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CHARACTERIZATION AND PURITY ANALYSIS OF A FEW AROMATIC INDIGENOUS RICE GENOTYPES OF BIHAR USING AN AROMA-SPECIFIC MARKER

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Abstract: The aromatic rice landraces in Bihar is cultivated throughout the state. In majority of the aromatic rice lines, fragrance is caused due to accumulation of the fragrant compound 2-Acetyl -1-pyrroline (2-ACP). The mutation in the *badh2* gene has been explored previously towards development of a perfect co-dominant marker to discriminate between aromatic and non-aromatic lines at molecular level. In the present study, we have conducted the morphological characterization and molecular screening for *badh2* allelic variation present in 9 rice landraces, collected directly from farmers' field of different parts of Bihar. Through exploring the co-dominant nature of the molecular marker, we have documented the existence of contamination in the seed lots of these local rice landraces. Hence, along with its ability to exhibit the *badh2* allelic variation, we advocate the utility of perfect molecular marker of *badh2* locus towards evaluation of purity of rice seed lots, at an early growth stage.
Keywords: Aroma; Betain aldehyde dehydrogenase 2; Genetic purity; Multiplex PCR; Rice landraces.

Introduction

Consumers all over the world prefer aromatic rice due to its flavour and palatability. Long grained Basmati is premium quality aromatic rice but high demand in domestic and international market and acute shortage result in its high price. Beside basmati rice, several aromatic short-grained rice landraces are grown in specialized pockets of the states like Bihar, Orissa, Madhya Pradesh, West Bengal, Chhattisgarh and Uttar Pradesh. These landraces are grown by small group of farmers of a particular area, mainly for their own consumption and certain religious rituals (Agnihotri and Palni, 2007; Bhagawat *et al.*, 2008 and Sing *et al.*, 2000). These aromatic landraces have been reported to be superior in quality, fineness, aroma, taste and nutritional contents than Basmati rice and have wider adaptability to local conditions. Additionally, these landraces possess high genetic diversity and different alleles for various agronomically important traits (McCouch *et al.*, 1997; Jackson, 1999;

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Loresto *et al.*, 2000 and Choudhury *et al.*, 2013) and therefore can be utilized in rice breeding programmes. Out of several volatile flavour compounds, which contribute to rice aroma, 2-acetyl-1-pyrroline (2-ACP) has been identified as the principle compound for distinctive fragrance in Basmati and Jasmine rice (Bourgis *et al.*, 2008; Kovach *et al.*, 2009 and Hashemi *et al.*,2013). The sensory or chemical methods to determine the rice fragrance involve smelling leaf tissue and grains after heating with water or reacting with KOH or I_2-KI_8 solutions (Sood and Siddiq, 1978). However, this method can be personally biased and less reliable because, smelling after cooking becomes ineffective in successive analysis due to saturation of nostril senses. Gas chromatography has been reported to be a reliable method to determine 2-ACP (Tanchotikul and Hsieh, 1991), but it is time consuming, expensive and requires large samples of tissue for analysis. In this situation, DNA markers have advantages over other methods for the identification of fragrance because they are cheap, reproducible, free from environmental effects, and can be used even in early growth stages of the crop. Among the different types of DNA markers, functional markers or perfect markers, based on gene sequences, are considered to be more precise due to their ability to display functional polymorphism and direct correlation with the concerned phenotype (Kottearachchi, 2013). The *badh2* (*b*etain *a*ldehyde *d*ehydrogenase 2) locus of rice on chromosome 8 constituting the *fgr* gene has been documented to be the major determinant of fragrance (Bradbury *et al.*, 2005a; Bradbury *et al.*, 2005b and Chen *et al.*, 2006) in most of the aromatic rice lines. Sequence alignment of fragrant and non-fragrant genotypes using the *badh2* sequence has revealed an 8 base pair deletion and 3 Single Nucleotide Polymorphisms (SNPs) in the 7^{th} exon of the *badh2* allele present in the aromatic rice lines. Thus, the non-fragrant rice varieties possess a fully functional copy of the gene encoding BADH2 while fragrant varieties possess a mutant gene encoding a non-functional BADH2 enzyme (due to creation of a premature termination codon through the 8 bp deletion in the 7^{th} exon). The loss-of-function of BADH2 subsequently causes accumulation of the aromatic compound 2-ACP. In order to document this allelic variation, a multiplex PCR using 2 pairs of sense and antisense primers have been suggested (Bradbury *et al.*, 2005b). The primers have been designed to amplify a common band in both aromatic and non-aromatic rice lines, along with a differential band (on the basis of the 8 bp deletion in the 7^{th} exon of *badh2*) in case of aromatic and non-aromatic rice lines. Being co-dominant in nature, the marker system has also been exploited to identify heterozygous (in context to the *badh2* locus) plants in rice population.

Bihar is very rich in genetic resource of aromatic rice lines which is grown all over the state with Bhagalpur, Magadh and Champaran divisions in particular. However, the contamination in the local landraces is the major cause for loss and/or deterioration in many characteristics like aroma, grain texture, colour and market value. These impurities are more caused due to physical factors (like, due to the lack of the knowledge regarding the practice of roughing) than cross pollination (less than 1%) at flowering (Deb, 2006). At the same time, lack of in-depth characterization often raises the question of duplication of the same landrace in different names in different locations. Hence, characterization of local aromatic landraces of Bihar demands research initiatives. In the present study, we have characterized 9 rice landraces of Bihar, collected from the farmer's field. Through exploring the perfect co-dominant molecular marker for *badh2* gene, we have documented the allelic status of *badh2* in these lines and have also identified the existence of contaminations (in respect to *badh2* allele) in some of these aromatic entries. Thus, the utility of the perfect co-dominant molecular markers for *badh2* gene has been documented to identify genetic contamination in the local aromatic landraces, even at an early stage of growth.

Materials and Methods

Plant Materials and Phenotypic Evaluations

The present study was conducted in the year 2013-14 at the University Farm of Bihar Agricultural University, Sabour, Bhagalpur, Bihar. A total of 9 indigenous rice accessions of Bihar (namely Katarni, Jasua, Burma Bhusi, Malida, Kishanganj Basmati, Champaran Basmati, Marcha, Sonachur, and Hafsal) and a high yielding aromatic short grained released variety Rajendra Kasturi were collected from the farmers' field from different locations. Morphological data on different important agronomic traits of these 10 accessions were recorded on 5 plants' basis. The grains type was determined on the basis of IRRI (1996) classification.

PCR Amplification

Genomic DNA from the genotypes (10 collected aromatic lines, 7 released aromatic genotypes namely, Basmati 370, Kalanamak, Rajendra Suwasini, Rajendra Bhagwati, Sabour Surbhit, Sugandha and Badshahbhog as positive control and IR64 as negative control) was extracted from the leaves of two-week old rice seedlings, germinated in petri plates, using CTAB method suggested by Doyle and Doyle (1990). Genomic DNA was also isolated from 4/5 individual single plants of the genotypes Jasua, Kalanamak, Katarni, Marcha and Burma Bhusi in field for testing purity of the concerned entries. The isolated genomic DNA was

checked for quality and quantity through electrophoresis in 0.8% agarose gel. Each genotype was subjected to multiplex PCR using 4 specific primers (Bradbury *et al.*, 2005b), namely External Sense Primer (ESP: TTGTTTGGAGCTTGCTGATG), Internal Fragrant Anti-sense Primer (IFAP: CATAGGAGCAGCTGAAATATATACC), Internal Non-fragrant Sense Primer (INSP: CTGGTAAAAAGATTATGGCTTCA) and External Antisense Primer (EAP: AGTGCTTTACAAAGTCCCGC). For each sample, a total volume of 12µl of PCR mixture contained 2µl (~100ng) of extracted genomic DNA, 1.2 µl of 10X PCR buffer, 0.125 mM of dNTPs, 0.8 µM of ESP and EAP primers, 0.4 µM of INSP and IFAP primers and 0.3 U of *Taq* DNA polymerase (Xcelris). Amplification was performed in an ABI thermal cycler under the temperature profile consisting of an initial denaturation at 94 ˚C for 4 min followed by 35 cycles of 40 s at 94 ˚C, 30 s at 58 ˚C (primer annealing), 40 s at 72 ˚C, and ended with final extension at 72 ˚C for 10 min followed by hold at 4 ˚C. Amplified PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide and imaged through gel documentation system. PCR amplification was repeated twice for the accessions that exhibited the presence of both the fragrant and non-fragrant alleles, to confirm the result.

Results and Discussion

Phenotypic Characterization of the Aromatic Rice Genotype

Mean performance (Fig.1) and range of the 10 rice genotypes for 8 important morphological traits {as per the Indian guidelines for distinctiveness, uniformity and stability (DUS) testing of variety registration [Anonymous, 2007]} are indicated in Table 1.

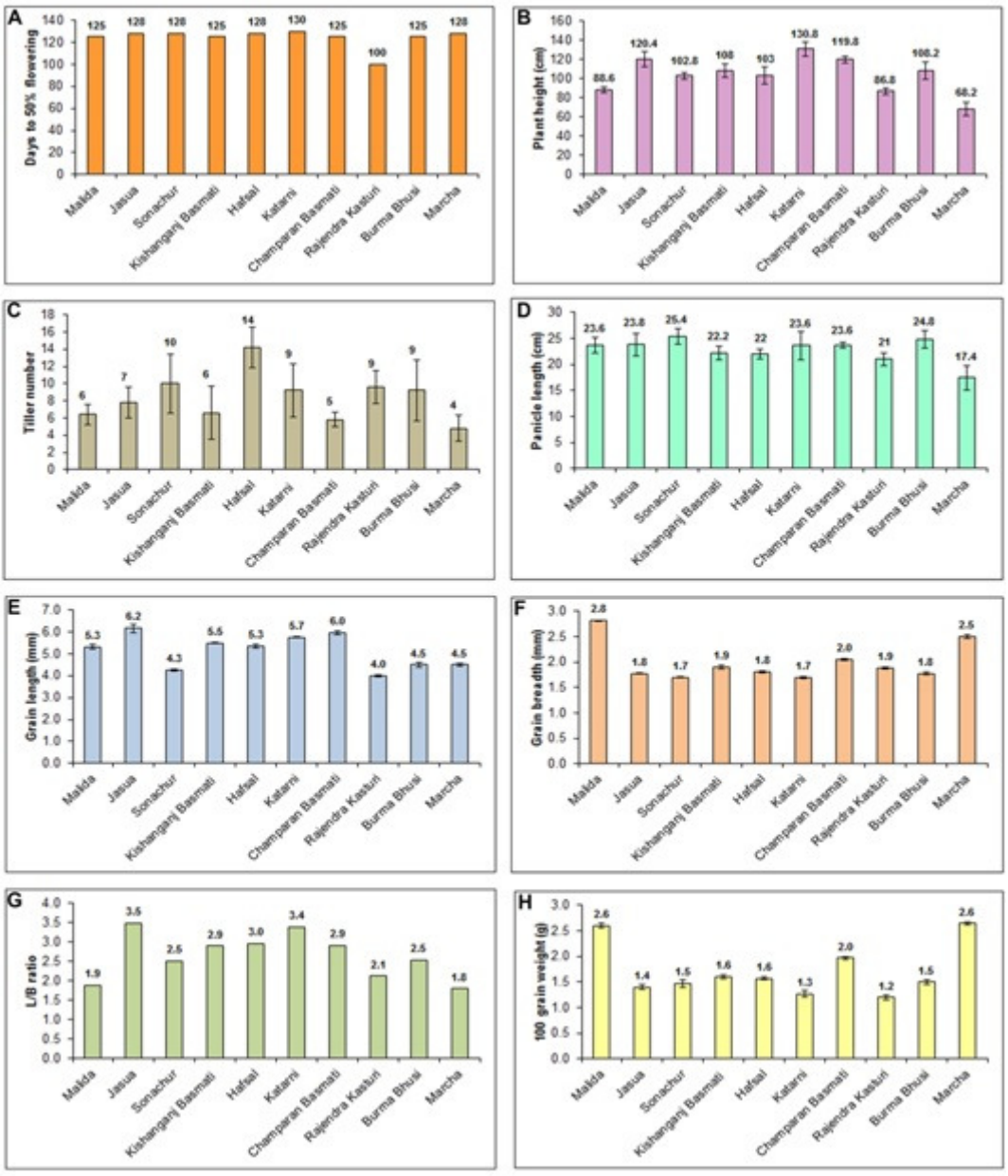

Fig.1 Bar diagram on mean value for the eight morphological observations of the collected 9 aromatic rice landraces and 1 released aromatic short-grained rice (Rajendra Kasturi). **A** Days to 50% flowering. **B** Plant height. **C** Tiller number. **D** Panicle length. **E** Grain length. **F** Grain breadth. **G** L/B ratio. **H** 100 grain weight. Error bars indicate standard deviation (SD) values

Table 1 Mean performance and range of 9 local aromatic landraces of rice on different important morphological parameters

Trait	Mean performance of all genotypes	Highest ranking genotype (Mean ± SD[1])	Lowest ranking genotype (Mean ± SD)
Days to 50% flowering	124	Katarni (130days)	Rajendra Kasturi (100 days)

Plant Height(cm)	103	Katarni (130.8 ± 7.73)	Marcha (68.2 ± 6.61)
Tiller No.	8	Hafsal (14 ± 2.39)	Marcha (4 ± 1.48)
Panicle length(cm)	23	Sonachur(25.4±1.52)	Marcha (17.4 ± 2.30)
Grain length (L) (mm)	5.1	Jasua (6.2 ± 0.19)	Rajendra Kasturi (4.0 ± 0.06)
Grain breadth (B) (mm)	1.9	Malida(2.8 ± 0.01)	Katarni (1.7 ± 0.02)
L/B ratio	2.65	Jasua(3.5)	Marcha (1.8)
100 grain weight(g)	1.72	Marcha (2.6 ± 0.03) and Malida(2.6 ± 0.04)	Rajendra Kasturi (1.2 ± 0.04)

[1]SD = Standard deviation

On the basis of average performance of eight morphological traits, the genotypes were categorised into late maturing (124 days), with moderate panicle length (23 cm), low in 100 seed weight (1.72 g) and medium slender grain type (L/B ratio: 2.65) with very short grain length (5.1mm) and very narrow grain width (1.98mm). All the collected aromatic landraces were found to take ≥ 125 days for attending 50% flowering, where the genotype Katarni took maximum number of days (130). Among the tested genotypes, Katarni was found to be the tallest (plant height 131±7.73 cm) followed by Jasua (120.4 ± 8.08 cm). Hafsal was observed to have the highest tillering ability (14±2.39) while panicle length was highest for Sonachur (25.4±1.51cm). Based on the grain length (L) and L/B ratio, Jasua (L = 6.2 ± 0.19 mm; L/B = 3.5), Katarni (L = 5.7 ± 0.02 mm; L/B = 3.4) and Hafsal (L = 5.3 ± 0.08 mm; L/B = 3.0) were categorised as 'Medium Slender Grain' type; Sonachur (L = 4.3 ± 0.06 mm; L/B = 2.5), Rajendra Kasturi (L = 4.0 ± 0.06 mm; L/B = 2.1) and Burma Bhusi (L = 4.5 ± 0.12 mm; L/B =2.5) were categorised as 'Short Medium' type; Kishanganj Basmati (L =5.5 ± 0.04 mm; L/B = 2.9) and Champaran Basmati (L = 6.0 ± 0.08 mm; L/B =2.9) were categorised as 'Medium Gain' type; and Malida (L = 5.3± 0.11 mm; L/B =1.9) and Marcha (L =4.5 ± 0.05 mm; L/B = 1.8) were categorised as 'Short Bold Grain' type. Among the 9 collected landraces, 100 grain weight was found to be maximum in the genotypes Malida (2.6 ± 0.04 g) and Marcha (2.6 ± 0.03 g), whereas minimum for the genotype Katarni (1.3 ± 0.07 g).

Molecular Characterization of the Aromatic Rice Genotypes

Following morphological characterization, the 10 aromatic rice genotypes along with 7 positive controls (Basmati 370, Kalanamak, Rajendra Suwasini, Rajendra Bhagwati, Sabour

Surbhit, Sugandha and Badshahbhog) and one negative control (IR 64) were subjected to molecular characterization using the *badh2* gene specific perfect co-dominant marker through multiplex PCR. As per the previous study (Bradbury, 2005b), the external primers ESP and EAP generated a common band of ~580 bp in all the 18 samples which serve as positive control for the PCR reaction (Fig. 2).

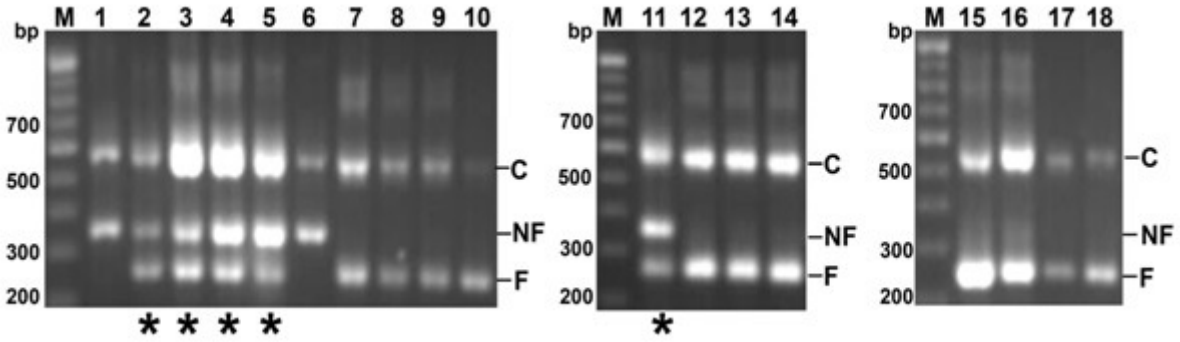

Fig. 2 Multiplex PCR amplicons of 18 rice genotypes using *badh2* gene specific primers. Lane 1 = IR 64; lane 2 = Katarni; lane 3 = Burma Bhusi; lane 4 = Jasua; lane 5 = Kalanamak; lane 6 = Malida; lane 7 = Basmati 370; lane 8 = Rajendra Suwasini; lane 9 = Rajendra Bhagwati; lane 10 = Sabour Surbhit; lane 11 = Marcha; lane 12 = Sonachur; lane 13 = Hafsal; lane 14 = Champaran Basmati; lane 15 = Sugandha; lane 16 = Kishanganj Basmati; lane 17 = Rajendra Kasturi; lane 18 = Badshabhog. Presence of the ~580 bp common band, ~355 bp non-fragrance allele-specific band and ~257 bp fragrance allele-specific band are denoted by C, NF and F, respectively. Co-existence of non-fragrance allele-specific band and fragrance allele-specific band is indicated by asterisk mark. Lane M = 100 bp DNA ladder, where size (in bp) of 4 bands are shown.

The primer combination ESP and IFAP (complementary to deleted allele) generated the fragrance allele-specific ~257 bp band in 11 genotypes (4 out of the 9 collected aromatic landraces, Rajendra Kasturi, and 6 out of the 7 positive controls). On the other hand, the primer combination INSP (complementary to undeleted allele) and EAP generated the expected non-fragrance-specific allele (~355 bp) in case of the negative control IR 64 as well as in Malida. In this multiplex PCR, the external sense and antisense primer is consumed twice (for amplification of positive control band and either 355/257bp band with internal primers), hence the concentration of external primers was kept twice as compared with the internal primers.

Apart from this, the multiplex PCR revealed presence of both the fragrance-specific and non-fragrance-specific alleles (along with the common band) in 5 genotypes (4 out of 9 collected aromatic landraces and the genotype Kalanamak). This result was found to be consistent through repeating the experiment (data not shown), indicating the possibility of mixture in

these genotypes. To check this hypothesis, we went for isolating genomic DNA from 4/5 individual single plants of theses genotypes and subjected them to the same PCR condition. As per the expectation, we found the problem of co-existence of both the fragrance- and non-fragrance-specific alleles to be resolved this time (Fig. 3).

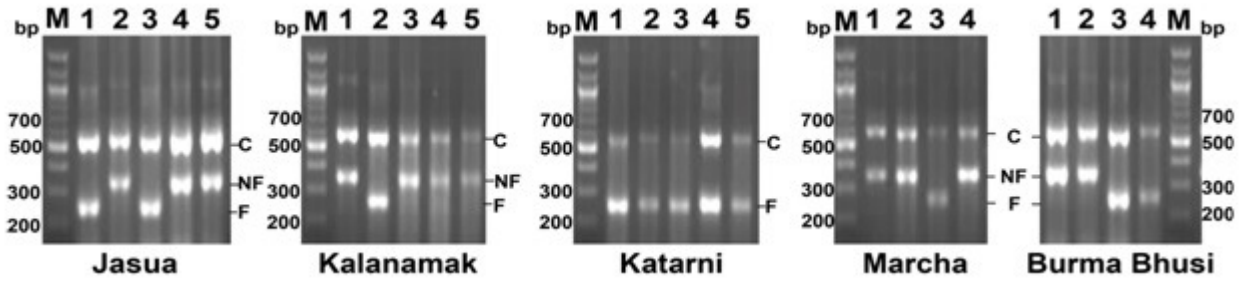

Fig. 3 Multiplex PCR amplicons obtained using genomic DNA isolated from 5 individual single plants of the genotypes Jasua, Kalanamak and Katarni and 4 individual single plants of genotypes Marcha and Burma Bhusi using the *badh2* gene specific primers. Presence of the ~580 bp common band, ~355 bp non-fragrance allele-specific band and ~257 bp fragrance allele-specific band are denoted by C, NF and F, respectively. Lane M = 100 bp DNA ladder, where size (in bp) of 4 bands are shown.

Using the genomic DNA isolated from individual single plants as template, we found 2 out of 5 plants of the genotype Jasua, 1 out of 5 plants of the genotype Kalanamak, 5 out of 5 plants of the genotype Katarni, 1 out of 4 plants of the genotype Marcha and 2 out of 4 plants of the genotype Burma Bhusi to contain the fragrance-specific allele (Fig. 3). This result also clarified the reason behind obtaining both the fragrance- and non-fragrance-specific alleles in our previous experiment (Fig. 2), where we used genomic DNA isolated from a group of germinated seedling as template for the PCR.

Conclusions

Genetic purity of a seed lot is of utmost importance, both from breeders' and farmers' point of view. Purity of a seed lot is generally tough to identify through morphological traits only and is generally assayed using the grow out test (GOT). However, GOT is time consuming, space demanding and often doesn't allow the unbiased identification of genotypes (Rajuguru, 2011). In the present study, we have characterized 9 local aromatic landraces collected from different parts of Bihar and have used the *badh2* gene-specific perfect co-dominant marker system to identify impurities in these genotypes. The utility of this marker system for marker assisted selection (MAS) in breeding programmes targeting the development of aromatic rice varieties has been well documented before (Bradbury, 2005b and Yeap *et al.*, 2013). Through

our present study, we advocate the utility of this marker system also for testing purity of aromatic rice seed lots.

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