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Screening of rice (*Oryza sativa* L.) cultures for biotic and abiotic stress using SSR markers

Hemant S Sawant, RS Deshpande, SV Sawardekar, SG Mahadik, Samiksha C, Mahendra KC and Govind P

Abstract
The current research employed 18 simple sequence repeat (SSR) markers to screen 27 rice cultures for biotic (bacterial blight, blast, gall midge, brown plant hopper) and abiotic stress tolerance (salinity, drought) and to develop rice culture profiles using SSR markers. DNA was extracted from twenty-seven rice cultures and molecular profiling was performed using 18 SSR markers. MVSP software was used to determine genetic profiles. Blast tolerant linked alleles were not found in any of the 27 rice genotypes investigated. Bacterial Blight tolerant linked alleles were discovered in cultures Nom-2102, Nom-2104, Nom-2107, Nom-2108, Nom-2110, Nom-2111, Nom-2118, and Nom-2119. Gall midge tolerant linked alleles were found in the cultures Nom-2112, Nom-2114, Nom-2115, Nom-2116, Nom-2117, Nom-2118, Nom-2119, Nom-2121, Nom-2122, Nom-2124, Nom-2127, and Nom-2132. Brown Plant Hopper tolerant linked alleles were found in Nom-2117, Nom-2118, Nom-2119, and Nom-2123 cultures. There were no drought-tolerant cultures found. Salt tolerance linked allele were found in Nom-2102, Nom-2103, Nom-2104, Nom-2105, Nom-2108, Nom-2109, Nom-2110, Nom-2111, Nom-2112, Nom-2116, Nom-2117, Nom-2132, and Nom-2133. All 18 primers amplified and exhibited 100% polymorphism. There was a total of 75 alleles detected, with an average of 4.16 alleles per primer. The polymorphism information content (PIC) values ranged from 0.314 to 0.927, with an average PIC value of 0.619 per primer. The genetic distance varied from 0.053 to 0.818 and indicated a wide range of variation. All 27 rice cultures were categorized into two clusters: I, which had 10 cultures, and II, which contained 17 cultures by UPGMA. This leads to the conclusion that molecular screenings of rice crops employing SSR markers gave sufficient knowledge on biotic and abiotic tolerance traits at the molecular level. This research will be used in future strategic crop enhancement breeding programs.

Keywords: Molecular screening, MAS, polymorphism, SSR, biotic and abiotic stress

Introduction
Rice is a staple meal for 33 percent of the total population, and it occupies almost one-fifth of the entire geographical area covered by grains. It has the scientific name *Oryza sativa* L. and the chromosomal number 2n = 24. It belongs to the Poaceae family and subfamily Oryzoidea. Rice is one of the most important food crops, and it requires a lot of water during its life cycle compared to other crops (Wang *et al*., 2012) [71]. In comparison to maize, wheat, and potatoes, raw, long-grain white rice provides a comparatively rich source of energy carbohydrates, calcium, iron, thiamine, pantothenic acid, and vitamin E. Rice is an excellent model plant for investigating grass hereditary characteristics and genome association because to its diploid hereditary traits and genome association. The genome size of the rice plant is 430 Mb, which is significantly smaller (Causse *et al*., 1994; Kurata *et al*., 1994) [12, 41]. The main concerns that address the need for sustainable crop development and resilience to biotic and abiotic stresses are the emergence of new diseases and insect pests as well as the changing climate. Understanding the genetic/molecular basis of target attributes needs to be fully explored in order to accurately manipulate the genetics of complex quantitative traits like yield, resistance to biotic/abiotic stresses, quality, etc. Abiotic and biotic stresses can both be detrimental to rice. Examples of biotic stresses include fungus, insect pests, and bacteria, whereas abiotic stresses like salt and drought have been thoroughly examined in rice and have been proven to reduce average yields by more than 50% (Bray *et al*., 2000 and Iqbal *et al*., 2013) [11, 28]. The advent of novel pests and illnesses, as well as environmental changes, provide substantial challenges that necessitate research into abiotic and biotic stress tolerance (Hasan *et al*., 2015) [25]. It has recently been possible to transfer genes that impart resistance to biotic stresses (blast, bacterial blight, gall midge, etc.) and abiotic stresses (salinity, drought, etc.) due to technical advancements and the introduction of DNA-based molecular markers.

With the advancements achieved in the field of molecular markers, it is now feasible to identify plants with two or more resistance genes by identifying the path of markers that are linked to or tagged to each gene for resistance. For genetic characterisation, a variety of molecular markers are used. Microsatellites and Simple Sequence Repeats (SSR) are simple tandemly repeated, di- to tetra-nucleotide sequence designs delimited by distinct sequences among several molecular markers that are co-dominant and highly polymorphic (Hamada *et al*., 1982) [23]. SSR markers are thus a superior option for molecular screening of rice. Genetic diversity research is critical for such improvements, as yield and grain quality decline over time as a result of both biotic and abiotic stresses (Nadia *et al*., 2014) [49]. Molecular analysis of available cultures is useful for determining the genetic potential of rice crops and for preventing erosion, which can be interpreted as a loss in genetic variability over time (Manifesto *et al*., 2001) [44]. There are around 40,000 rice types reported globally, however only a small percentage have been employed in breeding. Rice breeding will benefit from a better knowledge of the genetic composition of different rice cultivars. Crop progress is limited unless screening and genetic variety are preserved in plant breeding programmes, as well as the application of novel biotechnological technologies. Rice has a lot of genetic variation among and amongst wild genotypes, which means there's a lot of room for future crop enhancement. Molecular screening of rice genotypes for biotic and abiotic stresses using microsatellites offers enormous potential for expediting breeding studies. The use of diverse markers in the molecular study of significant biotic and abiotic stress resistance imparting traits across genotypes would be advantageous in the selection of parents for hybridization and marker assisted selection to boost productivity even under adverse conditions. Molecular analysis will assist breeders in developing strategic breeding programmes that will result in improved and tolerant rice cultivars. The purpose of this research was to use SSR markers linked with tolerance traits or QTLs to screen cultures for biotic and abiotic stresses and to determine the best cultures to be used as a donor for tolerance to multiple stresses in future breeding programmes for the development of new rice varieties that are equally beneficial to farmers and the scientific community. In the findings presented here, 18 SSR markers were employed to identify 27 rice cultures. SSR data were used to determine genetic relationships among accessions and to measure the extent of genetic diversity within rice cultures.

Materials and Methods

The research work was carried out in the laboratory of Plant Biotechnology Centre, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist. – Ratnagiri, Maharashtra, India during the period of 2021-2022. For the present investigation, the seeds of 27 rice cultures were obtained from Agricultural Research Station, Shirgaon, Dist- Ratnagiri, Maharashtra, India. Details of cultures are listed in Table 1.

Table 1: Details of cultures used in the study

Sr. No.	Name of cultures	Sr. No.	Name of cultures
1.	Nom-2101	15.	Nom-2117
2.	Nom-2102	16.	Nom-2118
3.	Nom-2103	17.	Nom-2119
4.	Nom-2104	18.	Nom-2121
5.	Nom-2105	19.	Nom-2122
6.	Nom-2107	20.	Nom-2123
7.	Nom-2108	21.	Nom-2124
8.	Nom-2109	22.	Nom-2127
9.	Nom-2110	23.	Nom-2128
10.	Nom-2111	24.	Nom-2129
11.	Nom-2112	25.	Nom-2131
12.	Nom-2114	26.	Nom-2132
13.	Nom-2115	27.	Nom-2133
14.	Nom-2116		

DNA Extraction

The DNA was isolated using the protocol described by Edwards *et al*., (1991) [17]. The young newly flushed 10–12-day old leaves were taken and disinfected with 70% ethanol to avoid contamination for DNA extraction. Leaf tissue (100 mg) was cut into small pieces and put in a 1.5 ml Eppendorf tube to ensure that the sample size was consistent. Tissue was macerated for 15 seconds at room temperature without buffer with a Mortar and pestle. Extraction buffer (500 µl) was added, and the leaf tissue was gently macerated for a few seconds and then kept in a hot water bath at 65 °C for 45 minutes. The sample was cooled to room temperature and centrifuged at 10000 rpm for 10 minutes. The aqueous layer was transferred to a new Eppendorf tube, and 200 µl of chloroform: iso-amyl alcohol (24:1) was added and mixed by gentle inversion for 5-6 times. After that, the mixture was centrifuged for 10 minutes at 8000 rpm. A double amount of chilled iso-propanol was added to the supernatant and incubated at -20 °C overnight. The solution was centrifuged at 8000 rpm for 10 minutes the next day, and pellets were collected. The pellet was rinsed with 100 µl of 70% ethanol and then centrifuged for 10 minutes at 8000 rpm. The pellet was dried, re-suspended in 50 µl of 1x TE buffer, incubated for 30 minutes at 37 °C in a water bath, and then kept at -20 °C for further use. Using the Agarose gel electrophoresis technique, the quality of the extracted DNA was verified.

Microsatellite assay

In order to conduct a molecular screening of rice genotypes, 18 different trait-specific microsatellites, or SSR markers, that are evenly dispersed throughout all 12 rice chromosomes, were used (Table 2). The 'Gramene' database (http://www.gramene.org) was used to determine these SSR

markers' physical locations on the 12 chromosomes of rice, as well as their relationship to several genes that confer resistance to bacterial blight, blast, gall midge and tolerance to salt and drought. Thermocycler was used to perform the PCR reactions, which had a total volume of 20 µl and contained 10 ng of genomic DNA, 10X assay buffer, 10mM of dNTPs, 25 mM $MgCl_2$, 10 pmol of forward and reverse primers, 3 U Taq polymerase enzyme, and molecular grade water. The PCR cycles were set at 94 °C for 5 minutes, 94 °C for 20 seconds, 55 °C for 30 seconds, 72 °C for 45 seconds for 35 cycles, and 72 °C for 7 minutes for final extension. The amplified products were separated on 1.8% agarose gel prepared in 1X TAE buffer and stained with Ethidium bromide. The gel was run for 100 minutes in 1X TAE buffer at a constant voltage of 80 V.

Table 2: List of SSR primers with their sequences and linked gene

Sr. No	Primer	Sequence Forward primer	Sequence Reverse Primer	Chromosome No.	Linked gene	Reference
1.	RM 140	TGCCTCTTCCCTGGCTCCCCTG	GGCATGCCGAATGAAATGCATG	1	Saltol	Karmarkar *et al*., 2012 [75]
2.	RM 1287	CCATTTGCAGTATGAACCATGC	ATCATGCAATAGCCGGTAGAGG	1	Saltol	Ganie *et al*., 2016 [20]
3.	RM 562	GGAAAGGAAGAATCAGACACAGAGC	GTACCGTTCCTTTCGTCACTTCC	1	Saltol	Ganie *et al*., 2016 [20]
4.	RM 3412	AAAGCAGGTTTTCCTCCTCC	CCCATGTGCAATGTGTCTTC	1	Saltol	Islam *et al*., 2015 [31]
5.	RM 6775	AATTGATGCAGGTTCAGCAAGC	GGAAATGTGGTTGAGAGTTGAGAGC	6	Bph25	Myint *et al*., 2012 [48]
6.	RM 309	CACGCACCTTTCTGGCTTTCAGC	AGCAACCTCCGACGGGAGAAGG	12	Bph26	Myint *et al*., 2012 [48]
7.	RM 5926	ATATACTGTAGGTCCATCCA	AGATAGTATAGCGTAGCAGC	11	Pi1	Thippeswamy *et al*., 2015 [68]
8.	RM 8225	GCGTGTTCAGAAATTAGGATACGG	GATCTCGCCACGTAATTGTTGC	6	Pi-z	Thippeswamy *et al*., 2015 [68]
9.	RM 206	ATCGATCCGTATGGGTTCTAGC	GTCCATGTAGCCAATCTTATGTGG	11	Pi-kh	Kumar *et al*., 2013 [40]
10.	RM 212	AAGGTCAAGGAAACAGGGACTGG	AGCCACGAATTCCACTTTCAGC	1	Dr	Ashfaq *et al*., 2014 [5]
11.	RM 302	TGCAGGTAGAAACTTGAAGC	AGTGGATGTTAGGTGTAACAGG	1	Dr	Ashfaq *et al*., 2014 [5]
12.	RM 3825	CCACTAGCAGATGATCACAGACG	GAGCACCTCATAAGGGTTTCAGC	1	Dr	Kanagraj *et al*., 2010 [35]
13.	RM 201	CTCGTTTATTACCTACAGTACC	CTACCTCCTTTCTAGACCGATA	9	Dr	Kanagraj *et al*., 2010 [35]
14.	RM 1233	ATGGGCACGTGTAATTCATTCG	ATCCTCGAAAGTAGGAGTAGGAAAG	11	Pi-1	Ramadevi *et al.* 2015 [60].
15.	pTA248	AGACGCGGAAGGGTGGTTCCCGGA	AGACCGGGTAAT CGAAAGATGAAA	11	Xa21	Sabar *et al*., 2016 [64]
16.	RM 122	GAGTCGATGTAATGTCATCAGTGC	GAAGGAGGTATCGCTTTGTTGGAC	5	Xa5	Sabar *et al*., 2016 [64]
17.	RM 22709	CGCGTGGGCGAGACTAATCG	CCTTGACTCCGAGGATTCATTGTCC	8	Gm8	Mohapatra *et al*., 2016 [46]
18.	RM 547	TTGTCAAGATCATCCTCGTAGC	GTCATTCTGCAACCTGAGATCC	8	Gm4	Kalpana *et al*., 2016 [33]

Data analysis and scoring

Using the 100 bp DNA ladder as a reference size, the sizes of the amplified fragments were determined using the Uvi-tec Fire Reader software by Gel documentation system. Markers were scored based on whether the corresponding band existed among the cultures (1) or not (0). UPGMA cluster analysis was employed to generate a Dendrogram using Jaccard's similarity coefficient matrices calculated from SSR markers across 27 rice cultures. With the Multivariate Statistical Package (MVSP), a pairwise similarity index (SI) was estimated and a UPGMA-based dendrogram of 27 rice cultures was generated. To assess the in formativeness of the markers, the polymorphism information content (PIC) for each SSR marker was calculated using the formula,

$$\text{Percent polymorphism} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \text{ X } 100$$

The Polymorphism Information Content (PIC) value was determined using a formula developed by Powell *et al*., (1996) [58]

$$PIC = 1-\sum P_{ij}^2$$

Where, P_{ij} is the frequency of i^{th} and j^{th} locus, summed across the entire locus over all lines. PIC values range from 0 (monomorphic) to 1 (very highly discriminative, with many alleles each in equal and low frequency) were estimated for each profile generated across 27 rice cultures.

Results and Discussion

Biotic Stresses

Blast: The hemi biotrophic, filamentous heterothallic ascomycetous fungus *Pyricularia grisea*, sometimes referred to as *Magnaportha oryzae*, is what causes the blast disease of rice Divya *et al* (2014) [16]. It is the most devastating fungus that affects rice, greatly reducing rice productivity and presenting a serious danger to global food security (Miah *et al*., 2013) [45]. The annual loss of rice output brought on by the explosion may provide for the annual food needs of 60 million people (Parker *et al*., 2008) [54]. One of the most crucial methods for disease control in rice is the exploitation of resistant gene resources.

There are now over 100 blast resistance genes known, and many of them have been cloned and described, including *Pik*, Pb1, Pid3, Pia, Pib, Pid2, Pik-h/Pi54, Pik-m, Pik-p, Pit, Pita, Piz-t, Pish, Pi2/Piz-5, Pi1, Pi5, Pi9, Pi21, Pi56, Pi25, Pi36, Pi35, Pi37, Pi64, Pi63 (Devanna *et al*., 2014) [15]. There are several resistant rice cultivars created to withstand rice blast, however the resistance is short-lived because to the high pathogen plasticity (Bonman *et al*., 1986; Lang *et al*., 2009) [10, 42]. The development of long-lasting disease tolerant methods against a wide range of blast pathogens is required. The discovery of many resistance genes in plants is best accomplished through the use of molecular breeding techniques. Due to the poor precision in the identification of suitable genotypes and the tedious and time-consuming procedure, this is challenging to achieve using conventional breeding techniques.

In this study, 27 rice cultures were used to identify blast resistance linked genes using four SSR primers, RM-8225, RM-206, RM-5926, and RM-1233. Primer RM-206, which is specific to *Pi-kh* resistance linked allele, conferred absence of the resistance linked allele as no fragment was amplified at 140 bp in the cultures. By using the marker RM-206, Kumar *et al*. (2013) [40] tested rice genotypes for blast resistance associated alleles particular to the gene *pi-kh*.

Using the gene-specific primer RM-8225, which is predicted to amplify a 221 bp fragment in the genotypes harbouring the resistance linked allele, the cultures were examined for the

presence of the blast resistance gene, *Pi-z*. The blast resistance gene *Pi-z* for the primer RM-8225 was not found in any culture to have the resistance linked allele. By utilising the marker RM-8225, Fjellstrom *et al*., (2004) [18] found resistance related alleles unique to gene *Pi-z*.

By visualising amplicons of 170 bp segments using the microsatellite marker RM-1233, the cultures were examined for the existence of the resistance linked allele for gene *Pi-1*. The findings showed that no cultures for the gene *Pi-1* specific marker RM-1233 suggested the presence of resistance linked alleles. Researchers Ashkani *et al*., (2011) [6], Ramadevi *et al*., (2015) [60], and Yadav *et al*., (2017) [73] reported using the primer RM-1233, which is specific to the gene *Pi-1*, to test rice genotypes for blast resistance.

The tolerant linked allele *pi-1* (176 bp) in the primer RM-5926 was not found in any of the 27 rice cultures. Thippeswamy *et al*., (2015) [68] used the marker RM-5926 to check rice genotypes for the presence of resistance linked alleles unique to the resistance gene *Pi-1*. Anupam *et al*., (2017) [4], Randive *et al*., (2019) [61], Jayawardana *et al*., (2014) [32] SSR primers were also used by Bhagwat *et al*., (2017) [8] to analyse the various cultivars' levels of blast resistance.

Bacterial Blight

The Bacterial Leaf Blight is caused by *Xanthomonas oryzae pv Oryzae*. The cultivar that is used and the environment have a significant impact on the yield losses brought on by bacterial blight. In tropical regions, Indonesia, India, and the Philippines reported productivity reductions of up to 75% (Nino-Liu *et al*., 2006) [51]. Exploring elite bacterial blight resistance genes in rice is crucial. Nine of the 35 related genes that have been identified so far—Xa21, Xa23, Xa27, Xa29, Xa30, Xa32, Xa35, and Xa36—come from wild rice. According to Phuc *et al*. (2005) [57], marker-assisted selection proved effective at enhancing the resistance of rice cultivars to BLB.

Twenty-seven rice cultures were examined in the current study to assess their resiliency to the bacterial blight resistance genes, *Xa5 and Xa21*, using the PCR-based SSR markers RM-122 and pTA-248, respectively. Resistance associated alleles were found in the genotypes Nom-2102, Nom-2104, Nom-2107, Nom-2108, Nom-2110, Nom-2111, Nom-2118, and Nom-2119 when the *Xa5* resistance gene was screened by the amplification of the SSR marker RM-122, which was used to track the resistant amplicons of 240-250 bp. For the *Xa5* gene specific marker RM-122, Islam *et al*., (2015) [31], Ullah *et al*., (2012) [70], and Khan *et al*., (2015) [31] similarly found the existence of resistance linked alleles around 240–250 kb. This suggests that the unique RM-122 marker for the bacterial blight gene *Xa5* is successful in identifying the presence of resistance-linked alleles. The marker pTA-248 failed to find any amplicons unique to resistance-linked alleles for the *Xa21* gene. This suggests that none of the 27 cultures included any of the gene *Xa21*'s resistance linked alleles. By using the marker pTA-248, which is unique to the gene *Xa21*, Kumar *et al*., (2013) [40], Xiao-Deng *et al*., (2012) [72], Singh *et al*., (2015) [67], and Amgai *et al*., (2015) [3] additionally checked a number of rice entries for the existence of bacterial blight resistance linked alleles (of 1040 bp). These findings concur with research by Patel *et al*., (2015) [55], Sabar *et al*., (2016) [64], Bhagwat *et al*., (2017) [8], and Randive *et al*., (2019) [61].

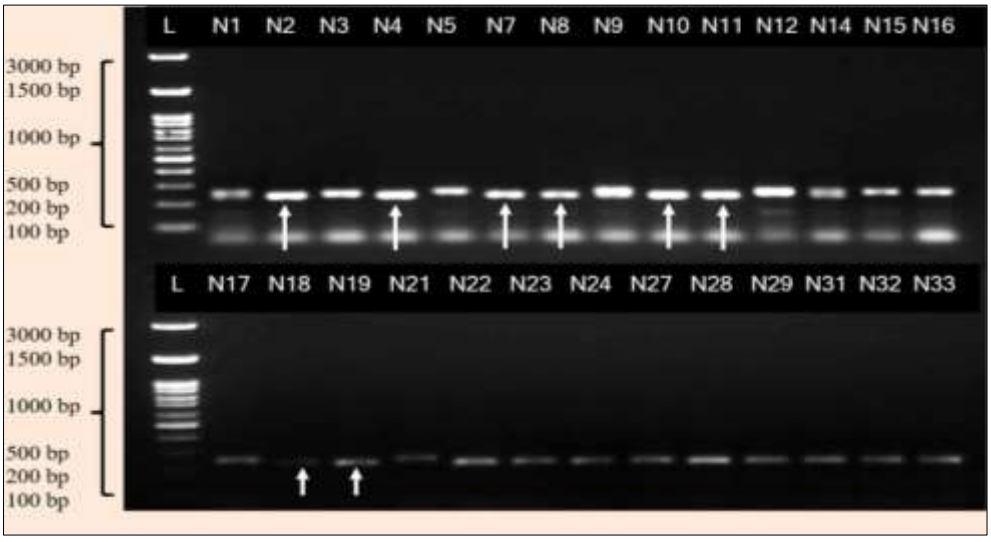

Plate 1: Amplified DNA fragments of 27 rice cultures using SSR primer RM-122 linked to Bacterial blight tolerant trait. L= 3000 bp ladder. (Arrow indicates presence of the tolerant

Brown Plant Hopper

In Asia, where the majority of the world's rice is grown, such as China, India, Philippines, Japan, Korea, Vietnam, etc., the brown plant hopper (BPH) is a damaging insect pest (Khush *et al*., 1985) [38]. The key to understanding the genetics of BPH tolerance and accurately identifying tolerance genes is appropriate evaluation of rice genotypes for BPH tolerance. Important breeding techniques to prevent BPH damage include the discovery and insertion of novel BPH resistance genes from wild rice into contemporary cultivars. A better method for finding the BPH resistance gene is marker assisted selection (MAS).

Using the microsatellite markers RM-6775 and RM-309, the 27 rice cultures were tested for Brown Plant Hopper resistance. Among them, the resistance associated alleles in the cultures Nom-2117, Nom-2118, Nom-2119, and Nom-2123 were detected by marker RM-6775 (of 192 bp), which is unique to the resistant gene *Bph25*. The lack of resistance-

linked alleles was detected in all 27 cultures using the marker RM-309 (at 152 bp), which is exclusive to the resistant gene *Bph26*. These genotypes may either be devoid of resistance associated alleles for any of the *Bph* genes or they may have resistance linked alleles for additional *Bph* resistant genes that may be identified using gene-specific primers. The *Bph25* and *Bph26* genes were also searched for in rice genotypes by Myint *et al*., (2012) [48] using the markers RM-6775, RM-309, and RM-5479. Many additional researchers have published comparable marker studies for *Bph* genes, including Harini *et al*., (2013) [24], Shabanimofrad *et al*., (2015 a and b) [66], Bhogadhi *et al*., (2015) [9], Bhagwat *et al*., (2017) [8], and Randive *et al*., (2019) [61].

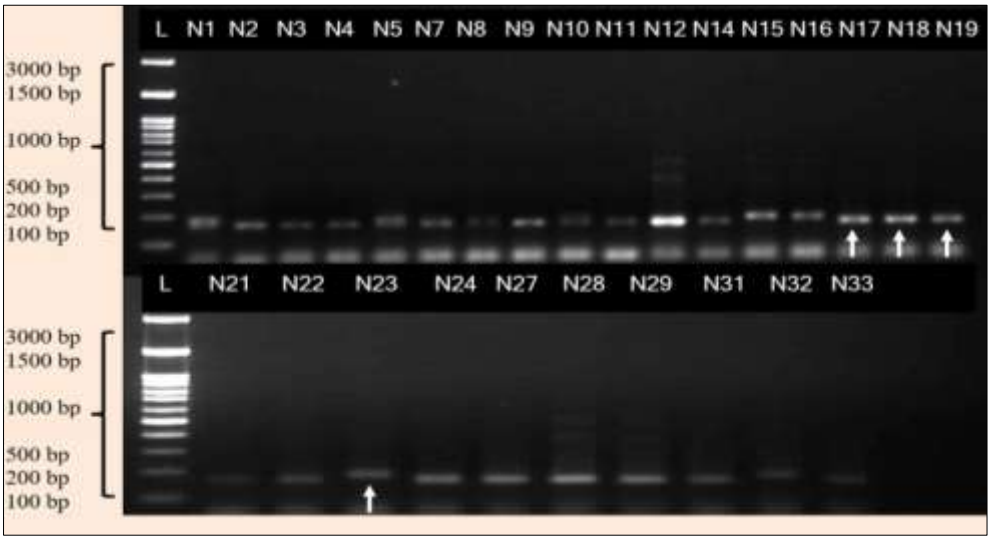

Plate 2: Amplified DNA fragments of 27 rice cultures using SSR primer RM- 6775 linked to brown plant hopper tolerant trait. L= 3000 bp ladder. (Arrow indicates presence of the tolerant lined allele)

Gall midge

In Asia, the rice gall midge *Orseolia oryzae* is a damaging pest. The insect is widespread and is regarded as a serious obstacle to rice production in India as well. The most practical solution to manage the pest has been to use resistant types, and there are various sources of resistance in cultivated rice. Breeding programmes can be expedited by using molecular markers linked to the resistance genes. In rice, 11 gall midge tolerance genes have been examined (Himabindu *et al*., 2010) [26]. Eight genes-*Gm1, Gm2, Gm4, Gm5, Gm6, Gm7, Gm8, and Gm11*—have been identified and mapped among these (Yasala *et al*., 2012) [74].

Two SSR markers, namely RM-547 and RM-22709 were used in this investigation. The population of 27 rice cultures was screened for gall midge resistance using RM-547 and RM-22709. Resistance linked alleles *Gm4* (270 bp) for the primer RM-547 were found in the genotypes Nom-2112, Nom-2117, and Nom-2132, as well as resistance linked alleles Gm8 (160 bp-170 bp) for the primer RM-22709 in the genotypes Nom-2112, Nom-2114, Nom-2115, Nom-2116, Nom-2117, Nom-2118, Nom-2119. Further usage in breeding programmes is possible for genotypes showing the existence of resistance-related alleles. Mohapatra *et al*., (2016) [46], Sama *et al*., (2012) [65], Kumar *et al*., (2013) [40], Bhagwat *et al*., (2017) [8], and Randive *et al*., (2019) [61] explored the significant correlation of trait specific makers which are in assent with this study.

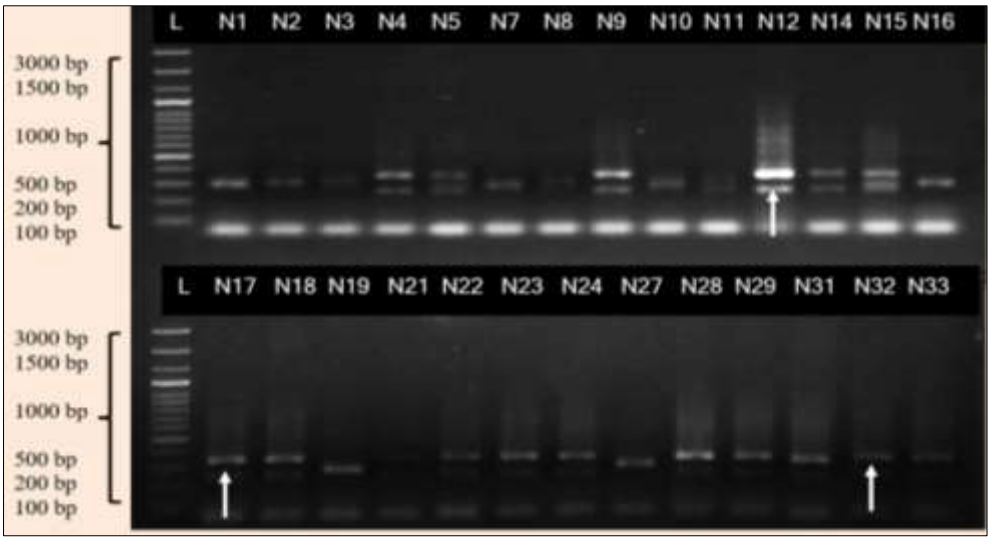

Plate 3: Amplified DNA fragments of 27 rice cultures using SSR primer RM- 547 lined to Gall midge tolerant traint. L= 3000 bp ladder. (Arrow indicate presence of the tolerant linked alkele)

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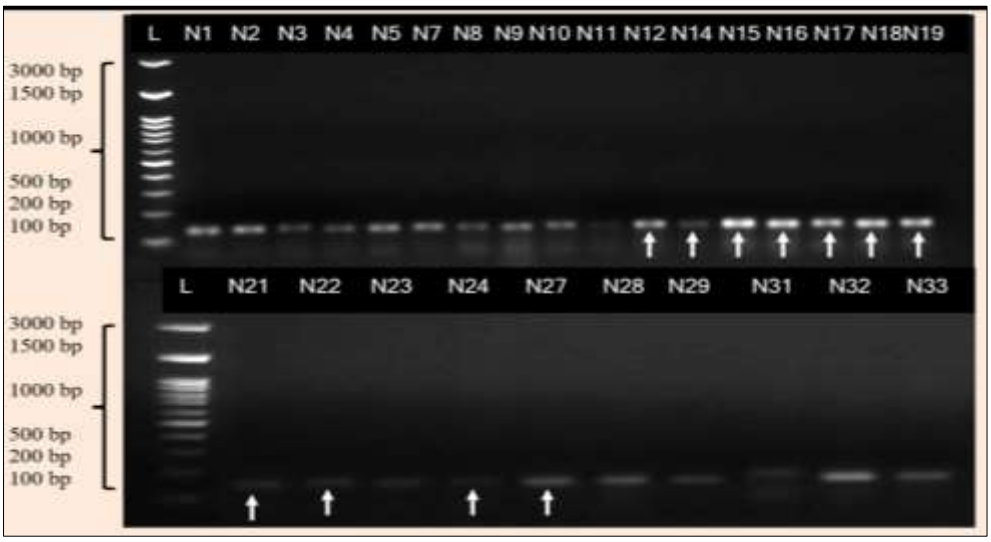


Plate 4: Amplified DNA fragments of 27 rice cultures using SSR primer RM- 22709 linked to gall midge tolerant trait. L= 3000 bp ladder. (Arrow indicates presence of the tolerant lined allele)

Abiotic Stresses

Drought stress

Drought is one of the major limiting factors that lowers rice's yield stability and productivity in rain-fed environments, among other abiotic stresses. The identification of drought tolerant genotypes for use in upcoming breeding programmes requires the use of microsatellite markers associated to drought tolerance in rice. The findings demonstrated that SSR primers could distinguish between rice genotypes that were drought resistant.

Four SSR primers, RM-212, RM-302, and RM-3825, are placed on rice's chromosome 1 and RM-201 is found on its chromosome 9, respectively, at positions 135 bp, 140 bp, 147 bp, and 220 bp. All cultures lacked the resistance-linked alleles Dr (220 bp), Dr (135 bp), Dr (140 bp), and Dr (147 bp), which correspond to the primers RM-201, RM-212, RM-302, and RM-3825, respectively. We may recommend the use of alternative drought-related marker combinations to test the genotypes for drought resistance because these genotypes saw poor results for these markers.

For the purpose of screening rice genotypes for QTL, Kanagaraj *et al*. (2010) [34-35] and Noorzurain *et al*., (2013) [52] also used the RM-212 primer. The markers RM-302 and RM-212 were shown to be helpful for choosing lines that were drought resistant by Freeg *et al*., (2016) [19] using a MAS method. Similar investigations using a microsatellite marker unique to drought tolerance were conducted by Niksiar *et al*., (2013) [50], Ramadan *et al*., (2015) [59], Bhagwat *et al*., (2017) [8], and Randive *et al*., (2019) [61].

Salt Stress Tolerance

Along with other abiotic stresses, salt stress is a key abiotic source of stress in the country's coastal regions, lowland areas that receive rain, and rain fed places. Agriculture as well as rice production and yield will be severely harmed by an increase in soil salinity and salt intrusion. Microsatellite marker screening is very promising to designate the main gene locus for salt tolerance in order to generate new cultivars through enhanced breeding programmes.

The salt resistance associated SSR primers RM-140, RM-1287, RM-562, and RM-3412 were employed in this work and are situated on rice chromosome 1 between 260 bp, 160 bp, 243 bp, and 211 bp, respectively. The salt resistance alleles were detected by the primer RM-140 at 260 bp were found in cultures Nom-2102, Nom-2103, Nom-2105, Nom-2108, Nom-2109, Nom-2110, Nom-2111, Nom-2116, Nom-2117, and Nom-2132. The *Saltol* QTL was found in all four of the cultures Nom-2110, Nom-2111, Nom-2112, and Nom-2116 cultures at (160 bp). The *Saltol* QTL, which was located by the marker RM-3412, was seen at (at 211 bp) in the cultures Nom-2104, Nom-2111, Nom-2112, Nom-2117, and Nom-2133. The lack of the resistance-linked alleles was revealed by *Saltol* alleles in the primer RM-562 in all cultures. SSR primers RM-140, RM-1287, and RM-3412 were utilised by Islam *et al*. (2012) [30] in their QTL study to find the salt-tolerant rice cultivars. Using the primer RM-3412, Aliyu *et al*., (2011) [2] detected salt tolerance in QTL analysis. The microsatellite markers used in this investigation were successful in identifying *Saltol* QTL in a number of the genotypes examined.

All 27 of the cultures were amplified using the four SSR *Saltol* linked primers used in this investigation. The primers that showed polymorphism were compared to the rice QTLs for salt tolerance that had previously been identified. The microsatellite primers for salt tolerance that were used in this investigation were generated from a study (Thomson *et al*., 2010) [69] that examined QTLs for seedling stage salinity tolerance that were derived from Pokkali in order to be used

in the MAS technique.

These markers are highly polymorphic and beneficial for *Saltol*, according to a number of researchers like Karmakar *et al*., (2012); Iqbal *et al*., (2013) [28]; Ganie *et al*., (2016) [20]; Linh *et al*., (2012) [43]; Al-Amin *et al*., (2013) [1]; Chattopadhyay *et al*., (2014) [13]; Rubel *et al*., (2014) [62]; Bhagwat *et al*., (2017) [8] and Randive *et al*., (2019) [61].

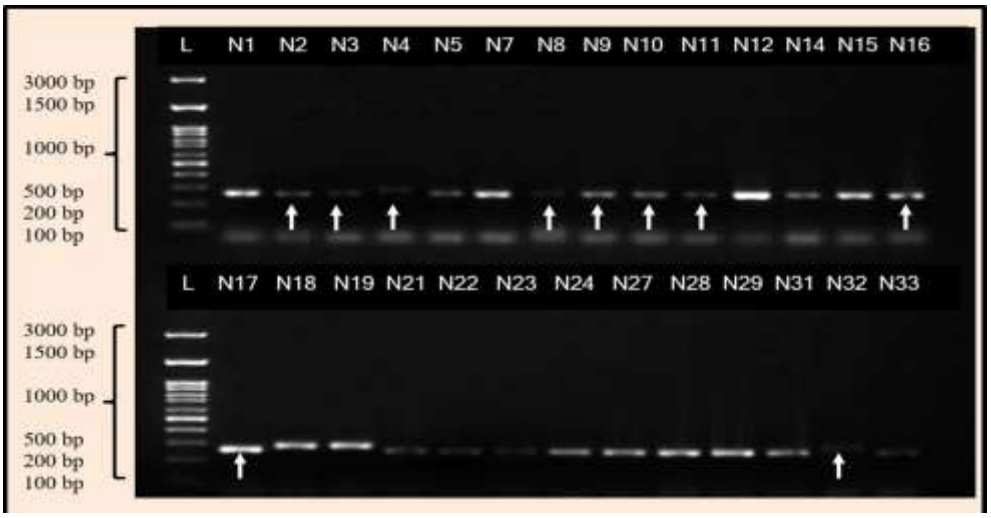


Plate 5: Amplified DNA fragments of 27 cultures using SSR primer RM- 140 linked to salt tolerant trait. L= 3000 bp ladder. (Arrow indicates presence of the tolerant lined allele)

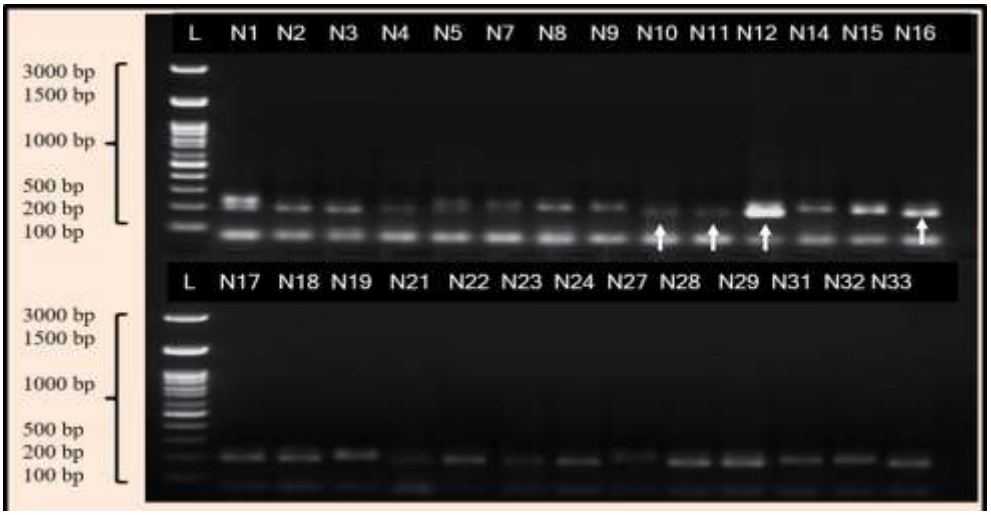


Plate 6: Amplified DNA fragments of 27 rice cultures using SSR primer RM- 1287 linked to salt tolerant trait. L= 3000 bp ladder. (Arrow indicates presence of the tolerant linked allele)

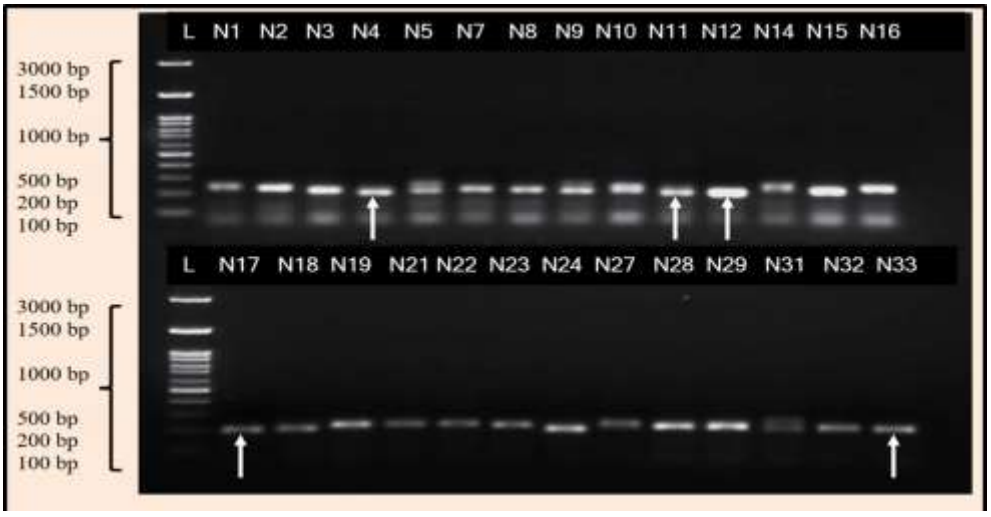


Plate 7: Amplified DNA fragments of 27 rice cultures using SSR primer RM- 3412 linked to salt tolerant trait. L= 3000 bp ladder. (Arrow indicate presence of the tolerant lined allele)

Genetic Diversity

SSR Polymorphism

All 27 rice cultures were genotyped using 18 trait-linked SSR markers, and they were chosen based on its ability to produce amplified products, detect polymorphism levels between cultures, and maintain pattern consistency. These markers yielded a total of 75 alleles, with an average of 4.16 alleles per marker, and 100% of them were found to be polymorphic. There were between 2 to 9 alleles found for each marker. The most alleles were generated by the primer pTA-248 (9) while the marker RM-309 produced the fewest alleles (2). Chungada *et al*., (2016) [14] and Okello *et al*., (2017) [53] reported similar findings and noted that the primer RM-309 yielded the fewest alleles (5).

Amplified fragments varied in size from 125 to 1024 bp. The majority of microsatellite markers used to study diversification are typically polymorphic in nature. The current work is also in line with other results by Bhagwat *et al*., (2017) [8], who utilised 18 primers for the examination of polymorphism in 50 rice germplasms, and Randive *et al*., 2019 [61], who employed 19 SSR markers.

Polymorphic Information Content (PIC)

Polymorphism information content (PIC) values ranged from 0.314 to 0.927 in this study, with an average PIC value of 0.619 per primer. Randive *et al*., (2019) [61] also noted that the average PIC value is (0.65) identical to this study. The SSR primer, pTA-248 revealed highest (0.927) PIC value; whereas the primer RM 3825 revealed lowest (0.314). 75 alleles in all, with an average of 4.16 alleles per marker, were found in this study ranged from 2 to 9 per locus. It was demonstrated that primers that saw fewer alleles revealed less gene variability than those that observed more alleles, which revealed more gene diversity.

According to Aliyu *et al*., (2011) [2], primers with PIC values of 0.5 or above are tremendously helpful in determining the polymorphism rate of a primer at a particular locus and are highly informative for genetic investigations. Consequently, it was discovered that primers PTA-248, RM-547, and RM-302 were highly informative (Table 3). Since only PIC values higher than 0.5 indicate high polymorphism, the SSR primers employed in this work showed an average PIC value of 0.619, indicating that they were highly informative.

The high PIC value found in this study indicated that there is significant genetic variation among rice cultivars. The genetic diversity of the accessions used is typically a factor in the PIC values (Pervaiz *et al*., 2009) [56] for the particular research. The best markers for molecular characterization and genetic diversity study are those that can identify a large number of identifiable alleles (Islam *et al*., 2008) [29].

The present PIC value (0.619) is greater than the PIC values reported in earlier papers by Islam *et al*., (2012) [30], Hoque *et al*., (2014) [27], Gholizadeh *et al*., (2014) [21], Freeg *et al*., (2016) [19], Krupa *et al*., (2017) [39], and Bhagwat *et al*., (2017) [8], which are, respectively, 0.57, 0.54, 0.45, 0.52, 0.49, and 0.41.

Genetic distance values between rice varieties

The alleles were transformed to binary scores based on their presence (1) or absence (0) based on the screening of SSR scoring. With the use of the software Multivariate Statistical Package (MVSP), this data was selected for a similarity-based analysis to calculate the Jaccard's coefficient matrices, or an estimate of how similar the twenty-seven cultures are to one another.

The results for pairwise similarity ranged from 0.053 to 0.818. Maximum similarity value (0.818) was found between Nom 2128 & Nom 2129 while, the minimum similarity value (0.053) was obtained between (Nom 2105 and Nom 2129) and (Nom 2112 and Nom 2129). According to this study, rice cultures are more distinct, which suggests that a sizable portion of their genomes may differ. However, the great discriminating power of microsatellite markers is indicated by the genetic diversity found using molecular markers in the current study. For the purpose of resolving issues relating to breeding for heterosis, this is crucial.

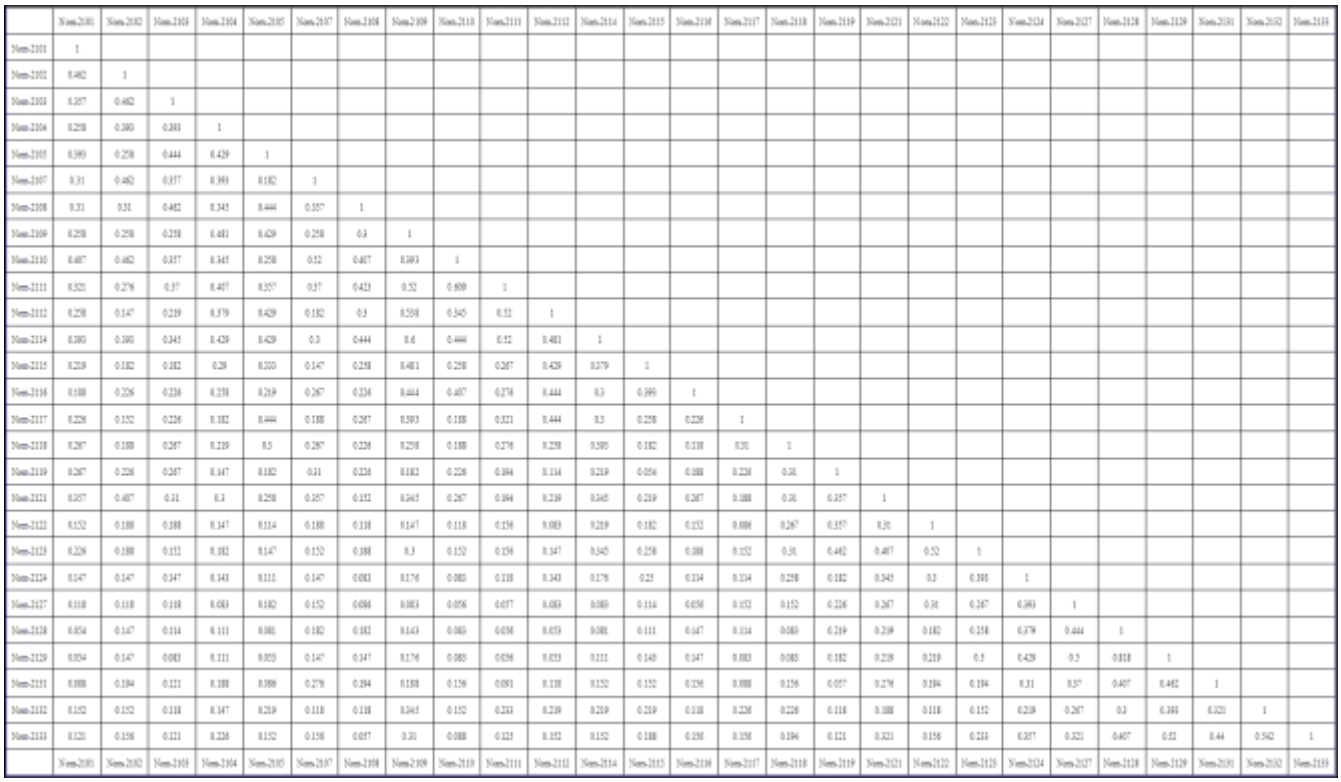
Cluster analysis based on Dendrogram pattern:

Multivariate Statistical Package (MVSP) was used to create the UPGMA based Dendrogram of 27 rice cultures. Two primary clusters, I and II, were obtained by the clustering pattern of the Dendrogram created using the pooled molecular data of 18 SSR primers of 27 cultures. The Dendrogram showed that the variations that are genetically linked variations of the same kind are grouped together in a single cluster.

The minor cluster-I comprised 10 rice cultures, and was further found to be divided into two sub clusters (IA and IB). Sub Cluster IA was further subdivided into two sub-sub clusters (IA (a) and IA (b)). Sub-sub cluster IA (a) included of two cultures i.e., Nom-2133 and Nom-2132. Sub-sub cluster IA (b) consisted of five cultures i.e., Nom-2131, Nom-2129, Nom-2128, Nom-2127 and Nom-2124. Sub Cluster IB was further subdivided into two sub-sub clusters (IB (a) and IB (b)). Sub-sub cluster IB (a) consisted of two cultures i.e., Nom-2123 and Nom-2122. Sub-sub cluster IB (b) consisted of one culture i.e., Nom-2119.

The major cluster-II comprised 17 rice cultures, and was further found to be divided into two sub clusters (IIA and IIB). Sub Cluster IIA was further subdivided into sub-sub clusters [IIA (a)]. Cluster IIA(a) included of two cultures i.e., Nom-2118 and Nom-2117. Sub Cluster IIB was further subdivided into two sub-sub clusters [IIB (a) and IIB (b)]. Sub-sub cluster IIB (a) was further sub divided into sub-sub-sub clusters [IIB (aa) and IIB (ab)]. Sub-sub-sub cluster IIB (aa) consisted of two cultures i.e., Nom-2116 and Nom-2115. Sub-sub-sub cluster IIB (ab) consisted of nine cultures i.e., Nom-2111, Nom-2110, Nom-2112, Nom-2114, Nom-2109, Nom-2104, Nom-2105, Nom-2108 and Nom-2103. Sub-sub cluster IIB (b) consisted of four cultures i.e., Nom-2121, Nom-2107, Nom-2102 and Nom-2101.

Table 3: Genetic distances based on SSR pooled over 18 primers in 27 rice cultures



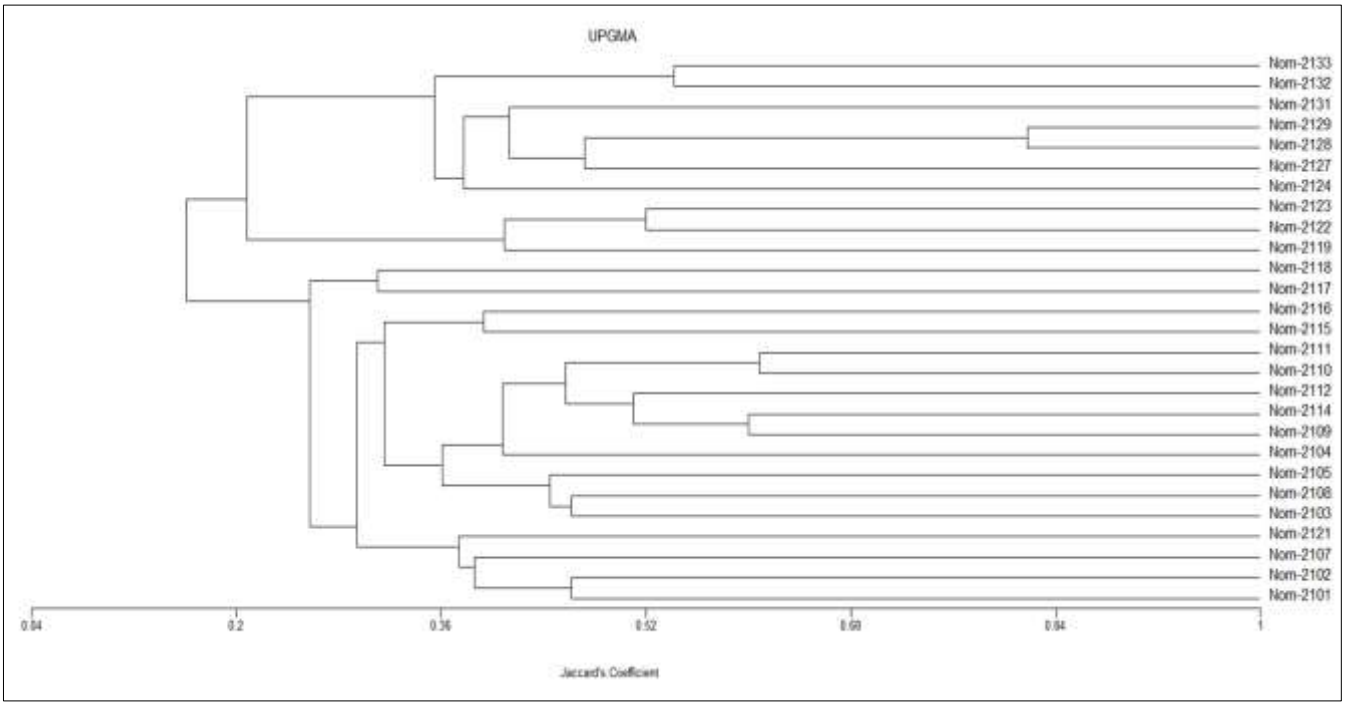


Fig 1: Dendrogram constructed using Jaccard's Similarity Coefficient

By creating Dendrograms and doing cluster analyses, other authors similarly revealed the diversity among distinct rice types. The 88 rice varieties, which comprised landraces, farmer's variations, and well-known Basmati lines, were divided into four clusters at a dissimilarity coefficient of 0.58 and two major clusters at a dissimilarity coefficient of 0.55 by Yadav *et al*., (2013) [73]. Based on the Nei's genetic distance estimated from 27 SSRs generated from the 30 types of rice, Mohiuddin *et al*., (2014) [47] created a dendrogram. Singh *et al*., (2016) [76] examined 132 different genotypes of Indian wild rice and found that there was cumulative variance of 66.53 percent and 77.65 percent, respectively, using SSR and SNP markers. By utilising 18 SSR primers and creating a Dendrogram, Okello *et al*., (2017) [53] determined the diversity across 48 rice types. He claimed that rice genotypes were dispersed in two primary clusters and then split into four smaller clusters, with IA including 12 genotypes, IB comprising 12 genotypes, and IIA comprising 8 genotypes and IIB comprising 16 genotypes. Using 18 microsatellite markers and the 50 rice germplasms, Bhagwat *et al*., (2017) [8]

created a dendrogram. By employing 19 SSR markers and creating a dendrogram, Randive *et al*., (2019) [61] also reported the diversity across 50 rice genotypes.

Because of the polyallelic nature of microsatellite markers, they have the advantage of discriminating the individuals more precisely. The SSR primer gave more clusters with fewer varieties in each cluster and therefore, large variation within each cluster was observed. In the present study, the rice genotypes were grouped into two major clusters.

Conclusion

In order to identify the parental genotype with the tolerant genes that will be accessed for MAS to breed the most stable elite lines for stresses, it is determined that screening the rice cultures for abiotic and biotic stresses using the SSR is crucial. Microsatellite markers were used for molecular screening of 27 rice cultures, and the results revealed enough information on molecular tolerance traits. The great degree of variation across 27 rice cultures was shown through polymorphism.

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