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Original Research Article

ASSESSMENT OF GENETIC DIVERSITY IN RICE (*ORYZA SATIVA*) GERMPLASM USING SALTOL AND AROMATIC SSR MARKERS

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Abstract *Rice (Oryza sativa) serves as a staple food for nearly half of the global population. Soil salinity has a major effect on plant growth and the production of rice all over the world. The aromatic properties of the rice are another important aspect from a market perspective. The experiment was conducted at Rice Research Institute Kala Shah Kaku. The randomized complete block design with three replications. Molecular markers in genetics are DNA segments linked to a region of the genome. To differentiate one DNA sequence from an unknown DNA pool, molecular markers are used. Most plant genomes include simple sequence repeats (SSRs), which are widely distributed and highly polymorphic sequences. These are often employed in population genetic research, paternity testing, and DNA fingerprinting. Four Salinity resistant linked gene SSR markers (RM-7075, RM-562, RM-152, RM-WN-13903), the results of these markers show that, accession 27141, 17146, 27731, 27745, and 27789 show all the resistant genes, and 27727 shows least resistant gene. Two aromatic SSR markers (Badex and RM-23-120) were used and 27131, 27141, 27146, 27161, 27166, 27202, 27207, 27731, 27745, and 27769 show highly aromatic properties to screen out salt-tolerant and enhanced aromatic accessions and 27141, 27146, 27731, and 27745 have both highly salt resistant and aromatic genes. From this experiment, it is identified that the accessions are significantly high-yielding, and have salinity-resistant genes the accessions show both salt-tolerant genes and have aromatic properties used further in breeding programs and go under the verity development process.*

Keywords*:* *rice; polymorphic sequences; salt-tolerant; aromatic genes; SSR markers*

Introduction

Rice (*Oryza sativa*) belongs to the Poaceae family, which is also known as the grass family (Yuan et al., 2021). Rice has 2n=24 chromosomes (Liu et al., 2023). Nearly half of all people on the planet use rice as a staple diet (Bin Rahman & Zhang, 2023). Over 3.5 billion individuals globally get at least 20% of their daily calories from rice (Hashimoto et al., 2022). With 90% of the world's rice consumption coming from Asia, this region is the biggest consumer worldwide (Samal et al., 2022). According to Samal et al. (2022) the continent of Africa is experiencing the fastest growth in rice consumption. Africa has not been able to produce enough rice to support itself (van Eekelen, 2020). but increased rice output and increased rice acreage have allowed the globe to continue meeting the demand for rice (Yuan et al., 2022). Among the cereal crops, rice is the second most important source of foreign exchange profits and is one of Pakistan's staple food grain crops (Javed et al., 2020). Genetic development has led to a huge rise in rice productivity (Kumar et al., 2021). In lowland rice production systems, significant gains in grain output have been noted, especially during the Asian "Green Revolution" era (Yuan et al., 2022). Improved management techniques, inputs like fertilizer and irrigation, as well as rice cultivars with higher grain production potential and input responsiveness, have all been credited for this improvement (Mallareddy et al., 2023). Rice is one of the most significant crops, providing more than half of the food on Earth, particularly in Asia (Mohidem et al., 2022). Over 100 countries in Asia cultivate and generate 90% of the world's production (Wang et al., 2023) (Yuan et al., 2022). With a combined 75% of the world's rice production, China, India, Indonesia, Bangladesh, Vietnam, and Thailand are the top producers in the globe (Sporchia et al., 2021). Calories, magnesium, phosphorus, manganese, and selenium are all found in good amounts in rice (Zahra and Jabeen, 2020). Harvested from 158

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million hectares each year, its output value in poor nations is double that of any other food crop (Bin Rahman and Zhang, 2023). Over 150$ billion rice is sold annually on the global market (Durand-Morat and Mulimbi, 2024). In many countries in Africa and Latin America, rice has long been a staple food, and its significance is only increasing (Yuan et al., 2024). However, depending on the scenario of climate change, some study indicates that African rice yields might vary from −24% to +18% by 2070 compared with 2000 (Cai et al., 2024).

Understanding and making use of the genetic variety of rice germplasm can be beneficial, particularly for characteristics like fragrance and salt tolerance (Haque et al., 2021a). These characteristics are critical for rice yield, quality, and adaptability to various markets and conditions (Hussain et al., 2020). The researchers may find and choose rice genotypes with favorable genes for salt tolerance and scent using Saltol and aromatic SSR markers, and they can employ these genotypes in the next breeding operations (Haque et al., 2021b). This may result in the creation of enhanced rice cultivars that can handle salt stress and satisfy consumer demands for flavorful rice. To advance agricultural techniques, increase food security (Dhama et al.), and support resilient and sustainable rice production in the face of climatic and environmental uncertainty (Shahid et al., 2021), it is essential to examine the genetic diversity in rice germplasm using Saltol and Aromatic SSR markers (Suvi et al., 2020). The study was performed to assess the genetic diversity and population structure of rice germplasm by employing aromatic and Salt SSR markers. To determine which aromatic and salt-tolerant genes in rice germplasm are linked to fragrance and salt tolerance**.**

Materials and Method

Experimental Site

The experiment was carried out at the Rice Research Institute (31.72140 N, 74.27020 E) at Kala Shah Kaku, Lahore, Pakistan. The average rainfall was around 220 mm, the temperature was approximately 36 degrees Celsius, and the soil type was clay loamy.

Experimental Design

The Randomization Complete Block Design (RCBD) was used to conduct this experiment. There were three replications, Row x Row distance was 9 Inches, Plant x Plant distance also was 9 inches, and there were 30 Plants in a row. Following accessions were used for experimental material (Table 1).

table 1: accession used in the experiment

Sr.	Accession No.	Sr.	Accession No.
1	27131	14	27750
2	27137	15	27759
3	27141	16	27769
4	27146	17	27779
5	27151	18	27784
6	27156	19	27789
7	27161	20	27794
8	27166	21	27799
9	27202	22	27805
10	27207	23	27814
11	27727	24	37405
12	27731	25	37412
13	27745	26	37419

Genetic analysis

For genotypic analysis, the accessions were grown in trays, ten seeds of each accession were grown separately. The seeds were socked, into the water for one day. The waster was removed, and the tissue was placed in the tray each accession seeds were placed on it and every box was labeled with the accession number and socked with water with the help of a spray bottle for germination. After six to seven days of sowing the leave size was about two to three inches and these leaves were taken for DNA extraction (Figure 1).

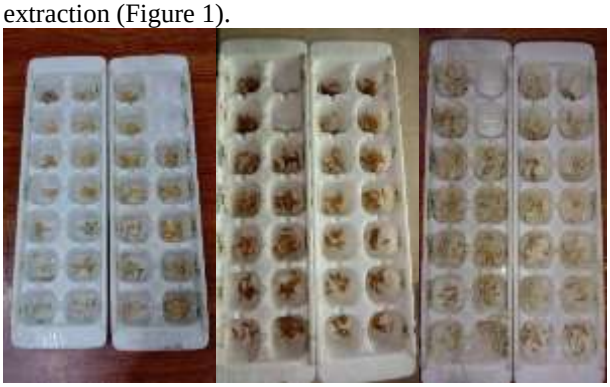

Figure 1. Seed sowing and germination in trays for genetic analysis

Primers Designing

The primers were designed for PCR amplification utilizing the https://archive.gramene.org/db/markers/ genomic database. The RM-7075 markers are linked to chromosome number 2, RM-152 is linked to chromosome number 8, RM-562 is linked to chromosome number 1, RM-10793 is linked to chromosome number 1, and RM-WN-13903 is linked to chromosome number 2. The given specific saltol markers were used for the PCR amplification (Table 2 & 3).

Table 2. Salinity SSR markers

SSR Marker	Primer Sequence		Annealing temperature	Band size	Chromosome Number
RM-7075	Foreword	GACTTGCCAACTCCTTCAATTCG	55	155	Chr. 1
	Reverse	TCGTCGAGTAGCTTCCCTCTCTACC			
RM-152	Forward	GAAACCACCACACCTCACCG	61	243	Chr. 8
	Reverse	CCGTAGACCTTCTTGAAGTAG			
RM-562	Forward	CACAACCCACAAACAGCAAG	55	151	Chr. No. 1
	Reverse	TCGTCGAGTAGCTTCCCTCTCTACC			
RM-WN-13903	Forward	TTCTAATACAGTCCTCCTCCATCC	63.3	1000	Chr. No.2
	Reverse	GTGTCACATCCAATCAAAGTACCC			

Table 3. Aromatic SSR Markers

SSR Marker	Primer Sequence		Annealing Temperature	Band Size	Chromosome number
RM-23-120	Foreword	GGCTGGAGGAGGAGGAG	53	580bp	Chr. No.8
	Reverse	GAGGAGGAGGAGGAGGTT			
Badex	Foreword	TCTTGCTCCAGCTTCCAG	54	95-103	Chr. No.4
	Reverse	ACGCTTGCTTGCTTGCTT			

Results

RM-7075, RM-563, RM-152, and RM-WN-13903 are the four polymorphic SSR markers against salinity that were utilized in this experiment to screen for salt-tolerant accession identification. These accessions were evaluated for their aromatic qualities using the aromatic SSR markers. Positive and negative identification and comparison checks were employed to describe these accessions. When an accession's band size is shown in comparison to a positive check, it indicates that the accession carries a particular gene that protects against salt stress. The experiment employed IR-29 as a negative check to test for salinity, and FL-478, N-22, Morobrecan, Shaheen Basmati, and N-22 as positive checks. Super Gold was the positive check and PK-86 was the negative check for fragrance.

RM-7075

This polymorphic genetic marker has a band size of 155 bp, is associated with the QTL against salinity found on chromosomal number 1, and its annealing temperature for polymerase chain reaction (PCR) was around 55°C (Figure 2).

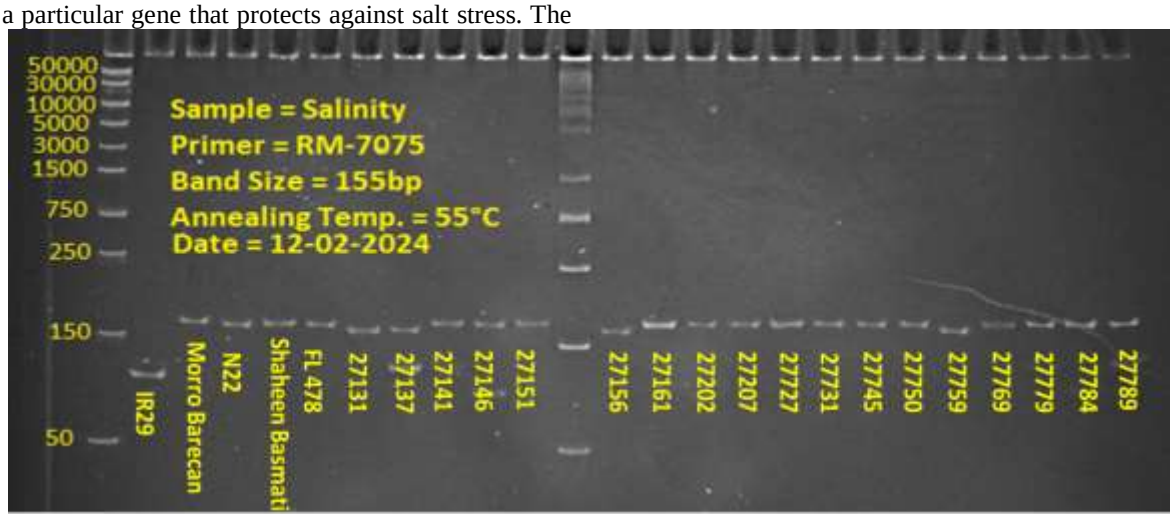

Figure 2. Molecular Results For RM-7075

The positive checks that were employed, according to this gel electrophoresis picture, are Morr Barrecan, N-22, Shaheen Basmati, and FL-478, which displays the bands at around 155 bp. After analyzing this image, the accessions 27131, 27137, 27141, 27146, 27151, 27156, 27161, 27202, 27207, 27727, 27745, 27750, 27759, 27769, 27779, 27784, and 27789 display their bands at the exact level of the positive check at 155 bp, as calibrated from the ladder were used in the gel electrophoresis. The accessions also show bands that are slightly smaller than the 155 bp bands. These accessions can be used after the field trails.

RM-562

This polymorphic genetic marker has a band size of 243 bp, is associated with the QTL against salinity found on chromosomal number 1, and its annealing temperature for polymerase chain reaction (PCR) was around 55°C (Figure 3).

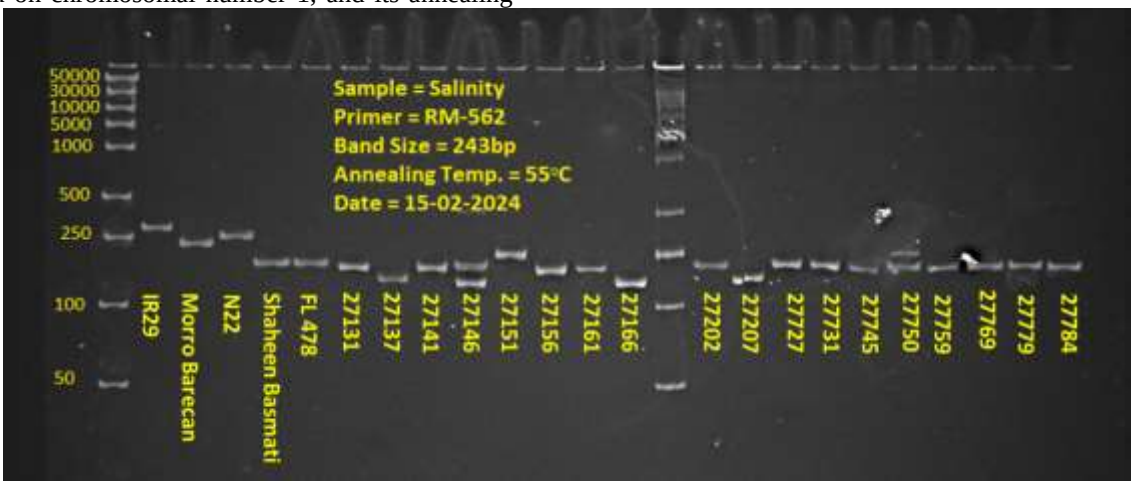

Figure 3. Molecular Results For RM-562

The bands on this gel electrophoresis picture are around 243 bp in length, and N-22 and FL-478 exhibit bands that are somewhat larger than the resistance gene band size Shaheen Basmati. These positive checks are Morr Barrecan and N-22.After analyzing this image, the accessions 27131, 27137, 27141, 27146, 27151, 27156, 27161, 27202, 27727, 27731, 27745, 27750, 27759, 27769, 27779, 27789, 27814, 37405, 37412, and 37419 show their bands at the almost level of the positive check at 243 bp as calibrated from the ladder were used in the gel electrophoresis. Meanwhile, the accessions 27207, 27799, and 27805 show bands slightly lower than the 243 bp bands. These accessions can be used after the field trails.

RM-152

This polymorphic genetic marker has a band size of 151 bp, is associated with the QTL against salinity found on chromosome 8, and its annealing temperature for polymerase chain reaction (PCR) was around 55°C (Figure 4).

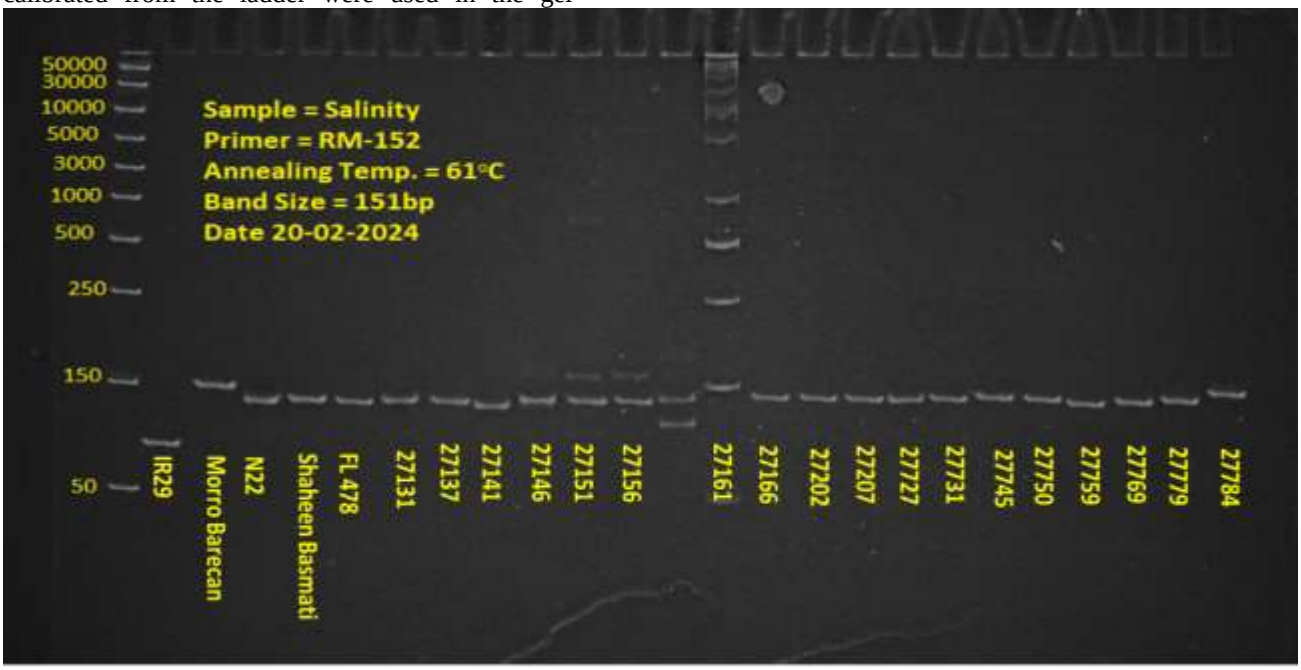

Figure 4. Molecular results for RM-152

The positive checks that were employed, according to this gel electrophoresis picture, are FL-478, N-22, Shaheen Basmati, and Morro Barrecan, which exhibit the bands at around 151 bp. After examining this picture, it can be seen that the bands for N-22, FL-478, and Shaheen Basmati are just below the 150 bp threshold.It is known that these varieties carry the RM-152 marker against the salt stress in their genomes, which serves as a positive check. The following accession numbers: 27131, 27137, 27141,

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27146, 27151, 27156, 27161, 27202, 27207, 27727, 27731, 27745, 27750, 27759, 27769, 27779, 27784, 27789, 27794, 27799, 27805, 27814, 37405, 37412, and 37419. These accessions display the same band as N-22, Shaheen Basmati, and FL-478." That particular gene, which protects the genome from salt stress, is evidently present in these accessions and is known to be RM-152 salinity resistant.

RM-WN-13903

This marker is linked to the QTL against salinity that present on the chromosome number 2, the band size of this polymorphic genetic marker was 1000bp, and the Annealing temperature for polymerase chain reaction (PCR) was about 63.3°C (Figure 5).

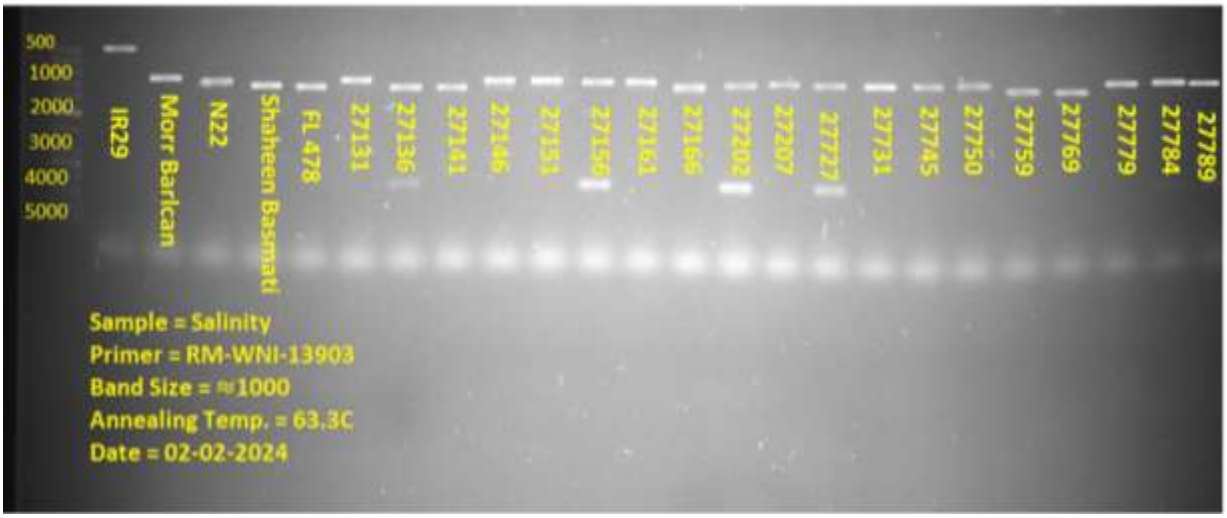

Figure 5. Molecular Results for RM-WN-13903

The bands at around 1000 bps are shown in this gel electrophoresis picture, which indicates that Morro Barrecan, N-22, Shaheen Basmati, and FL-478 are the positive checks that were employed .Following analysis of this image, the following accession numbers were found to have bands at the 1000bp: 27131, 27137, 27141, 27146, 27151, 27156, 27161, 27202, 27207, 27727, 27731, 27745, 27750, 27759, 27769, 27779, 27784, 27789, 27794, 27799, 27805, 27814, 37405, 37412, and 37419. The employed marker's band size was also 1000 bps. Positive checks confirm the presence of the particular resistance gene in the particular verity. All of the accessions utilized in the experiment had particular DNA that is resistant to the salinity-resistant gene, as shown by the gel electrophoresis data. This indicates that every accesssion used in the experiment included the particular marker that was utilized in it. Each of these accessions will demonstrate resistance to the salt stress and the presence of the particular resistant gene. further aid in enhancing the program for rice breeding.

Badex

This polymorphic genetic marker, which is located on chromosomal number 4, is associated with the QTL against rice aroma. Its band size ranges from 93 to 103 bp, and the annealing temperature for the polymerase chain reaction (PCR) was around 54°C (Figure 6).

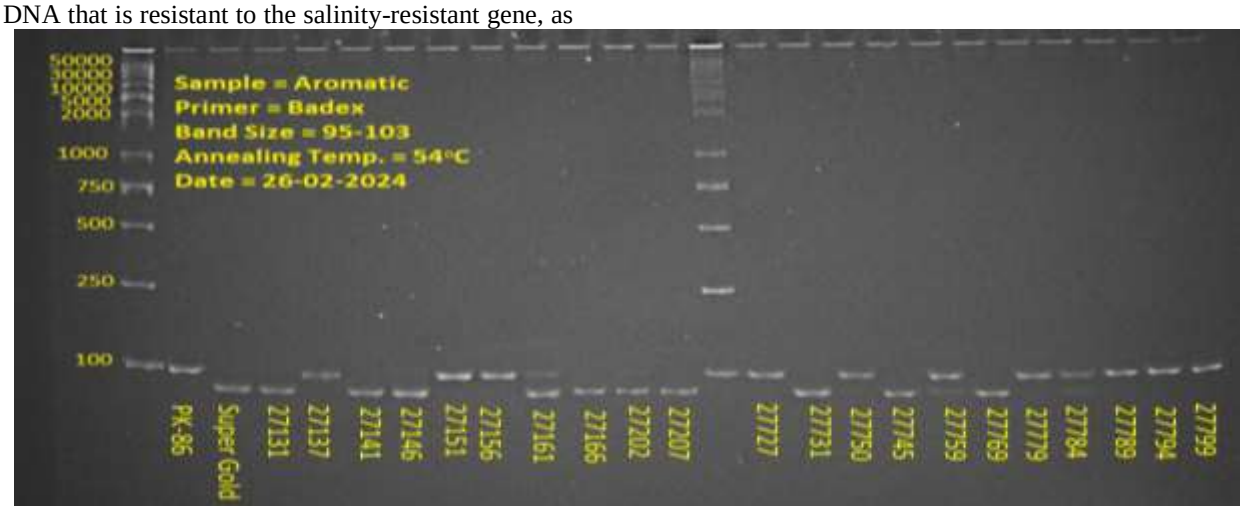

Figure 6. Molecular results for Badex

This gel electrophoresis picture, which displays a band size of around 93 bp, indicates that Super Gold was utilized as the positive check. In this reaction, PK-86 was utilized as the negative check to determine the aromatic characteristics of the chosen accessions. After examining this picture, it was found that the accessions 27131, 27141, 27146, 27161, 27166, 27202, 27207, 27750, and 27769 show their band at the exact level of the positive check at 93 bps, which was determined by calibrating the ladder that was used in the gel electrophoresis. The accessions 27137, 27151, 27156, 2727, 27745, 27759, 27779, 27784, and 27789 show a slightly higher band at a band size of 100 bps to 103 bps. That makes it evident that these accessions carry the particular gene linked to the aromatic qualities of cooked rice. The accession numbers 27794 and 27799 have bands at the PK-86 band size, indicating an absence of that particular aromatic gene in their genomes.

RM-23-120

This polymorphic genetic marker, which is located on chromosomal number 4, is associated with the QTL against rice aroma. Its band size ranges from 580 bp to 355 bp, and its annealing temperature for PCR was around 53°C. If the band appears at 355 bp, it indicates that the accessions lack the unique QTL or gene in their genome that regulates the aromatic qualities of the various rice accessions. At 580 bp, the accessions have that particular gene of aroma in the selected accessions (Figure 7).

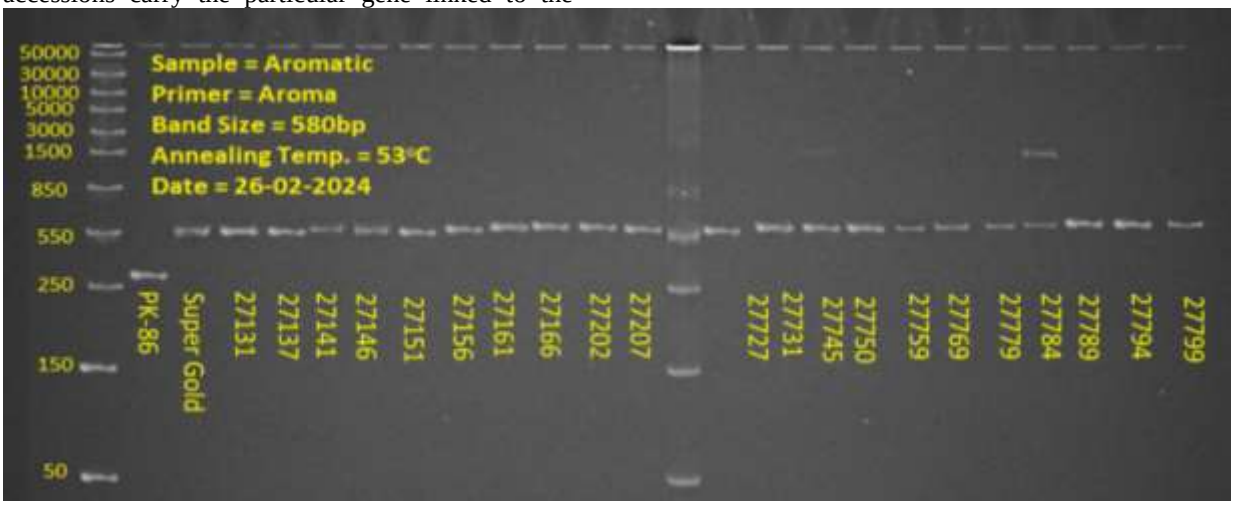

Figure 7. Molecular Results for RM-23-120

This gel electrophoresis picture, which displays a band size of around 580 kb, indicates that Super Gold positive checks were employed. The PK-86 negative check, which was utilized in this reaction to determine the non-aromatic characteristics of the chosen accessions, showed a band at 355 bp for this SSR marker. After examining the picture, it can be seen that the Accessions 27131, 27141, 27146, 27161, 27166, 27202, 27207, 27750, 27769, 27137, 27151, 27156, 2727, 27745, 27759, 27779, 27784, and 27789 exhibit a band at a 580 bp band size, in addition to the super gold band. This indicates that all of the accessions were screened against the aroma SSR marker, and that each and every one of the chosen accessions had the marker-linked gene. The only sample that exhibits non-aromatic qualities is PK-86, which also served as a negative check with its band at 355 bp.

Discussion

It is now known that DNA markers are a very useful and high-quality method for determining genetic variation at the molecular level. Important information about genetic diversity and potential link with resistance to salt stress is provided by the molecular markers' findings (Amiteye, 2021). Many genes dispersed across the genome, each with a little influence, control the majority of genetic variants against environmental stress (Stange et al., 2021). Following the phenotypic assessment, a genotypic screening process was carried out based on the two traits: aroma and salinity resistance. The RM-WN-13903, RM-152, RM-7075, and RM-562 were employed for the salinity screening. Two markers, Bedex and Aroma, were utilized to screen against the Aroma. These markers were applied to the chosen accessions to screen and characterize them. The resistant and susceptible groups of accessions are distinguished by the polymorphic SSR molecular marker RM-7050. To separate the accession pairs, band size at the same location as the tests for the particular markers that exhibit resistance other than the fact that those accession pairs did not exhibit salinity resistance must be considered. This marker has a band size of around 155 bp. RM-562 is a polymorphic SSR molecular marker that characterizes the accessions into the resistant and susceptible categories. In order to segregate the

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accessions, accordance to the band size of the accession at the same position as the checks for the specific markers shown have resistance other than that those accession did not have the salinity resistance. The band size for this marker is about 243bp. According to this gel electrophoresis image the positive checks were used are Morro Barrecan, N-22 shows there band slightly higher to the band size for the resistant gene band size Shaheen Basmati, and FL-478 show the bands at about 243bp and these checks are known that they have resistance to the salinity. After analyzing this image, the accessions 27131, 27137, 27141, 27146, 27151, 27156, 27161, 27202, 27727, 27731, 27745, 27750, 27759, 27769, 27779, 27789, 27814, 37405, 37412, and 37419 show their bands at the almost level of the positive check at 243 bp as calibrated from the ladder were used in the gel electrophoresis. Meanwhile, the accessions 27207, 27799, and 27805 show bands slightly lower than the 243 bp bands. These accessions can be used after the field trails. It is known that these varieties carry the RM-152 marker against the salt stress in their genomes, which serves as a positive check. The following accession numbers: 27131, 27137, 27141, 27146, 27151, 27156, 27161, 27202, 27207, 27727, 27731, 27745, 27750, 27759, 27769, 27779, 27784, 27789, 27794, 27799, 27805, 27814, 37405, 37412, and 37419. These accessions display the same band as N-22, Shaheen Basmati, and FL-478." That particular gene, which protects the genome from salt stress, is evidently present in these accessions and is known to be RM-152 salinity-resistant. Accessions are classified into resistant and susceptible groups using the polymorphic SSR molecular marker RM-WN-13903 (Abbas et al., 2024a; Abbas et al., 2024b). With the exception of that accession that lacked salinity resistance, the accessions were divided based on their band size at the same location as the tests for the particular markers that were shown to have resistance. This marker's band is around 1000 bps in width. This gel electrophoresis picture indicates that the positive checks that were employed include FL-478, N-22, Morro Barrecan, and Shaheen Basmati. These checks are known to have the tolerance to salt and display bands at about 1000 bp. The Bedex polymorphic single-strand break marker is utilized to identify or exclude accessions according to whether or not they have an aromatic gene present in their genome. The native check is the PK-86, and the positive check is the Super Gold. About 93 t0 103 bp is the band size of the Bedex SSR marker. The gel electrophoresis picture indicates that Super Gold was employed as a positive check, and it displays a band size of around 93 bp. To determine the aromatic characteristics of the chosen accessions, PK-86 was employed as the negative check in this reaction. The Aroma polymorphic single-strand break marker is utilized to identify or exclude accessions according to whether or not the genome contains the aromatic gene. The native check is the PK-86, and the positive check is the Super Gold. About 580 bps and 355 bps make up the Aroma SSR marker's band sizes. Whereas the 355 bp bands show that the accessions are not aromatic, the 580 bp band size indicates that the chosen accessions include an aroma-related gene. The 355 bp band is also known as the aroma SSR marker or non-aromatic band size. This gel electrophoresis picture, which displays a band size of around 580 kb, indicates that Super Gold positive checks were employed. The PK-86 negative check, which was utilized in this reaction to determine the non-aromatic characteristics of the chosen accessions, showed a band at 355 bp for this SSR marker (ALI, 2022; Fatima et al., 2023; Javed et al., 2024; Junaid and Gokce, 2024).

After examining the picture, it can be seen that Accessions 27131, 27141, 27146, 27161, 27166, 27202, 27207, 27750, 27769, 27137, 27151, 27156, 2727, 27745, 27759, 27779, 27784, and 27789 exhibit a band at a 580 bp band size, in addition to the super gold band. This indicates that all of the accessions were screened against the aroma SSR marker, and that each and every one of the chosen accessions had the marker-linked gene. The only sample that exhibits non-aromatic qualities is PK-86, which also served as a negative check with its band at 355 bp.

Conclusion

This study offers the genotypic variance in 26 different rice accessions and these accessions were screened out against the Saltol and Aromatic SSR markers. The Saltol markers used in this experiment were RM-7075, RM-562, RM-152, and RM-WN-13903 and the aromatic SSR used was Badex and RM23120. The significant variances were measured against the genetic variance in this experiment, some of the accessions were performed upright and some were performed ordinary. Most of the accessions show salt-resistant genes in their genome during the screening against saltol markers and the aromatic genes were also present for better aromatic properties. This study helps us to better understand which of the accessions perform better and have salt tolerance and, in the future, this study help breeders to understand and create a batter yielding salt tolerant verities for the salt-affected areas to increase the rice production area and have improved yield.

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Conflict of interest

There is no conflict of interest among the authors of the manuscript.