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DNA marker based evaluation of aromatic rice genotypes of Chhattisgarh region

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Abstract
Among different strategies to detect aroma in rice, fragrance linked –DNA markers have potential to improve the efficiency and precision being simple, inexpensive and co-localized with aroma gene. In this study the F_2 plants of two mapping population developed by crossing aromatic parent Tarunbhog with local non aromatic variety safri17 and aromatic variety Dubraj were evaluated for a set of 13 yield attributing traits and further, we evaluated the discrimination ability of 29 gene tagged functional DNA marker for genotypic selection of aromatic plants. All the F2 plants were categorized into two additional categories indicated involvements of more than two gene .out of 29 markers 2 were showed polymorphism.

Keywords: Aroma, functional DNA marker

Introduction
Rice (*Oryza sativa* L.) is one of the most important crops that provide food for more than half of the world population. India has a long history of rice cultivation and stands first in rice area and second in rice production, after China. Aromatic or fragrant rice varieties constitute a small but economically important group of rice as they fetch a premium price in agricultural markets for their superior aroma and grain quality. There are 2 groups of aromatic rice: the long-grained basmati type and the small and medium-grained indigenous aromatic varieties or landraces. Differences in aroma occur in aromatic genotypes arising from diverse origins and there is no consensus on the nature of the exquisite fragrance of rice yet (Sun *et al*., 2008) [13]. In terms of unique fragrance and grain quality, a number of small- and medium-grain scented rice varieties are cultivated in India in addition to the traditional long-grain basmati, which is restricted to North India.
Chhattisgarh is very rich for biodiversity but the resources are not yet properly utilized. This geographical region has vast diversity of aromatic rice. Some of the local rice or land races having unique identity and taste are very much in demand by traders and consumers. The research efforts have been focused to develop high yielding dwarf rice varieties having resistance to biotic stresses but less emphasis has been given to improve the local aromatic rice of Chhattisgarh. Though the land races are being maintained by the farmers traditionally, they are not released as varieties and are not notified and not in seed production chain. Thus the present study was conducted to identify the fragrance gene in F_2 population to develop elite aromatic rice lines.

Materials and methods
Genotypic characterization using functional/gene tagged markers.
3.4.1. Genomic DNA isolation
Total rice genomic DNA was extracted from single tagged plant during wet season 2015, by MiniPrep method (Doyle and Doyle, 1987) and used for PCR amplification to test the presence of gene and allelic pattern using functional/gene tagged markers. A total of 29 aroma specific markers colocalized in chromosome # 3, 4 & 8 were selected for aroma genotyping. Through detailed literature survey and using experiments. Among 29 markers, & were validated functional and gene tagged marker. The details of marker used in the present investigation & primer sequences were given in Table 1.

Table 1: List of 29 markers used for genotypic characterization

S. No.	Name of marker	Marker type	linkage group	Reference
1	SCU-Rice-SSR-1	SSR	8	Garland *et al*., 2002 [6]
2	RM42	SSR	8	Garland *et al*., 2002 [6]
3	RM223	SSR	8	Garland *et al*., 2002 [6]
4	SCU015RM	SSR	8	Cordeiro *et al*., 2002 [5]
5	ESP + IFAP + INSP + EAP	ASA	8	Bradbury *et al*., 2005 [3]
6	10L03_FW	SSR	8	Wanchana *et al*., 2005
7	BO3_127.8	SSR	8	Wanchana *et al*., 2005
8	CP04133	EST	8	Wanchana *et al*., 2005
9	L05	STS	8	Chen *et al*., 2006 [4]
10	L06	STS	8	Chen *et al*., 2006 [4]
11	Aro7	SSR	8	Sun *et al*., 2008
12	RM23120	SSR	8	Sun *et al*., 2008
13	RM3459	SSR	8	Sun *et al*.,2008
14	nksbad2	Functional	8	Amarawathi *et al*., 2008 [1]
15	RM5474	SSR	3	Amarawathi *et al*., 2008 [1]
16	RM5633	SSR	4	Amarawathi *et al*., 2008 [1]
17	RM273	SSR	4	Amarawathi *et al*., 2008 [1]
18	RM342	SSR	8	Kibria *et al*., 2008 [7]
19	RM515	SSR	8	Kibria *et al*., 2008 [7]
20	RG28L	STS	8	Lang and Buu, 2008 [8]
21	Fmbadh2-E2A	Functional	8	Shi *et al*., 2008 [12]
22	Fmbadh2-E2B	Functional	8	Shi *et al*., 2008 [12]
23	Fmbadh2-E7	Functional	8	Shi *et al*., 2008 [12]
24	ARSSR-3	SSR	8	Madhv *et al*., 2009 [9]
25	BADEX7-5	unctional	8	Sakthival *et al*., 2009 [11]
26	AR-5+AR-3	STS	8	Bourgis *et al*., 2008 [2]
27	AR-5+NAR-3	STS	8	Bourgis *et al*., 2008 [2]
28	MITE-5+MITE-3	STS	8	Bourgis *et al*., 2008 [2]
29	RM282	SSR	8	Amarawathi *et al*., 2008 [1]

Result and Discussion

Study II- genotypic characterization

Availability of high restriction molecular map and sequence data has allow the fine mapping and positional cloning gene for fragrance in rice genotypes. The sequence information of aroma locus was further employed for development of simple ready to use marker system for identification of presence of fragrance genes, alleles and its patterns diversity at aroma locus and identification of new gene. In the present study a total of 29 functional/gene tagged marker of aroma locus on chromosome # 3, 4 and 8 were employed for detection of aroma in the panel of 140 aromatic rice genotypes and mapping population and further the ability of markers to discriminate short and medium grain varieties.

A set of 140 aromatic rice genotypes were genotyped using a set of 29 aroma specific functional/gene tagged markers and the genetic diversity parameters such as number of alleles per locus, major allele frequency, gene diversity and polymorphic information content (PIC) for each marker was estimated using software programmed powermarker version 3.25. A total of 55 alleles were detected in 140 germplasm accessions. The number of alleles per loci varied from 1 to 3 with an average of 2.1 alleles per locus. The highest number of alleles (3) was recorded for six markers *viz*., RM342, RM3459, L06, RM282 and ESP+IFAP+INSP+EAP whereas, the lowest number of allele (1) was obtained for 3 markers *viz*., ARSSR-3, SCU-Rice-SSR-1 and FMbadh2-E2A. PIC value represents the relative informativeness of each marker and in the present study the average PIC value was obtained as 0.272. Similar PIC values was obtained by other investigators using different marker system (Rai *et al*., 2015, Cordeiro *et al*., 2002, Chen *et al*., 2006, Amarawathi *et al*., 2008 and Shi *et al*., 2008) [10, 5, 4, 1, 12]. The PIC values ranged from 0.0 to 0.55 where 0.0 PIC value indicated presence of one allele per locus. Out of 23 markers, two (8.69%) markers were highly informative (PIC ≥ 0.5), 14 markers (60%) were reasonably informative (PIC = 0.25–0.5) and 7 markers (30.43%) were less informative (PIC ≤ 0.25). Heterozygosity was observed to be very low across 140 accessions which may be attributed to the autogamous nature of rice. Expected heterozygosity or Gene diversity computed according to Nei (1973) and varied from 0.0 to 0.79 with an average of 0.396 (Table 2). The major allele frequency across 140 accessions ranged from 0.48 to 0.966 with the mean major allele frequency 0.743. However one major allele was obtained for single allele marker, thus the 140 accessions used in this study have wide genetic diversity and are good candidates for candidate gene specific association studies for aroma.

Table 2: Details of functional/gene tagged aroma locus specific markers used for genotyping I the 140 aromatic rice genotypes and their genetic diversity parameters using power marker V 3.25 program

S. no.	Marker	Chr no.	Position (cM)	Minimum Molecular weight	Maximum Molecular weight	No. of alleles	Major Allele Frequency	Gene Diversity	Heterozygosity	PIC
1	RM5474	3	14.08	90	110	2	0.8309	0.2810	0.0000	0.24
2	RM282	3	51.21	124	138	3	0.6159	0.5277	0.0000	0.46
3	RM5633	4	48.42	203	225	2	0.5276	0.4985	0.0000	0.37
4	RM273	4	83.1	210	220	2	0.6214	0.4705	0.0000	0.36
5	SCU-Rice- SSR-1	8	76.29	125	125	1	1.0000	0.0000	0.0000	0.00
6	RM42	8	74.4	160	175	2	0.5789	0.4875	0.0000	0.37

7	RM223	8	76.48	150	160	2	0.7574	0.3675	0.0000	0.30
8	ESP + IFAP+ INSP + EAP	8	80.3	257	580	3	0.8726	0.2295	0.2075	0.22
9	10L03_FW	8	80.08	186	200	2	0.8413	0.2671	0.0000	0.23
10	BO3_127.8	8	80.3	122	134	2	0.6022	0.4791	0.0000	0.36
11	CP04133	8	80.67	421	483	2	0.6786	0.4362	0.1667	0.34
12	L05	8	80.35	316	368	2	0.7752	0.3485	0.0465	0.29
13	L06	8	80.39	325	376	3	0.6719	0.4507	0.0469	0.36
14	Aro7	8	80.3	290	300	2	0.5969	0.4812	0.0000	0.37
15	RM23120	8	75.1	393	460	2	0.9669	0.0639	0.0000	0.06
16	RM3459	8	75.7	180	197	3	0.4818	0.6276	0.0000	0.55
17	Nksbad2	8	80.3	82	90	2	0.9500	0.0950	000	0.09
18	RM342	8	78.4	132	150	3	0.5038	0.5865	0.0000	0.50
19	RM515	8	75.12	200	230	2	0.7319	0.3925	0.0000	0.32
20	RG28L	8	76.29	125	125	2	0.6861	0.4307	0.0000	0.34
21	FMbadh2-E7	8	80.3	260	270	2	0.9663	0.0651	0.0000	0.06
22	ARSSR-3	8	80.6	160	160	1	1.0000	0.0000	0.0000	0.00
23	BADEX7-5	8	80.3	95	108	2	0.5954	0.4818	0.6260	0.37
24	AR-5+AR-3	8	80.38	210	220	2	0.8182	0.2975	0.0000	0.25
25	AR-5+NAR-3	8	80.38	200	250	3	0.6571	-	0.000	-
26	FMbadh2- E2A	8	80.37	125	125	1	1.0000	0.0000	0.0000	0.00

Table 3: Mean diversity analysis of 140 aromatic rice genotypes using functional/gene tagged for aroma locus in rice

S. No	Diversity Traits	Number	Range	
1	Total no. of alleles	55	Maximum	Minimum
2	Mean no. of alleles per locus	2.7	3	1
4	Mean major allele frequency	0.743	1.00	0.48
4	Mean Gene Diversity	0.33	0.62	0.0
5	Mean PIC	0.2.7	0.55	0.0

As obtained in the present study, Rai *et al*., (2015) [10] also reported an average of two alleles per locus and the PIC value ranged from 0 to 0.91, with an average of 0.61 while studying 24 rice genotypes using a set of 30 aroma locus specific markers. Similar results were arrived by many investigators while studying aroma locus specific markers. (Rai *et al*., 2015, Cordeiro *et al*., 2002, Chen *et al*., 2006, Amarawathi *et al*., 2008 and Shi *et al*., 2008) [10, 5, 4, 1, 12].

Genotypic characterization of aromatic rice genotypes

A total of 29 aroma specific markers were employed for genotyping of *BADH2* locus. The result revealed that the all aromatic rice genotypes were fragrant genotypes. Among 29 markers 26 markers characterized the aromatic rice genotypes. Among 26 markers, eight marker *viz*. RM515, Nksbadh2, RM273, RG28L, RM223, SCU-Rice-SSR-1, RM282, RM5474 characterized all the aromatic rice genotypes. Among 8 markers alleleic variation was obtained in RM515, RM273, RM3459, RM223, RM282, RM5474 and RG28L. The alleleic variation signifies presence of different sequence pattern in aroma locus. This can be identified employing resequencing approach.

Table 4.5: Details of functional/gene tagged aroma locus specific markers used for genotyping in the 140 aromatic rice genotypes and their genetic diversity parameters using power marker V 3.25 program.

Genetic diversity

A total of 55 alleles were detected through agarose gel electrophoresis with 29 primers pairs, which were further subjected to cluster analysis. The genotypic data was used to calculate Jaccard‟s similarity coefficients incorporated in GGT software. The resulting similarity matrix was used to construct an unweighted pair group method (UPGMA) based dendogram. The UPGMA dendogram grouped 140 aromatic rice accessions into three major clusters. Cluster I contained 7 fragrant genotypes (Basmati Jamuna, Bantaphool, Kadamphool, Pant sugandh dhan15, Bindli and karigilas) whereas cluster 2 contain only 2 genotypes (Jeeradhan and nawabbhog). In cluster 3, a total of 131 aromatic genotypes were clustered forming different sub-sub groups. In the cluster II Jeeradhan aromatic variety of Chhattisgarh region separated with nawabbhog. in cluster III basmati and its derived varieties and modern varieties were grouped separately, the short and medium grained fragrant landraces were separated from rest of the genotypes within the same cluster.

Genotyping of F2 individual of mapping populations

The genotypic data of a total of 29 aroma specific marker were used for genotyping of individual F_2 plants. Among 29 markers, 26 markers amplified the F_2 individuals of mapping population. Parental polymorphism survey of F_2 individual of Dubraj X Tarunbhog revealed polymorphism between parent for two marker namely CP04133 and an allele specific PCR marker ESP+IFAP+INSP+EAP located on Chromosome number 8, using this marker a total of 27 heterozygous aromatic, 11 homozygous non aromatic and 8 homozygous aromatic F2 individual were obtained, indicating slight distortion from expected 1:2:1 codominant monogenic inheritance genotypic ratio. As the parents used in the present study were aromatic genotypes, the aroma could be rendered by different alleleic variation at *BADH2* locus. The marker CP04133 is EST based marker, therefore the cDNA sequence used for designing of this marker may results in biasness towards specific parent to which it shows sequence similarity. Therefore in the present study the F_2 individual were distorted towards one of the parent used to develop the cross. The distortion could be attributed tothe involvement of more than one gene for expression of aroma in rice. The Tarunbhog used as a parent in the study was identified as non aromatic genotype by most of the functional or aroma locus specific markers (Rai *et al*., 2015) [10]. Irrespective of being aromatic genotype.

A single tube allele specific PCR marker also termed as allele specific amplification PCR (ASP) developed by Bradburry *et al*., (2005) [3], at *BADH2* locus is a perfect marker system for screening of grain aroma in basmati and Jasmine rice variety,

in the present study an attempt was made to test the screening ability of marker for F_2 individuals obtained by crossing of short grain aromatic rice varieties. Parental polymorphism survey revealed difference in parent as Dubraj amplified 580 bp positive control band and 257 bp fragrant band, however along with positive band Tarunbhog amplified 355 bp band. In heterozygous individual 355 bp band, in heterozygous individual 355, 257 and 580 bp bands were produced indicating non fragrant plant. A total of 12 homozygous fragrant, 16 homozygous non fragrant and 34 heterozygous non fragrant individual were obtained, indicating a nearly perfect segregation of codominant and monogenic inheritance, however as evident from previous investigation, Tarunbhog being aromatic rice genotype was not identified as aromatic using ASA marker (Rai *et al*., 2015, Sakthival *et al.*, 2009) [10, 11]. Therefore all homozygous individuals harbouring Tarunbhog allele could not be referred as aromatic. In order to resolve this contradiction high resolution genome wide mapping becomes prerequisite for identification of aroma gene in Tarunbhog and individual with Tarunbhog allele. The F_2 individuals of Safri17 X Tarunbhog were screened using 29 aroma locus specific markers, parental polymorphism survey revealed no differences between the parents, as 29 markers used in the study mostly were colocalized within aroma gene nearby to aroma locus and some of were located on chromosome number 3 and 4, for which no difference were obtained between parent indicated presence of different loci for expression of aroma, the individuals were, however phenotypically aroamatic. in order to identify the gene responsible for aroma in Tarunbhog genotype, Genome wide association mapping or next generation based transcriptone sequencing could be the best strategy. The present result were in accordance to the findings of Rai *et al.*, where markers BADHEX-5, RM5474, nksbad2, ESP+IFAP+INSP+EAP, FMbadh2-E7, and RM42 not confirmed the the presence of fragrant gene in Tarunbhog genotype.

Study III- Survey of promoter of aroma

1 kb upstream sequences of aroma gene (LOC_0s0832870) was obtained using GRASSIUS database. The obtained nucleotide sequence was then used as query for the detection of MITE and other upstream regulatory elements.

Identification of MITE elements in promoters

To identify the MITE element in 1 kb upstream sequence of aroma gene. MITEdigger programme was used. A total of 3 MITE insertion were obtained in 1 kb upstream sequence using the plantCARE database (http://bioinformatics.psb. ugentbe/ webtools/ plantcare /html/).

MITE in the promoter region of *BADH2* locus was analyzed for its insertion/deletion. MITE is a transposable genetical element and is reported to be a major characteristic of Indica variety (Bourgies *et al*., 2008) and rare in japonica variety. In particular the study suggested altogether absence of MITE in temperate aromatic rice variety. Therefore the variation in the frequency of MITE in rice collection reflects a pattern similar to other genetic marker derived from isozyme or RFLP where one allele is specific to one group. Acting regulatory elements were identified in 1 kb upstream sequence of aroma gene. The analysis revealed presence of heat shock elements, light responsive regulatory elements and MYB, MYC with different frequencies (table 4.6). The presence of regulatory elements in promoter of aroma locus indicates spatio temporal regulation of expression of aroma is dependent of geographical location, topography, soil types and storage condition. Therefore presence of such element can help the fragrant gene in plants to modulate their expression in response to changing environments.

Table 4: MITE elements present in 1 Kb upstream of aroma gene

Elements	Motif	Frequency
MITE	TE	3
MYB	C/TAACG/TG	2
MYC	CACATG/CATGTG	2
HSE	AAAAAAATTTC	2
MBS	CAACTG	1
4C1-CMA2B	TCTCACCAACC	1

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