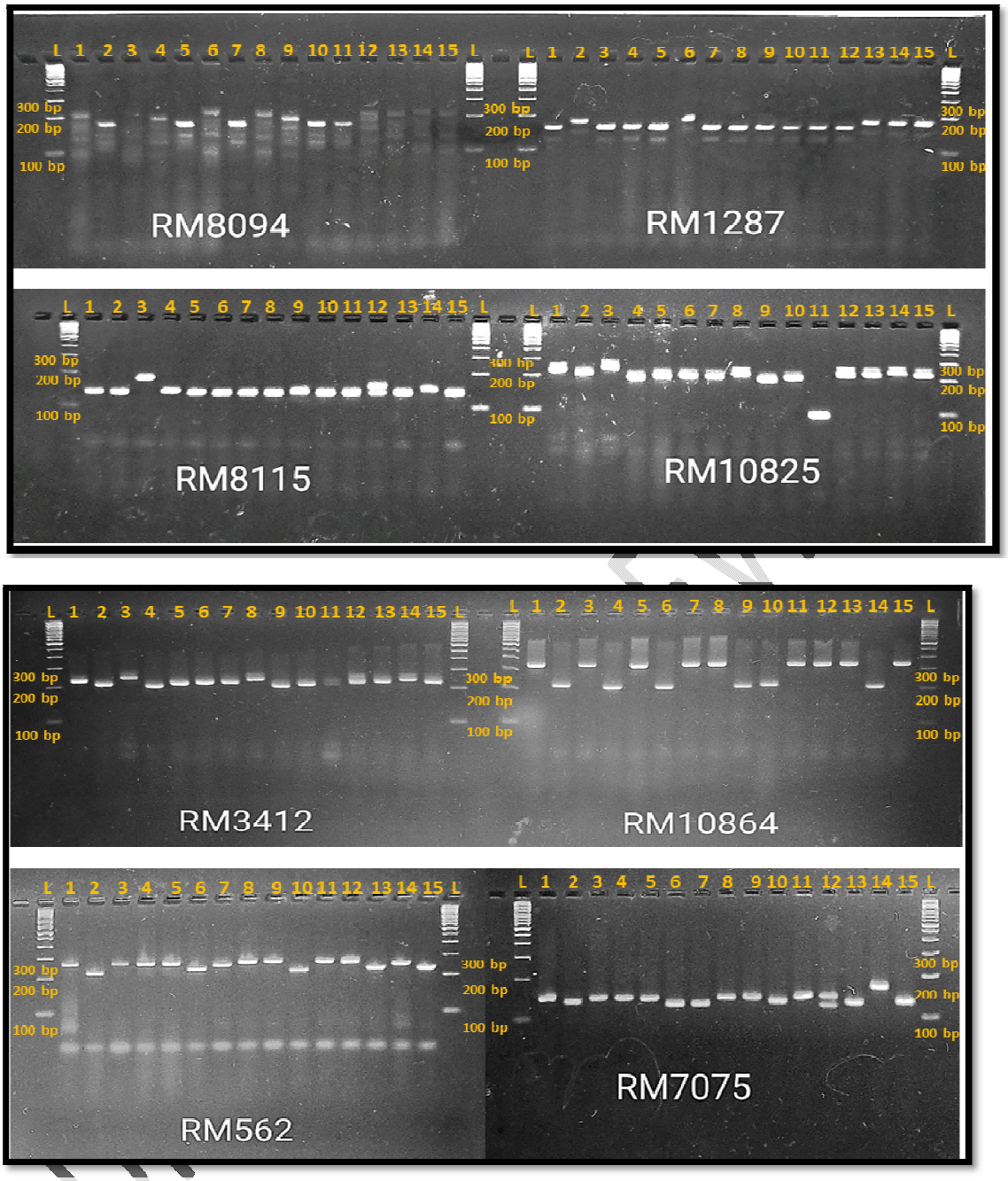

Photo 1: Molecular profiling of SSR markers in 15 rice genotypes

1.	GR 17	2.	IR55179	3.	IR 28	4.	Dandi	5.	FL478
6.	Pokkali	7.	NVSR 2272	8.	Nona bokra	9.	GR 19	10.	GNR 5
11.	GR 25	12.	Devlikolam	13.	*Oryza nivara*	14.	Purna	15.	Lalkada gold

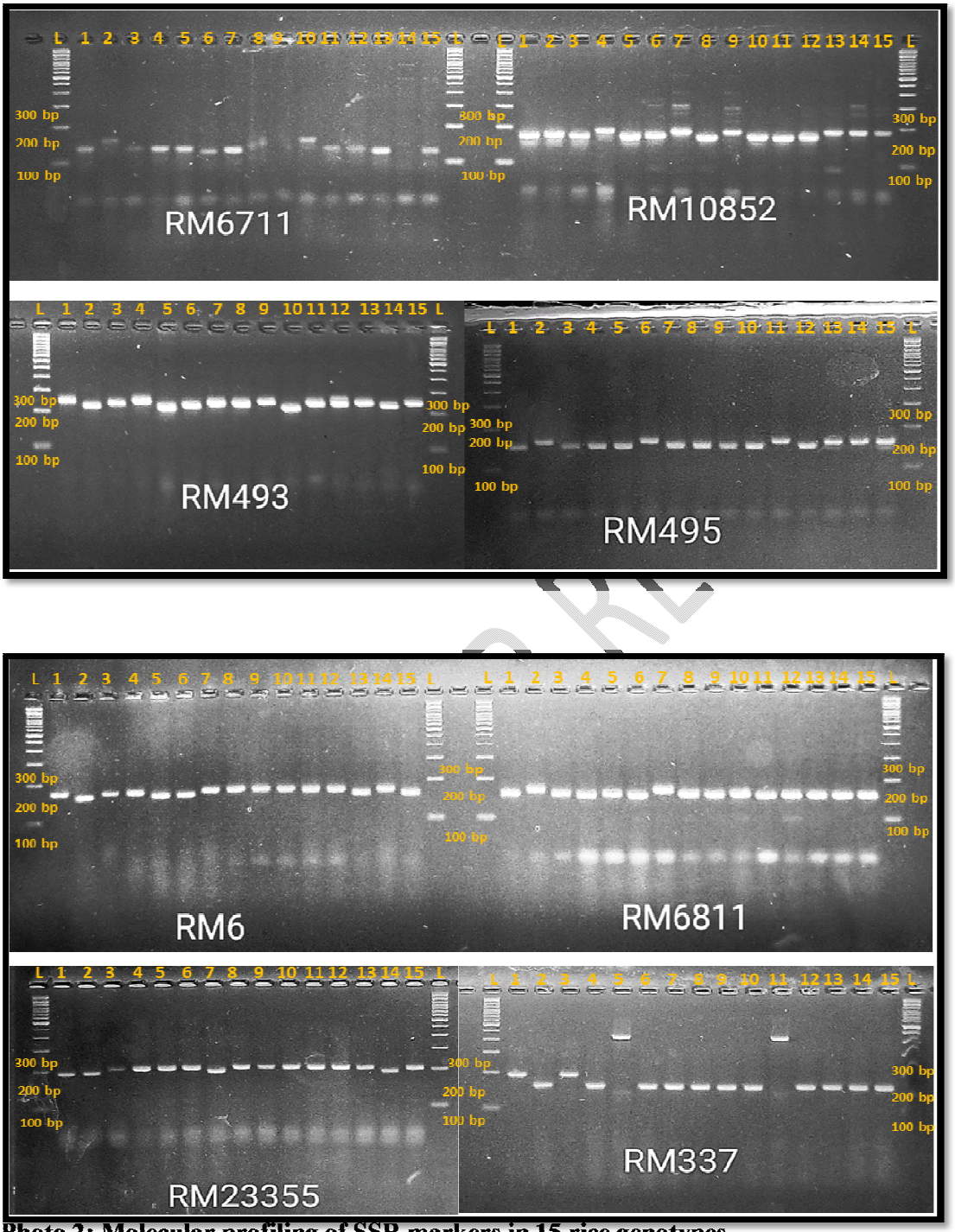

Photo 2: Molecular profiling of SSR markers in 15 rice genotypes

1.	GR 17	2.	IR55179	3.	IR 28	4.	Dandi	5.	FL478
6.	Pokkali	7.	NVSR 2272	8.	Nona bokra	9.	GR 19	10.	GNR 5
11.	GR 25	12.	Devlikolam	13.	*Oryza nivara*	14.	Purna	15.	Lalkada gold

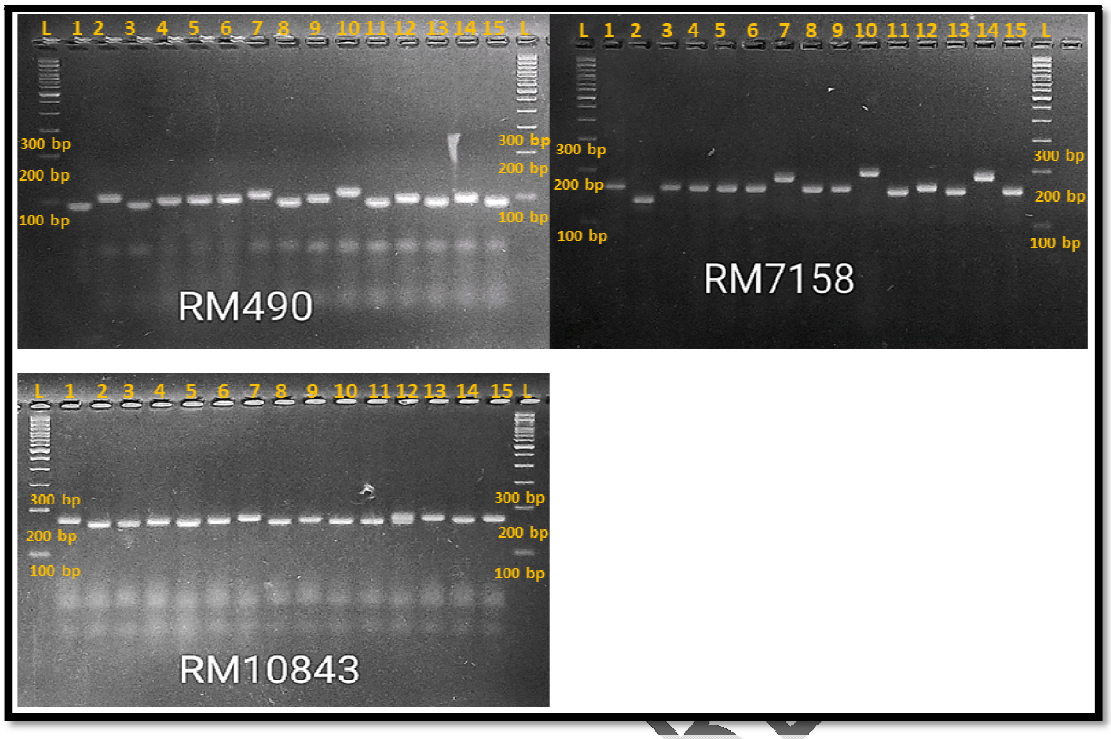

Photo 3: Molecular profiling of SSR markers in 15 rice genotypes

1.	GR 17	2.	IR55179	3.	IR 28	4.	Dandi	5.	FL478
6.	Pokkali	7.	NVSR 2272	8.	Nona bokra	9.	GR 19	10.	GNR 5
11.	GR 25	12.	Devlikolam	13.	*Oryza nivara*	14.	Purna	15.	Lalkada gold

These differences can be attributed to the use of different sets of SSR markers and the genetic variability among the genotypes studied. The higher average number of alleles per locus reported by Darshna *et al.* (2018) suggested that the markers used in their study were more polymorphic or that their sample population had greater genetic diversity. Conversely, the lower average numbers reported by Ali *et al.* (2014), Anyomi*et al.* (2018) and Adak *et al.* (2020) indicate less polymorphism or genetic diversity in their respective studies.

The variation in the average number of alleles per locus among different studies highlights the importance of marker selection and the genetic diversity of the sample population. The current investigation shows a higher average number of alleles per locus compared to some studies, indicating a broader genetic base and possibly more effective marker selection. However, the results from Darshna *et al.* (2018) suggested that even higher polymorphism can be achieved with different markers and genotypes, emphasizing the need for careful selection of SSR markers to maximize genetic diversity assessment.

Table 5: Results of SSR marker analysis

Marker	Amplicon size	Major allele frequency	No. of allele	Gene diversity	Heterozygosity	PIC
RM6	158-198	0.47	7.00	0.73	0.00	0.70
RM6811	146-163	0.60	4.00	0.58	0.00	0.53
RM490	96-118	0.40	7.00	0.76	0.00	0.74
RM495	151-168	0.40	5.00	0.72	0.00	0.67
RM7158	135-189	0.33	7.00	0.80	0.00	0.77

RM337	149-412	0.47	4.00	0.68	0.00	0.62
RM562	225-300	0.33	6.00	0.79	0.00	0.76
RM7075	132-180	0.30	8.00	0.80	0.27	0.77
RM8115	124-175	0.40	7.00	0.73	0.07	0.69
RM3412	208-244	0.40	6.00	0.74	0.07	0.70
RM10864	199-329	0.53	7.00	0.68	0.00	0.65
RM8094	121-240	0.33	7.00	0.78	0.00	0.75
RM1287	156-192	0.60	5.00	0.59	0.13	0.55
RM493	209-245	0.40	5.00	0.75	0.00	0.71
RM6711	125-158	0.33	7.00	0.80	0.00	0.77
RM10825	87-250	0.27	7.00	0.81	0.00	0.78
	Mean	0.41	6.19	0.73	0.03	0.70
	Total	6.57	99.00	11.73	0.53	11.19
	Min	0.27	4.00	0.58	0.00	0.53
	Max	0.60	8.00	0.81	0.27	0.78

With a mean major allelic frequency of 0.41, the values ranged from 0.27 (RM10825) to 0.60 (RM6811 and RM8115). This indicates a moderate to high level of polymorphism among the markers used. The highest gene diversity was observed for RM10825 (0.81), while the lowest was for RM6811 (0.58), with an overall mean gene diversity of 0.73.

When compared to previous studies, such as Adak *et al.* (2020), who reported gene diversity values ranging from 0.0894 to 0.8178 with an average of 0.4711, the current study shows a higher average gene diversity (0.73). This suggests that the SSR markers used in this study were more effective in capturing the genetic variability among the rice genotypes. The higher gene diversity observed in the current study could be due to the inclusion of a more diverse set of genotypes or the selection of highly polymorphic markers. The findings underscore the importance of using a well-selected set of markers to accurately assess the genetic diversity within a population. High gene diversity is crucial for the success of breeding programs as it provides a broader genetic base for selection and improvement. This study demonstrates the utility of SSR markers in evaluating genetic diversity and highlights the genetic potential of the rice genotypes screened for salinity tolerance.

Rice is a self-pollinated crop, which reduces gene flow between populations, thereby preventing the combination of gene pools, decreasing genetic heterozygosity and increasing homozygosity. PIC values of markers provide an estimate of their discriminating power in a set of accessions, taking into account both the number of alleles and the relative frequencies of each allele.

In the present study, PIC values for SSR markers ranged from 0.53 (RM6811) to 0.78 (RM10825). All most markers were highly informative, with PIC values exceeding 0.6, The average PIC value of SSR markers in this study was 0.70, which is slightly higher than the 0.4482 reported by Adak *et al.* (2020) for screening salinity tolerance in rice, with values ranging from 0.0854 to 0.7939. However, it is slightly lower than the average PIC value of 0.74 reported by Ali *et al.* (2014), which ranged from 0.67 to 0.84.

Out of sixteen marker RM490, RM7158, RM562, RM3412, RM493, RM7075, RM8094, RM6711 and RM10825 are the most proficient descriptors to screen salt tolerant genotypes with higher PIC (more than 0.75) value and significantly distinguished salt tolerant genotypes. Ali *et al.* (2014) reported that RM8094, RM493, RM7075 and RM3412 higher PIC value. Adak *et al.* (2020) also reported that RM7075, RM8094 and RM562 have higher PIC value.

The higher PIC value in this study could be attributed to the high genetic diversity among the 15 rice genotypes evaluated. Additionally, the high PIC value and large number of alleles per marker may also be due to the nature of the materials studied, as suggested by Ramu *et al*. (2013). The high PIC values indicate the effectiveness of the selected SSR markers in distinguishing among the rice genotypes, making them valuable tools for genetic studies and marker-assisted selection in rice breeding.

3. Genetic relationship among fifteen rice genotypes based on SSR markers

Genetic diversity is commonly measured by genetic distance or genetic similarity, both of which imply that there are either differences or similarities at genetic level. Molecular marker based genetic diversity analysis also has potential for assessing changes in genetic diversity over time and space. Among the molecular markers, microsatellites or SSRs (Simple Sequence Repeats) were chosen as one of the best marker systems for genotyping of germplasm collections due to their high polymorphic information content (PIC), co-dominant inheritance, locus specificity, extensive genome coverage and simple detection using labelled flank primers. The dendrogram depicted here (Figure 1), represents the genetic relationships among 15 rice genotypes based on their salt tolerance.

The dendrogram illustrates the genetic relationships among different rice genotypes based on molecular markers. The clustering of genotypes into different groups indicates their genetic similarities and differences.

Cluster 1: Lalkada gold, *Oryza nivara*, Purna having high bootstrap value (100) suggests strong genetic similarity. Phenotypically these genotypes are highly susceptible to salt stress. Except Lalkada gold, which are in susceptible genotype.

Cluster 2: GR 25 (Mahatma) and Devli Kolam had same cluster, but we found phenotypically that GR 25 is moderately tolerant, wherever Devli Kolam is a susceptible genotype.

Cluster 3: GR 19 and Nona bokra are tolerant genotypes, with GR 19 and Nona bokra showing close genetic similarity (bootstrap 29).

Cluster 4: FL478 and Dandi are shared in to common cluster, but phenotypically FL478 found tolerant, whereas dandi found as moderate tolerant genotype.

Cluster 5: IR 28, GR 17 are susceptible genotypes, with IR 28 closely related to GR 17 (bootstrap 46), indicating some level of shared genetic background. But phenotype class of IR 28 is susceptible and GR 17 founded as a moderate genotype. GNR 5 also correlated with this cluster, indicating intermediate genetic traits.**Cluster 5** is phenotypically correlated with **cluster 2.**

Cluster 6: IR55179-3B-11-3, Pokkali are highly tolerant genotypes, with IR55179-3B-11-3 showing close similarity to Pokkali. Bootstrap 44 highlighting moderate genetic similarity.**Cluster 6** is phenotypically correlated with **cluster 3** and **cluster 4.**

Cluster 7: NVSR 2272 stands alone but still related to **Cluster 1**, indicating high susceptibility.

The genetic dendrogram in question shows some exceptions where the genotypes do not cluster according to their phenotypic classification (salt tolerance levels). This discrepancy can be attributed to several factors, each with a basis in genetic and molecular biology principles.

Genetic background and recombination: Random recombination during meiosis can result in genetic shuffling, causing progeny to inherit a mix of alleles from both parents. This shuffling can obscure the clear phenotypic traits observed in parents, leading to genotypes clustering differently than expected (Lander *et al.*,1989)

Multiple mechanisms of salt tolerance: Salt tolerance in rice is a complex trait governed by multiple genetic mechanisms, including ion transport regulation, osmotic

adjustment, and stress-responsive gene expression. Different genotypes may possess different mechanisms for salt tolerance, leading to varied genetic profiles despite similar phenotypic traits (Munns *et al.,* 2008).

Marker density and coverage: The genetic markers used in the study may not cover all regions associated with salt tolerance. Incomplete marker coverage can result in genotypes clustering based on regions not related to salt tolerance, leading to discrepancies between phenotypic and genetic clustering (Collard *et al.,* 2005)

Epistatic Interactions: Interactions between different genes (epistasis) can influence the expression of salt tolerance. These interactions might not be captured by the markers used, causing genotypes with similar phenotypic traits to appear genetically distinct (Phillips *et al.,* 2008)

Environmental influence: Environmental factors can influence the expression of traits phenotype critical environments contributing to discrepancies between phenotypic and genetic data (Marais *et al*., 2013)

Specific exceptions in the dendrogram: IR55179, Pokkali, FL478, Nona Bokra, GR 19 are known tolerant genotypes but do not cluster together. This could be due to different mechanisms of salt tolerance, segmental recombination and different genetic background, which are not captured by the few markers used. GR 17, Dandi, GNR 5 and GR 25 are moderately tolerant genotypes cluster differently, possibly due to their unique genetic backgrounds and the influence of minor QTLs not marked in the study. IR 28, Lalkada gold are susceptible genotypes clustering apart suggests that their susceptibility might be due to different genetic causes or environmental factors influencing their expression. Devlikolam, Purna, *Oryza nivara* and NVSR 2272 are highly susceptible genotypes might have diverse genetic backgrounds with different pathways leading to high susceptibility, hence their differences are visible in the dendrogram.

The discrepancies between the phenotypic classification and genetic clustering in the dendrogram highlight the complexity of genetic traits such as salt tolerance. Factors such as random recombination, multiple tolerance mechanisms, incomplete marker coverage, epistatic interactions and environmental influences all contribute to these exceptions. Understanding these nuances is crucial for refining breeding programs and developing robust salt-tolerant rice varieties.

4. Correlation between matrices

A genetic dissimilarity matrix is a tool used in genetics to quantify the genetic differences between pairs of genotypes. Here is a breakdown of the key points and how to interpret this specific matrix. Matrix structure is the symmetric, meaning the dissimilarity between genotype " *i* " and genotype "j" is the same as between " *j* " and " *i* ". The diagonal elements (all zeroes) indicate that the genetic dissimilarity between a genotype and itself is zero. Values range from 0 to 1, where 0 indicates identical genetic makeup and 1 indicates complete dissimilarity. The values represent the proportion of genetic difference between pairs of genotypes.

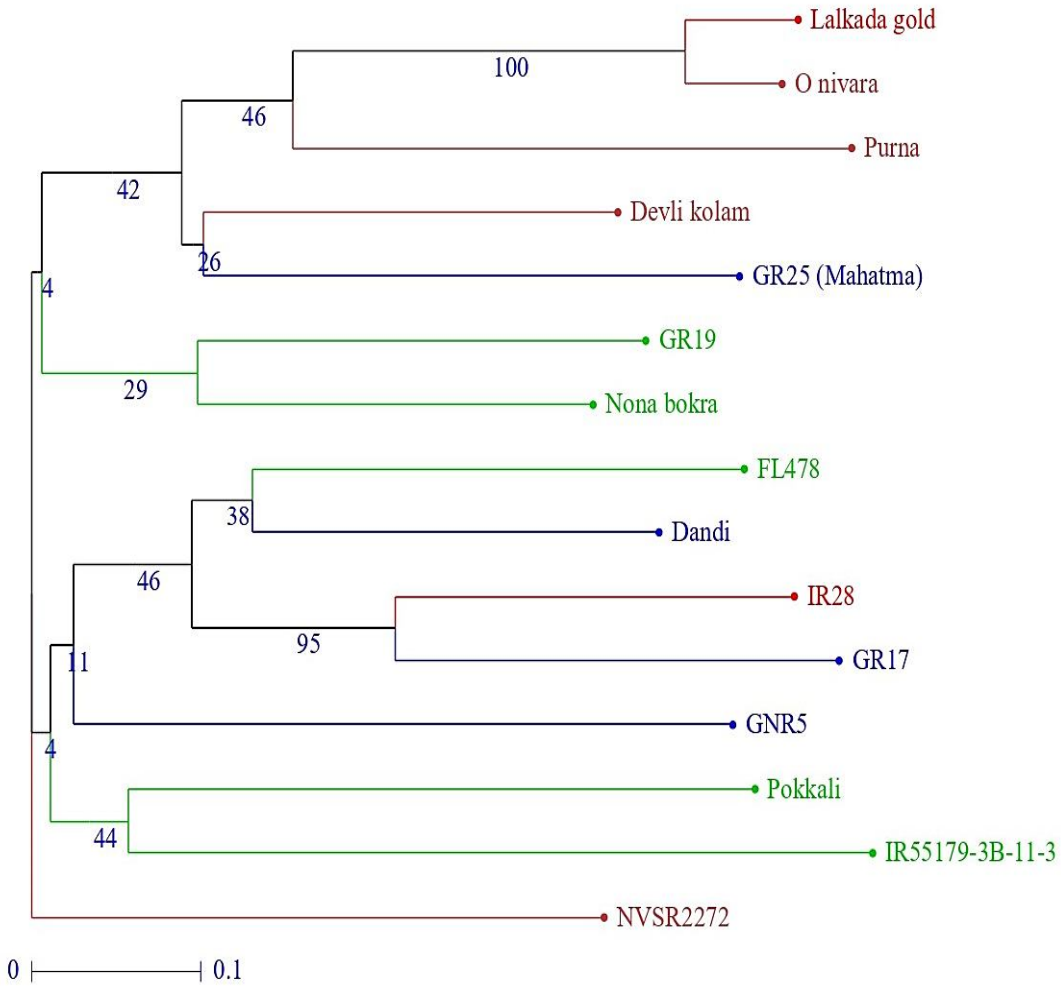

Figure 1: Dendrogram showing relationship among 15 rice genotypes generated by DARwin using molecular marker data

Table 6: Dissimilarity matrix for Nei's genetic distance of 15 rice genotypes based on pooled SSR analysis

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0														
2	1	0													
3	0.5	1	0												
4	0.688	0.875	0.563	0											
5	0.781	0.875	0.656	0.531	0										
6	0.938	0.813	0.875	0.688	0.75	0									
7	0.75	0.875	0.813	0.75	0.688	0.75	0								
8	0.844	0.875	0.781	0.781	0.813	0.75	0.563	0							
9	0.844	0.813	0.781	0.656	0.75	0.813	0.813	0.5	0						
10	0.813	0.813	0.813	0.75	0.813	0.875	0.813	0.813	0.688	0					
11	0.875	0.938	0.844	0.844	0.844	0.938	0.781	0.719	0.781	0.75	0				
12	0.844	0.938	0.781	0.594	0.688	0.781	0.625	0.625	0.781	0.844	0.563	0			
13	0.875	0.875	0.938	0.813	0.938	0.938	0.813	0.75	0.875	0.813	0.625	0.563	0		
14	0.938	0.938	0.938	0.938	0.938	0.75	0.813	0.75	0.813	0.938	0.813	0.75	0.625	0	
15	0.875	0.875	0.938	0.875	0.938	0.938	0.813	0.75	0.813	0.875	0.625	0.625	0.125	0.625	0

1. GR 17
2. IR55179-3B-11-3
3. IR 28
4. Dandi
5. FL478
6. Pokkali
7. NVSR 2272
8. Nona bokra
9. GR 19
10. GNR 5
11. GR 25
12. Devlikolam
13. *Oryza nivara*
14. Purna
15. Lalkada gold

Based on the results of dissimilarity matrix (Table 6), those genotypes having value close to 0, indicate high genetic similarity. For example, between GR 17 and IR 28 (0.5) or between genotype NVSR 2272 and Nona bokra (0.563). Those genotypes having high values close to 1, indicate high genetic dissimilarity. For example, between GR 17 and Pokkali and Purna (0.938). GR 17 and IR55179-3B-11-3 have a dissimilarity value of 1, indicating they are completely dissimilar. GR 17 and Dandi have a dissimilarity value of 0.688, suggesting they have moderate genetic similarity. Purna and Lalkada gold have a dissimilarity value of 0.625, indicating they have a higher similarity compared to other pairs like GR 17 and IR55179-3B-11-3.

This matrix can be used to create dendrograms (as shown in previous), helping to visualize genetic relationships. It helps in identifying clusters of genotypes with similar genetic backgrounds and understanding the genetic diversity within a set of genotypes. The genetic dissimilarity matrix is a crucial tool in genetic analysis, providing detailed insights into the genetic relationships between different genotypes. By quantifying genetic differences, it aids in various applications, creating variability by crossing genetically dissimilar parents, identify genotypes having different salt tolerance mechanisms to develop durable tolerant genotype, from breeding to conservation, ensuring informed decision-making based on genetic data.

Conclusion

In sense, the markers RM490, RM7158, RM562, RM3412, RM493, RM7075, RM8094, RM6811 and RM10825 were particularly proficient in screening for salinity tolerance in different rice genotype due to more PIC value. The dendrogram generated reveals the genetic similarities and differences among the genotypes, forming several distinct clusters. The discrepancies between phenotypic classifications and genetic clustering can be attributed to several factors: Including genetic shuffling during meiosis can obscure clear phenotypic traits, leading to unexpected clustering. Different genotypes may utilize distinct genetic mechanisms for salt tolerance, resulting in varied genetic profiles despite similar phenotypes.Incomplete coverage by genetic markers can cause clustering based on regions unrelated to salt tolerance. Interactions between genes can affect the expression of salt tolerance, potentially causing genetic distinctions despite similar phenotypes.Environmental factors can impact trait expression, contributing to discrepancies between phenotypic and genetic data.Specific exceptions in the dendrogram, such as the clustering of known tolerant genotypes (IR55179, Pokkali, FL478, Nona Bokra, GR 19) and the separation of susceptible genotypes (IR 28, Lalkada gold), highlight the complexity of genetic traits like salt tolerance. The study emphasizes the importance of considering these nuances for refining breeding programs and developing robust salt-tolerant rice varieties. The dissimilarity matrix revealed significant genetic relationships among the 15 rice genotypes, for instance, GR 17 and IR 28 (0.5) are highly similar, whereas GR 17 and IR55179-3B-11-3 (1.0) are completely dissimilar. These findings, visualized through dendrograms, highlight clusters of genotypes with similar genetic backgrounds and underscore the genetic diversity within the set. The matrix is a valuable tool for breeding programs, aiding in the selection of genetically diverse parents for crossing, identifying genotypes with different salt tolerance mechanisms, and ultimately developing robust, salt-tolerant rice varieties.

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Details of the AI usage are given below:

1. Goggle scholar

2. Sci-hub

3. ChatGPT for language correction

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