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Evaluation of molecular markers linked to fragrance and genetic diversity in Indian aromatic rice

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Abstract: DNA-based markers have the potential to improve the efficiency and precision of breeding programs based on marker-assisted selection. In the present study we evaluated the predictive abilities of previously reported PCR-based simple sequence repeat and functional markers related to fragrance in a set of 24 rice genotypes, including traditional basmatis, evolved basmatis, and aromatic indigenous landraces. High-resolution melting analysis with 3 markers was also performed to detect the presence of SNPs and length polymorphism in monomorphic amplicons. All genotypes showed expected marker patterns according to their fragrance classification except 5 fragrant genotypes: Kalanamak 3119, Kasturi Basmati, Basmati LC 74-3, Thurunbhog, and Jeeraga Samba. The genetic diversity analysis based on fragrance locus grouped the genotypes broadly into 2 major clusters representing fragrant and nonfragrant genotypes. Kalanamak 3119, Kasturi Basmati, and Basmati LC 74-3 comprised the fragrant cluster, while Thurunbhog and Jeeraga Samba clustered with nonfragrant genotypes. None of the functional markers could identify these 5 genotypes as fragrant, which supports the assumption of the existence of a second gene for fragrance in rice. Our work validates previously identified markers, namely nksbad2, L06, BADEX7-5, and ESP + IFAP + INSP + EAP, suitable for use in marker-assisted selection programs.

Key words: Functional markers, landraces, high-resolution melting analysis, geographical indicator, validation

1. Introduction

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). Basmati rice cannot be grown beyond stipulated tracts as it loses aroma and essential quality traits. Thus, the areas under cultivation and production have stagnated. To maintain its position in the global scented rice market, India needs to offer new aromatic rice varieties to boost its exports. 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. Some non-basmati scented rice varieties are traded in the international market, but many others are not despite their eminent qualities such as elongation after cooking, aroma, and agronomic characters (Singh et al., 2003).

Rice breeders need a simple and inexpensive assay to identify the fragrance trait in breeding populations. Fragrance can be detected by tasting the associated flavor in individual seeds or assessing the aroma of leaf tissue or grains after either heating in water or reacting with solutions of KOH. The principal component responsible for fragrance is 2-acetyl-1-pyrroline (2AP) (Lorieux et al., 1996). The developments of PCR-based markers for fragrance have the advantages of being inexpensive, simple, rapid, and only requiring small amounts of tissue. The compound 2AP is produced through a single recessive allele (*fgr*) at a locus on chromosome 8 (Lorieux et al., 1996; Chen et al., 2006), which corresponds to the gene that encodes betaine aldehyde dehydrogenase (*BADH2*). An 8-bp deletion in exon 7, introducing a premature stop codon upstream of key coding regions, makes this gene nonfunctional (*badh2*), and consequently the mutant *badh2* transcript leads to 2AP accumulation in aromatic rice (Hashemi et al., 2013). Based on this locus, several PCR-based codominant markers have been developed.

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However, these markers are located physically away from the gene and therefore may or may not be efficient in marker-assisted selection (MAS) for aroma. Although some functional markers have also been developed based on sequence variations within the *fgr* gene (Amarawathi et al., 2008; Shi et al., 2008; Sakthivel et al., 2009), their evaluation in Indian aromatic germplasm has not been done so far. Moreover, these markers were developed for basmati and/or jasmine rice and their usefulness in short- and medium-grained indigenous landraces has not been tested yet.

High-resolution melting (HRM) analysis is fast becoming the method of choice for genotyping, discovering mutations, and tracking SNPs (Han et al., 2012). HRM combines PCR and melting behavior of amplicons using a double-stranded DNA (dsDNA) binding dye. The changes in fluorescence can be monitored when dsDNA dissociates in single-stranded DNA through melting and shifts in melting curves can be attributed to variations in amplicon sequences caused by SNPs, insertions/deletions, or simple sequence repeat (SSR) variants and amplicon length (Lehmensiek et al., 2008). This technique has been used extensively in medical diagnostic applications to locate mutations in human genetics (Sinthuwiwat et al., 2008). It has recently been applied in the SNP genotyping of plant species, including barley (Lehmensiek et al., 2008), wheat (Botticella et al., 2011), rice (Li et al., 2011), *Brassica rapa* (Lochlainn et al., 2011), and *Capsicum* (Jeong et al., 2012).

In the current study, PCR-based markers linked to fragrance were evaluated in traditional basmatis, evolved basmatis, and short/medium-grained aromatic landraces to test their ability to discriminate fragrant and nonfragrant genotypes accurately and to relate it to the diversity observed at the *BADH2* locus. Additionally, HRM analysis was performed with 3 markers that were monomorphic on agarose gels to determine their efficiency to resolve variations caused by SNPs or amplicon length.

2. Materials and methods

The plant material consisted of 3 nonaromatic and 21 aromatic rice genotypes including 10 basmati genotypes and 11 non-basmati aromatic landraces (Table 1). All the genotypes were grown in plastic portrays and young leaves were collected from 2-week-old seedlings. The genomic DNA was extracted following the CTAB method (Doyle and Doyle, 1987). Twenty-eight previously described PCR-based markers known to be linked to the fragrance gene in rice were selected for evaluation (Table 2). The comparative positions of the markers at chromosome 8 are shown in Figure 1a.

2.1. Phenotyping of fragrance

Aroma was detected by sniffing following a KOH-based method (Nagaraju et al., 1991). Each sample was scored on a 1–4 scale, where 1 stands for absence of aroma, 2 for slight aroma, 3 for moderate aroma, and 4 for strong aroma. The samples were evaluated by a panel of 5 experts in aromatic rice breeding and quality evaluation.

2.2. Polymerase chain reaction (PCR) and gel electrophoresis

A PCR mixture of 15 µL containing 50 ng of genomic DNA, 10 pM each of forward and reverse primers, 100 µM dNTPs (Bangalore Genei, Bangalore, India), 1.5 mM $MgCl_2$, 1X PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$], and 1 U of Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA) was prepared for each reaction. Amplifications were performed in a Mastercycler gradient (Eppendorf, Germany) with a thermal profile of 94 °C for 5 min followed by 30 cycles of 30 s at 94 °C, 30 s at the annealing temperatures from 50 to 60 °C for individual primers, and 30 s at 72 °C with a final extension for 7 min. The size of amplified products was determined on 3.0% Metaphor agarose gels (Lonza, Allendale, NJ, USA) prepared in 1X TAE buffer [40.0 mM Tris-base, 16.65 M acetic acid, 0.5 M EDTA (pH 8.0)] at a constant voltage of 65 V for 3–4 h. Thereafter, gels were stained with ethidium bromide (0.5 µg/mL) and visualized using a gel documentation system (Gel Doc XR+ Imager, Bio-Rad, Hercules, CA, USA). In order to determine the molecular size of the amplified products, each gel was also loaded with 1 µg of DNA of 50-bp DNA size marker (Thermo Scientific).

2.3. HRM Analysis

HRM analysis was performed using a LightCycler 96 (Roche Applied Science, Penzberg, Germany) in a total volume of 20 µL with only those primers that were not polymorphic on agarose gel. The reaction mixture contained 50 ng of total DNA, 2.5 mM $MgCl_2$, 0.2 µM forward and reverse primers, and 1X HRM Master Mix (Roche Applied Science). Before performing HRM analysis, preincubation at 95 °C for 10 min was followed by 50 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 10 s, and extension at 72 °C for 20 s. The amplification cycles were immediately followed by the following HRM steps: 95 °C for 60 s, cooling to 40 °C for 60 s, a temperature increase to 65 °C, and a continuous increase to 97 °C with 0.2 °C s^{-1} increments. During the incremental melting step, fluorescence data were acquired continuously. LightCycler 96 Gene Scanning Software was used for the HRM analysis. It analyzed the *Tm* of the individual curves, calculated the relative fluorescence signal differences between the genotypes, and autogrouped those with similar melting curves. The genotypes of the same group were considered to have the same allele and those of different groups to have different alleles.

2.4. Data analysis

The markers were scored for the presence (1) or absence (0) of the corresponding band among the 24 genotypes.

Table 1. A list of the genotypes used in this study.

Sample number	Genotypes	Origin	Fragrance	Remarks
1	IR-64	IRRI	None	Variety
2	Swarna	Andhra Pradesh	None	Variety
3	Jaya	Punjab	None	Variety
4	Basmati-370	Punjab	Strong	Traditional basmati
5	Ranbir Basmati	J&K	Strong	Traditional basmati
6	Taroari Basmati	Haryana	Strong	Traditional basmati
7	Type-3	Uttarakhand	Strong	Traditional basmati
8	Pusa Basmati-1	IARI	Strong	Evolved basmati
9	Kasturi Basmati	Punjab	Strong	Evolved basmati
10	Mahi Sugandha	Rajasthan	Strong	Evolved basmati
11	Yamini	Haryana	Strong	Evolved basmati
12	Basmati LC 71-1	Uttar Pradesh	Strong	Local basmati
13	Basmati LC 74-3	Uttar Pradesh	Moderate	Local basmati
14	HUR-105	Madhya Pradesh	Moderate	Aromatic variety
15	Adam Chini	Uttar Pradesh	Strong	Aromatic landrace
16	Kanakjeera 26-1	Uttar Pradesh	Strong	Aromatic landrace
17	Kalanamak 3119	Uttar Pradesh	Strong	Aromatic landrace
18	Shakkarchini	Uttar Pradesh	Strong	Aromatic landrace
19	Tulsimanjari	Bihar	Strong	Aromatic landrace
20	Dubraj	Madhya Pradesh	Strong	Aromatic landrace
21	Jawaphool	Madhya Pradesh	Strong	Aromatic landrace
22	Badshahbhog	Assam	Strong	Aromatic landrace
23	Thurunbhog	Orissa	Moderate	Aromatic landrace
24	Jeeraga Samba	Tamil Nadu	Slight	Aromatic landrace

Data from HRM analysis were converted into binary data and used with gel electrophoresis data for further analysis. The combined data were analyzed using NTSYS-pc version 2.11 (Rohlf, 1997). The SIMQUAL program was used to calculate Jaccard's similarity coefficients. The resulting similarity matrix was used to construct an unweighted pair group method with arithmetic mean (UPGMA)-based dendrogram. Polymorphic information content (PIC) for each SSR marker was calculated according to Anderson et al. (1993) as per the following formula:

$$\mathrm{PIC} = 1 - \sum_{i=1}^{k} P_i^2$$

where P_i is the frequency of the ith allele and k is the total number of different alleles at the specific locus.

3. Results

3.1. Fragrance in tested genotypes

The genotypes Basmati-370, Taraori Basmati, and Type-3 were used as controls to detect the presence of the fragrance gene by phenotyping as well as by using molecular markers. The negative controls were IR-64, Swarna, and Jaya, which are nonfragrant genotypes. All the genotypes were categorized into 4 groups based on their fragrance: nonfragrant, strongly fragrant, moderately fragrant, and slightly fragrant (Table 1). Among the 21 fragrant genotypes, 17 were categorized as strongly fragrant, 3 (Basmati LC 74-3, HUR-105, and Thurunbhog) as moderately, and 1 (Jeeraga Samba) as slightly.

3.2. Marker evaluation

Most of the markers showed only 2 alleles (Table 2) as expected, but nonfragrant alleles were also amplified in some

Table 2. A list of the molecular markers and distribution of alleles among fragrant and non-fragrant genotypes.

Reference/marker name	Marker type	Linkage group	PIC**	No. of alleles#	Approximate allele size (bp)			
					Range	Basmati- 370	Kalanamak	IR-64
Garland et al., 2000								
RM85	SSR	8	0.74	2	90–110	90	90	110
SCU-Rice-SSR-1	SSR	8	0	1 (4)	125	125	125	125
RM42	SSR	8	0.72	2	160–175	160	160	175
RM223	SSR	8	0.71	2	150–160	150	150	160
Cordeiro et al., 2002								
SCU015RM	SSR	8	0	0	–	-	-	-
Bradbury et al., 2005								
ESP + IFAP + INSP + EAP	ASA*	8	0.48	2	355–580	257/580	355/580	355/580
Wanchana et al., 2005								
10L03_FW	SSR	8	0.84	4	186–200	190	190	190
BO3_127.8	SSR	8	0.71	2	122–134	134	122	122
CP04133	EST	8	0.73	2	421–483	483	421	421
Chen et al., 2006								
L05	STS	8	0.71	2	316–368	316	316	368
L06	STS	8	0.71	2	325–376	376	376	325/355
Sun et al., 2008								
Aro7	SSR	8	0.69	2	290–300	290	290	300
RM23120	SSR	8	0.67	2	393–460	416	393	416
RM3459	SSR	8	0.71	2	180–197	180	180	197
Amarawathi et al., 2008								
nksbad2	Functional	8	0.72	2	82–90	82	90	90
RM5474	SSR	3	0.72	2	90–110	90	110	110
RM282	SSR	3	0.75	2	124–138	124	124	138
RM5633	SSR	4	0.91	3	203–225	218	203	225
RM273	SSR	4	0.69	2	210–220	210	210	220
RM80	SSR	8	0.89	3	115–137	125	125	115/137
Kibria et al., 2008								
RM342	SSR	8	0.88	3	132–150	150	140	140
RM515	SSR	8	0.80	3	200–230	230	230	230
Lang and Buu, 2008								
RG28L	STS	8	0	1 (4)	125	125	125	125
Shi et al., 2008								
FMbadh2-E2A	Functional	8	0	1	125	125	125	125
FMbadh2-E2B	Functional	8	0.37	1	NA†/410	410	NA	410
FMbadh2-E7	Functional	8	0.72	2	260–270	260	270	270
Mahdav et al., 2009								
ARSSR-3	SSR	8	0	1 (3)	160	160	160	160
Sakthivel et al., 2009								
BADEX7-5	Functional	8	0.72	2	95–108	95	103	103
			0.61	55 (66)				

*ASA: allele-specific amplification; **PIC: polymorphic information content; †NA: null allele; #: figures in parentheses indicate HRM alleles.

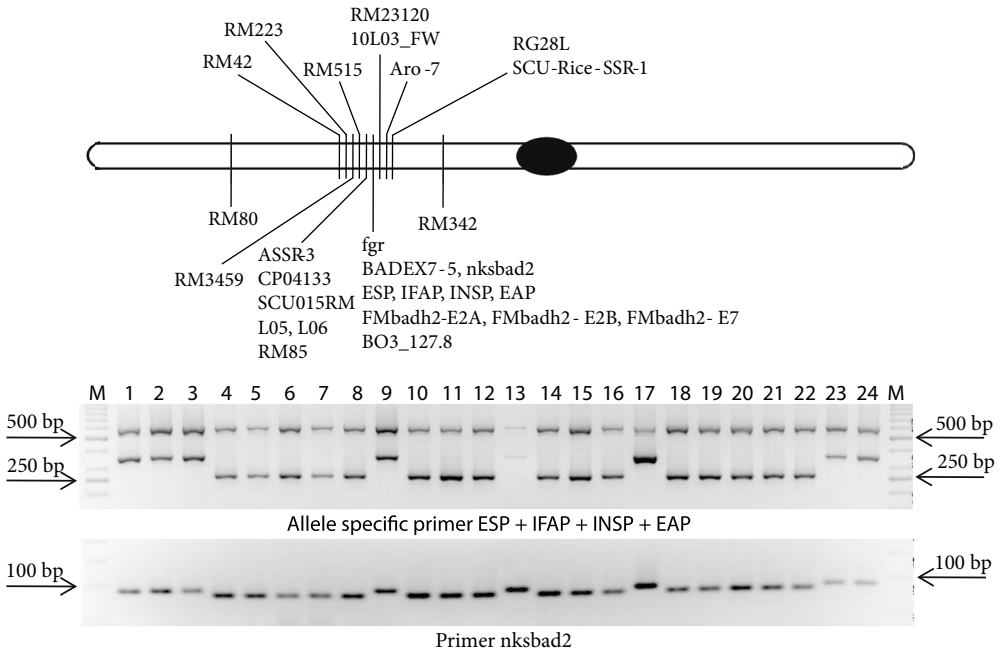

Figure 1. (a) Comparative position of SSRs and functional markers associated with rice fragrance on chromosome 8; (b) agarose gel electrophoretic patterns of 24 rice genotypes generated by allele-specific primer (ESP + IFAP + INSP + EAP) and nksbad2. M is 50-bp DNA size marker and numbers correspond to the genotypes listed in Table 1.

fragrant genotypes by all the markers. The highest number of alleles (4) was found in 10L03_FW, followed by RM515, RM5633, RM80, and RM342 (3), and the average was 2.36 alleles per marker (Table 2). The PIC values ranged from 0 to 0.91, with an average of 0.61. The highest PIC value (0.91) was found for the RM5633 locus, followed by RM80 (0.89), RM342 (0.88), and RM515 (0.80). Out of 21 fragrant genotypes, marker RM273 confirmed the presence of the fragrance gene in 18 genotypes (exceptions were Kasturi Basmati, Basmati LC 74-3, and Jeeraga Samba, which showed nonfragrant alleles as shown by 3 nonfragrant genotypes). Markers RM223, RM3459, L05, and B03_127.8 could confirm the presence of the fragrance gene in 17 genotypes (exceptions were Kasturi Basmati, Basmati LC 74-3, Thuran Bhog, and Jeeraga Samba), while markers BADEX7-5, RM5474, nksbad2, ESP + IFAP + INSP + EAP, FMbadh2-E7, and RM42 confirmed the presence of the fragrance gene in 16 genotypes (exceptions were Kasturi Basmati, Basmati LC 74-3, Kalanamak 3119, Thuran Bhog, and Jeeraga Samba). The gel images generated by 2 markers, ESP + IFAP + INSP + EAP and nksbad2, are shown in Figure 1b. Markers ASSR-3, RG-28L, and SCU-Rice-SSR-1 could not discriminate between fragrant and nonfragrant genotypes. Primer SCU015RM failed to amplify any PCR product (Table 2).

3.3. HRM curve analysis

Analyses of the amplification curves and melting curves revealed that the CT values of ASSR-3, RG-28L, and SCU-Rice-SSR-1 were no more than 30 cycles, and no exceptional melting peaks were found when performing *Tm* calling analysis. In addition, these markers gave clear major bands in subsequent agarose gel electrophoresis of HRM-PCR products. A clear difference in fluorescence was observed among the nonfragrant genotypes, the fragrant genotypes, and some aromatic landraces. The normalized melting curves and difference plot curves of ASSR-3 and SCU Rice SSR-1 amplicons are shown in Figure 2. The melting curves of the ASSR-3 amplicons yielded 3 groups that represented nonfragrant, basmati type, and non-basmati type fragrant genotypes. The melting curves of SCU Rice SSR-1 and RG-28 amplicons yielded 4 groups. Thus, HRM analysis of amplicons of all primers separated traditional and evolved basmati genotypes (exceptions were Kasturi Basmati and Mahi Sugandha) from nonfragrant genotypes and other aromatic landrace genotypes.

3.4. Genetic relationship

A total of 66 alleles were detected through agarose gel electrophoresis and HRM analysis with 28 primer pairs, which were further subjected to cluster analysis. The UPGMA dendrogram grouped 24 genotypes into 2 major clusters with the similarity coefficients ranging from 0.21 to 1.00. Cluster I contained all 3 nonfragrant genotypes along with 2 fragrant non-basmati genotypes (Jeeraga Samba and Thurunbhog), while cluster II contained all of the traditional basmatis along with modern varieties

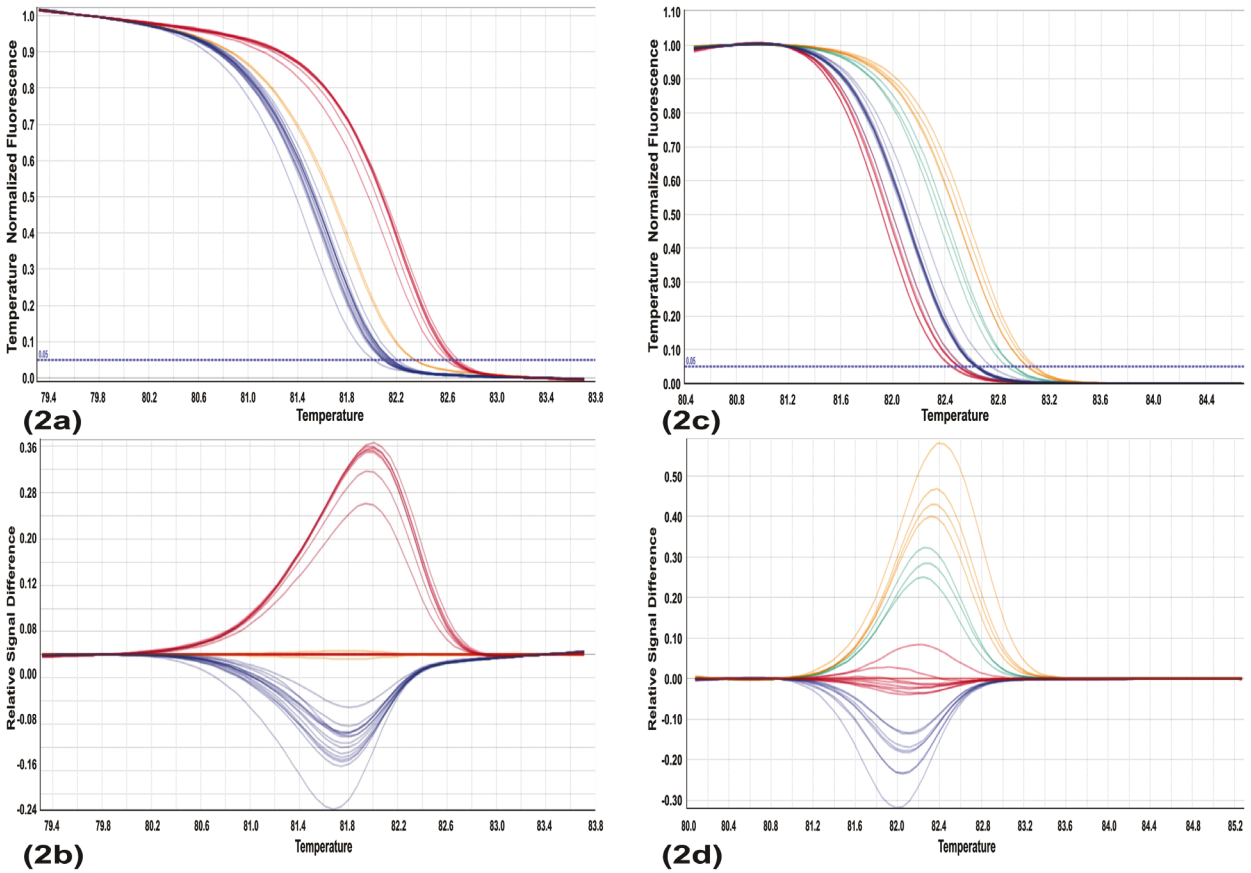

Figure 2. High-resolution melting analysis of 24 rice genotypes with markers ASSR-3 (a, b) and SCU Rice SSR-1 (c, d). (a) and (c) show normalized melting profiles, while (b) and (d) show the difference plot of 24 genotypes.

derived from basmatis and indigenous aromatic landraces (Figure 3). In cluster I, Jeeraga Samba was grouped with IR-64 while Thuran Bhog was separated from the rest. In the second cluster 4 traditional basmatis (Basmati 370, Ranbir Basmati, Taraori Basmati Type-3, and the modern variety Pusa Basmati-1) were indistinguishable from each other (similarity coefficient of 1.00). Other modern varieties derived from traditional basmati such as Yamini, Kasturi Basmati, and Mahi Sugandha were separated from traditional basmatis. Interestingly, the medium-grained highly fragrant landrace Kalanamak was separated from the rest of the medium-grained aromatic genotypes, although within the same cluster (Figure 3).

4. Discussion

The basic objective of plant breeding is the selection of specific plants bearing the target gene at an early stage of plant growth. The practical application of MAS requires the development of tightly linked, cost-effective, and easy-to-use molecular markers. This study illustrates the utility of markers to identify the presence of the *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. In the present study, none of the SSRs or functional markers could differentiate all the fragrant genotypes from the nonfragrant varieties with 100% efficacy. Eight SSR and STS markers (RM273, RM223, RM42, RM3459, RM5474, B03_127.8, L05, and L06) and 4 functional markers (nksbad2, BADEX7-5, ESP + IFAP + INSP + EAP, and FMbadh2-E7) could differentiate fragrant from nonfragrant genotypes with the following exceptions: Kasturi Basmati, Basmati LC 74-3, Kalanamak 3119, Thuran Bhog, and Jeeraga Samba. Therefore, these markers can be used for identification, discrimination, and MAS for aroma in traditional basmatis, evolved basmatis, and local aromatic landraces, excluding Kasturi Basmati, Basmati LC 74-3, Kalanamak 3119, Thuran Bhog, and Jeeraga Samba. MAS has been successfully used for the improvement of aromatic rice varieties (Yi et al., 2009; Singh et al., 2011). The successful utilization of MAS was done by Singh et al. (2011) for the development of Improved Pusa Basmati 1 and the improved versions of PRR78 by utilizing the nksbad2 marker for aroma. They

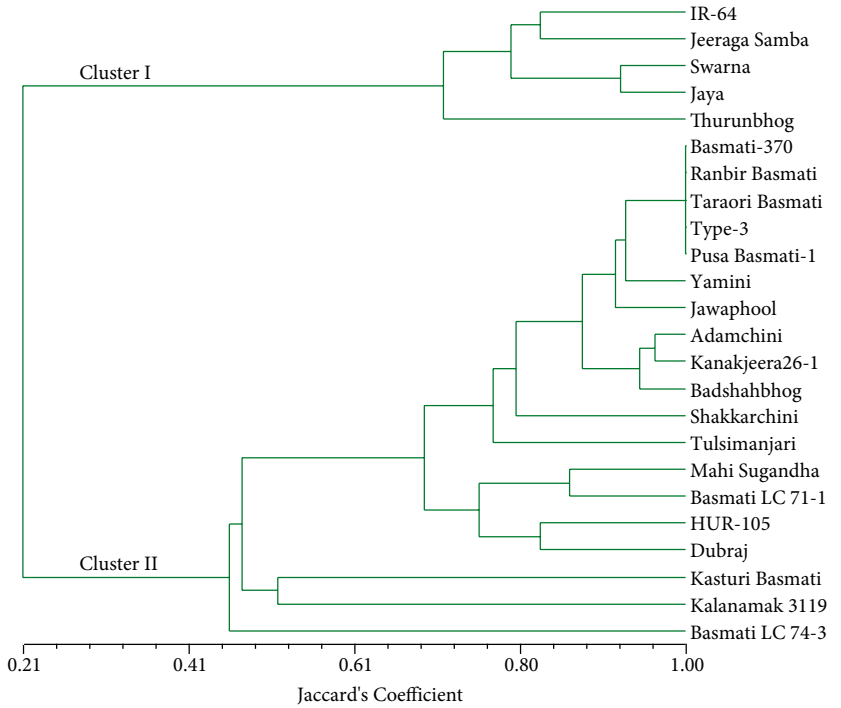

Figure 3. UPGMA-based dendrogram of 24 rice genotypes based on 28 SSR and functional markers.

successfully validated marker nksbad2 in 25 aromatic and 28 nonaromatic genotypes. In our study nksbad2 could only identify 16 out of 21 aromatic genotypes with an efficacy of 76%. Interestingly, in the study of Singh et al. (2011), nksbad2 generated both fragrant and nonfragrant alleles in Kalanamak 3119, but in the present study a single band of 90 bp corresponding to nonfragrance was amplified (Figure 1b), which places Kalanamak 3119 in the nonfragrant group. Other functional markers were also not able to identify it as a fragrant genotype. This supports the hypothesis of a second gene for fragrance in some non-basmati aromatic genotypes such as Kalanamak 3119, as suggested by Fitzgerald et al. (2008).

In the present study, the traditional basmati landraces were readily separated from the evolved basmati varieties as well as the non-basmati fragrant genotypes. The cluster analysis showed great diversity among fragrant genotypes. 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, 3 evolved basmati genotypes (Yamini, Kasturi Basmati, and Mahi Sugandha) were separated from traditional basmatis. This result is in agreement with Steele et al. (2008), who studied genetic diversity of 24 rice varieties with 41 indel markers and were able to differentiate traditional basmatis from other fragrant varieties.

A novel fragrant rice genotype, Kalanamak 3119, was separated distantly from all the medium-grained fragrant genotypes in the second cluster. None of the functional markers could recognize it as a fragrant genotype (Table 2), but phenotypic analysis reconfirmed it as a highly fragrant genotype. 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). Additionally, several aromatic accessions did not carry any mutation in the coding segments (Singh et al., 2010; Myint et al., 2012), and therefore the cause of their fragrant nature is unknown. Fitzgerald et al. (2008) postulated that production of 2AP could be driven by alleles at 2 different genes besides the different alleles of *BADH2*. Rice has indeed a second BADH enzyme, which acts in a similar way to BADH2, but is regulated differently. Furthermore, Thurunbhog and Jeeraga Samba were clustered with nonfragrant genotypes, although they were either slightly or moderately fragrant. Thurunbhog and Jeeraga Samba were from Orissa and Tamil Nadu, respectively, and they

are adapted for warm climates. This also indicates the existence of some other gene responsible for fragrance other than *badh2*. Annotation of the rice genome database between *aro4-1* QTL intervals indicates the presence of a gene for *BADH1*, which could be a likely candidate gene for aroma due to its similar molecular function to the *badh2* gene on chromosome 8 (Singh et al., 2010). This further reinforced the theory that there should be at least one more locus leading to the production of 2AP in rice as suggested by Fitzgerald et al. (2008).

The possibility of other loci controlling 2AP accumulation was reported on chromosomes 3 (Amarawathi et al., 2008), 4, and 12 (Lorieux et al., 1996), but with a smaller effect. Data mining into the QTL on chromosome 4 localized the 2AP ortholog, *BADH1*, within the region, although no genetic validation was reported for the existence and roles of this gene in the biosynthesis and accumulation of 2AP (Singh et al., 2010). In the present study, marker RM273 was linked with aroma QTL '*aro4-1*' at chromosome 4 and could identify the fragrant allele in 18 fragrant genotypes, including Kalanamak 3119 and Thurunbhog. This further supports the probable existence of other locus/loci responsible for fragrance in rice.

HRM analysis showed a clear grouping of the genotypes according to their fragrance nature. All the traditional basmatis and modern varieties were clustered in one group, except for Kasturi Basmati and Mahi Sugandha, which showed a clear distinction with nonfragrant and indigenous fragrant landraces. The HRM analysis supported the results obtained from agarose gel electrophoresis. This suggests that this approach could be applied in order to identify SNPs and length polymorphisms without the requirement of a post-PCR procedure, as has been required in traditional SSR/functional marker analysis. In this study we showed that SSRs/functional markers combined with HRM analysis could provide a fast, accurate, and close-tubed way for genotyping large populations with reduced costs as described earlier by Li et al. (2011).

In terms of unique fragrance and grain quality, a number of novel fragrant rice landraces are cultivated in India. Kalanamak, well known for its aroma and distinct taste, was awarded the Geographical Indication (GI) tag recently (Kalanamak Scented Paddy Production & Conservation Society, 2012). It surpasses basmati rice in every aspect except grain length. From plant type to grain quality, it is very different from other fragrant genotypes. The strong, unique fragrance and the inability of functional markers to identify it as a highly fragrant genotype may be explained by the hypothesis that some genotypes can be fragrant despite a low level of 2AP, probably because of higher levels of other unknown compounds (Sakthivel et al., 2009; Hashemi et al., 2013). Therefore, a comprehensive biochemical, molecular, and transcriptome analysis is required to elucidate the cause of fragrance in Kalanamak 3119 and other aromatic landraces.

In conclusion, none of the 28 markers could discriminate all fragrant genotypes from the nonfragrant ones. Kalanamak 3119, Kasturi Basmati, Thurunbhog, and Jeeraga Samba were not identified as fragrant genotypes by any functional marker, which supports the assumption of a probable existence of a second gene for fragrance in rice other than *badh2*. Four markers, namely nksbad2, L06, BADEX7-5, and an allele-specific amplification primer (ESP + IFAP + INSP + EAP), were validated in the rest of the genotypes and determined suitable for use in MAS for improvement of those genotypes. In this study, HRM analysis provided a fast and simple approach for genotyping of individuals to detect SNPs or length variations.

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References

Amarawathi Y, Singh R, Singh AK, Singh VP, Mohapatra T, Sharma TR (2008). Mapping of quantitative trait loci for basmati quality traits in rice (*Oryza sativa* L.). Mol Breed 21: 49–65.

Anderson JA, Churchill GA, Autrique JE, Tanksley SD, Sorrels ME (1993). Optimizing parental selection for genetic linkage maps. Genome 36: 181–186.

Botticella E, Sestili F, Hernandez-Lopez A, Phillips A, Lafiandra D (2011). High-resolution melting analysis for the detection of EMS induced mutations in wheat *SbeIIa* genes. BMC Plant Biol 11: 156.

Bradbury LMT, Henry RJ, Jin Q, Reinke RF, Waters DLE (2005). A perfect marker for fragrance genotyping in rice. Mol Breed 16: 279–283.

Chen SH, Wu J, Yang Y, Shi WW, Xu ML (2006). The *fgr* gene responsible for rice fragrance was restricted within 69 kb. Plant Sci 171: 505–514.

Cordeiro GM, Christopher MJ, Henry RJ, Reinke RF (2002). Identification of microsatellite markers for fragrance in rice by analysis of the rice genome sequence. Mol Breed 9: 245–250.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19: 11–15.

Fitzgerald MA, Hamilton NRS, Calingacion MN, Verhoeven HA, Butardo VM (2008). Is there a second fragrance gene in rice? Plant Biotechnol J 6: 416–423.

Garland S, Lewin S, Blakeney A, Reinke R, Henry R (2000). PCR-based molecular markers for the fragrance gene in rice (*Oryz sativa* L.). Theor Appl Genet 101: 364–371.

Han YH, Khu DM, Monteros MJ (2012). High-resolution melting analysis for SNP genotyping and mapping in tetraploid alfalfa (*Medicago sativa* L.). Mol Breed 29: 489–501.

Hashemi FSG, Rafii MY, Ismail MR, Mahmud TMM, Rahim HA, Asfaliza R, Malek MA, Latif MA (2013). Biochemical, genetic and molecular advances of fragrance characteristics in rice. Crit Rev Plant Sci 32: 445–457.

Jeong HJ, Kwon JK, Pandeya D, Hwang J, Hoang NH, Bae JH, Kang BC (2012). A survey of natural and ethyl methane sulfonate-induced variations of *eIF4E* using high-resolution melting analysis in *Capsicum*. Mol Breed 29: 349–360.

Kalanamak Scented Paddy Production & Conservation Society (2012). Kalanamak Rice. Geographic Indications Application Number 205. Uttar Pradesh, India: Kalanamak Scented Paddy Production & Conservation Society.

Kibria K, Islam MM, Begum SN (2008). Screening of aromatic rice lines by phenotypic and molecular markers. Bangladesh J Bot 37: 141–147.

Kovach MJ, Calingacion MN, Fitzgerald MA, McCouch SR (2009). The origin and evolution of fragrance in rice. P Natl Acad Sci USA 106: 14444–14449.

Lang NT, Buu BC (2008). Development of PCR based markers for aroma (*fgr*) gene in rice (*Oryza sativa* L.). Omonrice 16: 16–23.

Lehmensiek A, Sutherland MW, McNamara RB (2008). The use of high resolution melting (HRM) to map single nucleotide polymorphism markers linked to a covered smut resistance gene in barley. Theor Appl Genet 117: 721–728.

Li J, Wang X, Dong R, Yang Y, Zhou J, Yu C, Cheng Y, Yan C, Chen J (2011). Evaluation of high-resolution melting for gene mapping in rice. Plant Mol Biol Rep 29: 979–985.

Lochlainn SO, Amoah S, Graham NS, Alamer K, Rios JJ, Kurup S, Stoute A, Hammond JP, Ostergaard L, King GJ et al. (2011). High-resolution melt (HRM) analysis is an efficient tool to genotype EMS mutants in complex crop genomes. Plant Methods 7: 43.

Lorieux M, Petrov M, Huang N, Guiderdoni E, Ghesquière A (1996). Aroma in rice: genetic analysis of quantitative trait. Theor Appl Genet 93: 1145–1151.

Mahdav MS, Pandey MK, Kumar PR, Sundaram RM, Prasad GSV, Sudarshan L, Rani NS (2009). Identification and mapping of tightly linked SSR marker for aroma trait for use in marker assisted selection in rice. Rice Genet Newsl 25: 38–39.

Myint KM, Courtois B, Risterucci AM, Frouin J, Soe K, Thet KM, Vanavichit A, Glaszmann JC (2012) Specific patterns of genetic diversity among aromatic rice varieties in Myanmar. Rice 5: 20.

Nagaraju M, Mohanty KK, Choudhary D, Gangadharan C (1991). A simple technique of detecting scent in rice. Oryza 28: 109–110.

Rohlf FJ (1997). NTSYSpc. Numerical Taxonomy and Multivariate Analysis System. Vol. 2.0. Setauket, NY, USA: Exeter Software.

Sakthivel K, Sundaram RM, Rani NS, Balachandran SM, Neereja CN (2009). Genetic and molecular basis of fragrance in rice. Biotechnol Adv 27: 468–473.

Shi W, Yang Y, Chen S, Xu M (2008). Discovery of a new fragrance allele and the development of functional markers for the breeding of fragrant rice varieties. Mol Breed 22: 185–192.

Singh A, Singh B, Panda K, Rai VP, Singh AK, Singh SP, Chouhan SK, Rai V, Singh PK, Singh NK (2013). Wild rices of eastern Indo-Gangetic plains of India constitute two sub-populations harbouring rich genetic diversity. Plant Omics 6: 121–127.

Singh A, Singh PK, Singh R, Pandit A, Mahato AK, Gupta DK, Tyagi K, Singh AK, Singh NK, Sharma TR (2010). SNP haplotypes of the *BADH1* gene and their association with aroma in rice (*Oryza sativa* L.). Mol Breed 26: 325–338.

Singh AK, Gopalakrishnan S, Singh VP, Prabhu KV, Mohapatra T, Singh NK, Sharma TR, Nagarajan M, Vinod KK, Devinder S et al. (2011). Marker assisted selection: a paradigm shift in Basmati breeding. Indian J Genet Plant Breed 71: 120–128.

Singh RK, Singh US, Singh N, Singh HN, Singh G (2003). Kalanamak: a scented black pearl of eastern Uttar Pradesh. In: Singh RK, Singh US, editors. A Treatise on the Scented Rices of India. New Delhi, India: Kalyani Publishers, pp. 421–431.

Sinthuwiwat T, Poowasanpetch P, Wongngamrungroj A, Promso S, Auewarakul C, Mooney S, Tocharoentanaphol C (2008). High resolution melting curve analysis for genotyping of common SNP in *MTHFR* gene using fixed-cell suspension. Mol Cell Probe 22: 329–332.

Steele KA, Ogden R, McEwing R, Briggs H, Gorham J (2008). InDel markers distinguish Basmatis from other fragrant rice varieties. Field Crops Res 105: 81–87.

Sun SH, Gao FY, Lu XJ, Wu XJ, Wang XD, Ren GJ, Luo H (2008). Genetic analysis and gene fine mapping of aroma in rice (*Oryza sativa* L. Cyperales, Poaceae). Genet Mol Biol 31: 532–538.

Wanchana S, Kamolsukyunyong W, Ruengphayak S, Toojinda T, Tragoonrung S, Vanavichit A (2005). A rapid construction of a physical contig across a 4.5 cM region for rice grain aroma facilitates marker enrichment for positional cloning. Sci Asia 31: 299–306.

Yi M, Nwea KT, Vanavichit A, Chai-arree W, Toojinda T (2009). Marker assisted backcross breeding to improve cooking quality traits in Myanmar rice cultivar Manawthukha. Field Crops Res 113: 178–186.