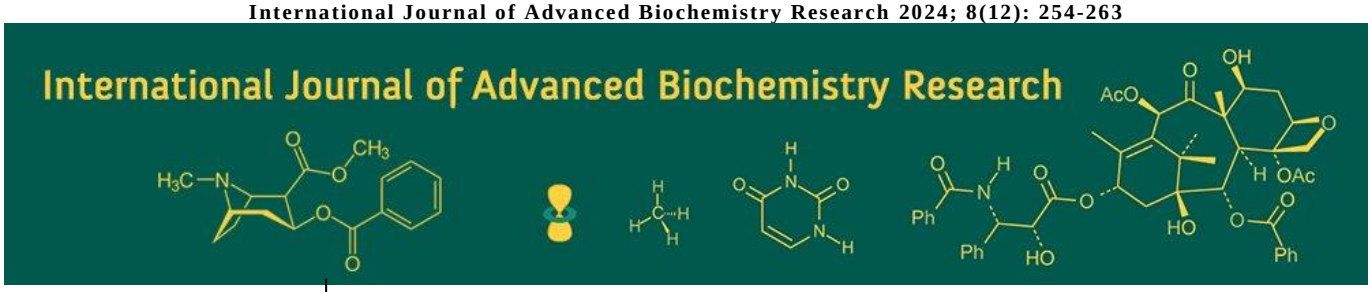

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Identification of corresponding markers responsible for expression of 2 acetyl 1 pyrroline in landraces and improved varieties of Rice (*Oryza sativa* L)

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Abstract
The demand of aromatic rice is increasing in the world every day. This fragrant rice has high economic value in the markets. There are many traditional landraces and improved basmati, short/medium-grain aromatic rice varieties which have aromatic trait but in initial stage of plant identification aroma is difficult. Many conventional methods have been utilized by plant breeders for detection of grain aroma in the field level for varietal selection, which are time consuming, labour intensive and more over unreliable due to saturation of sensory organs. Since past few decades, with the availability of molecular markers to discriminate between aromatic and non-aromatic genotypes, marker assisted selection approach is being widely employed to separate aromatic lines/genotypes from the non-aromatic ones. Hence, the present study was carried out with 29 traditional landraces and improved basmati, short/medium-grain aromatic rice varieties with 19 microsatellite or SSR and functional markers.

For the molecular analysis 29 rice genotypes seedling used for DNA extraction. The received product were amplified and run with 19 markers (13 SSR and 6 functional) were used to detect the aromatic gene in traditional landraces and improved basmati, short/medium-grain aromatic rice varieties. The markers showed 2-5 alleles per marker. The average PIC value calculated of all the markers was 0.396. Out of 19 markers fifteen the markers (14 SSR and 5 Functional) showed good amplification which allowed to differentiate between the aromatic and non-aromatic varieties. The functional markers BADEX7-3, BADEX7-4, BADEX7-2, BADEX7-5, BADEX7-1 showed clear and distinct banding pattern as they were closely linked to the *fgr* locus. They easily distinctive differentiation of the genotypes based on the product size which was either of aromatic or non-aromatic.

The ten SSR marker SCU017RM, RM342A, RM223, CP04133, BO3_127.8, 10L03_FW, RM515, ARSSR-3, F05_103.0, E11_44.5 showed amplification in all the genotypes and markers which are very closely linked to the aroma gene *fgr*. The genotypes like Kothambiri sal, Tilsha, CSR-27, Chinor, Karjat shatabdhi, Kasturi, Jeeraga Samba, Karnal local, Kachari kothambir, Kanak jeera, Ghansal, Pusa Basmati-1. Pusa Basmati-376, Basmati-306, Pusa Sugandha-2, Assam Basmati, Pakisthan Basmati, Manohar sal, Ambemohor and Indrayani were observed to be highly aromatic based on molecular studies, similarly the genotypes Kasbai, Pusa Basmati-63, Pusa Basmati-6524, Pusa Sugandha-1, Pusa Sugandha-3, Pusa Sugandha-5,Mahi Sugandha were observed absence of aroma. Which supports the assumption of a probable existence of a second gene for fragrance in rice other than *badh2*. Hence, the confirmation of aroma controlled by different genes within the rice genome was done.

The presence of different genes and QTLs for aroma was confirmed. The dendrogram of the 29 genotypes showed the similarities and grouped them according to their type of aroma. The present investigation concluded that the genes present in rice on chromosome 8 are responsible for the aroma and can be detected using molecular technique and this data can be used for the further development of the aromatic rice varieties and to design more specific marker system for detection of aromatic germplasm.

Keywords: Aromatic Rice, PIC, Polymorphism, SSR and function markers

Introduction
Rice (*Oryza sativa* L.) is the world's most popular food crop and a primary food source for more than one third of world's population, belongs to family Poaceae (Gramineae). It is a monocotyledonous angiosperm with a small genome of 430Mb across 12 chromosomes (Arumuganathan and Earle, 1991). All members of the Oryza genus are diploid (2n=24).

It is one of the few crop species provided with richest genetic diversity across the world. There are 24 recognized species in the genus Oryza out of which only two are cultivated; *O. sativa* or Asian rice and *O. glaberrima* or African rice (Chang, 1976) [6]

Asia produces and consumes most of the world's rice which has greater than half of the world's population (Chakravarthy and Naravaneni, 2006) [5]. Nearly 640 million tonnes of rice are grown in Asia, representing 90 percent of global production and 90 percent of global rice consumption.

Rice is the second most important crop after sorghum in Maharashtra, which is grown over an area of 15.97 lakh hectares with rice production of 35.10 lakh tonnes and the average productivity of the state 2.198 t/ha (Department of Agriculture, Government of Maharashtra, 2019). Maharashtra has 13th position in rice production in the country. The average productivity of other rice growing states viz., Punjab, Tamil Nadu, Haryana, Andhra Pradesh etc.is high as compared to Maharashtra state. In Maharashtra, Konkan coast area, the Ghats and some eastern parts are the places were rice is grown. Some of the important rice producing districts of Maharashtra are Raigad, Thane Chandrapur, Gondia, Bhandara and Kolhapur.

The Basmati and Jasmine rice have the aromatic and fragrant flavours associated with them which are believe to be the most important rice flavours. Basmati rice belong to a genetically distinct cluster, known as group V (Glaszmann, 1987) [10] also called as aromatic rice. These aromatic rice have their centre of origin from the foothills of Himalayas in the Indian states of Bihar and Uttar Pradesh (UP), and Tarai region of Nepal.

The organoleptic qualities of aromatic rice such as, taste, mouth feel and aroma have special importance. Aromatic rice are considered to be the best in quality even if they compose of a small but special group of rice varieties and landraces. The high economic value and preferred flavour of fragrant rice have encouraged rice breeders to develop varieties of fragrant rice and to discover rice fragrance genes.

In some cereal products 2AP is formed through the Maillard reaction whereas in scented rice varieties it is synthesized in its aerial parts from amino acids enzymatically. 1-pyrroline and 2-oxopropanal are important intermediates in the generation of 2AP. The genetic analysis revealed that a recessive gene, *fgr* on chromosome 8 is responsible for rice fragrance. It was reported that the betaine aldehyde dehydrogenase (BADH2) gene is associated with rice fragrance which comprises of 15 exons and 14 introns on chromosome 8. The Badh2 allele of aromatic rice contains an 8-bp deletion and 3 SNPs in exon 7 (badh2-E7) compared to the Badh2 allele of non-fragrant rice, which leads to functional loss of the encoding BADH2 protein.

Landraces can be defined as geographically or ecologically distinctive population which is largely diverse in their genetic composition. Thus such traditional agricultural systems serve to maintain the landraces, 'farmers' varieties', 'local varieties' or 'traditional varieties' which have been bred and selected by farmers.Apart from basmati, other short and medium scented non-basmati rice is grown in India for centuries. India has rich genetic variation of aromatic rice landraces. These indigenous varieties are grown locally in every almost all state of the country.

Many conventional methods have been utilized by plant breeders for detection of grain aroma in the field level for varietal selection, which are time consuming, labour intensive and more over unreliable due to saturation of sensory organs. Since past few decades, with the availability of molecular markers to discriminate between aromatic and non-aromatic genotypes, marker assisted selection approach is being widely employed to separate aromatic lines/genotypes from the non-aromatic ones. However, use of markers could not resolve the intensity of aroma in these genotypes which may be due to varied level of different physico-chemical factors (Buttery *et al.,* 1983; Yoshihashi *et al.*, 2002; Bradbury *et al.*, 2005b) [6, 22].

The accurate assessment and selection of suitable, recessive traits of fragrance within individual plants is the main barrier to breeding high yielding fragrant rice cultivars, whether basmati, jasmine or in adapted backgrounds. There are many studies related with chemical and genetic components of the rice fragrance in order to cope with the requirement for an accurate and reliable method for determining the fragrance phenotype of rice plants. The studies which would help the decreasing landrace, varieties or germplasms with good quality traits like as aroma by using the molecular makers based on BADH2 enzyme-bioassay and SNPs or SSRs markers and genetic data to improve them are important and necessary. In view of above point this study have been taken with objective to characterize genes responsible for aroma production based on PCR markers in traditional landraces and improved basmati, short/medium-grain aromatic rice and test their ability to discriminate fragrant and nonfragrant genotypes.

Materials and Methods

The present investigation entitled "Identification of corresponding markers responsible for expression of 2 Acetyl 1 Pyrroline in landraces and improved varieties of Rice (*Oryza sativa.* L)" was carried out at plant Biotechnology centre, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli. And rice quality analysis facilities are available at Regional Agricultural Research Station, Karjat during the year 2023-24. The details of materials used and methods followed in the present study are mentioned under following subheadings.

Materials

Plant material

The plant materials used in this study comprised of twenty-nine genotypes comprising traditional landraces and improved basmati, short/medium-grain aromatic rice collected from Regional Agricultural Research Station, Karjat Dist. Raigad. The list of genotypes used in the present investigation is given in Table 1

Table 1: The list of rice genotypes

Sr. No.	Coding accession	Genotypes/Varieties	Fragrance	Remarks
1	S1	Kothambiri sal	Strong	Aromatic landrace
2	S2	Tilsha	Strong	Aromatic landrace
3	S3	CSR-27	Strong	Aromatic landrace
4	S4	Chinor	Mild	Aromatic landrace
5	S5	Karjat shatabdhi	Mild	Aromatic landrace
6	S6	Kasturi	Strong	Aromatic landrace
7	S7	Jeeraga Samba	Mild	Aromatic landrace
8	S8	Karnal local	Mild	Aromatic landrace
9	S10	Kasbai	Mild	Aromatic landrace
10	S9	Kachari kothambir	Mild	Aromatic landrace
11	S11	Kanak jeera	Mild	Aromatic landrace
12	S12	Ghansal	Mild	Aromatic landrace
13	S13	Pusa Basmati-1	Strong	Traditional basmati
14	S14	Pusa Basmati-63	Strong	Traditional basmati
15	S15	Pusa Basmati-376	Strong	Traditional basmati
16	S16	Pusa Basmati-6524	Strong	Traditional basmati
17	S17	Basmati 306	Strong	Traditional basmati
18	S18	Pusa Sugandha-1	Strong	Evolved basmati
19	S19	Pusa Sugandha-2	Strong	Evolved basmati
20	S20	Pusa Sugandha-3	Strong	Evolved basmati
21	S21	Pusa Sugandha-5	Strong	Evolved basmati
22	S22	Mahi Sugandha	Strong	Evolved basmati
23	S23	Assam Basmati	Strong	Local basmati
24	S24	Pakistan Basmati	Strong	Local basmati
25	S25	Manohar sal	Mild	Aromatic variety
26	S26	Ambemohar	Strong	Aromatic variety
27	S27	Indrayani	Strong	Aromatic variety
28	S28	Karjat-3	None	Improved Variety
29	S29	Karjat-7	None	Improved Variety

To characterize the aromatic gene in the rice genotypes the extracted genomic DNAs were subjected for PCR analysis using different primers.

Markers

Table 2: SSR and functional markers used in the study

Sr. No.	Name of Marker	Marker Sequence 5'-3'	Chromosome	STD. Annealing temp. (ºC)	Reference
1	RM42	F- ATCCTACCGCTGACCATGAG R- TTTGGTCTACGTGGCGTACA	8	**55.5**	Garland *et al.*, (2000) [10].
2	SCU015RM	F- GGTTCATTCAAGCCTCCAGC R- TTTTCCCACCAGCCAAACAT	8	**54**	Cordeiro *et al.*, (2002) [7].
3	SCU017RM	F-AGGTGCCTTAGATCGAACAG R-CAAAATCGTACTTACCTTGC	8	**54.5**	Cordeiro *et al.*, (2002) [7].
4	F05_103.0	R- CTCCGTGCAAAGGAGGTACT F-CCATACCCCAGCTTCTCTCA	8	**55.5**	Wanchana *et al.*, (2005) [20].
5	10L03_FW	F-AACTAAGCACAACGCAAGGC R-TCACTCTAATTGGCCTGGTTTT	8	**56.8**	Wanchana *et al.*, (2005) [20].
6	BO3_127.8	F-CGTGGCTCGACCTTTTTAAT R-TCAAACCCTGGTTACAGCAA	8	**51**	Wanchana *et al.*, (2005) [20].
7	CP04133	F-CAGAGGCAATGCAAGAACAG R-CGATCATCCCGAATTCATTT	8	**55.5**	Wanchana *et al.*, (2005) [20].
8	E11_44.5	F-TCATGATATGGGCACTGCTG R-TCGATCCAGGAGGAGAGCTA	8	**55.5**	Ahn *et al.*, 1992 [1]
9	E03_92.0	F-GCCATGGCTAAGCTAGGATTC R-ATCCGCGTACTCTCTCCTCA	8	**59**	Ahn *et al.*, 1992 [1]
10	RM223	F-GAGTGAGCTTGGGCTGAAAC R-GAAGGCAAGTCTTGGCACTG	8	**60**	Begum *et al.*. 2006 [2]

11	RM342A	F-CCATCCTCCTACTTCAATGAAG R-ACTATGCAGTGGTGTCACCC	8	**54.5**	Begum *et al.* 2006 [2]
12	RM515	F-TAGGACGACCAAAGGGTGAG R-TGGCCTGCTCTCTCTCTCTC	8	**59.5**	Begum *et al.*, 2006 [2]
13	ARSSR-3	F-GACACGCACCTCTGTCTAGC R- GTTTAATTGGTGAGGAAGTGG	8	**56**	Madhav *et al.*, 2010 [13]
14	BADEX7-1	F-TTGTTTGGAGCTTGCTGATG R-TTTTTCCACCAAGTTCCAGTG	8	**59.5**	Sakthivel *et al.* 2009 [15]
15	BADEX7-2	F-TGCTCCTTTGTCATCACACC R-TTCCACCAAGTTCCAGTGAA	8	**54.5**	Sakthivel *et al.* 2009 [15]
16	BADEX7-3	F-AGGACTTGTTTGGAGCTTGC R-AACCATAGGAGCAGCTGAAG	8	**59.5**	Sakthivel *et al.* 2009 [15]
17	BADEX7-4	F-TGCTCCTTTGTCATCACACC R-TGGAAACAAACCTTAACCATAGG	8	**60**	Sakthivel *et al.* 2009 [15]
18	BADEX7-5	F-TGTTTTCTGTTAGGTTGCATT R- ATCCACAGAAATTTGGAAAC	8	**54.5**	Sakthivel *et al.*, 2009 [15]
19	BADEX7-6	F-GGTTGCATTTACTGGGAGTTATG R- AACCATAGGAGCAGCTGAAG	8	**56.8**	Sakthivel *et al.*, 2009 [15]

Methods

Isolation of genomic DNA from leaves

For molecular marker analysis, 29 genotypes were used. The genomic DNA of 29 rice genotypes was extracted following the Cetyl trimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1990) [8] with some modifications.

Procedure

1.	Extraction buffer was prepared and warmed at 65 ºC in a water bath
2.	Leaf tissues (500mg) were grinded using liquid nitrogen into a fine powder with mortar and pestle.
3.	The crushed powder was transferred to 650 µl extraction buffer (10% CTAB, 1.4 M NaCl, 1 M Tris pH 8.0, 0.5 M EDTA pH 8.0, 0.2% mercaptoethanol) pre-warmed at 65 ºC in water bath.
4.	The tubes were completely mixed by inverting and incubated for 60 minutes in a water bath at 65 ºC. During incubation, tubes were frequently inverted for 8-10 minutes to prevent the tissue separation from extraction buffer.
5.	Equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifugation was carried out at 12000 rpm for 10 min at 4 ºC.
6.	After centrifugation the aqueous phase was collected in fresh tubes (1.5 ml) and 300 µl chilled isopropanol was added and mixed by inversion.
7.	Tubes were incubated at -20 ºC in deep refrigerator for about 30 min.
8.	After 30 min tubes were removed and centrifugation was carried out at 12000 rpm for 5min at 4 ºC.
9.	After centrifugation supernatant obtained was discarded and pellet was washed with 100 µl of 70% ethanol tap well and then centrifuge at 5000 rpm for 5 min at 4 ºC.
10.	After centrifugation supernatant obtained was discarded and pellet was air dried.
11.	100 µl sterile distilled water or TE buffer was added to pellet and stored to -20 ºC.

Photography and gel documentation

The gels were photographed under UV light. The Gel documentation systems (Uvitec Fire Reader Software) took images of the gel and recorded them in the computer for subsequent study.

Molecular screening of genotypes: The gels were systematically examined, and amplicons that had only been produced once for a certain variant were identified as containing the band for that variant. In addition, two genotypes fragments were marked, which when combined with additional bands obtained with different primers, constituted the screening.

Data analysis

Cluster analysis

Data obtained from all the polymorphic markers were used to determine genetic relationships. As a dominant expression, each amplification result was considered an SSR marker and was scored across all samples. The presence (+/1) or absence (-/0) of bands was determined. The size of each allele was assessed by utilizing software to run a DNA ladder at the same time (Uvi-tech, Fire-reader software version 15.12). Using the tool MVSP-A (Multivariate Statistical Package - 5785 Version 3.1), the data was used for similarity-based analysis. To produce a dendrogram, similarity coefficients were utilized to construct UPGMA (unweighted pair group method with average).

The distance matrix and dendrogram were created using the UPGMA (Unweighted Pair Group Method of Arithmetic Means), a computer tool for distance estimation, based on the diversity coefficient produced from pooled data.

Results and Discussion

The genotypes comprised of some traditional landraces and some improved varieties of rice. The molecular study included 29 genotypes. The results obtained from experiments are discussed below.

Isolation of genomic DNA

In the present study DNA from 29 genotypes of rice were isolated from the young leaves of 3-4 weeks old seedlings by following the modified CTAB method given by Doyle and Doyle (1990) [8]. For the intactness of DNA bands, the DNA was treated with RNase, which showed good quality bands. The quality of extracted genomic DNA was checked by 0.8% agarose gel electrophoresis (Plate 4.1.).

Molecular characterization of aromatic genes

In this study simple sequence repeats (SSR) and functional markers which were specific to the gene being characterized

were used. They were used to study the molecular characterization of rice genotypes for aroma genes.

Marker analysis

The genomic DNA was isolated from 29 genotypes and subjected to further PCR amplification using 19 markers. Annealing temperature of each of the marker was used as per published literature.

Out of 19 (13 SSR + 6 Functional) markers fifteen markers were showing amplification with the alleles in the range of 2-5 per marker. The 15 markers were found polymorphic with an average of 4.52 alleles per marker (Table 4.2). The PIC range was 0.067 to 0.923 with an average of 0.396. Almost all the markers amplified the alleles at expected size except RM42. SCU015RM, E03_92.0, BADEX7-6. The details of the markers are given the following tables -

Table 3: Analysis of rice genotypes with the markers used

Sr. No.	Analysis	Observations
1	Total number of markers pairs used	19
2	Number of markers amplifying DNA	19
3	Total number of polymorphic markers	19
4	Range of alleles	2-10
5	Total alleles	86
6	Amplified product size	56-532 bp

Table 4: Details of amplification using different markers

Sr. No.	Marker	Marker type	Total number of polymorphic band	No. of alleles amplified	Product size (kb)	Allele frequency	PIC
1	SCU017RM	SSR	41	10	160-495	0.689	0.524
2	RM342A	SSR	25	5	130-234	0.862	0.256
3	RM223	SSR	27	5	134-238	0.793	0.370
4	CP04133	SSR	33	4	410-514	0.896	0.196
5	BO3_127.8	SSR	27	4	135-218	0.931	0.133
6	10L03_FW	SSR	29	3	165-290	0.965	0.067
7	RM515	SSR	25	5	200-304	0.862	0.256
8	ARSSR-3	SSR	26	6	145-270	0.896	0.196
9	F05_103.0	SSR	30	4	160-243	0.896	0.196
10	E11_44.5	SSR	26	4	123-290	0.896	0.196
11	BADEX7-3	Functional	23	2	265-273	0.793	0.370
12	BADEX7-4	Functional	26	2	187-195	0.896	0.196
13	BADEX7-2	Functional	20	9	391-399	0.655	0.570
14	BADEX7-5	Functional	27	5	95-103	0.931	0.133
15	BADEX7-1	Functional	18	2	481-489	0.620	0.614
16	RM42	SSR	13	5	145-270	0.448	0.799
17	SCU015RM	SSR	17	7	160-453	0.448	0.799
18	E03_92.0	SSR	8	2	475-516	0.275	0.923
19	BADEX7-6	Functional	15	2	56-97	0.517	0.732
	Total no. of alleles		458	86	--	--	--
	Average		24.10	4.52	--	0.751	0.396

Amplification using SSR marker SCU017RM

Marker SCU017RM showed amplification in all the 19 genotypes with expected product size ranged from 160 bp to 495 bp except Pusa Sugandha-5, Mahi Sugandha, Pakistan Basmati, Manohar Sal, Indrayani, Karjat-3 and Karjat-7 genotypes which showed absence of amplification (Plate 4.2). The SCU017RM marker showed amplification at 47.7 ºC temperature as given in Cordeiro *et al.* (2002) [7] and showed high intensity bands for the genotypes. The SCUO17RM was found highly polymorphic between the rice genotypes. Total Forty-one bands were scored from the SCUO17RM marker. The number of alleles detected was ten. The allelic variation shows the presence of different sequences and or different allele at aroma locus analysed. The genotypes in the range of expected product size are having the recessive non-functional aromatic gene which imparts fragrance to the rice. The PIC value of marker SCU017RM is 0.524.

Amplification using SSR marker RM342A

RM342A showed amplification in all the 19 genotypes for which expected product size is ranging between 130 bp to 234 bp except Manohar Sal, Karjat-3 and Karjat-7 genotypes which showed absence of amplification (Plate 4.3). RM342A marker showed amplification at 55 ºC temperature as given in Kibira *et al.* (2008) and showed high intensity bands for the genotypes RM342A also showed polymorphic pattern in all the genotypes. The PIC value of marker RM342A was 0.256.

Amplification using SSR marker RM223

The marker RM223 showed amplification in all genotypes and confirmed the presence of fragrance. The genotypes Pusa Sugandha-5, Pakistan Bamati, Ambemohar, Indrayani, Karjat-3 and Karjat-7 showed absence of amplification. The marker showed god results at 55ºC. The number of alleles observed for this marker was five (Plate 4.4). The PIC value of RM 223 is 0.370. The SSR marker RM223 covers the most effective QTL, aro8-1 located on chromosome 8 (Amrawathi *et al.* 2008 Ahn *et al.* (1992) [1] and Lorieux *et al.* (1996). RG28 the first mapped RFLP marker for aroma lies between RM42 and RM223. The *fgr* locus is close to RM223 marker (Garland *et al.* 2000) [9].

Amplification using SSR marker CP04133

The marker CP04133 had the expected product size ranging from 410 bp to 514 bp (Plate 4.5). The genotypes having the product size between the said ranges was counted as fragrant. The marker amplified at 55ºC with intact bands for all the genotypes. The genotypes Kasbai, Pakistan Basmati, Karjat-3 and Karjat-7 showed no amplification. The PIC value of CP04133 was 0.196.

Amplification using Functional marker BADEX7-2

BADEX7-2 is functional marker developed by Sakthivel *et al.* (2009) [15]. The aromatic genotypes produced 391 bp (with 8 bp deletion) whereas a 399 bp pair was produced by non-aromatic ones. Hence, the said marker only showed 2 alleles in all the genotypes. The PIC value of BADEX7-2 is 0.570. The genotypes Tilsha, CSR-27, Chinor, Ambemohar and Indrayani showed the band of 391 bp i.e the presence of aromatic gene and genotype Karjat-3 and Karjat-7 showed the product size of 399 bp similar to the results by Sakhtivel *et al.* (2009). The genotypes Kothambiri sal, Kasbai, Pusa Bamati-63, Pusa Basmati-6524, Pusa Sugandha-3, Mahi Sugandha and Manohar sal showed absence of amplification while all the other genotypes showed presence of amplification with good and intact bands at 59 ºC (Plate 4.6).

Amplification using Functional marker BADEX7-5

BADEX7-5 is functional marker pair distinctly separated the aromatic with non-aromatic genotypes with few exceptions. The aromatic genotypes produced 95 bp (with 8 bp deletion) whereas a 103 bp pair was produced by non-aromatic ones. Hence, the said marker has showed 5 alleles in all the genotypes. The PIC value of BADEX7-5 is 0.133. The genotypes, Pakistan Basmati, Manohar sal, Karjat-3 and Karjat-7 showed absence of amplification while all the other genotypes showed presence of amplification with good and intact bands at 59 ºC (Plate 4.7).

Amplification using Functional marker BADEX7-1

BADEX7-1 is functional marker pair distinctly separated the aromatic with non-aromatic genotypes with few exceptions. The aromatic genotypes produced 481 bp (with 8 bp deletion) whereas a 489 bp pair was produced by non-aromatic ones. Hence, the said marker showed only 2 alleles in all the genotypes. The PIC value of BADEX7-1 is 0.614. The genotypes Kothambiri sal, Kasbai, Pusa Bamati-63, Pakistan Basmati, Manohar sal, Karjat-3 and Karjat-7 showed absence of amplification while all the other genotypes showed presence of amplification with good and intact bands at 60 ºC (Plate 4.8).

Molecular analysis

The SSR and functional markers together showed amplification in all the genotypes. The data derived from the molecular studies was correlated with aromatic genotypes like Kothambiri sal, Tilsha, CSR-27, Chinor, Karjat Shatabdhi, Kasturi, Jirga Sambha, Karnal local, Kasbai, Kachari kothambir, Kanak jeera, Ghansal, Pusa Basmati-1, Pusa Basmati-63, Pusa Basmati -376, Pusa Basmati-6524, Pusa Basmati-306, Pusa Sugandha-1 Pusa Sugandha-2, Pusa Sugandha-3, Pusa Sugandha-5, Mahi Sugandha, Assam Basmati, Manohar sal, Ambemohar, Indrayani, and non-aromatic genotypes Karjat-3 and Karjat-7 showed amplification of expected allele. The functional markers showed distinctive differentiation between the aromatic and non-aromatic genotypes than the SSR markers. All these markers were situated on chromosomes 8.

Genetic diversity analysis by molecular data

The genetic diversity was observed in the 29 rice genotypes for the grouping based on their similarities and dissimilarities. To visualize the inherent relationship among the 29 rice genotypes, a dendrogram was constructed based on the neighbour joining method using the UPGMA (unweighted pair group method with average) program is presented in Fig.1. The cluster information from the dendrogram is given in Table 4.3.

Based on the cluster analysis using different markers used in the study, the genotypes were grouped in only one major clusters namely I include Kothambir sal genotype. The First cluster was again divided in to 2 sub clustered IA (i) and IA(ii) and 5 sub-sub clusters namely IA (ia), IA (ib) and IA (iia), IA (iib) and IA (iic) respectively.

Table 5: Distribution of rice genotypes into different clusters

Sr. No	Cluster	Sub-cluster	Sub- sub cluster	Genotypes	Remarks
1	I	IA(i)	IA (ia)	Pakistan Basmati, Assam Basmati, Pusa Sugandha-2 Mahi Sugandha Pusa Sugandha-6, Pusa Sugandha-3 and Pusa Sugandha-1,	Highly aromatic genotypes
				Karjat-3 and Karjat-7,	Absent of aromatic genotypes
			IA(ib)	Pusa Sugandha-1,	Strong aromatic genotypes
		IA(ii)	IA(iia)	Indrayani, Ambemohar, Manohar sal	Regional aromatic genotypes
			IA(iib)	Ghansal, Pusa Basmati-1, Pusa Basmati-63, Pusa Basmati-376, Pusa Basmati-6524, Pusa Basmati-306, Kanak jeera, Kachari kothambir,Kasbai, Kasturi	Moderately aromatic genotypes
			IA(iic)	Karnal local, Jeeraga Samba, Chinor, Karjat Shatabdhi, CSR27, Tilsha,	Local aromatic genotypes

The genotypes in sub-sub cluster IA (ia) include two highly aromatic genotypes namely Pakistan Basmati and Assam Basmati. The sub-sub cluster IA(ib) show absent of aromatic genotypes which include Karjat-3 and Karjat-7 and include stongly aromatic genotypes Pusa Sugandha-1, Mahi Sugandha, Pusa Sugandha-2, Pusa Sugandha-3 Pusa Sugandha-5. Whereas the cluster IA(iia) shows the regional aromatic genotypes which are Indrayani, Ambemohar and Manohar sal. The cluster IA(iib) includes the moderately aromatic genotypes which are Ghansal, Pusa Basmati-1, Pusa Basmati-63, Pusa Basmati-376, Pusa Basmati-6524, Pusa Basmati-306, Kanak jeera, Kachari kothambir, Kasbai and Kasturi. The sub cluster IA(iic) includes local aromatic genotypes namely Karnal local, Jeeraga Samba, Chinor, Karjat Shatabdhi, CSR27 and Tilsha.

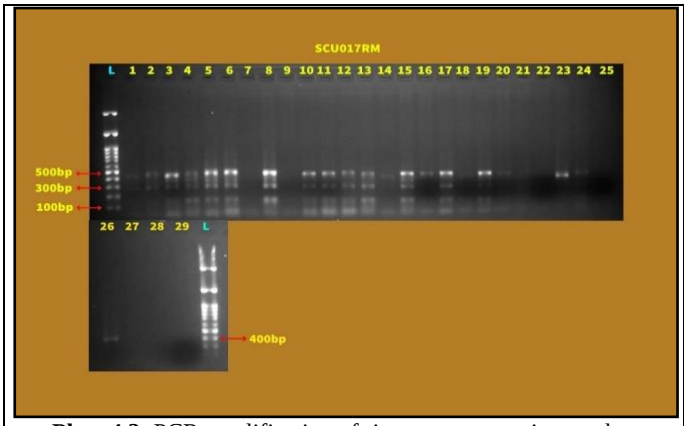

Plate 4.2: PCR amplification of rice genotypes using marker SCUO17RM

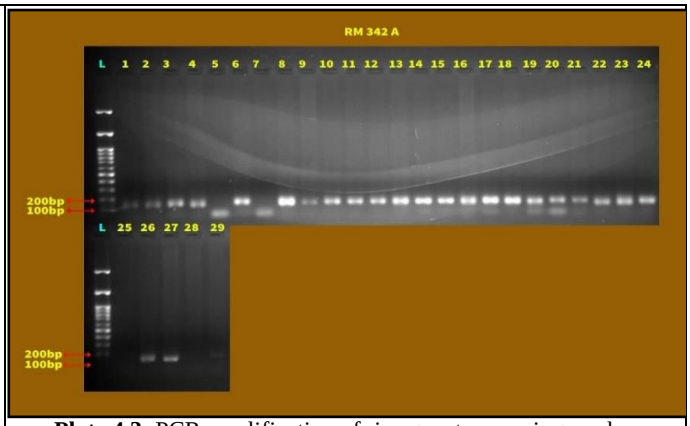

Plate 4.3: PCR amplification of rice genotypes using marker RM342A

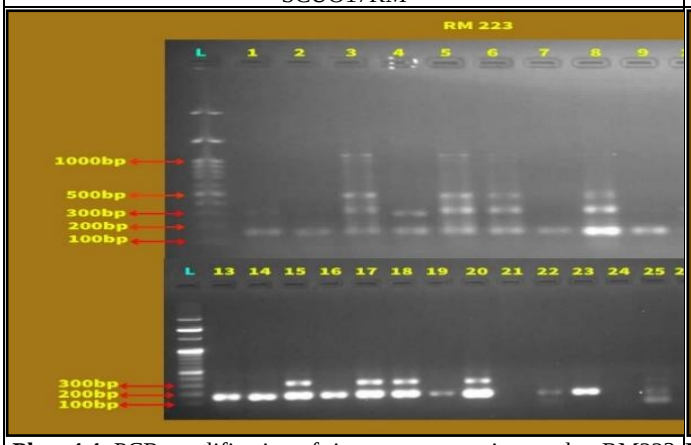

Plate 4.4: PCR amplification of rice genotypes using marker RM223

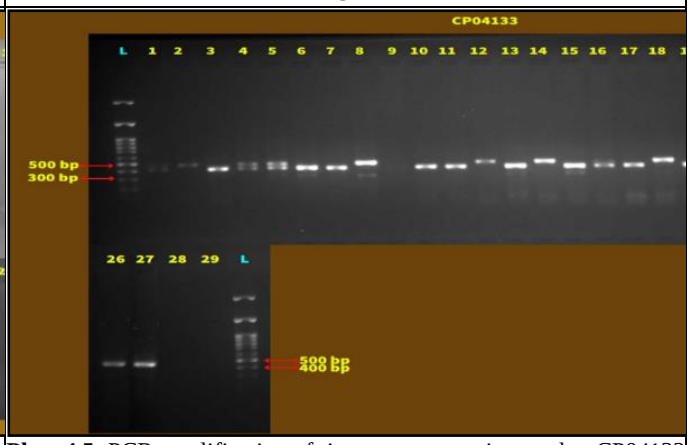

Plate 4.5: PCR amplification of rice genotypes using marker CP04133

Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes
1	Kothambir Sal	5	Karjat Shatabdhi	9	Kasbai	13	Pusa Basmati-1	17	Pusa Basmati-306	21	Pusa Sugandha-5	25	Manohar Sal	29	Karjat-7
2	Tilsha	6	Kasturi	10	KachariKothambir	14	Pusa Basmati- 63	18	Pusa Sugandha-1	22	Mahi Sugandha	26	Ambemohar		
3	CSR27	7	Jeeraga Samba	11	Kanak Jeera	15	Pusa Basmati-376	19	Pusa Sugandha-2	23	Assam Basmati	27	Indrayani		
4	Chinor	8	Karnal Local	12	Ghansal	16	Pusa Basmati-6524	20	Pusa Sugandha-3	24	Pakisthan Basmati	28	Karjat -3		

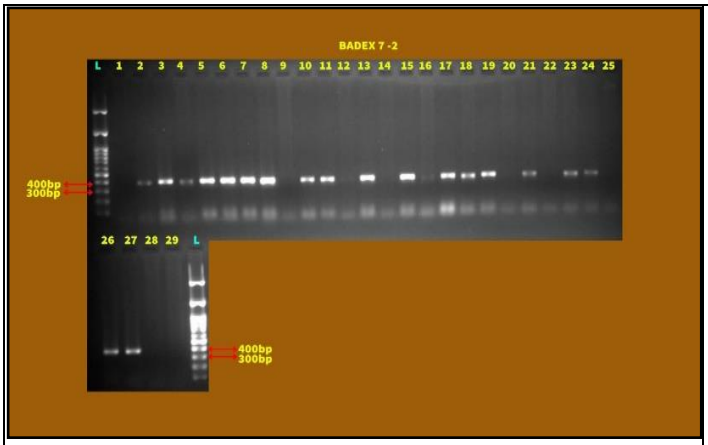

Plate 4.6: PCR amplification of rice genotypes using marker BADEX7-2

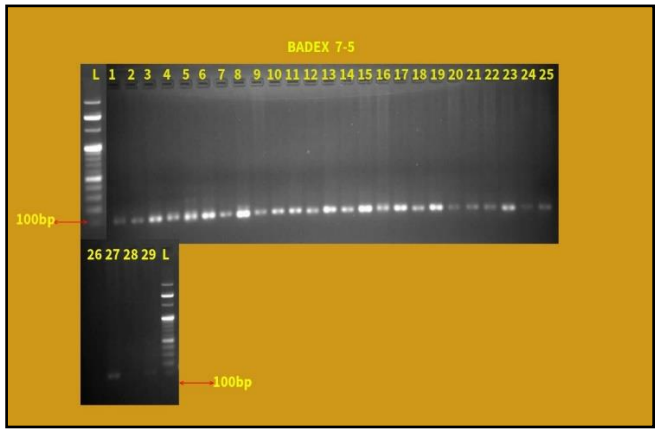

Plate 4.7: PCR amplification of rice genotypes using marker BADEX7-5

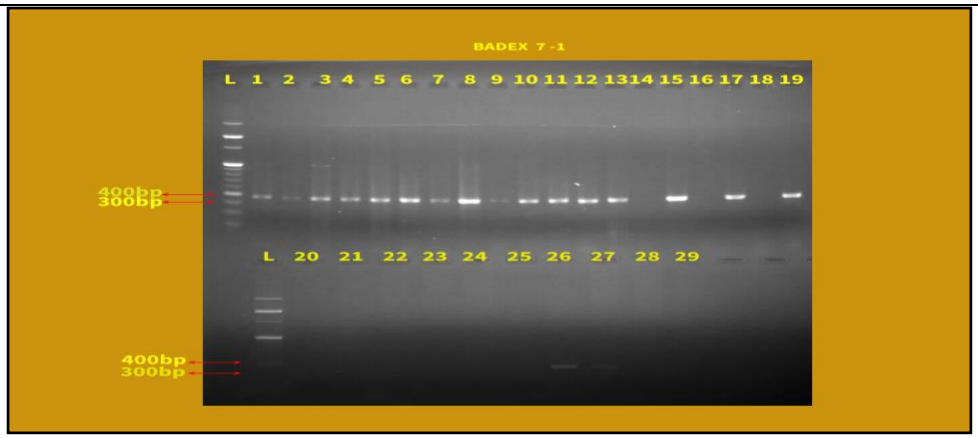

Plate 4.8: PCR amplification of rice genotypes using marker BADEX 7- 1

Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes
1	Kothambir Sal	5	Karjat Shatabdhi	9	Kasbai	13	Pusa Basmati-1	17	Pusa Basmati-306	21	Pusa Sugandha-5	25	Manohar Sal	29	Karjat-7
2	Tilsha	6	Kasturi	10	KachariKothambir	14	Pusa Basmati-63	18	Pusa Sugandha-1	22	Mahi Sugandha	26	Ambemohar		
3	CSR27	7	Jeeraga Samba	11	Kanak Jeera	15	Pusa Basmati-376	19	Pusa Sugandha-2	23	Assam Basmati	27	Indrayani		
4	Chinor	8	Karnal Local	12	Ghansal	16	Pusa Basmati-6524	20	Pusa Sugandha-3	24	Pakisthan Basmati	28	Karjat -3		

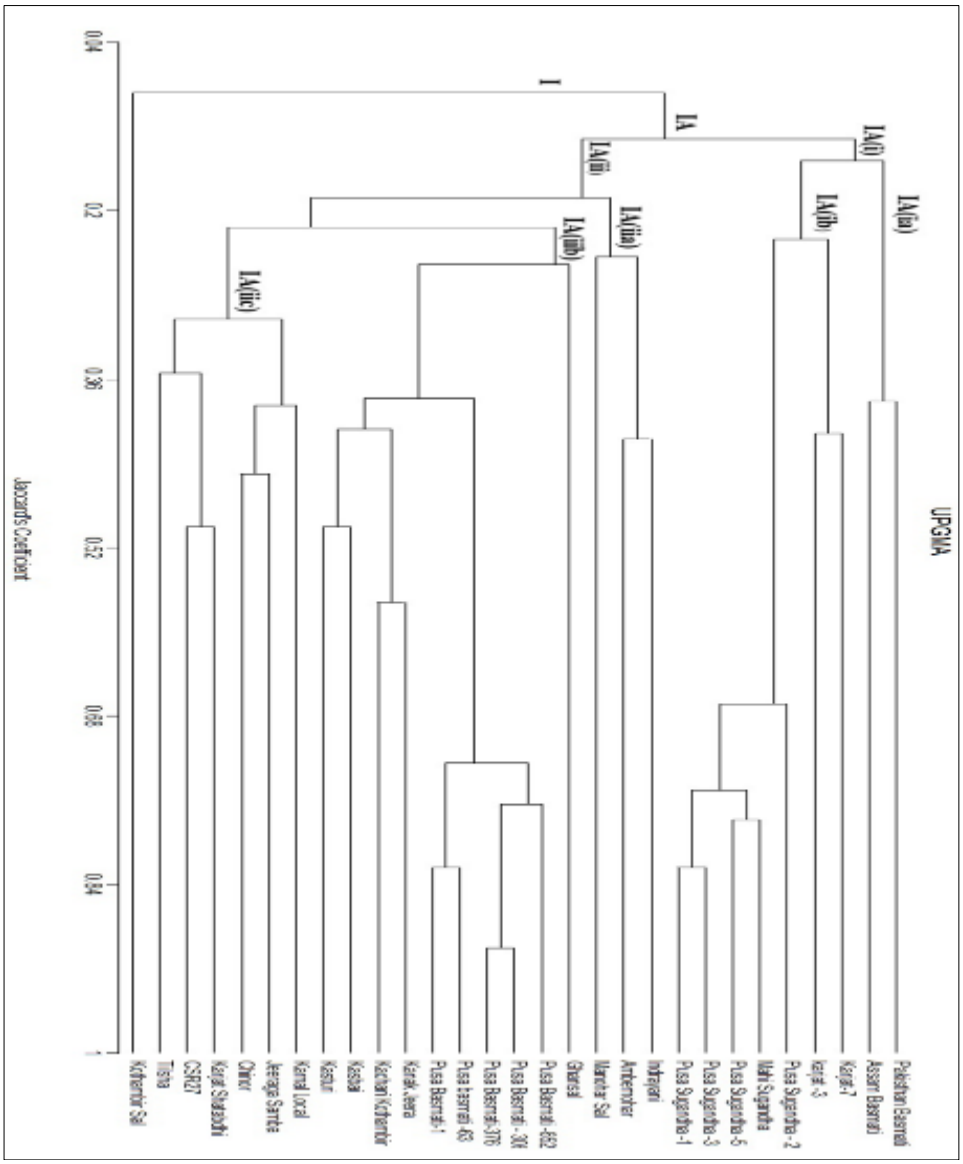

Fig 1: Dendogram of 27 aromatic and 2 non- aromatic entries based on average similarity coefficient for 19 primer pairs dependent allelic diversity

Discussion

The results from all the above study elaborated the benefit of markers to identify the presence of the fragrant (*fgr)* gene in rice and the genetic relationships among various fragrant and nonfragrant genotypes. The genotypes were carefully selected from fragrant Indica rice varieties with different geographical origins. The SSRs or functional markers were differentiating in all fragrant genotypes from the nonfragrant varieties with 100% efficacy. Thirteen SSR markers (SCU017RM, RM342A, RM223, CP04133, BO3_127.8, 10L03_FW, RM515, ARSSR-3, F05_103.0, E11_44.5, RM42, SCU015RM and E03_92.0) and six functional markers (BADEX7-3, BADEX7-4, BADEX7-2, BADEX7-5, BADEX7-1 and BADEX7-6) could differentiate fragrant from nonfragrant genotypes with the following exceptions: Kasbai, Pusa Basmati-63, Pusa Basmati-6524, Pusa Sugandha-1, Pusa Sugandha-3, Pusa Sugandha-5, Mahi Sugandha. Therefore, these markers can be used for identification, discrimination for aroma in traditional landraces and improved basmati, short/medium grain aromatic rice genotypes including Kothambiri sal, Tilsha, CSR-27, Chinor, Karjat shatabdhi, Kasturi, Jeeraga Samba, Karnal local, Kachari kothambir, Kanak jeera, Ghansal, Pusa Basmati-1. Pusa Basmati-376, Basmati-306, Pusa Sugandha-2, Assam Basmati, Pakistan Basmati, Manohar sal, Ambemohor and Indrayani. This results is in agreement with Yi *et al*., 2009; Singh *et al.*, 2011.

India, due to its vast size, experiences large variations in climate from region to region and is endowed with a great diversity of rice in the Indo-Gangetic plains (Singh *et al.*, 2013). Therefore, the genetic diversity of Indian fragrant rice varieties is expected to be high because of its rich ecological diversity. Among traditional basmatis, all markers were monomorphic, indicating that they share a common gene for fragrance. Interestingly, four evolved basmati genotypes (Pusa Sugandha-1, Pusa Sugandha-2 Pusa Sugandha-3 Pusa Sugandha-5) were separated from traditional basmatis. This result is in agreement with Steele *et al.* (2008), who studied genetic diversity of 44 rice varieties with 33 ideal markers and were able to differentiate traditional basmatis from other fragrant varieties.

Results revealed that all the used primers showed distinct polymorphism among the in traditional landraces and improved basmati, short/medium grain aromatic rice genotypes indicating the robust nature of SSRs and functional markers. The molecular studies and cluster analysis indicates that the 29 traditional landraces and improved basmati aromatic rice genotypes were grouped into one major cluster and which were divided into two sub clusters i.e**.** IA(i), and IA (ii) cluster (Fig.1). By comparing all the data the highly aromatic varieties (Kothambiri sal, Tilsha, CSR-27, Chinor, Karjat shatabdhi, Kasturi, Jeeraga Samba, Karnal local, Kachari kothambir, Kanak jeera, Ghansal, Pusa Basmati-1. Pusa Basmati-376, Basmati-306, Pusa Sugandha-2, Assam Basmati, Pakistan Basmati, Manohar sal, Ambemohor and Indrayani), absence of aroma varieties (Karjat-3 and Karjat-7) and other slightly and moderately aromatic genotypes (Kasbai, Pusa Basmati-63, Pusa Basmati-6524, Pusa Sugandha-1, Pusa Sugandha-3, Pusa Sugandha-5, Mahi Sugandha) were determined. The molecular studies confirmed the cluster analysis grouped all these genotypes either varieties according to their similarities and relationships.

Many previous studies on the diversity of the *BADH2* gene in a large collection of accessions have shown that an 8-bp deletion in the seventh exon is present in most aromatic accessions, but other less frequent mutations associated with fragrance were also detected (Shi *et al*., 2008; Kovach *et al*., 2009; Myint *et al.,* 2012) [12, 14]. Additionally, several aromatic accessions did not carry any mutation in the coding segments (Singh *et al.*, 2010; Myint *et al.*, 2012) [14], therefore the cause of their fragrant nature is unknown.

Conclusion

Among the 19 markers (13 SSR + 6 Functional) three SSRs (RM42 SCU015RM, E03_92.0,) and one Functional (BADEX7-6) could not discriminate fragrant in Kasbai, Pusa Basmati-63, Pusa Basmati-6524, Pusa Sugandha-1, Pusa Sugandha-3, Pusa Sugandha-5 and Mahi Sugandha. Which supports the assumption of a probable existence of a second gene for fragrance in rice other than *badh2*. The ten SSR markers (SCU017RM, RM342A, RM223, CP04133, BO3_127.8, 10L03_FW, RM515, ARSSR-3, F05_103.0, E11_44.5) and five functional markers (BADEX7-3, BADEX7-4, BADEX7-2, BADEX7-5, BADEX7-1) showed clear and distinct banding pattern as they were closely linked to the *fgr* locus. The presence of different genes and QTLs for aroma was confirmed. The dendrogram of the 29 genotypes showed the similarities and grouped them according to their type of aroma. The present investigation concludes that the genes present in rice on chromosome 8 are responsible for the aroma and can be detected using molecular technique and this data is useful for the further development of the varieties and to design more specific marker system for characterization of aromatic germplasm.

The study validates the practical utility of the PCR based assays, which allows determination of the genotypic status of an individual rice accessions for a specific trait like aroma. It will greatly help the breeders worldwide to analyse the breeding material at the early stage of the crop growth, preferably in the nursery itself. Moreover, the 20 individuals identified to be strongly aromatic can be useful for introgression of their fragment allele in the back ground of high yielding cultivars for genetic enhancement and improvement of aromatic short grain rice thereby providing higher economic gain to the farmers. The eleven genotypes which showed a heterozygous nature at molecular level and are still aromatic, will be useful towards understanding their genetic basis of aroma.

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