Isolation, Sequencing, *In silico* analysis of *badh* homologues/alleles and validation of DNA Markers for MAS in Aromatic Rice (*Oryza sativa* L.)

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Thesis

Submitted to

Faculty of Horticulture
Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir

in partial fulfillment of requirement for the award of the degree of

Master of Science in Biotechnology

2016

Dedicated
to my
beloved Parents

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Faculty of Horticulture, Division of Plant Biotechnology

Certificate – I

This is to certify that the thesis entitled, **"Isolation, Sequencing, *In silico* analysis of *badh* homologues/alleles and validation of DNA Markers for MAS in Aromatic Rice (*Oryza sativa* L.)"** submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science in Biotechnology**, to the **Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** is a record of bonafide research work carried out by **Ms. Aafreen Sakina (Regd. No. 2013-A-981-M)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that information received during the course of investigation has duly been acknowledged.

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Chairman
Advisory Committee

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Certificate – II

We, the members of the Advisory Committee of **Ms. Aafreen Sakina (Regd. No. 2013-A-981-M),** a candidate for the degree of **Master of Science in Biotechnology**, have gone through the manuscript of the thesis entitled, **"Isolation, Sequencing, *In silico* analysis of *badh* homologues/alleles and validation of DNA Markers for MAS in Aromatic Rice (*Oryza sativa* L.)"** and recommend that it may be submitted by the student in partial fulfillment of the requirements for the award of the degree.

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Certificate – III

This is to certify that the thesis entitled, **"Isolation, Sequencing, *In silico* analysis of *badh* homologues/alleles and validation of DNA Markers for MAS in Aromatic Rice (*Oryza sativa* L.)"** submitted by **Ms. Aafreen Sakina (Regd. No. 2013-A-981-M)** to the **Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** in partial fulfilment of the requirements for the award of the degree of **Master of Science in Biotechnology**, was examined and approved by the Advisory Committee and External Examiner on …………….

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ABSTRACT

Aroma trait imparts speciality to the rice and increases its demand in market. Genotypes of aromatic rice have been reported to possess a truncated version of betaine aldehyde dehydrogenase gene (badh2) located on chromosome no 8, which causes the production of aroma. Kashmir varieties need to be screened for the presence of variants of this fragrance imparting gene *badh2* for use in crop improvement programmes. In present study 10 genotypes of rice grown locally in Kashmir including special rice of Kashmir i.e Mushk Budji were assessed for allelic variants of exon 7 of *badh2* gene. Based on previous studies, primers were designed that targeted 8 bp deletion in exon 7 of badh2 gene and amplified a fragment of 95 bp in aromatic rice varieties and 103 bp fragment in non-aromatic varieties. To validate the results 463 bp region covering the target deletion was amplified by another set of primers (OsBadh2) and subsequently the bands were eluted and got sequenced by Sanger method. Software program T-COFEE was used for creating multiple sequence alignment in the region of interest. The analysis showed presence of a deletion of 8 bps "GATTATGG" and three SNPs were in exon 7 of aromatic rice genotypes which could be the possible reason of truncated betaine aldehyde dehydrogenase enzyme and subsequently aroma. No such deletion was found in non-aromatic rice varieties. Hence functional markers for badh2 allele were validated in all accessions of Mushk-budji, Pusa sugandh-3 and Basmati 1509 (aromatic varieties) which can facilitate development of fragrant rice varieties through MAS. Surprisingly aroma was not detected in a so-called accession claimed to be Kamad which had been collected from a farmer's field in year 2009. This so called Kamad didn't show any aroma in the fragrance test of Berner and Hoff and same was corroborated by evidence from PCR results and sequencing. It doesn't possess the 8 bp deletion! The reason could be that the concerned farmer was growing a mixture of aromatic and non aromatic genotypes in his field, and his claim was wrong.

Key words: Rice (*Oryza sativa* L.), Mushkbudji, Aroma, Sequencing, Functional markers.

Signature of Student　　　　　　　　Signature of Major Advisor

Dated:____________　　　　　　　　Dated:_________________

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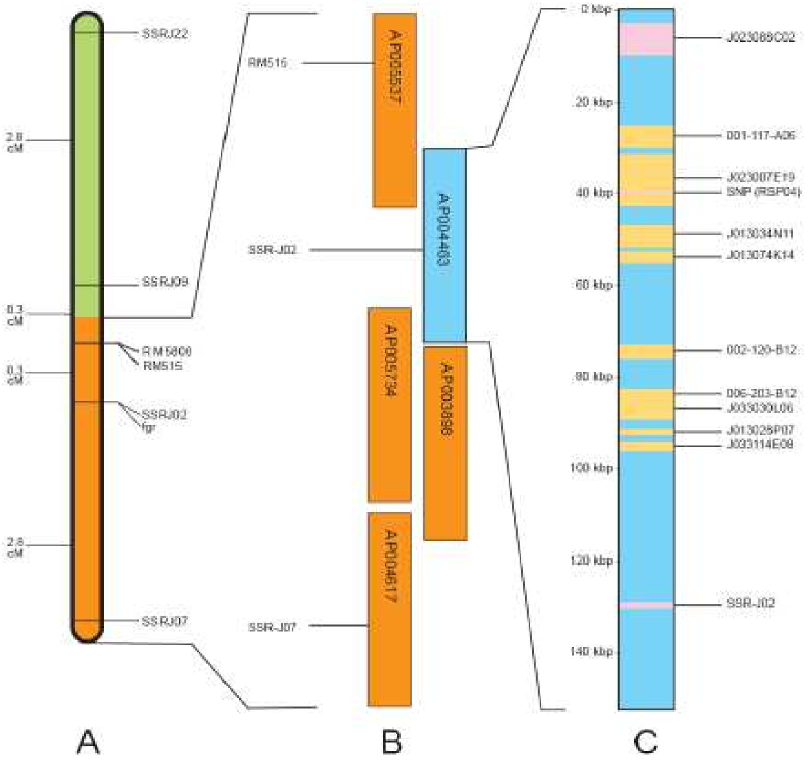
Chapter - 1

INTRODUCTION

Rice is undoubtedly a dominant and important staple food worldwide. The genus *Oryza* L. is classified under the tribe Oryzeae, subfamily Oryzoideae, of the grass family Poaceae (Gramineae) (Lu, 1999). This genus includes two cultivated species and more than 20 wild species, distributed in tropical Asia, Africa, Australia, Central and South America (Khush, 1997). The two cultivated rice species, *Oryza sativa* L. and *O. glaberrima* Steud., belong to a species group called *Oryza sativa* complex together with the five wild taxa, *O. rufipogon* (sensu lato), *O. longistaminata* Chev. Roehr., *O. barthii* A. Chev., *O. glumaepatula* Steud., and *O. meridionalis* Ng. Rice is now the model plant for genetic research among crop plants. Rice (2n = 12) has a genome size of 389 Mbp’s, and its importance as a staple food for about half of the world population is well recognized (IRGSP, 2005).

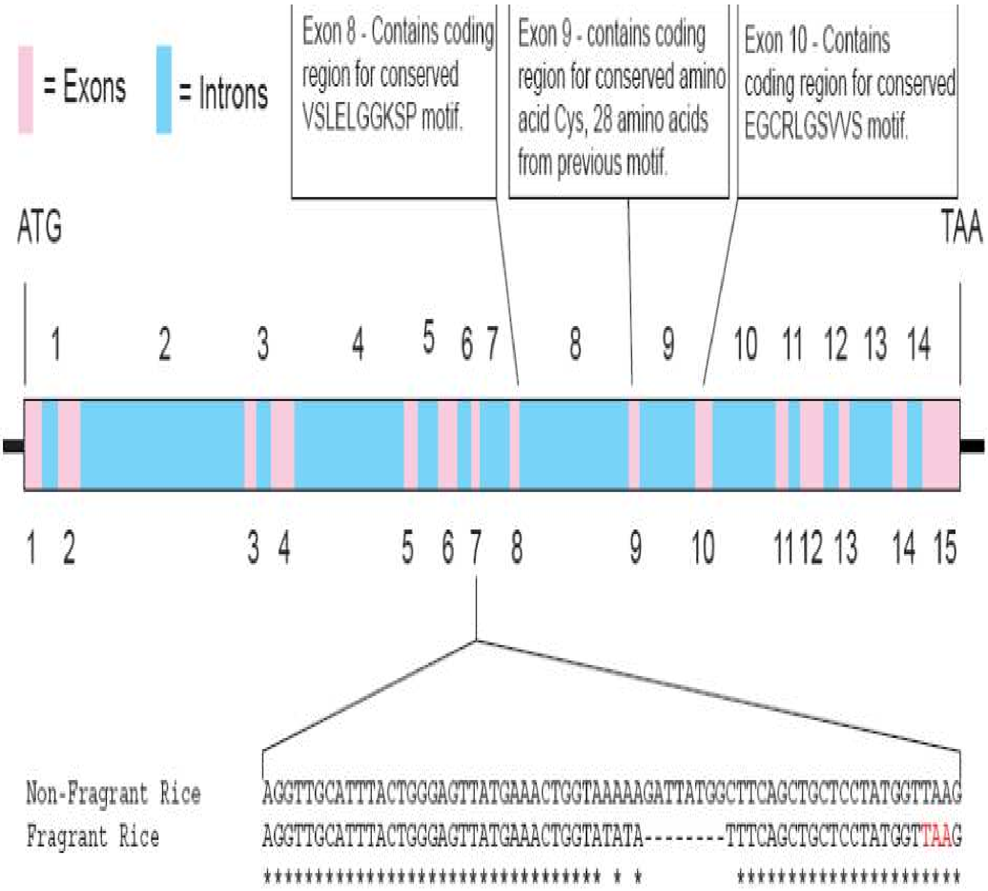
Rice is life for Asians in general and Indians in particular. Asia cultivates 137 million ha of rice of which Indians share 42.56 million ha. Its annual production and productivity is 95.33 million tonnes and 2.22 t ha^{-1}, respectively (D.O.E.S., 2013). Genetic diversity available in rice in India is quite impressive and local landraces/primitive cultivars play an important role in maintaining this diversity. Rice is the staple diet of the majority of people in Jammu and Kashmir. Rice crop ranks 2nd, both in area under cultivation and production. At present, rice in the state is cultivated on an area of 274 thousand hectares, its production in 2013-14 was 9044 thousand quintals (0.55 Mt) and productivity was 3.238 t ha^{-1} (D.O.E.S., 2013). This part of Himalayan region of India (33°17′-37°20′ N latitude and 73°25′-80°30′ E longitude) harbour land races which are important reservoirs of valuable traits viz. taste, aroma, nutrition, medicinal properties and other special uses. Grain aroma is the most attractive characteristic of high-quality rice. Aromatic rice varieties constitute a small but special group of rice and have gained greater importance with the worldwide increase in the demand for fine quality rice. Among the most prominent aromatic indigeneous rice cultivars of Kashmir Mushkbudji, stands at the top followed by Kamad (Parray *et al.*, 2008). Aromatic rice is rare and so precious that in some countries, it is considered a national asset and pride.

Fragrant rice has been in great demand in the Asian rice market for decades. Not only is aroma one of the most important characteristics for determining good quality rice but aromatic varieties have comparable or superior nutritional values and better amino acid profiles. Aroma characteristics were determined by Kovach *et al.* (2009) in three of the distinguished genetic subpopulations of rice: *Group V* (Sadri and Basmati), *indica* (Jasmine), and tropical *japonica*. Buttery *et al.* (1982) with his coworkers successfully identified 2-acetyl-1-pyrroline [2AP; IUPAC name 5-acetyl-3, 4-dihydro- 2*H*-pyrrole] as the compound responsible for the unique and pleasant fragrance of the aromatic rice. Genetic analysis revealed that the primary fragrance trait is controlled by recessive monogenic inheritance, independent of cytoplasmic genes. Studies on rice varieties showed, some are under monogenic control (Yano *et al.,* 1991) whereas, in some others it is found to be a quantitative trait and many genes are involved in its expression (Hien *et al.,* 2006) thus, indicating complex genetic control of the aroma trait. A single recessive gene located on chromosome 8 has been found to play significant role in aroma development, and has been identified by different techniques viz. RFLP (Ahn *et al.*, 1992), translocation and trisomics lines from non aromatic rice cultivar IR36 (Li *et al.*, 1996) and by using SSR markers RM210 and RM515 (Ren *et al.*, 2004). Bradbury *et al.* (2005) identified the gene responsible for fragrance in rice. A recessive gene (*fgr*) on chromosome 8 of rice has been linked to this important trait. This gene encodes an enzyme betaine aldehyde dehydrogenase (BADH2) and has significant polymorphisms in the coding region of fragrant genotypes relative to non-fragrant genotypes. Betaine aldehyde dehydrogenase (BADH2) is an enzyme capable of converting γ-aminobutyraldehyde (GABald), a four carbon aminoaldehyde derived from proline via putrescine oxidation into γ-aminobutyric acid (GABA). An eight base pair deletion in *Badh2* leads to the generation of a premature stop codon that would, if translated, produce a truncated non-functional protein. This results in abrogation of the function of the enzyme, Betaine aldehyde dehydrogenease, which consequently accumulates substrate 2AP in fragrant varieties. The functional *badh2* gene codes for a mature protein with 503 amino acids (Wanchana *et al.*, 2005). In aromatic varieties γ-aminobutyraldehyde (GABald), is not metabolized by BADH enzyme (truncated protein) and it cyclizes spontaneously into Δ1-pyrroline, which accepts acetyl group from methyl glyoxal, resulting in the formation of 2-acetyl 1-pyrroline (2AP). Identification of the gene for fragrance and availability of large aromatic rice gene pool has created a world-wide interest to look for allelic variants at this locus (Amarawathi *et al.*, 2008). A 7-bp deletion in exon 2 (Shi *et al.*, 2008), absence of MITE (miniature interspersed transposable element) in promoter (Bourgis *et al.*, 2008), two new SNPs in the central section of intron 8 (Sun *et al.*, 2008), a TT deletion in intron 2 and a repeated (AT)n insert in intron 4 of *badh2* were reported in various fragrant varieties (Chen *et al.*, 2008). The sequencing of the *badh2* locus derived from aromatic rice cultivars can uncover the presence of different *badh2 alleles*. Bradbury *et al.* (2005) reported that *badh2.1* allele consists of 8-bp deletion and 3 single nucleotide polymorphisms (SNPs) in exon 7 that leads to the introduction of premature stop codon to produce a truncated protein.



Location of fragrance gene in; A) a section of rice chromosome 8, showing the genetic distance between fgr and chromosomal markers B) BAC clones covering the region of rice chromosome 8 flanked by the chromosomal markers RM515 and SSR-JO7 where the fgr gene was calculated to reside, and C) the AP004463 BAC clone showing the genes in this region including the predicted fgr gene and chromosomal markers SSR-J02 and RSP04

[Source : Bradbury *et al.*, 2005]



[Source : Bradbury *et al.*, 2005]

Structure of the fragrance gene (fgr) showing initiation codon (ATG), 15 exons, 14 introns and the ATT termination site. The nucleotide sequence of exon 7 is shown for fragrant as well as non-fragrant rice varieties. The fragrant rice variety shows a large deletion and 3 SNPs which then terminate prematurely (stop codon in red), within this exon. The truncated protein encoded in fragrant rice varieties would therefore lack the highly conserved sequences encoded by sequence 8, 9 and 10 and which are believed to be required for correct function of this protein.

BADH1 is homologous with *BADH2* (Bourgis *et al.*, 2008) and located on chromosome 4 in rice, both having similar molecular function (Amarawathi *et al.*, 2008) but its role in aroma is not yet confirmed (Hasthanasombut *et al.*, 2011).

The reduced expression of *badh2* or 2AP accumulation was studied by Vanavichit *et al.* (2005) using, RNA interference (RNAi) technology in Jasmine rice. They observed that, the strongest RNAi expression gave the strongest suppression and the highest accumulation of 2AP. In another study, transgenic rice containing RNAi by an inverted repeat of cDNA encoding Os2AP accumulated 2AP in considerable amounts (Chen *et al.*, 2008). Niu (2008) confirmed the reduced expression of the *badh2* using RNAi in rice, resulted in 2AP accumulation. Therefore, it is confirmed that *badh2* determines the accumulation of 2AP in rice. BADH2, is basically involved in synthesis of an osmolyte glycine betaine from betaine aldehyde (Livingstone *et al.*, 2003). But rice is a non-accumulator of glycine betaine (Rathinasabapath *et al.*, 1993). Bradbury *et al.* (2008) found that rice BADH2 has higher activity towards gamma amino butyraldehyde (GABald) and moderate activity on betaine aldehyde. Rice BADH2 was found to regulate metabolism of gamma-amino butyric acid (GABA) from GABald in rice. GABAld spontaneously cyclises to Δ1- pyrroline, the key precursor of 2AP. This was recently confirmed by the absence of 2AP in an aromatic variety after transformation with functional *badh2* (Chen *et al.*, 2008) and suppression of *badh2* transcript in a non-aromatic *japonica* rice callus (*O. sativa* japonica cv. Nipponbare) increases 2 AP levels.

Important grain quality trait i.e aroma, which often appears to be under simple genetic control is difficult to recover in high-yielding backgrounds. Availability of high-density molecular marker maps and genome sequences has allowed fine mapping and positional cloning of gene for fragrance in rice. Multiplex markers targeting the functional InDel polymorphism were reported for genotyping fragrance trait. Subsequently, a perfect marker system targeting 8-bp deletion was proposed for fragrance genotyping (Bradbury *et al.*, 2005). In this marker assay, although developed from functional region of the gene, four primers (two pairs) were multiplexed in a single PCR reaction. Use of this multiplex marker system was complex and tedious, so a new marker that was simple, co-dominant, functional marker was developed for fragrance trait, which can be resolved in an agarose gel and validated in Basmati and non-Basmati aromatic rice varieties and in a mapping population segregated for fragrance trait was developed by Sakhtivel *et al.* (2009). The marker targeted the InDel polymorphism in badh2 gene and amplified 95 and 103 bp fragments in fragrant and non-fragrant genotypes, respectively. This newly developed marker was highly efficient in discriminating all fragrant and non-fragrant genotypes and showed perfect co-segregation with the trait of fragrance in the mapping population hence could be amenable for routine marker-assisted selection (MAS) involving large breeding materials. This has led to major advances in marker-assisted selection and pyramiding of useful genes. Thus, the population can be screened at any stage of growth and in various environments and scent character can be rapidly incorporated into the breeding lines.

The local fragrant rice genotypes of Kashmir being land races and having a characteristic aroma may possess allelic variants for the gene *badh2*. These genotypes need to be screened for the presence of variants of fragrance imparting gene *badh2*. The study is important as it can be utilized in the fragrant rice improvement programme for J&K or elsewhere, and validation of the functional markers (developed by different workers) for *badh2* allele can be used in breeding of fragrant rice varieties through MAS. In light of the above facts, present investigation was carried out with the following research objectives:-

1. Isolation and sequencing of *badh2* gene homologues from fragrant and non fragrant rice genotypes grown in Kashmir.
2. *In silico* analysis of *badh* homologues/alleles for sequence similarity.
3. Validation of functional markers for badh2 allele which can facilitate development of fragrant rice varieties through MAS.

Chapter 2

REVIEW OF LITERATURE

A candidate gene (fgr/BADH2) homologous to betaine aldehyde dehydrogenase is responsible for aroma metabolism in fragrant rice varieties. The presence of a dominant BADH2 allele encoding betaine aldehyde dehydrogenase inhibits the synthesis of 2-acetyl-1-pyrroline (2AP), a potent flavor component in rice fragrance. By contrast, its recessive alleles, badh2, induce 2AP formation.

Cordeiro *et al.* (2002) in order to overcome the difficulties in the detection of fragrance carried out via sensory or chemical methods identified an (AT)40 repeat microsatellite or simple sequence repeat (SSR) marker for fragrant and non-fragrant alleles of the *fgr* gene. Identification of this marker was facilitated through use of both the publicly available and restricted access sequence information of the Monsanto rice sequence databases. Fifty F_2 individuals from a mapping population were genotyped for the polymorphic marker. This marker has a high polymorphism information content (PIC = 0.9). Other SSR markers linked to fragrance could be identified in the same way of use in other populations. This study demonstrated that analysis of the rice genome sequence is an effective option for identification of markers for use in rice improvement.

Lang *et al.* (2002) conducted studies on identification and fine mapping of SSR marker linked to fgr gene of rice. The genomic clone RG28, which is tightly linked to fgr gene in rice, provides to perform marker-aided selection in rice breeding program. This study aims at identifying and making fine mapping of SSR marker linked to fgr gene in rice which controls aromatic property. RG28 marker can be converted by sequencing into SSR and used as primers for PCR amplification of genomic DNA from rice varieties differing in the aromatic responsiveness. Fine genetic mapping was conducted at initial steps with an effort to apply marker-assisted selection in plant breeding. Using 12 selected DNA markers and an F_2 population including 250 plants, fgr was fine mapped to a genomic region <2 cM on the chromosome 8. Two markers RG28, RM223 closely linked to extremely aromatic phenotypes. DNA marker assisted selection was used to detect fgr gene. To examine the power of the identified SSR markers in predicting the phenotype of the fgr locus, the genotypes were determined of the F_2's individuals at this locus by performing progeny testing for fgr in the F3 generation. The results indicated an accuracy of more than 84% in identifying the resistant plants which was similar to that using RG28F-R. The results of the germplasm survey will be useful for the selection of parents in breeding program aimed at transferring this gene from one variety background to another using marker-assisted selection.

Lam *et al.*(2003) investigated development of volatile components in milled rice over 50 d of storage. The major volatiles were identified and measured by gas chromatography- mass spectrometry and their relative odor potencies were subsequently calculated as aroma values. Volatile concentrations were greater in partially milled rice than in fully milled rice. Concentrations of 2-nonenal, octanal and hexanal increased significantly during storage whereas heptanal, 3-penten-2-one and 2-pentylfuran did not. Octanal and 2-nonenal had the highest contributions to milled rice odor. The aroma values for hexanal and 2-pentylfuran changed by a larger factor relative to those for heptanal, 2-nonenal, 3-penten-2-one and octanal during early storage, whereas heptanal, 3-penten-2-one and 2-pentylfuran did not.

Jin *et al.* (2003) sequenced the whole rice genome to assist in the identification of a single nucleotide polymorphism (SNP) marker linked to the fragrance gene (fgr) in rice. Genes flanked by restriction fragment length polymorphism and microsatellite markers known to be linked to the fragrance gene were identified by DNA sequence alignment of EST sequences against BAC clones covering this region of chromosome eight. Re-sequencing and comparison of parts of these genes derived from a fragrant and a non-fragrant cultivar revealed only one SNP (a C/T transition) in more than 6 kbp of sequence from 14 genes. Ten of eleven fragrant genotypes and six of 14 non-fragrant genotypes tested carried the C allele. This approach indicated a generally low level of SNP polymorphism in cultivated rice suggesting that association of SNP with phenotypes should be an efficient path to gene discovery in cultivated rice.

Bradbury *et al.* (2005) reported that the flavour or fragrance of basmati and jasmine rice is associated with the presence of 2-acetyl-1-pyrroline. A recessive gene (fgr) on chromosome 8 of rice was linked to this important trait. They showed that a gene with homology to the gene that encodes betaine aldehyde dehydrogenase (BAD) has significant polymorphisms in the coding region of fragrant genotypes relative to non-fragrant genotypes. The accumulation of 2-acetyl-1- pyrroline in fragrant rice genotypes was explained by the presence of mutations resulting in a loss of function of the fgr gene product. The allele in fragrant genotypes has a mutation introducing a stop codon upstream of key amino acid sequences conserved in other BADs. The fgr gene corresponds to the gene encoding BAD2 in rice, while BAD1 is encoded by a gene on chromosome 4. BAD has been linked to stress tolerance in plants. However, the apparent loss of function of BAD2 does not seem to limit the growth of fragrant rice genotypes.

Chen *et al.* (2006) with their initial mapping efforts with SSR markers confirmed that a single recessive gene (fgr) on chromosome 8 is responsible for the production of 2AP and placed the fgr locus between RM8264 and RM3459 with the physical distance of ~ 800 kb. The fgr region was then saturated with high-density markers developed by exploiting sequence diversities between indica and japonica rice subspecies. After mapping with segregant populations consisting of totally 2891 individuals, the fgr locus was restricted to an interval of 69 kb flanked by the left marker L02 and the right marker L06. Furthermore, the fgr locus was confirmed by simultaneous investigation of both genotypes and 2AP levels for the key recombinants and their offspring. Sequence analysis of the fgr region revealed three candidate genes encoding respective eukaryotic-type carbonic anhydrase, 3-methylcrotonyl-CoA carboxylase beta chain, and betaine aldehyde dehydrogenase.

Bourgis *et al.* (2008) reported on characterisation of the *frg* gene in the Azucena variety, one of the few aromatic *japonica* cultivars. A RIL population from a cross between Azucena and IR64, a non-aromatic *indica*, the reference genomic sequence of Nipponbare (*japonica*) and 93-11 (*indica*) as well as an Azucena BAC library, was used to identify the major fragance gene in Azucena. They thus identified a betaine aldehyde dehydrogenase gene, *badh2*, as the candidate locus responsible for aroma, which presented exactly the same mutation as that identified in Basmati and Jasmine- like rices. Comparative genomic analyses showed very high sequence conservation between Azucena and Nipponbare *BADH2*, and a MITE was identified in the promoter region of the *BADH2* allele in 93-11. The *badh2* mutation and MITE were surveyed in a representative rice collection, including traditional aromatic and non-aromatic rice varieties, and strongly suggested a monophylogenetic origin of this *badh2* mutation in Asian cultivated rices.

Fitzgerald *et al.* (2008) in their study, quantified 2AP and determined presence or absence of the fragrance allele (*fgr*) in 464 samples of traditional varieties of rice from the T.T. Chang Genetic Resources Centre at the International Rice Research Institute. It was shown that a number of aromatic varieties, primarily from South and South-East Asia, do not carry the 8-bp deletion, but 2AP was identified in both raw and cooked rice of these varieties. They suggest that the 8-bp deletion in *fgr* is not the only cause of aroma, and at least one other mutation drives the accumulation of 2AP. The amount of 2AP in most uniform *fgr* genotypes was not significantly different from that in aromatic n*fgr* genotypes, but several *fgr* genotypes, primarily from South Asia, reproducibly accumulated exceptionally large amounts of 2AP. They suggested that the mutation leading to 2AP in aromatic n*fgr* varieties possibly originated several times and, through either domestication or evolution, the *fgr* gene and other alleles leading to 2AP have combined in South Asia, leading to several highly aromatic traditional varieties. The identification of multiple mutations for 2AP will enable rice breeding programmes to select actively for multiple genetic sources of 2AP, leading to the development of highly aromatic and, consequently, high-quality varieties of rice.

Lang *et al.* (2008) developed PCR-based DNA markers for precise and efficient transfer of the fgr gene into new elite improved lines. This was done with an informative RFLP marker RG28, and RM223, which showed the closest linkage to fgr gene in rice, provides means to perform marker-assisted selection in rice breeding program. The objective of this study was to investigate the possibility of generating a polymerase chain reaction PCR-based polymorphic marker that can distinguish aroma and non-aroma. Based on the sequence tagged site data (STSs) of RG28FL-RL and microsatellite RM223, pair wise primers were designed to amplify genomic DNA from C53/Jasmine 85 and C51/Jasmine 85 to identify polymorphic amplified products between the two rice lines OM4900 from C53/Jasmine 85 and OM6161 from C51/Jasmine 85. Two polymorphic markers, amplified with RG28 FL-RL and RM223 were identified. These results demonstrate the utility of STS and microsatellite markers for use in marker-assisted selection and breeding within cultivated rice.

Niu *et al.* (2008) provided the direct evidence demonstrating the functions of *OsBADH2* and elucidated the physiological roles of *OsBADH2*. Sequencing approach and RNA interference (RNAi) technique were employed to analyze allelic variation and functions of *OsBADH2* gene in aroma production. Semi-quantitative, real-time reverse transcription-polymerase chain reaction (RT-PCR), as well as gas chromatography-mass spectrometry (GC-MS) were conducted to determine the expression levels of *OsBADH2* and the fragrant compound in wild type and transgenic *OsBADH2*-RNAi repression lines, respectively. Their results showed that multiple mutations identical to *fgr* allele occur in the 13 fragrant rice accessions across China; *OsBADH2* is expressed constitutively, with less expression abundance in mature roots; the disrupted *OsBADH2* by RNA interference leads to significantly increased 2- acetyl-1-pyrroline production. Ultimately it was found that the altered expression levels of *OsBADH2* gene influence aroma accumulation, and the prevalent aromatic allele probably has a single evolutionary origin.

Sun *et al.* (2008) investigated inheritance and carried out gene fine mapping of aroma in crosses between the aromatic elite hybrid rice *Oryza sativa indica* variety Chuanxiang-29B (Ch-29B) and the non-aromatic rice *O. sativa indica* variety R2 and *O. sativa japonica* Lemont (Le). The F_1 grains and leaves were non-aromatic while the F_2 non-aroma to aroma segregation pattern was 3:1. The F3 segregation ratio was consistent with the expected 1:2:1 for a single recessive aroma gene in Ch-29B. Linkage analysis between simple sequence repeat (SSR) markers and the aroma locus for the aromatic F_2 plants mapped the Ch-29B aroma gene to a chromosome 8 region flanked by SSR markers RM23120 at 0.52 cM and RM3459 at 1.23 cM, a replicate F_2 population confirming these results. Three bacterial artificial chromosome (BAC) clones cover chromosome 8 markers RM23120 and RM3459. Molecular mapping data from the two populations indicated that the aroma locus occurs in a 142.85 kb interval on BAC clones AP005301 or AP005537,implying that it might be the same gene reported by Bradbury *et al.* (2005). The flanking markers Aro7, RM23120 and RM3459 identified could greatly accelerate the efficiency and precision of aromatic rice breeding programs.

Kovach *et al.* (2009) in order to find the origin and evolution of the betaine aldehyde dehydrogenase gene (*BADH2*) gene conducted a study. In their study they identified eight putatively non functional alleles of the *BADH2* gene and showed that these alleles have distinct geographic and genetic origins. Despite multiple origins of the fragrance trait, a single allele, *badh2.1*, is the predominant allele in virtually all fragrant rice varieties today, including the widely recognized Basmati and Jasmine types. Haplotype analysis allowed them to establish a single origin of the *badh2.1* allele within the Japonica varietal group and demonstrate the introgression of this allele from Japonica to Indica. Basmati-like accessions were nearly identical to the ancestral Japonica haplotype across a 5.3-Mb region flanking *BADH2* regardless of their fragrance phenotype, demonstrating a close evolutionary relationship between Basmati varieties and the Japonica gene pool. These results clarify the relationships among fragrant rice varieties and challenge the traditional assumption that the fragrance trait arose in the Indica varietal group.

Prathepha (2009) conducted a study to determine the presence of the recessive allele of the fragrance gene of the weedy rice population in an important rice growing area of North eastern Thailand. Among the 215 weedy rice plants examined, three genotypes, BADH2/BADH2, BADH2/badh2 and badh2/badh2 were detected. Frequencies of the badh2 allele showed a high value of 0.547.

Fragrance development in rice has been reported due to a 8-bp deletion in the exon 7 of badh2 gene located on Chromosome 8S by Sakhtivel *et al.* (2009). Multiplex markers targeting the functional InDel polymorphism was earlier reported for genotyping fragrance trait, but the marker was observed to be inconsistent and difficult to use. Sakhtivel *et al.* (2009) developed a simple, co-dominant, functional marker for fragrance trait, which can be resolved in an agarose gel and validated in Basmati and non-Basmati aromatic rice varieties and in a mapping population segregated for fragrance trait. The marker targets the InDel polymorphism in badh2 gene and amplifies 95 and 103 bp fragments in fragrant and non-fragrant genotypes, respectively.The newly developed marker was highly efficient in discriminating all fragrant and non-fragrant genotypes and showed perfect co-segregation with the trait of fragrance in the mapping population. They recommend the use of this simple, low-cost marker in routine genotyping for fragrance trait in large scale breeding materials and germplasm.

Singh *et al.* (2010) reported the discovery and validation of SNPs in the BADH1 gene located on chromosome 4 by re-sequencing of diverse rice varieties differing in aroma and salt tolerance. There were 17 SNPs in introns with an average density of one per 171 bp, but only three SNPs in exons at a density of one per 505 bp. Each of the three exonic SNPs led to changes in amino acids with functional significance. Multiplex SNP assays were used for genotyping of 127 diverse rice varieties and landraces. In total 15 SNP haplotypes were identified but only four of these, corresponding to two protein haplotypes, were common, representing more than 85% of the cultivars. Determination of population structure using 54 random SNPs classified the varieties into two groups broadly corresponding to indica and japonica cultivar groups, aromatic varieties clustering with the japonica group. There was no association between salt tolerance and the common BADH1 haplotypes, but aromatic varieties showed specific association with a BADH1 protein haplotype (PH2) having lysine144 to asparagine144 and lysine345 to glutamine345 substitutions. Protein modeling and ligand docking studies show that these two substitutions lead to reduction in the substrate binding capacity of the BADH1 enzyme towards gamma-aminobutyraldehyde (GABald), which is a precursor of the major aroma compound 2-acetyl-1-pyrroline (2-AP).

Kumari *et al.* (2012) demonstrated that the aroma or fragrance of Basmati rice is associated with the presence and content of the chemical compound, 2-acetyl-1-pyrroline and the trait is monogenic recessive. Several polymerase chain reaction (PCR)-based co-dominant markers based on RG28 locus were developed, which could differentiate between fragrant and non-fragrant rice cultivars. Biochemical analysis of aroma was performed with the 1.7% KOH solution and molecular analysis of aroma was carried out with microsatellite markers present on chromosome 8 (BAD2, BADEX7-5, SCUSSR1) to determine the extent of association between trait, marker and chromosome 8. Among these markers, BAD2 amplified aroma specific alleles having 256 bp in 72 lines, BADEX7-5 with 95 bp in 74 lines and SCUSSR1 with 129 bp in 79 lines. Aromatic and non-aromatic lines were almost common in three markers, indicating association of markers with the trait and chromosome 8. The results reveal that these markers could be used for marker assisted selection and RIL population for mapping of aroma QTLs/genes.

Myint *et al.* (2012) observed peculiar rice varieties in Myanmar, in terms of classification in varietal groups and of grain quality, focused on Myanmar varieties and analyzed variations at 19 microsatellite loci as well as sequences of the aroma gene. Microsatellites were able to retrieve the well-established classification into Indica (isozyme group 1), Japonica (group 6, comprising temperate and tropical forms) and specific groups from the Himalayan foothills including some Aus varieties (group 2) and some aromatic varieties (group 5). They revealed a new cluster of accessions close to, but distinct from, non-Myanmar varieties in group 5. With reference to earlier terminology, they propose to distinguish a group “5A” including group 5 varieties from the Indian subcontinent (South and West Asia) and a group “5B” including most group 5 varieties from Myanmar. In Myanmar varieties, aroma was distributed in group 1 (Indica) and in group 5B. New BADH2 variants were found. Some accessions carried a 43 bp deletion in the 3’ UTR that was not completely associated with aroma. Other accessions, all of group 5B, displayed a particular BADH2 allele with a 3 bp insertion and 100% association with aroma. With the new group and the new alleles found in Myanmar varieties, study shows that the Himalayan foothills contain series of non-Indica and non-Japonica varietal types with novel variations for useful traits.

Nadaf *et al.* (2014) reported 2-acetyl-1-pyrroline as major compound responsible for pleasant aroma in basmati and other scented rice varieties. The biosynthesis of this molecule is due to deletion in the betaine aldehyde dehydrogenase2 gene. This deletion leads in the accumulation of Δ^{1}-pyrroline which reacts non-enzymatically with methyl glyoxal to form 2-acetyl-1-pyrroline. Due to non-functionality of this gene that regulates the synthesis of Gamma-amino butyric acid, the plant species synthesizing 2-acetyl-1-pyrroline suffers for the yield losses, sterility and susceptibility to biotic and abiotic stresses. Thus the non-functionality of betaine aldehyde dehydrogenase2 gene coupled with 2-acetyl-1-pyrroline synthesis serves as a metabolic disease.

Shi *et al.* (2014) conducted a hybrid complementation test to demonstrate the association of a new fragrance allele without mutation in the coding region with flavor formation in a fragrant rice variety Nankai 138. The new allele (badh2-p-5'UTR) has a 3-bp deletion in the 5' untranslated region and an 8-bp insertion in the promoter (-1,314 site upstream from the initiation codon). Surprisingly, they also found that there is also an 8-bp insertion in the promoter of the badh2-E7 allele. A new sequence tagged site functional marker was developed to identify the badh2-p-5'UTR and badh2-E7 alleles according to the 8-bp insertion in their promoters. A cleaved amplified polymorphic sequence (AluI) functional marker targeting a common base substitution in the intron 2 of three badh2 alleles, viz. badh2-p- 5'UTR, badh2-E7 and badh2-E2, was developed to identify diverse genotypes for fragrance in rice. Based on the results of sequence alignments among the three badh2 alleles, they suggested that the badh2-E7 and badh2-p-5'UTR alleles may have the same genetic origin. In addition, the genetic distance between the badh2-E7 and badh2-p-5'UTR alleles may be closer than that between the badh2-E2 and the badh2-p- 5'UTR alleles, or between the badh2-E2 and the badh2-E7 alleles.

Tang *et al.* (2014) demonstrated a pivotal role of *OsBADH1* in stress tolerance without altering glycine betaine biosynthesis capacity, using the RNA interference (RNAi) technique. As an important osmoprotectant, glycine betaine (GB) plays an essential role in resistance to abiotic stress in a variety of organisms, including rice (*Oryza sativa* L.). However, glycine betaine content is too low to be detectable in rice, although rice genome possesses several orthologs coding for betaine aldehyde dehydrogenase (BADH) involved in plant GB biosynthesis. Rice BADH1 (OsBADH1) has been shown to be targeted to peroxisome and its overexpression resulted in increased glycine betaine biosynthesis and tolerance to abiotic stress. In this study, *OsBADH1* was ubiquitously expressed in different organs, including roots, stems, leaves and flowers. Transgenic rice lines downregulating *OsBADH1* exhibited remarkably reduced tolerance to NaCl, drought and cold stresses. The decrease of stress tolerance occurring in the *OsBADH1-RNAi* repression lines was associated with an elevated level of malondialdehyde content and hydrogen peroxidation. No glycine betaine accumulation was detected in transgene-positive and transgene-negative lines derived from heterozygous transgenic to plants. Moreover, transgenic *OsBADH1-RNAi* repression lines showed significantly reduced seed set and yield. In conclusion, the downregulation of *OsBADH1*, even though not causing any change of glycine betaine content, was accounted for the reduction of ability to dehydrogenate the accumulating metabolism-derived aldehydes and subsequently resulted in decreased stress tolerance and crop productivity. These results suggested that OsBADH1 possesses an enzyme activity to catalyze other aldehydes in addition to betaine aldehyde (the precursor of GB) and thus alleviate their toxic effects under abiotic stresses.

Wettewa *et al.* (2014) revealed that the gene responsible for the fragrance in rice is Betaine aldehyde dehydrogenase (badh2) gene. An 8-bp deletion and three single nucleotide polymorphisms in the exon 7 of badh2 gene, named as badh2.1 allele, was reported to result in accumulation of a major aromatic compound, 2-acetyl 1-pyrroline (2AP) in fragrant rice. Although badh2.1 is the predominant allele in virtually all fragrant varieties, exceptions such as involvement of another genetic loci or allele had been reported by several researchers. This study was conducted aiming at detecting presence or absence of badh2.1 allele in popular traditional Sri Lankan fragrant rice varieties. A 463-bp DNA fragment was amplified covering 7th exon region and sequenced. The results showed that “Lanka Samurdi”, the fragrant high yielding variety, possesses the 8bp deletion while highly fragrant three traditional rice varieties did not show the particular mutation. Hence it confirms that the aroma in most of Sri Lankan traditional fragrant varieties is not resulted by the badh2.1 allele genetic factor but by another factor.

Chapter 3

MATERIALS AND METHODS

The present investigation entitled “Isolation, Sequencing, *In silico* analysis of *badh* homologues/alleles and validation of DNA Markers for MAS in Aromatic Rice (*Oryza sativa* L.)” was carried out in the Centre for Plant Biotechnology, Division of Biotechnology, SKUAST-Kashmir, Shalimar campus. In this chapter, details of different materials used and methodologies followed for the study have been described.

3.1 Experimental material

The basic material for the present study consisted of 10 genotypes of rice (*Oryza sativa* L.).

3.2 Seed Germination

- Seeds of each sample were put inside a loose muslin cloth pouch. Seeds were loosely packed to allocate space for seeds to germinate.
- Pouches were tied together using a wire and put in a water bath.
- Temperature of water bath was set at 34°C for day and 11°C at night till radicles emerged.
- Water of the bath was changed daily to prevent accumulation of inhibitory substance that hampered seed germination.

After germination seedlings were transferred to pots in green house and were regularly watered twice a day to maintain submerged conditions. Temperature of green house was maintained at 30°C and then 20 days old seedlings were transplanted to field. Genotypes used in the study both fragrant and non-fragrant ones were grown in the experimental plot of SKUAST-K Shalimar in Kharief season 2014 and subsequently the plant material (leaf) was procured from experimental field. The list of selected genotypes are given in Table-1 and Plate-1.

Table- 1: Rice genotypes used in the present study

S. No.	Genotypes
1.	Mushkbudji Sel. 3
2.	Mushkbudji Acc. F
3.	Mushkbudji Sel. 6
4.	Kamad? (Sel_2009)
5.	Pusa Sugandh-3
6.	Basmati-1509
7.	Jehlum
8.	Shalimar Rice-2
9.	Kawa Kreed
10.	Loul Anzul

3.3 Olfactory evaluation of fragrance

Fragrance was evaluated according to Berner and Hoff (1986). The phenotype of all the genotypes were classified as fragrant or non-fragrant by tasting dehulled seed. At least 12 seeds from individual plants were chewed individually. Scoring for grain aroma was done by 5 individuals on an arbitrary scale of 0-5, with five as most aromatic and 0 as no aroma. The results were pooled to classify the genotypes into fragrant/ non-fragrant rice.

3.4 Isolation of DNA from rice

Fresh and young leaf samples were collected from 18 day old seedlings of rice cultivars and used for the isolation of genomic DNA. Harvested leaves were placed in glassine bags transported in ice and stored in -20°C. Plant DNA was

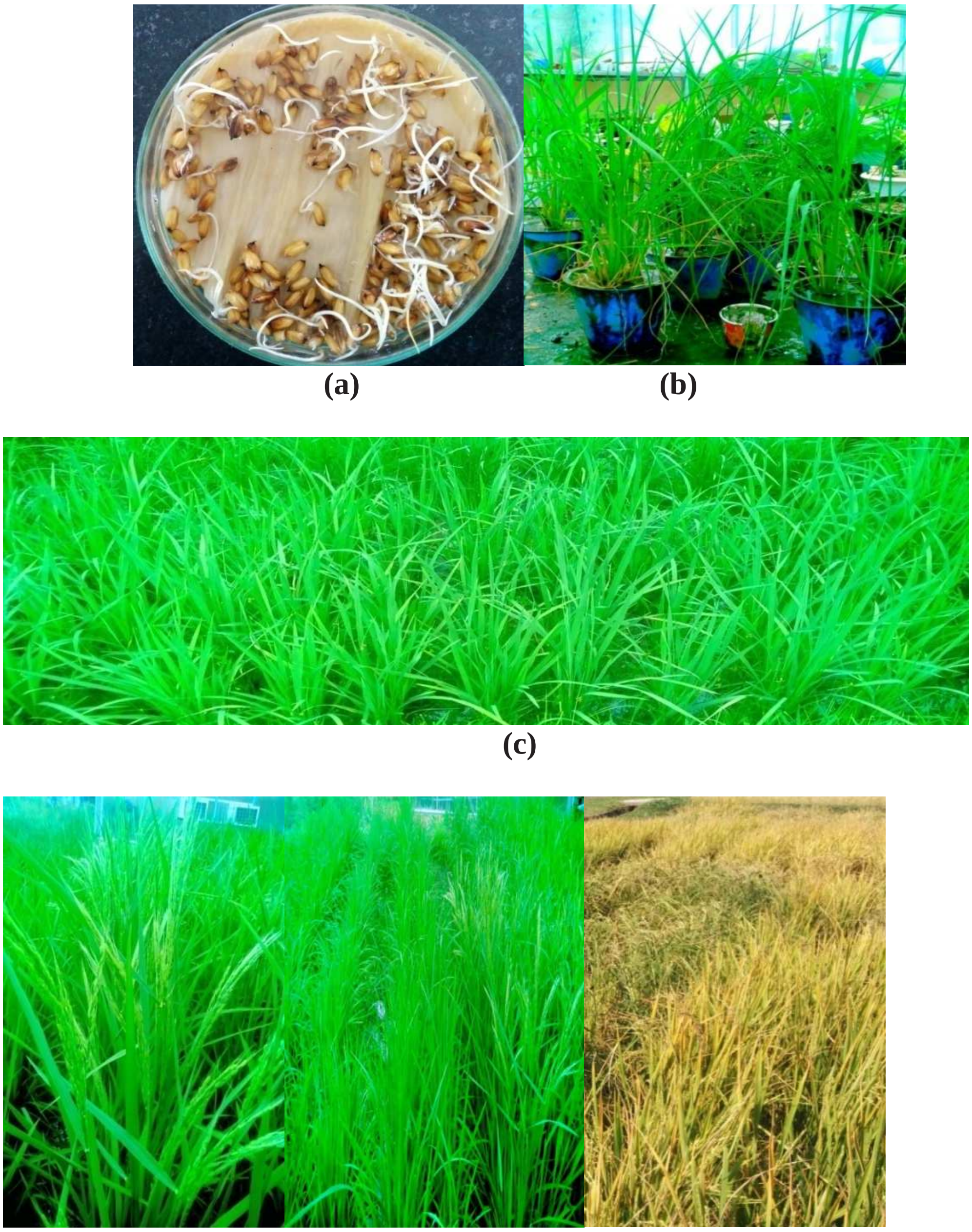
Jehlum **Shalimar Rice 2**

MushkBudji Sel. 3 **MushkBudji Acc. F**

Kamad? (Sel_2009) **Basmati 1509**

Pusa Sugandh-3 **MushkBudji Sel. 6**

Plate 1: Seeds of varieties selected for study



(a) (b)

(c)

(d) (e) (f)

Plate 2: Stages of growth a) Seed germination b) Tillering stage c) Panicle initiation d) Flowering stage e) Maturity stage

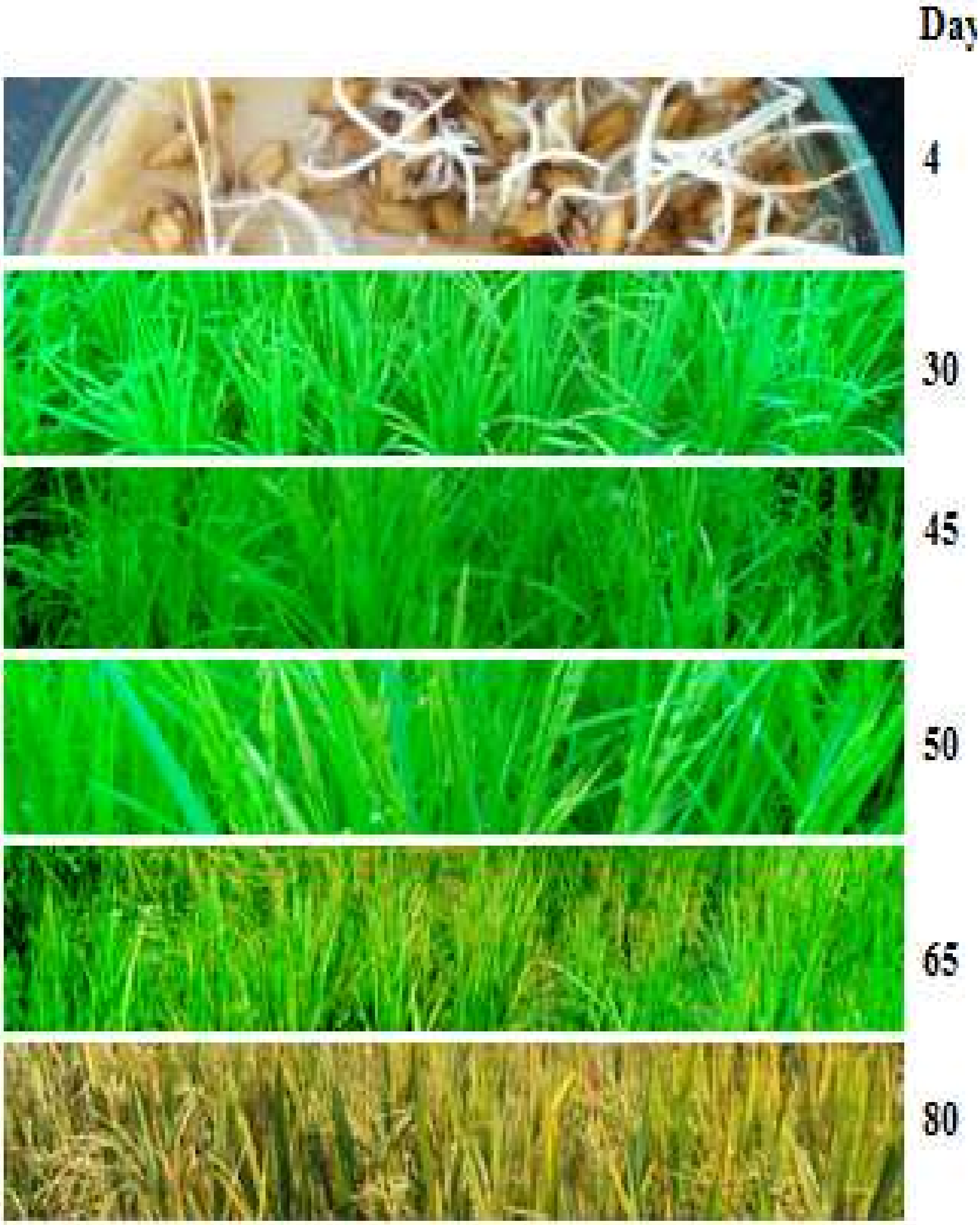


Plate 3: Growth phase of Mushk Budji

isolated using CTAB (Cetyl Trimethyl Ammonium Bromide) method as modified by Saghai-Maroof *et al.* (1994).

Preparation of Reagents

1M Tris (pH 8): 121.1g of Trizma base was added to about 800 ml distilled water. Solution was stirred vigorously using a magnetic stirrer. pH was adjusted to 8.0 by adding concentrated HCl. Solution was allowed to cool at room temperature and volume of solution was adjusted to 1 litre with double distilled water. Final solution was sterilized by autoclaving.

0.5M EDTA (pH 8): 186.1g of Na_2EDTA was added to about 800ml distilled water. Solution was stirred vigorously using a magnetic stirrer. 20 g of NaCl pellets were added for dissolution of EDTA. pH was adjusted to 8.0 by adding NaOH pellets. Solution was allowed to cool at room temperature and volume of solution was adjusted to 1 litre with double distilled water. Final solution was sterilized by autoclaving.

5M NaCl: 292 g of NaCl was added to about 800ml distilled water and final solution was made upto 1 litre. Solution was autoclaved at 121°C for 20 minutes.

1X TE: 1 ml of 1M Tris was taken in a volumetric flask and then 0.2 ml of EDTA was added to it and final volume was made upto 100ml by adding double distilled water. Solution was autoclaved at 121°C for 20 minutes.

50X TAE: 242 g of tris base (MW 121.1 g) was mixed with stir bar to dissolve in 600 ml of double distilled water. Then 100 ml of 0.5 M EDTA and 57.1 ml of Glacial acetic acid was added and pH was maintained to 8. Final volume was raised to 1 litre with double distilled water.

1X TAE: 100ml of 1X TAE was prepared by adding 2ml 50X TAE to 98 ml double distilled water.

Table 2: Composition of 1 litre 2X CTAB Extraction Buffer

Components	Grams/Volume added	Final concentration
CTAB	20g	2%
1M Tris HCl	100 ml	0.1M
5M NaCl	280 ml	1.4 M
0.5M EDTA	40 ml	0.02M
PVP	10 g	1%
ddH$_2$O	570 ml	

Procedure:

1. Healthy portions of youngest leaves were cut apart with sterile scissors and forceps.
2. Leaves were then washed well with sterile distilled water and ethanol and dried on fresh tissue paper to remove spore of micro-organisms and other DNA contaminants.
3. The leaves (0.5g) were ground to fine powder using pre-chilled pestle and mortar after adding liquid nitrogen to make leaves brittle as well as to stop DNase activity.
4. The powder was transferred immediately to a 2 ml autoclaved microfuge tube containing 1.0 µl of β-mercaptoethanol and 700 µl of pre-warmed (65°C) 2X CTAB extraction buffer. The composition of extraction buffer is given in Table 2.
5. The powder was suspended in the buffer by inverting and rotating the tubes properly.
6. The tubes were incubated at 65°C for 30-40 minutes in a water bath. The samples were mixed occasionally while maintaining a temperature of 65 ±1°C.
7. After incubation, equal volume of chloroform: isoamyl alcohol (24:1) was added and tubes were swirled gently for 15 minutes to ensure mixing

of contents followed by centrifugation at 5000 rpm for 10 minutes at room temperature.

8. The aqueous phase was transferred to another clean microfuge tube followed by addition of equal volume isopropanol and the tubes were inverted gently several times.
9. The DNA formed white cotton like precipitate and good quality DNA floated atop. The floating DNA was hooked out using a sterile hooked Pasteur pipette.
10. If the DNA was not hookable, it was pelleted by centrifugation 12000 rpm for 10 minutes.
11. The pelleted DNA was transferred into a clean sterile 2.0 ml microfuge tubes and was rinsed with 500 µl 70 per cent ethanol for five minutes so as to remove any residual salts followed by re-centrifugation 5000 rpm for 5 minutes.
12. Pellet was collected and the left over ethanol was dried up completely by turning down microfuge tubes on a blotting paper and was allowed to air dry (at room temperature) for one hour.
13. Then 50 µl of 1X TE was added. The tubes were left for few hours at room temperature and intermittently taped to allow DNA to dissolve.

3.5 RNase Treatment for Purification of DNA:

DNA and RNase were preheated in water bath maintained at temperature of 37°C for 15 mins.1 µl of RNase was added to 100 µl of DNA. Then the treated samples were incubated at 37°C for 1 hour in water bath. After incubation an aliquot was checked on 0.8% gel for confirmation.

3.6 Assessment of quality and quantity of DNA

Quantity of DNA was checked by Agarose gel electrophoresis.

1. 0.8 g of agarose was dissolved in 100 ml of 1X TAE electrophoresis buffer.

2. The mixture was heated till the agarose dissolved completely i.e. when solution became transparent and clear.
3. It was cooled down to 60°C at room temperature.
4. Ethidium bromide was added to a final concentration of 0.5µg/ml of buffer.
5. The agarose solution was poured into an already prepared gel mould with combs and was left for 20-30 min for solidification.
6. DNA samples for loading were prepared by adding 3 µl loading dye (1X) (0.25% w/v bromophenol blue, 50% glycerol in sterile water) to 3 µl DNA.
7. The DNA samples were loaded into wells with the help of micropipette. Along with the DNA samples, marker of known concentration (uncut λ DNA of 50 ng/µl concentration) was also loaded. The gel was run for about 1-2 hours at voltage of 5 V/cm.
8. The gel was then visualized under UV trans illuminator. Using photo Gel - documentation system and the DNA samples were photographed.
9. The intensity of fluorescence of each sample was indicative of the DNA concentration of each sample.
10. The purity of the DNA samples was ascertained by spectrophotometric method. The ratio of absorbance at 260 nm and 280 nm was calculated for each sample. A ratio of 1.7- 1.8 which is generally accepted as pure for DNA was obtained.

The quality of DNA samples was further judged based on whether DNA formed a single high molecular weight band (good quality) or a smear (degraded/ poor quality).

3.7 PCR analysis

3.7.1 Selection of Primers

Based on an extensive literature survey two primer pairs were chosen for PCR amplification of the target sequences (Table 3).

Table 3: PCR amplification of the target sequences

S. No.	Primer designation	Primer description	Primer sequence	Expected product size	Reference
1.	Os BADH2	For amplification of 7th exon region of badh2 gene	(F)5’ACATAGTGACTGGATTAGGTTCTG3’ (R)5’CATCAACATCATCAAACACCACT3’	463 bp covering 7th exon	Wetteva *et al.* (2014)
2.	BADEX 7-5	Gene specific marker that targets the InDel polymorphism in exon 7 of badh2	(F)5’TGTTTTCTGTTTAGGTTGCATT3’ (R)5’ATCCACAGAAATTTGGAAAC3’	95/103 bp	Sakhtivel *et al.* (2009)

3.7.2 Preparation of PCR cocktail

The components used to prepare PCR cocktail for primer Os-Badh2 are mentioned in Table 4. The total volume of PCR cocktail was 50µl for each sample.

Table 4: Stock and final concentration of different components used in PCR

Components	Stock conc.	Volume (µl)	Final conc.
Water	-	12.5	-
PCR buffer	10X*	5.0	1X
$MgCl_2$	25mM	5.0	2.5mM
DNTPs	1mM	10	0.2µM
Os-Badh2 (Forward Primer)	10µM	4.0	0.8µM
Os-Badh2 (Reverse Primer)	10µM	4.0	0.8µM
Taq Polymerase	5U/µl	0.20	1Unit
DMSO	100%	5	10%
DNA template	312.5ng/µl	4	25ng/µl
Total	**50**		

***10X PCR buffer :** 10mM Tris HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01 % Gelatin.

The components used to prepare PCR cocktail for primer BADEX-E7 are mentioned in Table 5. The total volume of PCR cocktail was 20µl for each sample.

Table 5: Stock and final concentration of different components used in PCR

Components	**Stock conc.**	**Volume (µl)**	**Final conc.**
Water	-	9.6	-
PCR buffer	10X*	2.0	1X
$MgCl_2$	25mM	1.2	1.5mM
DNTPs	1mM	4.0	0.2µM
BADEX-E7(Primer Forward)	10µM	1.0	0.5µM
BADEX-E7(Primer Reverse)	10µM	1.0	0.5µM
Taq Polymerase	5U/µl	0.20	1Unit
DNA template	500 ng/µl	1	25ng
Total	**20**		

***10X PCR buffer :** 10mM Tris HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01 % Gelatin.

3.7.3 PCR amplification profile

The PCR tubes were set on the wells of the thermocycler plate. Then the machine was run according to the following set up.

Table-6: Temperature profile used in PCR

Step	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	94	4 min	1
Denaturation	94	15 sec	30
Annealing	50	15 sec	
Elongation	72	30 sec	
Final Extension	72	30 mins	1
Hold	4		

3.7.4 Visualization of PCR products:

To 20 µl of the amplified product 3 µl of 1X loading dye was added. The PCR products were resolved on 3.5 per cent Electrophoresis Matrix low EEO agarose 1 (G Biosciences 92 well on parkway, St, Louis, MO 63043, USA) gel. 20 µl PCR product was loaded in each well. The gel was prepared in 1X TAE buffer. Ethidium bromide was added at concentration of 0.5µg/µl. The gel was run at 5V/cm, visualized under UV light and photographed using BIORAD gel documentation system. 2 µl of 100 bp DNA ladder was used to estimate PCR fragment size.

3.7.5 Sequencing

30 µl PCR product (200ng/µl) of approximately 450-470 bp size amplified by Os BADH2 primer was put in 2ml microfuge tubes and was sent for sequencing to SciGenomics labs, Kakanand-Cochin-Kerala. Primer Os-badh2 R/F (60ng/µl) 20 µl was also sent with the PCR product.

3.7.6 Sequence analysis:

In silico analysis was done using freely available software tools on portals of NCBI (http://www.ncbi.nlm.nih.gov) and ExPasy (www.expasy.org). ClustalW (www.genome.jp/tools/clustalw) and T-COFFEE software were used for creating multiple sequence alignment in the region of interest.

Chapter - 4

EXPERIMENTAL FINDINGS

The present investigation entitled “Isolation, Sequencing, *In silico* analysis of *badh* homologues/alleles and validation of DNA Markers for MAS in Aromatic Rice (*Oryza sativa* L.)” was undertaken to compare allelic variants for the exon 7 of *badh2* gene in fragrant genotypes of Kashmir and to validate functional markers for *badh2* allele which could then be used in breeding of fragrant rice varieties through MAS.

The pertinent information generated on different aspects of the study is presented in this chapter under the following sub-heads:

4.1 Berner and Hoff Test

Results of the test are presented in the Table 7:

Table 7 : Results of Berner and Hoff test

S. No.	Genotypes	Results
1.	Mushkbudji Acc. F	Fragrant
2.	Mushkbudji Sel-6	Fragrant
3.	Mushkbudji Sel-3	Fragrant
4.	Kamad? (Sel-2009)	Non - Fragrant
5.	Pusa Sugandh-3	Fragrant
6.	Basmati-1509	Fragrant
7.	Jehlum	Non-fragrant
8.	Shalimar Rice-2	Non-fragrant
9.	Loul-Anzul	Non-fragrant
10.	Kawa-Kreed	Non-fragrant

Results of Berner and Hoff test showed that all the accessions of Mushkbudji used for study were fragrant. Pusa Sugandh-3 and Basmati-1509 also showed fragrance. Kamad is a well known aromatic landrace of Kashmir, but the genotype that was collected from fields of a farmer in 2009 (which he claimed as Kamad) was found to be non-fragrant. Kawa Kreed, Shalimar Rice -2, Loul Anzul and Jehlum were found to be non-fragrant.

4.2 Allele Mining:

Gene specific marker that targets the InDel polymorphism in exon 7 of *badh2* namely BADEX-7-5 generated a fragment of approximately 96 bp in all fragrant rice accessions selected for this study (Table 7) indicating the presence of the fragrant allele, *badh2.1* (which possesses a 7 bp deletion and three SNPs in exon 7 of chromosome no 8). However BADEX-7-5 marker generated a 103 bp PCR product with the “Jehlum”, “Shalimar Rice 2”, “Loul Anzul” and “Kawa Kreed”, the non-fragrant genotypes. Kamad? (Sel_2009) which failed to show any fragrance in Berner and Hoff test also generated a 103 bp product predicting absence of *badh2.1* allele in this genotype. (Plate No 5)

4.3 Sequencing and *In silico* analysis

For amplification of 7th exon region of badh2 gene, a gene specific primer namely OsBadh2 was used which resulted in the amplification of a 463 bp region covering the target deletion in Exon 7 (Plate No 6). PCR amplified products were sequenced and analyzed using online software programmes. BLAST (Basic Local Alignment Search Tool), an algorithm for comparing primary biological sequence information was used to find regions of similarity between our five query sequences and the NCBI (National Center for Biotechnology Information) database of the biological sequences. Top hits with 100% sequence similarity in the region of interest are shown in (Plate No 9). Nucleotide BLAST analysis was carried out using algorithms BLASTN and MEGABLAST. Multiple sequence alignment created by ClustalW and T-

COFFEE software clearly shows the differences in the region of interest for the five varieties studied (Plate No 8). The expected 8 bp deletion “GATTATGG” and three SNP’s in exon 7 of aromatic rice genotypes Mushkbudji (Oryza sativa japonica), Pusa Sugandh-3 (Oryza sativa indica) can be clearly seen in the multiple sequence alignment, while the non-aromatic varieties Jehlum (Oryza sativa indica), Kawa kreed (Oryza sativa japonica) revealed no such deletion. The so called Kamad? (Sel_2009) also didn’t possess any 8bp deletion, which is consistent with the Berner and Hof test as well as genotyping results and hence clearly falsified the claim of the farmer in question. This shows the usefulness of the molecular techniques against false claims.

Mushk budji Sel-3 **Kamad? Sel2009** **Jehlum**

Shaimar Rice-2 **Pusa Sugandh-3**

Mushk Budji Sel-6 **Mushk Budji Acc.**

Plate 4: Panicle shape of the harvested genotypes

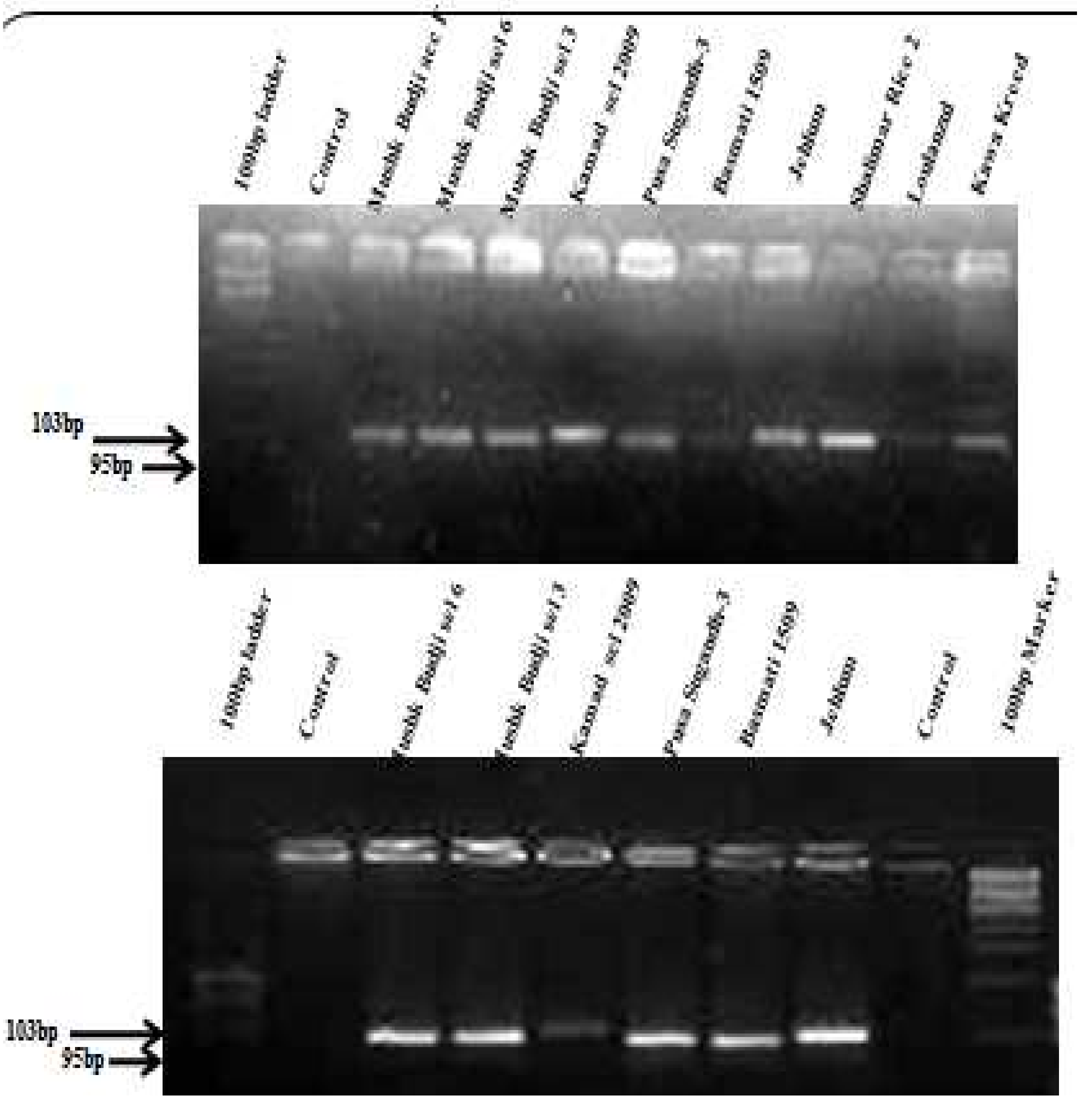


Plate 5: **PCR amplification of fragrant and non-fragrant rice varieties using the molecular marker BADEX7-5. Genomic DNA of all the tested fragrant rice varieties amplified a 95 bp fragment as compared to 103 bp in the non-fragrant varieties.**

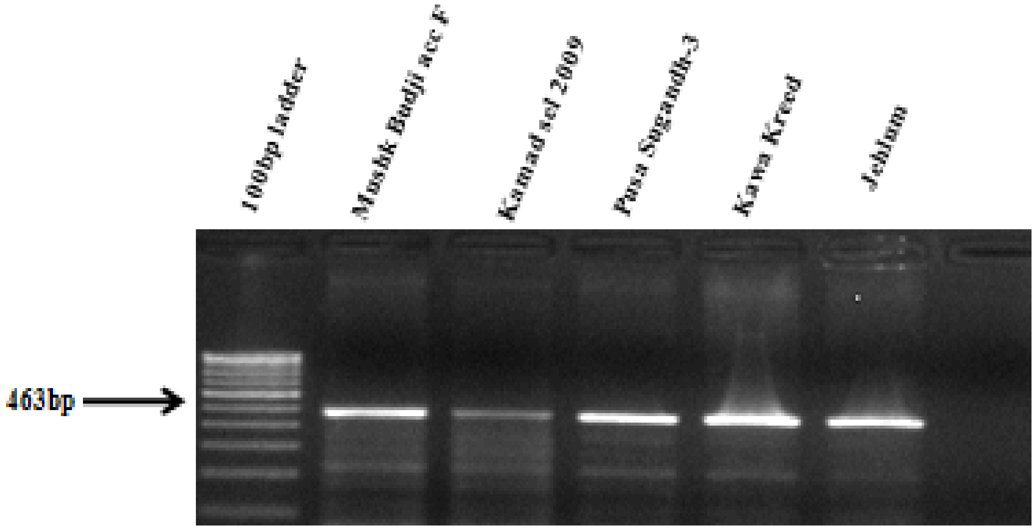


Plate 6: **PCR amplification of fragrant and non-fragrant rice varieties using *Os*Badh2.463 bp amplicons of Exon-7 of fragrant rice genotypes were generated by Osbadh2 specific primers.**

>Seq1[Organism=Oryza sativa] [Sub Species=japonica] [Country=India] [Altitude=1850m] [Cultivar=**Mushk Budji acc F.**]

T**ATG**TTTTCTGTTAG**GTTGCATTTACTGGGAGTTATGAAACTGGTATATATTTCAGCTGCTCCTATGGTTA**AGGTTTGTTTCCAAATT
TCTGTGGATATTTTTTGTTCTCTTTCTACTAACTCTCTATTATCAATTCTCAATGTTGTCCTTTTCTTTTAACTCCTTTACTTTTTAGAATTGT
GATCAAGACACTTTGAGCATCATTCTAGTAGCCAGTTCTATCCTGTTTCTTACCTTTTTATGGTTCGTCTTTTCTTGACAGCCTGTTTCACT
GGAACTTGGTGGAAAAAGTCCTATAGTGGTGTTTGA

>Seq1[Organism=Oryza_2_ORF1 Translation of Seq1[Organism=Oryza in frame 2, ORF 1, threshold 1, 52aa
MFSVRLHLLGVMKLVYISAAPMVKVCFQISVDIFCSLSTNSLLSILNVVLFF

> Seq2[Organism=Oryza sativa] [Sub Species=indica] [Country=India] [Altitude=1540m] [Cultivar=**Pusa_ Sugandh-3**]

CTCAATGGGGCTGATGAGTTGATGATGTTACATAGTAGACTGAACTATGCCTCCTGTAATCATGTATACCCCATCAATGGAAATGATAT
TCCTCTCAATAC**ATG**GTTT**ATG**TTTTCTGTTAG**GTTGCATTTACTGGGAGTTATGAAACTGGTATATATTTCAGCTGCTCCTATGGTT
A**AGGTTTGTTTCCAAATTTCTGTGGATATTTTTTGTTCTCTTTCTACTAACTCTCTATTATCAATTCTCAATGTTGTCCTTTTCTTTTAACTC
CTTTACTTTTTAGAATTGTGATCAAGACACTTTGAGCATCATTCTAGTAGCCAGTTCTATCCTGTTTCTTACCTTTTTATGGTTCGTCTTTT
CTTGACAGCCTGTTTCACTGGAACTTGGTGGAAAAAGTCCTATAGTGAAAAATAGATGATGTTGATGAT

>Seq2[Organism=Oryza_1_ORF1 Translation of Seq2[Organism=Oryza in frame 1, ORF 1, threshold 1, 88aa
LNGADELMMLHSRLNYASCNHVYPINGNDIPLNTWF**MFSVRLHLLGVMKLVYISAAPMVK**VCFQISVDIFCSLSTNSLLSILNVVLFF

> Seq3[Organism=Oryza sativa] [Sub Species=japonica] [Country=India] [Cultivar=**Kamad?sel 2009**]

CCCATTCGCTGATGATGTTGATGATGTTACATAGTGACTGGATTAGGTTCTGCTCAGTGGTGTTTGATGATGTTGATG**ATG**TTACATA
CCGACCGGTTTTGGTTCTGCACATTCTGTTAT**GTTGCCTTTACTGGGAGTTATGAAACTGGTAAAAAGATTATGGCTTCAGCTGCT
CCTATGGTTA**AGGTTTGTTTCCAAATTTCTGTGGATATTTTTTGTTCTCTTTCTACTAACTCTCTATTATCAATTCTCAATGTTGTCCTTTTC
TTTTAACTCCTTTACTTTTTAGAATTGTGATCAAGACACTTTGAGCATCATTCTAGTAGCCAGTTCTATCCTGTTTCTTACCTTTTTATGGT
TCGTCTTTTCTTGACAGCCTGTTTCACTGGAACTTGGTGGAAAAAGTCCTATAGTGGTGTTTGATGATGTTGATGATAAA

>Seq3[Organism=Oryza_1_ORF3 Translation of Seq3[Organism=Oryza in frame 1, ORF 3, threshold 1, 82aa
CYIVTGLGSAQWCLMMLMMLHTDRFWFCTFCYVAFTGSYETGKKIMASAAPMVKVCFQIS
VDIFCSLSTNSLLSILNVVLFF

>Seq3[Organism=Oryza_1_ORF5 Translation of Seq3[Organism=Oryza in frame 1, ORF 5, threshold 1, 41aa
SRHFEHHSSSQFYPVSYLFMVRLFLTACFTGTWWKKSYSGV

>Seq3[Organism=Oryza_2_ORF5 Translation of Seq3[Organism=Oryza in frame 2, ORF 5, threshold 1, 69aa
CYIPTGFGSAHSVMLPLLGVMKLVKRLWLQLLLWLRFVSKFLWIFFVLFLLTLYYQFSML
SFSFNSFTF

>Seq3[Organism=Oryza_3_ORF1 Translation of Seq3[Organism=Oryza in frame 3, ORF 1, threshold 1, 46aa
HSLMMLMMLHSDWIRFCSVVFDDVDDVTYRPVLVLHILLCCLYWEL

Plate 7: Badh-2 partial genomic DNA sequences of Exon 7 in *Oryza sativa* cultivars (highlighted in yellow)

Contd…

Plate-7 contd…

>Seq3[Organism=Oryza_4_ORF1 Translation of Seq3[Organism=Oryza in frame 4, ORF 1, threshold 1, 46aa
LSSTSSNTTIGLFPPSSSETGCQEKTNHKKVRNRIELATRMMLKVS

>Seq3[Organism=Oryza_5_ORF10 Translation of Seq3[Organism=Oryza in frame 5, ORF 10, threshold 1, 43aa
RQHNRMCRTKTGRYVTSSTSSNTTEQNLIQSLCNIINIISEWX

>Seq3[Organism=Oryza_6_ORF7 Translation of Seq3[Organism=Oryza in frame 6, ORF 7, threshold 1, 43aa
KKRTTLRIDNRELVEREQKISTEIWKQTLTIGAAEAIIFLPVS

BLAST-X CONSERVED SEQUENCE: 121 VAFTGSYETGKKIMASAAPMVK 186

> Seq4[Organism=Oryza sativa] [Sub Species=japonica] [Country=India] [Altitude=1850m] [Cultivar=**Kawa-Kreed**]

GCTATTCCTCCTGTAATCATGTATACCCCATCAATGGAAATGATATTCCTCTCAATACATGGTTT**ATG**TTTTCTGTTAG**GTTGCATTTACTGGGAGTTATGAAACTGGTAAAAAGATTATGGCTTCAGCTGCTCCTATGGTTAAG**GTTTGTTTCCAAATTTCTGTGGATATTTTTTGTTCTCTTTCTACTAACTCTCTATTATCAATTCTCAATGTTGTCCTTTTCTTTTAACTCCTTTACTTTTTAGAATTGTGATCAAGACACTTTGAGCATCATTCTAGTAGCCAGTTCTATCCTGTTTCTTACCTTTTTATGGTTCGTCTTTTCTTGACAGCCTGTTTCACTGGAACTTGGTGGAAAAAGTCCTATAGTGGTGT

>Seq4[Organism=Oryza_3_ORF1 Translation of Seq4[Organism=Oryza in frame 3, ORF 1, threshold 1, 81aa

YSSCNHVYPINGNDIPLNTWFMFSVRLHLLGVMKLVKRLWLQLLLWLRFVSKFLWIFFVLFLLTLYYQFSMLSFSFNSFTF

> Seq5[Organism=Oryza sativa] [Sub Species=indica] [Country=India] [Altitude=1600m] [Cultivar=**Jehlum**]

TCGATTCGCTGATGATGTTGATGATGTTACATAGTGACTGGATTAGGTTCTGCTCAGTGGTGTTTGATGATGTTGATG**ATG**TTACATAGGGACTGGATTAGGTTCAGCTCAGGGGGGTTT**GATGATGTTAATAATATTAATGAGCTGGGGAAAGATTATGGCTTCAGCTGCTCCTATGGTTAAG**GTTTGTTTCCAAATTTCTGTGGATATTTTTTGTTCTCTTTCTACTAACTCTCTATTATCAATTCTCAATGTTGTCCTTTTCTTTTAACTCCTTTACTTTTTAGAATTGTGATCAAGACACTTTGAGCATCATTCTAGTAGCCAGTTCTATCCTGTTTCTTACCTTTTTATGGTTCGTCTTTTCTTGACAGCCTGTTTCACTGGAACTTGGTGGAAAAAGTCCTATAGTGGTGTTTGATGATGTTGATGATG

>Seq5[Organism=Oryza_1_ORF3 Translation of Seq5[Organism=Oryza in frame 1, ORF 3, threshold 1, 52aa
CYIVTGLGSAQWCLMMLMMLHRDWIRFSSGGFDDVNNINELGKDYGFSCSYG

>Seq5[Organism=Oryza_2_ORF5 Translation of Seq5[Organism=Oryza in frame 2, ORF 5, threshold 1, 63aa
CYIGTGLGSAQGGLMMLIILMSWGKIMASAAPMVKVCFQISVDIFCSLSTNSLLSILNVV

>Seq5[Organism=Oryza_2_ORF7 Translation of Seq5[Organism=Oryza in frame 2, ORF 7, threshold 1, 41aa
SRHFEHHSSSQFYPVSYLFMVRLFLTACFTGTWWKKSYSGV

>Seq5[Organism=Oryza_3_ORF6 Translation of Seq5[Organism=Oryza in frame 3, ORF 6, threshold 1, 47aa
AGERLWLQLLLWLRFVSKFLWIFFVLFLLTLYYQFSMLSFSFNSFTF

Contd…

Plate-7 contd…

```
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frame 4, ORF 7, threshold 1, 52aa
KKRTTLRIDNRELVEREQKISTEIWKQTLTIGAAEAIIFPQLINIINIIKPP

>Seq5[Organism=Oryza_5_ORF1   Translation  of  Seq5[Organism=Oryza  in
frame 5, ORF 1, threshold 1, 45aa
SSTSSNTTIGLFPPSSSETGCQEKTNHKKVRNRIELATRMMLKVS
```

BLAST-X CONSERVED SEQUENCE:372 QPVSLELGGKSPIVVFDDVD 431

CDS is highlighted in Brown (and is based on annotation detail of *Oryza sativa* voucher MSB_6071 with Accession no JQ308421 in NCBI); Gene prediction for exons was done using GeneMark http://exon.gatech.edu/GeneMark/gmhmme.cgi; Sequence translations were generated by EMBOSS Sixpack, which reads a DNA sequence and outputs the three forward and three reverse translations. (https://www.ebi.ac.uk/Tools/st/emboss_sixpack/)

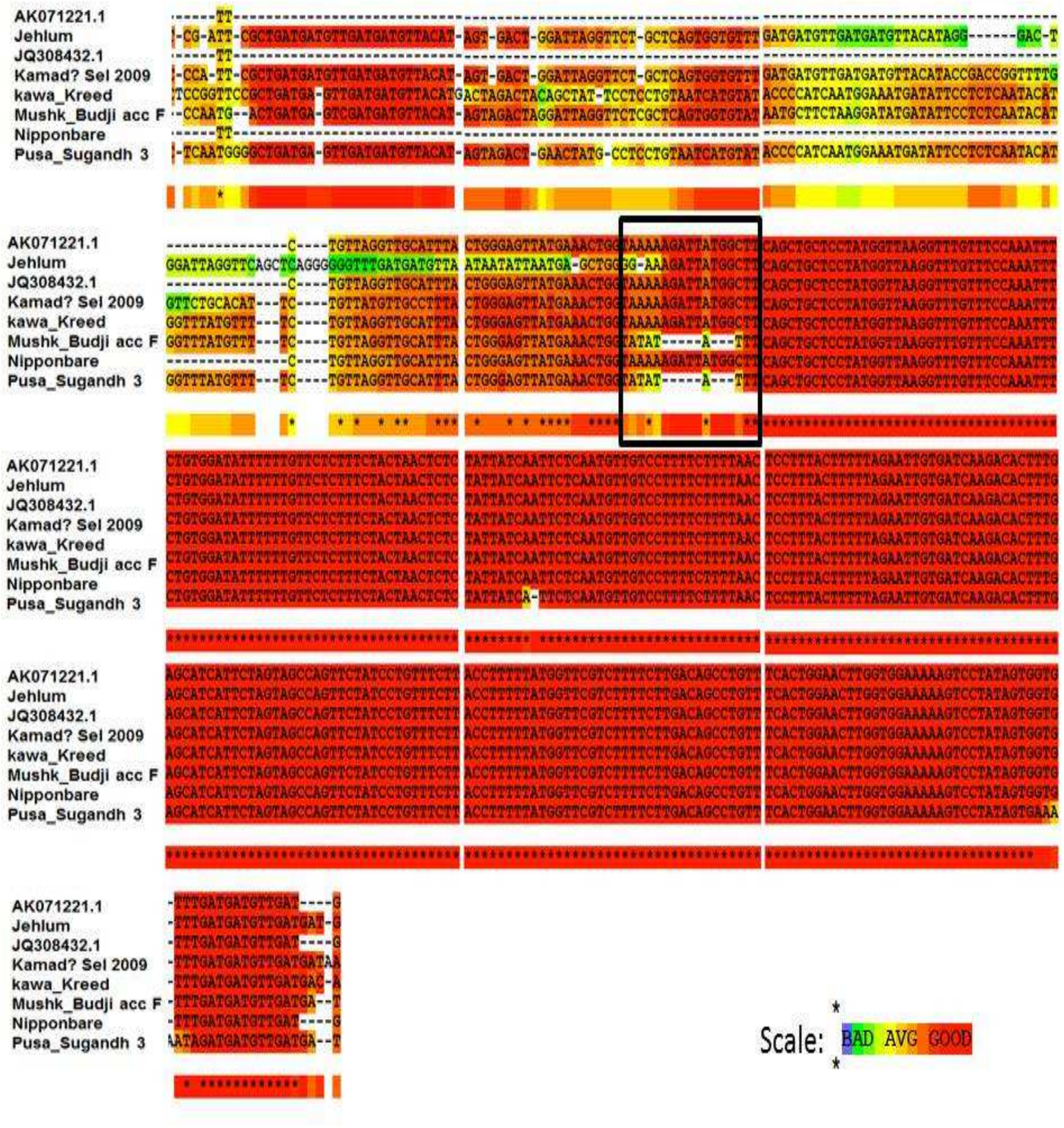
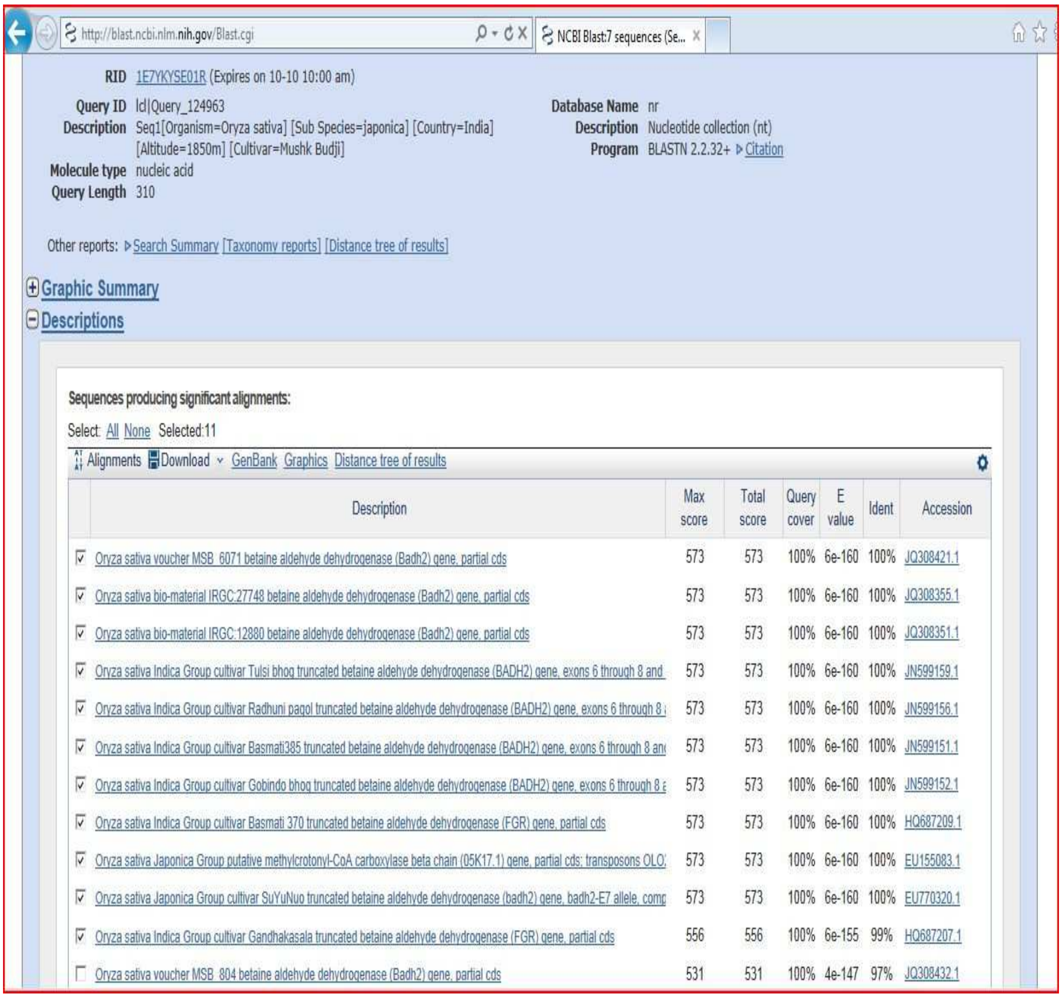


Plate 8: Multiple Sequence Alignment of selected rice genotypes for exon7 of badh-2 gene using T-COFFEE software (8 bp mutation and 3 SNPS are highlighted by black box)



RID 1E7YKYSE01R (Expires on 10-10 10:00 am)

Query ID lcl|Query_124963
Description Seq1[Organism=Oryza sativa] [Sub Species=japonica] [Country=India] [Altitude=1850m] [Cultivar=Mushk Budji]
Molecule type nucleic acid
Query Length 310

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.2.32+ ▷ Citation

Other reports: ▷ Search Summary [Taxonomy reports] [Distance tree of results]

⊞ Graphic Summary
⊟ Descriptions

Sequences producing significant alignments:

Select: All None Selected:11

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☑	Oryza sativa voucher MSB 6071 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	573	573	100%	6e-160	100%	JQ308421.1
☑	Oryza sativa bio-material IRGC:27748 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	573	573	100%	6e-160	100%	JQ308355.1
☑	Oryza sativa bio-material IRGC:12880 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	573	573	100%	6e-160	100%	JQ308351.1
☑	Oryza sativa Indica Group cultivar Tulsi bhog truncated betaine aldehyde dehydrogenase (BADH2) gene, exons 6 through 8 and	573	573	100%	6e-160	100%	JN599159.1
☑	Oryza sativa Indica Group cultivar Radhuni pagol truncated betaine aldehyde dehydrogenase (BADH2) gene, exons 6 through 8	573	573	100%	6e-160	100%	JN599156.1
☑	Oryza sativa Indica Group cultivar Basmati385 truncated betaine aldehyde dehydrogenase (BADH2) gene, exons 6 through 8 an	573	573	100%	6e-160	100%	JN599151.1
☑	Oryza sativa Indica Group cultivar Gobindo bhog truncated betaine aldehyde dehydrogenase (BADH2) gene, exons 6 through 8	573	573	100%	6e-160	100%	JN599152.1
☑	Oryza sativa Indica Group cultivar Basmati 370 truncated betaine aldehyde dehydrogenase (FGR) gene, partial cds	573	573	100%	6e-160	100%	HQ687209.1
☑	Oryza sativa Japonica Group putative methylcrotonyl-CoA carboxylase beta chain (05K17.1) gene, partial cds; transposons OLO	573	573	100%	6e-160	100%	EU155083.1
☑	Oryza sativa Japonica Group cultivar SuYuNuo truncated betaine aldehyde dehydrogenase (badh2) gene, badh2-E7 allele, comp	573	573	100%	6e-160	100%	EU770320.1
☑	Oryza sativa Indica Group cultivar Gandhakasala truncated betaine aldehyde dehydrogenase (FGR) gene, partial cds	556	556	100%	6e-155	99%	HQ687207.1
☐	Oryza sativa voucher MSB 804 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	531	531	100%	4e-147	97%	JQ308432.1

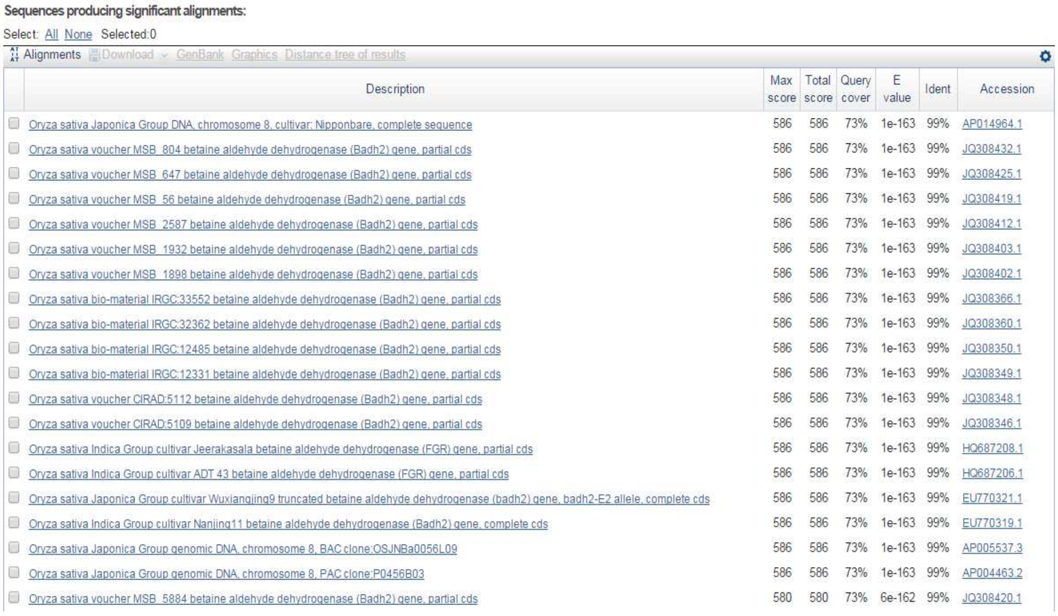
(a)

Plate 9: Top BLAST hits of the query sequences of varieties in NCBI

- **a) Top BLAST hits in NCBI with query sequence as Mushk Budji acc. F.**
- **b) Top BLAST hits in NCBI with query sequence as Kamad? Sel2009**
- **c) Top BLAST hits in NCBI with query sequence as Pusa-Sugandh-3**
- **d) Top BLAST hits in NCBI with query sequence as Kawa-Kreed**
- **e) Top BLAST hits in NCBI with query sequence as Jehlum**

Contd…

Plate 9 contd…

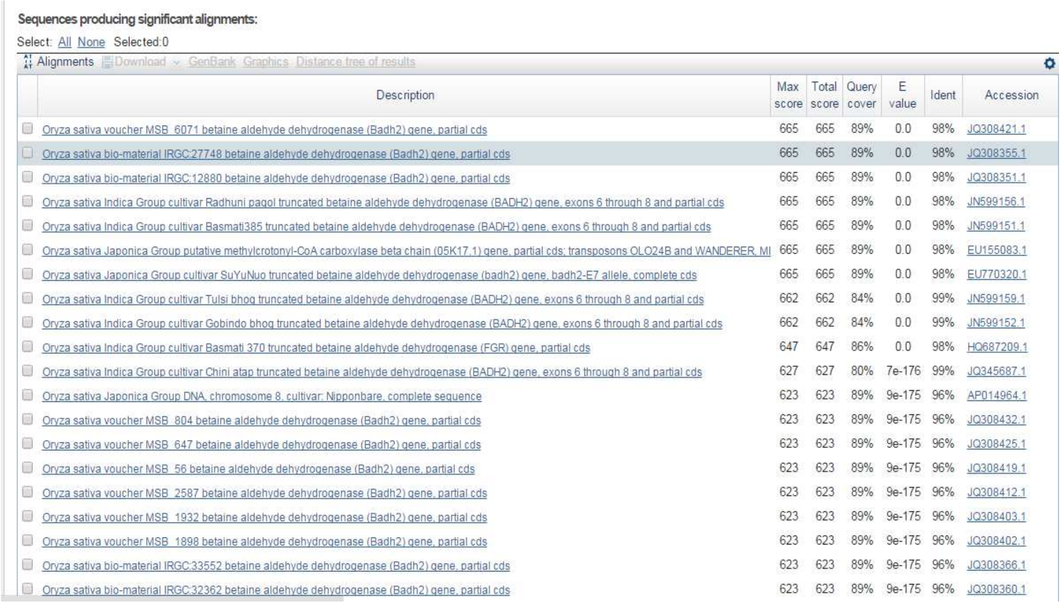


Sequences producing significant alignments:

Select: All None Selected:0

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Oryza sativa Japonica Group DNA, chromosome 8, cultivar: Nipponbare, complete sequence	586	586	73%	1e-163	99%	AP014964.1
☐	Oryza sativa voucher MSB 804 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	586	586	73%	1e-163	99%	JQ308432.1
☐	Oryza sativa voucher MSB 647 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	586	586	73%	1e-163	99%	JQ308425.1
☐	Oryza sativa voucher MSB 56 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	586	586	73%	1e-163	99%	JQ308419.1
☐	Oryza sativa voucher MSB 2587 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	586	586	73%	1e-163	99%	JQ308412.1
☐	Oryza sativa voucher MSB 1932 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	586	586	73%	1e-163	99%	JQ308403.1
☐	Oryza sativa voucher MSB 1898 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	586	586	73%	1e-163	99%	JQ308402.1
☐	Oryza sativa bio-material IRGC:33552 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	586	586	73%	1e-163	99%	JQ308366.1
☐	Oryza sativa bio-material IRGC:32362 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	586	586	73%	1e-163	99%	JQ308360.1
☐	Oryza sativa bio-material IRGC:12485 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	586	586	73%	1e-163	99%	JQ308350.1
☐	Oryza sativa bio-material IRGC:12331 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	586	586	73%	1e-163	99%	JQ308349.1
☐	Oryza sativa voucher CIRAD:5112 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	586	586	73%	1e-163	99%	JQ308348.1
☐	Oryza sativa voucher CIRAD:5109 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	586	586	73%	1e-163	99%	JQ308346.1
☐	Oryza sativa Indica Group cultivar Jeerakasala betaine aldehyde dehydrogenase (FGR) gene, partial cds	586	586	73%	1e-163	99%	HQ687208.1
☐	Oryza sativa Indica Group cultivar ADT 43 betaine aldehyde dehydrogenase (FGR) gene, partial cds	586	586	73%	1e-163	99%	HQ687206.1
☐	Oryza sativa Japonica Group cultivar Wuxiangjing9 truncated betaine aldehyde dehydrogenase (badh2) gene, badh2-E2 allele, complete cds	586	586	73%	1e-163	99%	EU770321.1
☐	Oryza sativa Indica Group cultivar Nanjing11 betaine aldehyde dehydrogenase (Badh2) gene, complete cds	586	586	73%	1e-163	99%	EU770319.1
☐	Oryza sativa Japonica Group genomic DNA, chromosome 8, BAC clone:OSJNBa0056L09	586	586	73%	1e-163	99%	AP005537.3
☐	Oryza sativa Japonica Group genomic DNA, chromosome 8, PAC clone:P0456B03	586	586	73%	1e-163	99%	AP004463.2
☐	Oryza sativa voucher MSB 5884 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	580	580	73%	6e-162	99%	JQ308420.1

(b)



Sequences producing significant alignments:

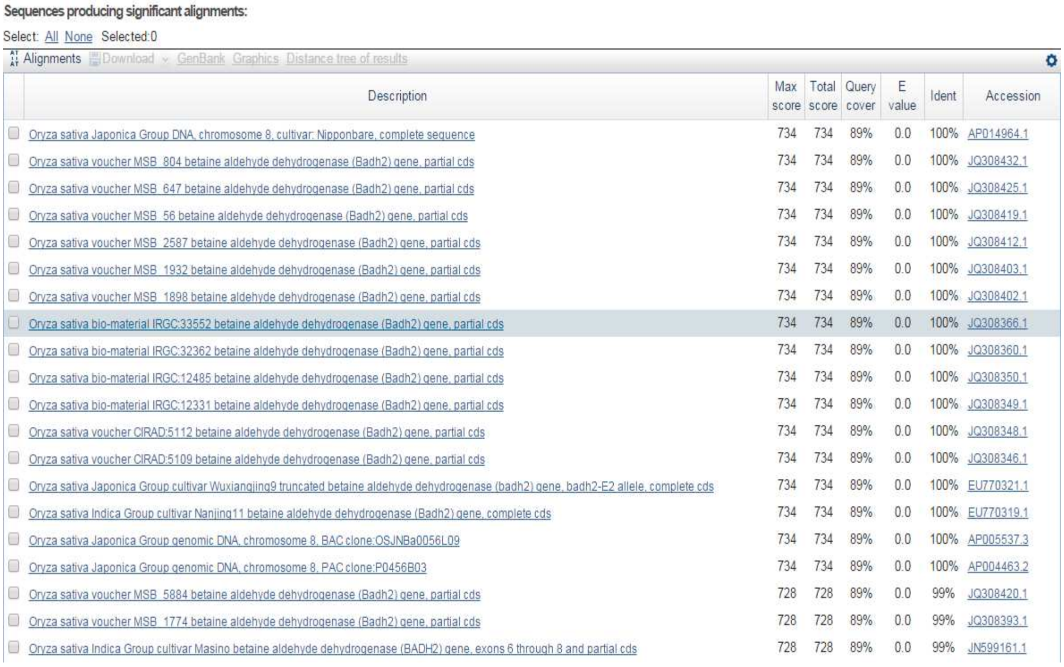
Select: All None Selected:0

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Oryza sativa voucher MSB 6071 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	665	665	89%	0.0	98%	JQ308421.1
☐	Oryza sativa bio-material IRGC:27748 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	665	665	89%	0.0	98%	JQ308355.1
☐	Oryza sativa bio-material IRGC:12880 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	665	665	89%	0.0	98%	JQ308351.1
☐	Oryza sativa Indica Group cultivar Radhuni pagol truncated betaine aldehyde dehydrogenase (BADH2) gene, exons 6 through 8 and partial cds	665	665	89%	0.0	98%	JN599156.1
☐	Oryza sativa Indica Group cultivar Basmati385 truncated betaine aldehyde dehydrogenase (BADH2) gene, exons 6 through 8 and partial cds	665	665	89%	0.0	98%	JN599151.1
☐	Oryza sativa Japonica Group putative methylcrotonyl-CoA carboxylase beta chain (05K17.1) gene, partial cds; transposons OLO24B and WANDERER, MI	665	665	89%	0.0	98%	EU155083.1
☐	Oryza sativa Japonica Group cultivar SuYuNuo truncated betaine aldehyde dehydrogenase (badh2) gene, badh2-E7 allele, complete cds	665	665	89%	0.0	98%	EU770320.1
☐	Oryza sativa Indica Group cultivar Tulsi bhog truncated betaine aldehyde dehydrogenase (BADH2) gene, exons 6 through 8 and partial cds	662	662	84%	0.0	99%	JN599159.1
☐	Oryza sativa Indica Group cultivar Gobindo bhog truncated betaine aldehyde dehydrogenase (BADH2) gene, exons 6 through 8 and partial cds	662	662	84%	0.0	99%	JN599152.1
☐	Oryza sativa Indica Group cultivar Basmati 370 truncated betaine aldehyde dehydrogenase (FGR) gene, partial cds	647	647	86%	0.0	98%	HQ687209.1
☐	Oryza sativa Indica Group cultivar Chini atap truncated betaine aldehyde dehydrogenase (BADH2) gene, exons 6 through 8 and partial cds	627	627	80%	7e-176	99%	JQ345687.1
☐	Oryza sativa Japonica Group DNA, chromosome 8, cultivar: Nipponbare, complete sequence	623	623	89%	9e-175	96%	AP014964.1
☐	Oryza sativa voucher MSB 804 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	623	623	89%	9e-175	96%	JQ308432.1
☐	Oryza sativa voucher MSB 647 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	623	623	89%	9e-175	96%	JQ308425.1
☐	Oryza sativa voucher MSB 56 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	623	623	89%	9e-175	96%	JQ308419.1
☐	Oryza sativa voucher MSB 2587 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	623	623	89%	9e-175	96%	JQ308412.1
☐	Oryza sativa voucher MSB 1932 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	623	623	89%	9e-175	96%	JQ308403.1
☐	Oryza sativa voucher MSB 1898 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	623	623	89%	9e-175	96%	JQ308402.1
☐	Oryza sativa bio-material IRGC:33552 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	623	623	89%	9e-175	96%	JQ308366.1
☐	Oryza sativa bio-material IRGC:32362 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	623	623	89%	9e-175	96%	JQ308360.1

(c)

Contd…

Plate 9 contd…



Sequences producing significant alignments:

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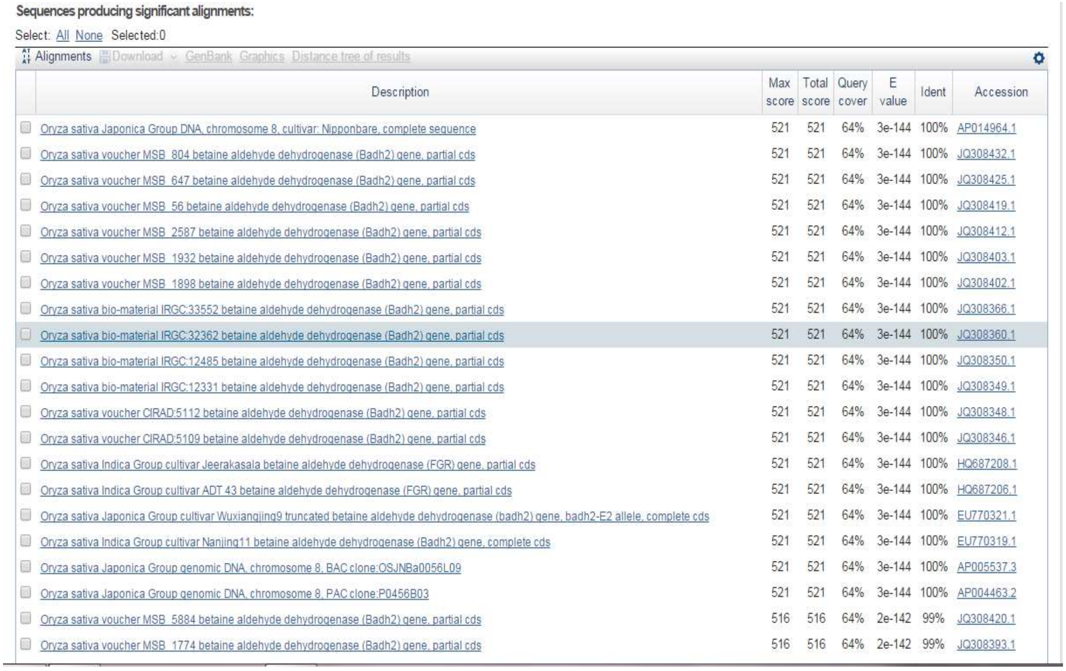
☐	Oryza sativa voucher MSB 804 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	734	734	89%	0.0	100%	JQ308432.1
☐	Oryza sativa voucher MSB 647 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	734	734	89%	0.0	100%	JQ308425.1
☐	Oryza sativa voucher MSB 56 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	734	734	89%	0.0	100%	JQ308419.1
☐	Oryza sativa voucher MSB 2587 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	734	734	89%	0.0	100%	JQ308412.1
☐	Oryza sativa voucher MSB 1932 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	734	734	89%	0.0	100%	JQ308403.1
☐	Oryza sativa voucher MSB 1898 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	734	734	89%	0.0	100%	JQ308402.1
☐	Oryza sativa bio-material IRGC:33552 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	734	734	89%	0.0	100%	JQ308366.1
☐	Oryza sativa bio-material IRGC:32362 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	734	734	89%	0.0	100%	JQ308360.1
☐	Oryza sativa bio-material IRGC:12485 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	734	734	89%	0.0	100%	JQ308350.1
☐	Oryza sativa bio-material IRGC:12331 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	734	734	89%	0.0	100%	JQ308349.1
☐	Oryza sativa voucher CIRAD:5112 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	734	734	89%	0.0	100%	JQ308348.1
☐	Oryza sativa voucher CIRAD:5109 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	734	734	89%	0.0	100%	JQ308346.1
☐	Oryza sativa Japonica Group cultivar Wuxiangjing9 truncated betaine aldehyde dehydrogenase (badh2) gene, badh2-E2 allele, complete cds	734	734	89%	0.0	100%	EU770321.1
☐	Oryza sativa Indica Group cultivar Nanjing11 betaine aldehyde dehydrogenase (Badh2) gene, complete cds	734	734	89%	0.0	100%	EU770319.1
☐	Oryza sativa Japonica Group genomic DNA, chromosome 8, BAC clone:OSJNBa0056L09	734	734	89%	0.0	100%	AP005537.3
☐	Oryza sativa Japonica Group genomic DNA, chromosome 8, PAC clone:P0456B03	734	734	89%	0.0	100%	AP004463.2
☐	Oryza sativa voucher MSB 5884 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	728	728	89%	0.0	99%	JQ308420.1
☐	Oryza sativa voucher MSB 1774 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	728	728	89%	0.0	99%	JQ308393.1
☐	Oryza sativa Indica Group cultivar Masino betaine aldehyde dehydrogenase (BADH2) gene, exons 6 through 8 and partial cds	728	728	89%	0.0	99%	JN599161.1

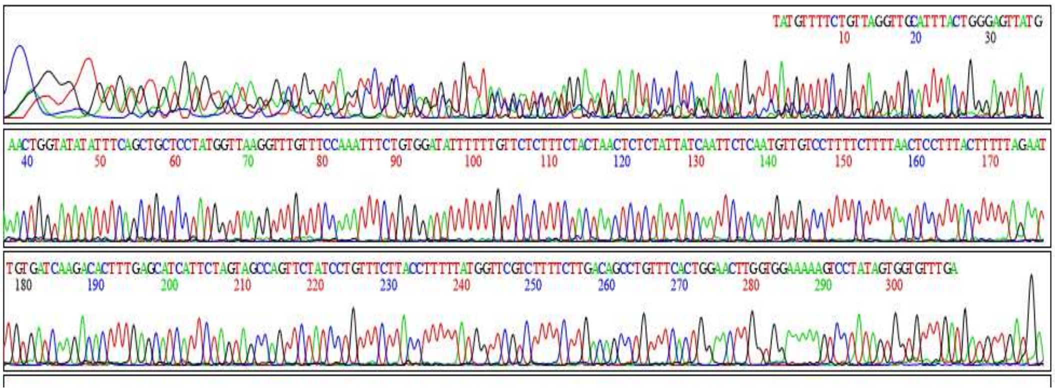
(d)



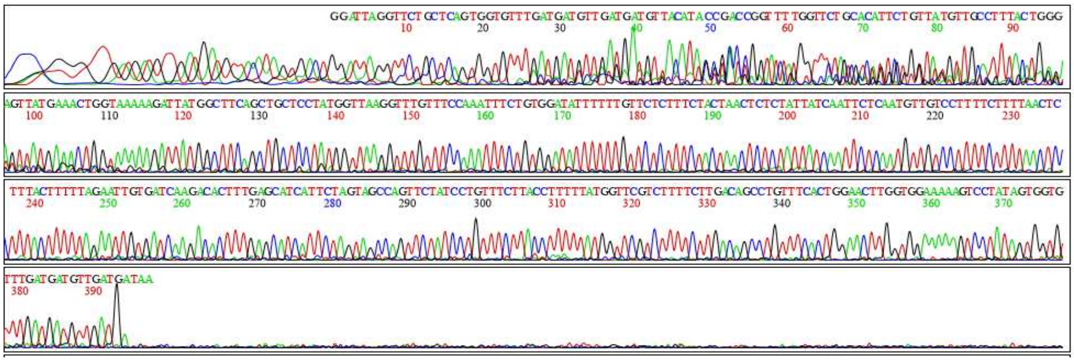
Sequences producing significant alignments:

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Oryza sativa Japonica Group DNA, chromosome 8, cultivar: Nipponbare, complete sequence	521	521	64%	3e-144	100%	AP014964.1
☐	Oryza sativa voucher MSB 804 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	521	521	64%	3e-144	100%	JQ308432.1
☐	Oryza sativa voucher MSB 647 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	521	521	64%	3e-144	100%	JQ308425.1
☐	Oryza sativa voucher MSB 56 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	521	521	64%	3e-144	100%	JQ308419.1
☐	Oryza sativa voucher MSB 2587 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	521	521	64%	3e-144	100%	JQ308412.1
☐	Oryza sativa voucher MSB 1932 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	521	521	64%	3e-144	100%	JQ308403.1
☐	Oryza sativa voucher MSB 1898 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	521	521	64%	3e-144	100%	JQ308402.1
☐	Oryza sativa bio-material IRGC:33552 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	521	521	64%	3e-144	100%	JQ308366.1
☐	Oryza sativa bio-material IRGC:32362 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	521	521	64%	3e-144	100%	JQ308360.1
☐	Oryza sativa bio-material IRGC:12485 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	521	521	64%	3e-144	100%	JQ308350.1
☐	Oryza sativa bio-material IRGC:12331 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	521	521	64%	3e-144	100%	JQ308349.1
☐	Oryza sativa voucher CIRAD:5112 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	521	521	64%	3e-144	100%	JQ308348.1
☐	Oryza sativa voucher CIRAD:5109 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	521	521	64%	3e-144	100%	JQ308346.1
☐	Oryza sativa Indica Group cultivar Jeerakasala betaine aldehyde dehydrogenase (FGR) gene, partial cds	521	521	64%	3e-144	100%	HQ687208.1
☐	Oryza sativa Indica Group cultivar ADT 43 betaine aldehyde dehydrogenase (FGR) gene, partial cds	521	521	64%	3e-144	100%	HQ687206.1
☐	Oryza sativa Japonica Group cultivar Wuxiangjing9 truncated betaine aldehyde dehydrogenase (badh2) gene, badh2-E2 allele, complete cds	521	521	64%	3e-144	100%	EU770321.1
☐	Oryza sativa Indica Group cultivar Nanjing11 betaine aldehyde dehydrogenase (Badh2) gene, complete cds	521	521	64%	3e-144	100%	EU770319.1
☐	Oryza sativa Japonica Group genomic DNA, chromosome 8, BAC clone:OSJNBa0056L09	521	521	64%	3e-144	100%	AP005537.3
☐	Oryza sativa Japonica Group genomic DNA, chromosome 8, PAC clone:P0456B03	521	521	64%	3e-144	100%	AP004463.2
☐	Oryza sativa voucher MSB 5884 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	516	516	64%	2e-142	99%	JQ308420.1
☐	Oryza sativa voucher MSB 1774 betaine aldehyde dehydrogenase (Badh2) gene, partial cds	516	516	64%	2e-142	99%	JQ308393.1

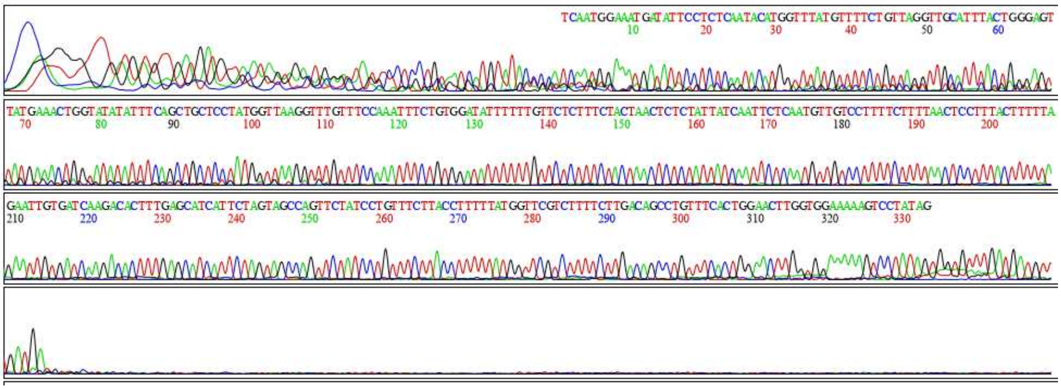
(e)



(a)



(b)



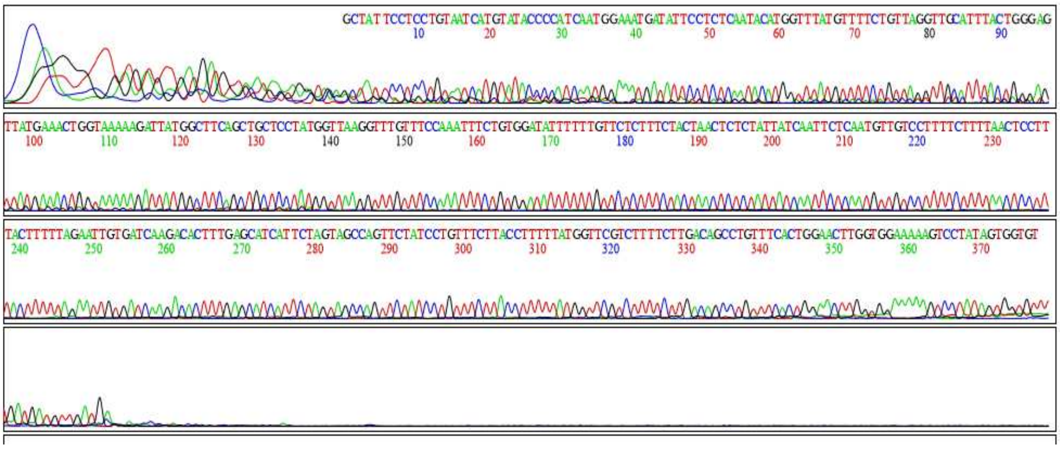
(c)

Plate 10: Sequence chromatograph of different rice varieties

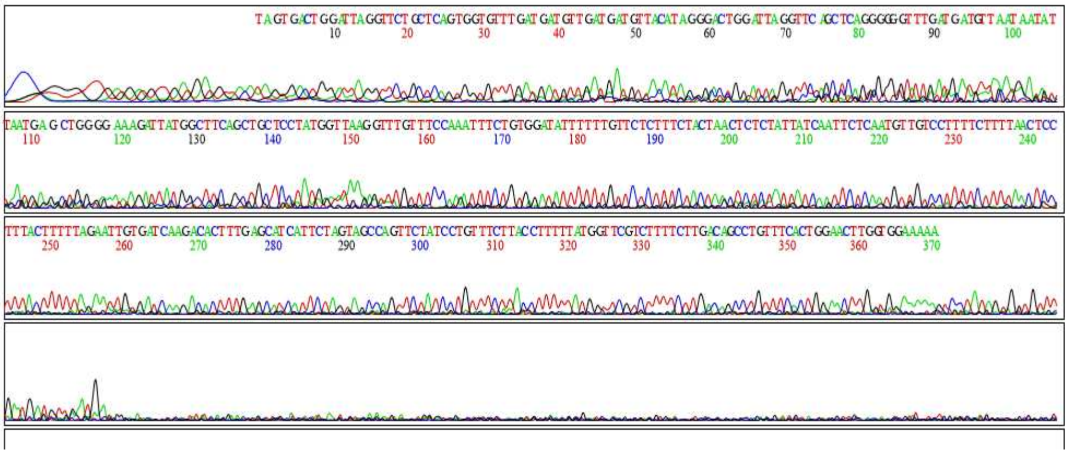
- **a) Sequence chromatograph of Mushk-Budji acc F.**
- **b) Sequence chromatograph of Kamad? Sel2009**
- **c) Sequence chromatograph of Pusa-Sugandh 3**
- **d) Sequence chromatograph of Kawa-Kreed**
- **e) Sequence chromatograph of Jehlum**

Contd…

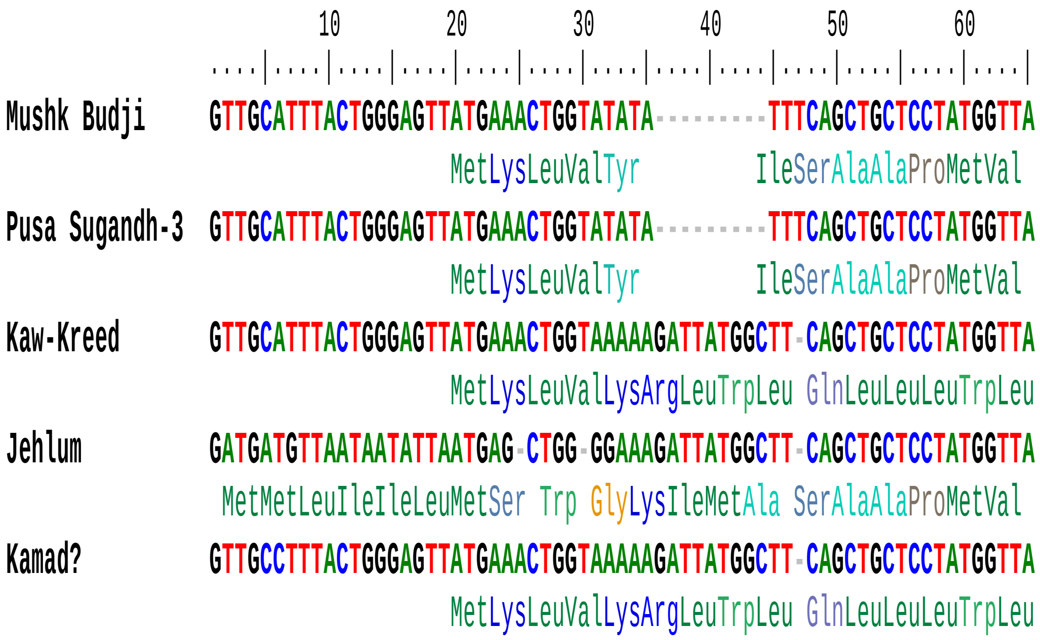
Plate 10 contd…



(d)

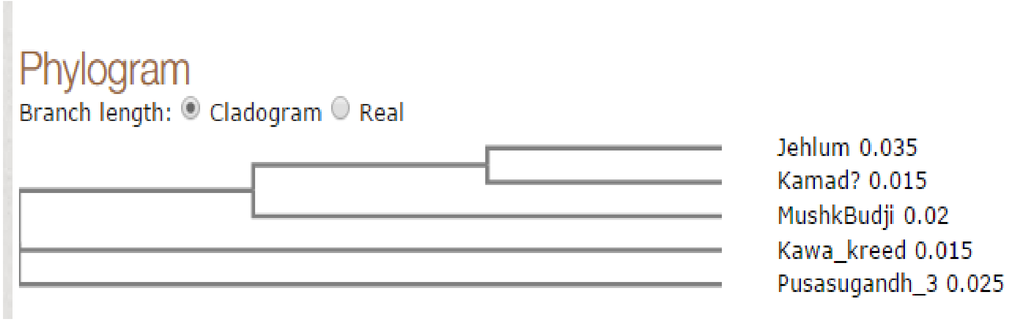


(e)



```
                        10        20        30        40        50        60
               ....|....|....|....|....|....|....|....|....|....|....|....|....|
Mushk Budji    GTTGCATTTACTGGGAGTTATGAAACTGGTATATA---------TTTCAGCTGCTCCTATGGTTA
                                  MetLysLeuValTyr        IleSerAlaAlaProMetVal
Pusa Sugandh-3 GTTGCATTTACTGGGAGTTATGAAACTGGTATATA---------TTTCAGCTGCTCCTATGGTTA
                                  MetLysLeuValTyr        IleSerAlaAlaProMetVal
Kaw-Kreed      GTTGCATTTACTGGGAGTTATGAAACTGGTAAAAAGATTATGGCTT-CAGCTGCTCCTATGGTTA
                                  MetLysLeuValLysArgLeuTrpLeu GlnLeuLeuLeuTrpLeu
Jehlum         GATGATGTTAATAATATTAATGAG-CTGG-GGAAAGATTATGGCTT-CAGCTGCTCCTATGGTTA
                MetMetLeuIleIleLeuMetSer Trp GlyLysIleMetAla SerAlaAlaProMetVal
Kamad?         GTTGCCTTTACTGGGAGTTATGAAACTGGTAAAAAGATTATGGCTT-CAGCTGCTCCTATGGTTA
                                  MetLysLeuValLysArgLeuTrpLeu GlnLeuLeuLeuTrpLeu
```

Plate 11: The predicted amino acids coded by the target sequence based on the most suitable reading-frame



Phylogram

Branch length: ● Cladogram ○ Real

Plate 12: **Phylogram showing genetic distance between different rice varieties**

	Aromatic Rice			Non-Aromatic Rice			
	JAPONICA	INDICA		JAPONICA		INDICA	
	Mushkbudji Accessions	Basmati 1509	Pusa Sugand-3	LoulAnzul	KawaKreed	Jehlum	Shalimar Rice-2
Berner and Hoff Test	Aroma	Aroma	Aroma	No aroma	No aroma	No aroma	No aroma
Marker validation	96 bpamplicon	96 bpamplicon		103 bpamplicon		103 bpamplicon	
Sequencing	8 bp deletion and 3 SNPS in exon 7			No such deletion in exon 7			

Plate 13: A pictorial over view of the major results

Chapter - 5

DISCUSSION

Rice is undoubtedly a dominant and important staple food worldwide. Grain aroma is the most attractive characteristics of high-quality rice. Fragrant rice has been in great demand in the Asian rice market for decades. Aromatic rice varieties constitute a small but special group of rice and have gained greater importance with the world wide increase in the demand for fine quality rice. Aromatic rice varieties also have superior nutritional values and better amino acid profiles. Aroma characteristics were determined by Kovach *et al.* (2009) in three of the distinguished genetic subpopulations of rice: *Group V* (Sadri and Basmati), *indica* (Jasmine), and tropical *japonica*. 2-acetyl-1-pyrroline [2AP; IUPAC name 5-acetyl-3, 4-dihydro- 2*H*-pyrrole] is the compound responsible for the unique and pleasant fragrance of the aromatic rice (Buttery *et al.*, 1982). In present study 10 genotypes were selected out of which Mushkbudji accessions, Kamad? (Sel_2009), Loul Anzul, Kawa Kreed belong to japonica group, while Shalimar Rice 2, Jehlum, Basmati 1509 and Pusa Sugandh 3 belong to Indica group. Mushkbudji and Kamad due to their unique characteristics and special use are very popular in Kashmir (Husaini *et al.*, 2013; Sultan and Subba Rao, 2013). These short bold grain aromatic rices have good cooking and organoleptic qualities (Husaini *et al.*, 2009). Basmati rice on the other hand due to its unique features such as extra long slender grain, lengthwise excessive elongation on cooking, soft and fluffy texture of cooked rice, and pleasant aroma have been characterized as speciality rice (Siddiq *et al.*, 2012). Basmati rices have been found to be rich in micronutrient especially for iron and zinc (Gregorio 2002) and also have low glycemic index (Foster *et al.*, 2002). Basmati is a prized commercial commodity (Aggarwal *et al.*, 2002). In present study the genotypes which were found positive for aroma in preliminary test of Berner and Hoff for aroma include Mushkbudji accessions from japonica group, Basmati 1509 and Pusa Sugandh 3 from Basmati and Sadri group. Due to absence of fragrance, phenotype of all other genotypes were classified as non- fragrant. Surprisingly aroma was not detected in a so-called accession claimed to be Kamad which had been collected from a farmer's field in year 2009. This so called Kamad didn't show any aroma in the fragrance test of Berner and Hoff. However, Mushkbudji not only smells good but also tastes better and has been relished by the local people since ages in Kashmir. Pusa-sugandh 3 which is a released basmati variety from Sugandh series obtained from crossing Pusa 1238-1 with Pusa 1238-81-6 is also a well-known aromatic rice variety (Siddiq *et al.*, 2012).

Fragrance in rice as revealed by genetic analysis suggest that this trait is controlled by recessive monogenic inheritance, independent of cytoplasmic genes. Studies show that some rice varieties are under monogenic control (Yano *et al.,* 1991) whereas, in some others it is found to be a quantitative trait and many genes are involved in its expression (Hien *et al.,* 2006) thus, indicating complex genetic control of the aroma trait. Genetic mapping showed that fgr locus on chromosome 8 is associated with rice fragrance. The recessive fgr imparts fragrance to rice varieties. A number of markers were identified by different workers that are closely linked to fgr (Ahn *et al.*, 1992; Jin *et al.*, 2003). Bradbury *et al.* (2005) sequenced 17 genes in the BAC possessing fgr and found sequence variation in Exon 7 of gene encoding putative betaine aldehyde dehydrogenase (BADH2) in fragrant varieties compared to non-fragrant varieties and suggested that gene encoding putative betaine aldehyde dehydrogenase (BADH2) imparts fragrance to aromatic rice. Chen *et al.* (2006) restricted fgr locus to 69 kb region flanked by the L02 and L06 makers confirming presence of badh2 gene in fgr locus. In addition to Badh2, two other genes which were located in this fgr region were Cah and Mccc2, encoding eukaryotictype carbonic anhydrase and 3-methylcrotonyl-CoA carboxylase b-chain, respectively. *Badh2* gene encodes an enzyme Betaine aldehyde dehydrogenase (BADH2) capable of converting γ-aminobutyraldehyde (GABald), a four carbon aminoaldehyde derived from proline via putrescine oxidation into γ-aminobutyric acid (GABA). The functional *badh2* gene codes for a mature protein with 503 amino acids (Wanchana *et al.*, 2005). However Bradbury *et al.* (2005) reported an eight base pair deletion in Exon 7 of *badh2* gene in aromatic rice which leads to the generation of a premature stop codon. This results in abrogation of the function of the enzyme, Betaine aldehyde dehydrogenease, which subsequently accumulates substrate 2AP in fragrant varieties. In aromatic varieties γ-aminobutyraldehyde (GABald), is not metabolized by BADH enzyme (truncated protein) and it cyclizes spontaneously into Δ1-pyrroline, which accepts acetyl group from methyl glyoxal, resulting in the formation of 2-acetyl 1-pyrroline (2AP) (Bradbury *et al.*, 2008). Several markers that target this functional InDel polymorphism were used by various workers for genotyping fragrance trait. In the present study a functional marker BADEX 7-5 developed by Sakhtivel *et al.* (2009) for fragrance in rice was used. Primer targeted 8 bp deletion in exon 7 of badh2 gene and amplified a fragment of 95 bp in Mushkbudji accessions, Basmati 1509 and Pusa- sugandh 3. 103 bp fragment was generated in Shalimar rice 2, Jehlum, Kamad, Loul Anzul and Kawa Kreed. BADEX 7-5 generated a product of 95 bp in aromatic varieties and 103 bp product in non-aromatic varieties. The results obtained are in line with the results obtained in the previous study on Traditional Basmati varieties (Basmati 217, Basmati 370, Basmati 386, Type-3, Taroari Basmati, Ranbir Basmati), Evolved Basmati varieties (Kasturi, Super Basmati, Haryana Basmati, Punjab Basmati, Mahi Sugandha, Pusa Basmati-1, Pusa 1121), Short grain aromatic varieties (Pankhari 203, Seetabhog, Tilakchandan, Tulasi Amrit, Tulsimanjari, Amritsari, Ambemohar, Badshah, Badshah bhog, Badshahbhog joha, Baspatri, Bansphool A, Chini kamini, Dubraj, Dhusara Badshah bhog, Govinda bhog , Jeeraga Samba, Kala namak , Kanak jeer, Katari bhog, Kola joha, Kunikuni joha), Long grain aromatic varieties (Acharmati, Amrutbhog, Dhan Prasad, Jirasar, Kala jira, Maguraphulla B, Maricha, Mugad Sugandha, PDKV Chinoor2, Sheetal Kani, Shyamjeer, Sugandhamati) (Sakhtivel *et al.*, 2009). The study carried out by Sakhtivel *et al.* (2009) using BADEX 7-5 generated a 95 bp product in 13 of these notified basmati varieties, 22 aromatic short grain aromatic varieties and 12 long grain aromatic landraces. It also generated a product of 103 bp in 13 non-aromatic varieties. In the present study, the fragrance in the Mushkbudji accessions, Basmati 1509 and Pusa- sugandh 3 was confirmed both at phenotypic as well as genotypic level. Hence the validation of this important functional marker for badh2 allele in all accessions of Mushk-budji, Pusa sugandh-3 and Basmati 1509 (aromatic varieties) opens the possibility of using it in Marker Assisted Breeding for the development of fragrant rice varieties for temperate and high altitude regions of Kashmir valley.

Bradbury *et al.* (2005) reported that *badh2.1* allele consists of 8-bp deletion and 3 single nucleotide polymorphisms (SNPs) in exon 7 and proved that this deletion leads to the introduction of premature stop codon to produce a truncated protein. Although molecular marker BADEX7-5 differentiated aromatic and non-aromatic varieties in the study undertaken by generating product of two different sizes 96 bp in aromatic and 103 in non-aromatic varieties and subsequently by the difference of bands obtained by electrophorosing two products on the 3.5% agarose gel. But in order to validate that it is the same deletion of 8 bp's that was observed by Bradbury *et al.* (2005), the target region possessing this deletion was amplified using another set of primers (Os Badh2) and subsequently sequenced using Sanger method. The sequencing of the *badh2* locus was carried out in aromatic rice cultivars as well as non-aromatic rice varieties to uncover the presence of *badh2.1 allele*. One genotype from each group was selected. Mushkbudji accession F. was selected from aromatic japonica group, Pusa Sugandh 3 from Sadri and Basmati (indica group), Kawa Kreed from non- aromatic japonica group, Jehlum from non- aromatic indica group and Kamad? (Sel_2009) was also selected due to its controversial behavior. Software program Clustal W (www.genome.jp/tools/clustalw) and T-COFEE software was used for creating multiple sequence alignment in the region of interest. The analysis showed presence of a deletion of 8 bps "GATTATGG" and three SNPs T and T in place of A and A before 8 bp deletion and T in place of C following 8 bp deletion in exon 7 in two of aromatic rice genotypes Mushkbudji 3 and Pusa Sugandh 3. However no such deletion was observed in non-aromatic varieties Kamad, Kawa Kreed and Jehlum. This 8 bp deletion in exon 7 of aromatic varieties could be the possible reason of aroma in these genotypes, which may be due to a truncated betaine aldehyde dehydrogenase enzyme. The study is in line with the results of Bradbury *et al.* (2005) which also observed same 8 bp deletion "GATTATGG" in his selected 14 diverse aromatic varieties namely $YRF_2$03, 00210-0-15, $YRF_2$07/1202, Yasmin, Amber, Dumsorhk, Dellmont, $YRF_2$07, $YRF_2$04, 00210-33, Basmati 370, Dragon Eyeball 100, Goolarah and Khao Dawk Mali 105. Watteva *et al.* (2008) also found 8 bp deletion and 3 SNPs as cause of aroma in two of his selected varieties Lanka Samurdi and Basmati 370. The 3 SNPs are present in the genotypes studied in the present study, too. Shi *et al.* (2008) also observed this mutation in twelve of his aromatic varieties namely Suyunuo, Wuxiangjing, Pangxiegu, Guanglingxiangnuo, Xiangxuenuo, XiangjingT37, Xiangjing20-18, Wuxiang075, Basmati385, Basmati370, Ganxiangnuo, and Meiguomolixiang. Sequencing results also clarified controversial behavior of so-called accession claimed to be Kamad which was collected from farmer field in year 2009. This so called Kamad earlier doesn't show any aroma in the fragrance test of Berner and Hoff and same was corroborated by evidence from PCR results. Sequencing results showed an intact region like other non-aromatic varieties confirming that this variety doesn't possess the 8 bp deletion. Hence it was confirmed at phenotypic as well as genotypic level that the accession was not Kamad. The reason that it was being called as Kamad could be that the concerned farmer was growing a mixture of aromatic and non-aromatic genotypes in his field. The origin of aroma in both aromatic varieties aromatic japonica variety Mushkbudji and Basmati variety Pusa-sugandh 3 was same 8 bp deletion in Exon 7. It can be deduced from the results that perhaps they share some commonness in origin. Kovach *et al.* (2009) in their study showed that across 5.3 Mb region flanking BADH2 Basmati cultivars were nearly identical to the ancestral japonica haplotype indicating that Basmati cultivars had close evolutionary relationship with japonica varietal group. Bradbury *et al.* (2008) suggested that it is loss of function mutation i.e 8 bp deletion in exon 7 of aromatic rice that lead to production of truncated protein which is incapable of metabolizing aroma compound 2-AP in rice but Chen *et al.*

(2008) observed that no such truncated protein is formed but this 8 bp deletion suppresses both transcription and translation of BADH2 gene resulting in 4-aminobutyraldehyde (ABald) accumulation which otherwise is metabolized by functional BADH2 enzyme. Even there are reports on deletion of base pairs in other exons a 7-bp deletion in exon 2 (Shi *et al.*, 2008), a TT deletion in intron 2, two new SNPs in the central section of intron 8 (Sun *et al.*, 2008), absence of MITE (miniature interspersed transposable element) in promoter (Bourgis, 2008), and a repeated (AT)n insert in intron 4 of *badh2* (Chen *et al.*, 2008). Whatever may be the case, either truncation of protein or inhibited transcription and translation, deletion in exon of badh2 gene imparts aromatic rice their characteristic aroma. Niu *et al.* (2008) conducted a study wherein downregulation of badh2 levels by RNA interference technique enhanced aroma production by accumulation of 2 AP thereby confirming the above said results.

Although 2AP is the major fragrant compound in aromatic rice, a range of volatile components, possibly different from 2AP-associated fragrance, gives every variety its own unique aroma. However, very little information is available about the association of these volatiles with aroma. Studies should be conducted to check the possible involvement of other genes in imparting particular aroma that is unique to each variety.

Microsatellite marker analysis is a promising approach to explore principal QTLs for aroma; therefore, it is beneficial for rice breeders to expand new aromatic rice varieties. Alternatively, marker-assisted selection can decrease the expenses and significantly accelerate the introgression of the fragrance gene to the target rice varieties. There are still some unresolved and ambiguous issues regarding the genetic basis of fragrance, and there is also little information about the biochemical pathway of rice aroma. Consequently, identifying the most important genes underlying the fragrance trait seems troublesome. Basic knowledge of aroma in local landraces of Kashmir has been provided by this study. But, there is a need to further map saturated markers in candidate chromosomal regions to identify both major and minor genes controlling rice aroma.

Chapter - 6

SUMMARY AND CONCLUSION

Fragrant rice is highly appealing to human beings; consequently, its global market value is tremendously increasing. Therefore, aroma is considered a prominent characteristic for numerous breeding programs. However the inadequate knowledge of the genetics of aromatic rice hinders the design of effective practical tools and methods for aromatic rice development programmes.

The present study was carried out to determine the presence of recessive allele of fragrance gene in the aromatic landrace of Kashmir “Mushkbudji” as well as in some other aromatic varieties grown locally. In present study 10 genotypes of rice grown locally in Kashmir including special rice of Kashmir i.e Mushkbudji were assessed for allelic variants of exon 7 of *badh2* gene. Based on some previous studies, primers were designed that targeted 8 bp deletion in exon 7 of badh2 gene and amplified a fragment of 95 bp in aromatic rice varieties (Mushkbudji sel. 3 and Pusa Sugandh 3, Basmati 1509, Mushkbudji Acc. F and Mushkbudji sel. 6) and 103 bp fragment in non-aromatic varieties (Jehlum, Shalimar Rice 2, Kamad? (Sel-2009), Loul Anzul and Kawa Kreed). Additionally a 463 bp region covering the target deletion was amplified by another set of primers (OsBadh2) and subsequently the bands were eluted and got sequenced by Sanger method. Software program Clustal W was used for creating multiple sequence alignment in the region of interest. The analysis showed presence of a deletion of 8 bps “GATTATGG” and three SNPs were in exon 7 of aromatic rice genotypes. No such deletion was found in non-aromatic rice varieties. Hence, functional markers for badh2 allele were validated in all accessions of Mushk-budji, Pusa sugandh-3 and Basmati 1509 (aromatic varieties) which can facilitate development of fragrant rice varieties through MAS. Surprisingly aroma was not detected in a so-called accession claimed to be Kamad which had been collected from a farmer’s field in year 2009. This so called Kamad didn’t show any aroma in the fragrance test of Berner and Hoff and same was corroborated by evidence from PCR results and sequencing. It doesn’t possess the 8 bp deletion! The reason could be that the concerned farmer was growing a mixture of aromatic and non-aromatic genotypes in his field, and his claim was wrong.

The results are summarized as:

- Primer BADEX 7-5 targeted 8 bp deletion in exon 7 of badh2 gene and amplified a fragment of 95 bp in Mushkbudji accessions, Basmati 1509 and Pusa Sugandh 3.
- Primer BADEX 7-5 generated a 103 bp fragment in Shalimar rice 2, Jehlum, Kamad?, Loul Anzul and Kawa Kreed.
- In short BADEX 7-5 generated a product of 95 bp in aromatic varieties and 103 bp product in non-aromatic varieties, and was therefore an effective functional marker that can accelerate MAS in rice for aroma.
- 463 bp region covering the target deletion was amplified in the selected varieties by another set of primers (OsBadh2) and subsequently the bands were eluted and got sequenced by Sanger method.
- The sequencing of the *badh2* locus was carried out in aromatic rice cultivars as well as non-aromatic rice varieties to check for the presence of *badh2.1* allele. Mushkbudji accession F. was selected from aromatic japonica group, Pusa Sugandh 3 from Sadri and Basmati group, Kawa Kreed from non- aromatic japonica group, Jehlum from non-aromatic indica group and the so called Kamad? (Sel-2009) was also selected due to its controversial behavior.
- In silico analysis identified the presence of a deletion of 8 bps “GATTATGG” and three SNPs T and T in place of A and A before 8 bp deletion and T in place of C following 8 bp deletion in exon 7 in two of aromatic rice genotypes Mushkbudji 3 and Pusa Sugandh 3. However, no such deletion was observed in non-aromatic varieties Kawa Kreed, Jehlum

and the so-called Kamad?.

- This 8 bp deletion in exon 7 of aromatic varieties could be the possible reason of truncated betaine aldehyde dehydrogenase enzyme and subsequently aroma.
- Sequencing results also clarified controversial behavior of so-called accession claimed to be Kamad which was collected from farmer field in year 2009. This so called Kamad, which did not show any aroma in Berner and Hoff test, showed an intact region like other non-aromatic varieties confirming that this accession doesn’t possess the 8 bp deletion.
- Hence it was confirmed at phenotypic as well as genotypic level that the accession was not Kamad. The reason that it was being called as Kamad could be that the concerned farmer was growing a mixture of aromatic and non-aromatic genotypes in his field.

Recommendations and Practical utility

- The present study not only augments our knowledge, but also helps fill the gaps in our understanding about the distribution of the *badh 2* allele in some important aromatic/ non-aromatic varieties of Kashmir.
- The present study could provide a stepping stone for developing effective rice development programs for Kashmir, especially for introgression of aroma.
- The study needs to be carried forward for a deeper understanding and exploration of the origins of aroma in Kashmir varieties.
- Metabolomic and genomic studies should be carried out to study molecular aspects of aroma development in rice.

LITERATURE CITED

Aggarwal, R.K., Shenoy, V.V., Ramadevi, J., Rajkumar, R. and Singh, L. 2002. Molecular characterization of some Indian Basmati and other elite rice genotypes using fluorescent-AFLP. *Theoretical and Applied Genetics* **105**:680-690.

Ahn, S.N., Bollich, C.N. and Tanksley, S.D. 1992. RFLP Tagging of a Gene for Aroma in Rice. *Theoretical and Applied Genetics* **84**: 85-828.

Amarawathi, Y., Singh, R., Singh, A.K., Singh, V.P. and Mohapatra, T. 2008. Mapping of quantitative trait loci for basmati quality traits in rice (*Oryza sativa* L.). *Molecular Breeding* **21**: 49-65.

Berner, D.K. and Hoff, B.J. 1986. Inheritance of scent in American long grain rice. *Crop Science* **26** : 876-878.

Bourgis, F., Guyot, R., Gherbi, H., Tailliez, E and Amabile, I. 2008. Characterization of the major fragrance gene from an aromatic japonica rice and analysis of its diversity in Asian cultivated rice. *Theoretical and Applied Genetics* **117**: 353-368.

Bradbury, L.M., Fitzgerald, T.L., Henry, R.J., Jin, Q. and Waters, D.L. 2005. The gene for fragrance in rice. *Plant Biotechnology* **3**: 363-370.

Bradbury, L.M.T., Gillies, S.A., Brushett, D.J., Waters, D.L.E. and Henry, R.J. 2008. Inactivation of an aminoaldehyde dehydrogenase is responsible for fragrance in rice. *Plant Molecular Biology*. **68**: 439-449.

Buttery, R.G., Ling, L.C. and Juliano, B.O. 1982. 2-acetyl-1-pyrroline; an important aroma component of cooked rice. *Chemical Industries* (*London)* **23**: 958-959.

Chen, S., Wu, J. Yang, Y., Shi, W. and Xu, M. 2006. The fgr gene responsible for rice fragrance was restricted within 69 kb. *Plant Science* **171**: 505-514.

Chen, S., Yang, Y., Shi, W., Ji, Q. and He, F. 2008. Badh2, encoding betaine aldehyde dehydrogenase, inhibits the biosynthesis of 2-acetyl-1-pyrroline, a major component in rice fragrance. *Plant Cell* **20**: 1850-1861.

Cordeiro, G.M., Christopher, M.J., Henry, R.J. and Reinke, F.R. 2002. Identification of microsatellite markers for fragrance in rice by analysis of the rice genome sequence. *Molecular Breeding* **9**: 245-250.

D.O.E.S., 2013. Agri-Statistics at a Glance 2013. Directorate of Economics and Statistics (D.O.E.S.), Ministry of Agriculture, Govt. of India.

Fitzgerald, M.A., Hamilton, N.R., Calingacion, M.N., Verhoeven, H.A. and Butardo, V.M. 2008. Is there a second fragrance gene in rice? *Plant Biotechnology Journal* **6**:416-423.

Foster-Powell, K., Holt, S.H.A. and Brand-Miller, J.C. (2002) International table of glycemic index and glycemic load values. *American Journal of Clinical Nutrition* **76**:5-56.

Gregorio, G.B. 2002. Progress in breeding for trace minerals in staple crops symposium: plant breeding: a new tool for fighting micronutrient malnutrition. *Journal of Nutrition* **132** : 5005-5025.

Hasthanasombut, S., Paisarnwipatpong, N., Triwitayakorn, K., Kirdmanee, C. and Supaibulwatana, C. 2011. Expression of OsBADH1 gene in Indic.a rice (*Oryza sativa* L.) in correlation with salt, plasmolysis, temperature and light stresses. *Plant Journal*4: 400-407.

Hien, N.L., Yoshihashi, T., Sarhadi, W.A. and Hirata, Y. (2006). Sensory test for aroma and quantitative analysis of 2-acetyl-l-Pyrroline in Asian aromatic varieties. *Plant Production Science* **9** : 294-297.

Husaini, A.M., Parray, G.A., Rather, A.G., and Sanghera, G.S. 2009. Performance of elite basmati rice varieties of subtropical India under valley temperate conditions of Kashmir. *International Rice Research Notes,* pp 1-5

Husaini, A.M., Parray, G.A., Wani, S.A. 2013. Biodiversity of rice germplasm and its conservation in Kashmir: Targets, accomplishments and way forward. In: *University Research in Kashmir Rice- A Historical Perspective,* SKUAST-Kashmir.

International Rice Genome Sequencing Project (IRGSP)-The map-based sequence of the rice genome, 2005 *Nature* **436**:793-800 (doi:10.1038).

Jin, Q., Waters, D., Cordeiro, G.M., Henry, R.J. and Reinke, R.F. 2003. A single nucleotide polymorphism (SNP) marker linked to the fragrance gene in rice (Oryza sativa L.). *Plant Science* **165** : 359-364.

Khush, G.S. (1997). Origin, dispersal, cultivation and variation of rice. *Plant Molecular Biology* **35** : 25-34.

Kovach, M.J., Calingacion, M.N., Fitzgerald, M.A. and Mccouch, S.R. 2009. The origin and evolution of fragrance in rice (*Oryza Sativa* L.). *Proc National Academy of Science USA* **106**: 14444-14449.

Kumari, P., Ahuja, U., Jain, S. and Jain, K.R. 2012. Fragrance analysis using molecular and biochemical methods in recombinant inbred lines of rice. *African Journal of Biotechnology* **11**: 15784-15789.

Lam, M.S. and Proctor, A. 2003. Milled rice oxidation volatiles and odor development. *Food Science* **68**: 1365-2621.

Lang, N.T. and Buu, B.C. 2002. Identification and fine mapping of SSR marker linked to fgr gene of rice. *Omonrice* **10** : 14-20.

Lang, N.T. and Buu, B.C. 2008. Development of PCR-based markers for aroma (*fgr)* gene in rice (*Oryza sativa* L.). *Omonrice* **16**: 16-23.

Li, J., Ku, D. and Li, L.F. 1996. Analysis of fragrance inheritance in scented rice variety, Shenxiangjing 4. *Acta Agriculturae Shanghai* **12**: 78- 81.

Livingstone, J.R., Maruo, T., Yoshida, I., Tarui, Y. and Hirooka, K. 2003. Purification and properties of betaine aldehyde dehydrogenase from *Avena sativa*. *Journal of Plant Resources* **116**: 133-140.

Lu, B.R. 1999. Taxonomy of the genus *Oryza* (Poaceae). Historical perspective and current status. *International Rice Research Notes* **24**:4-8.

Myint, K.M., Courtois, B., Risterucci, A., Frouin, J., Soe, K., Thet, K.M., Vanavichit, A. and Glaszmann, J.C. 2012. Specific patterns of genetic diversity among aromatic rice varieties in Myanmar. *Rice* **5**:20.

Nadaf, A.B., Wakte, K.V. and Zanan, R.L. 2014. 2-Acetyl-1-Pyrroline Biosynthesis: from Fragrance to a Rare Metabolic Disease. *Journal of Plant Science & Research* **1**:102

Niu, X., Tang, W., Huang, W., Ren, G. and Wang, Q. 2008. RNAi-directed downregulation of OsBADH2 results in aroma (2-acetyl-1-pyrroline) production in rice (*Oryza sativa* L.). *BMC Plant Biology* **8**: 100.

Parray, G.A., Shikari A.B., Ganai, M.A and Husaini, A.M. 2008. Local aromatic rice cultivars of north Himalayas - Present status and future strategies for their sustainable improvement. *Crop Resource* **36** (1,2,3) : 380-381.

Prathepha, P. 2009. The *badh2* Allele of the Fragrance (*fgr/BADH2)* Gene Is Present in the Gene Population of Weedy Rice (*Oryza sativa f. spontanea*) from Thailand. *American-Eurasian J. Agriculture & Environmental Science* **5**: 603-608.

Rathinasabapathi, B., Gage, D.A., Mackill, D.J. and Hanson, A.W. 1993. Cultivated and wild rices do not accumulate glycinebetaine due to deficiencies in two biosynthetic steps. *Crop Science* **33**: 534-538.

Ren, J.S., Xiao, P.C., Chen, Y., Huang, X. and Wu, X.J. (2004). Study on heredity of aroma genes in several maintainer lines of aromatic rice. *Seed* **25**: 778-781.

Saghai-Maroof, M.A., Biyashev, R.M., Yang, G.P., Zhang, Q. and Allard, R. 1994. Extraordinarily polymorphic microstatallite DNA in barley genome mapping : Species diversity, chromosomal location and population dynamics. *Proceeding of National Academy of Science USA* **91**: 5466-5470.

Sakthivel, K., Shobha Rani, N., Pandey, M.K., Sivaranjani, A.K.P., Neeraja, C.N. 2009. Development of a simple functional marker for fragrance in rice and its validation in Indian Basmati and non-Basmati fragrant rice varieties. *Molecular Breeding***24**:185-190.

Shi, W., Yang, Y.I., Chen, S. and Xu, M. 2008. Discovery of a new frangance allele and the development of functional markers for the breeding of fragrant rice varieties. *Molecular Breeding* **22**: 185-192.

Shi, Y., Zhao, G., Xu, X. and Li, J. 2014. Discovery of a new fragrance allele and development of functional markers for identifying diverse fragrant genotypes in rice. *Molecular Breeding* **33**:701-708.

Siddiq, E.A., Vemireddy, L.R. and Nagaraju, J. 2012. Basmati rices: genetics, breeding and trade. *Agriculture Resource* **1** : 25-36.

Singh, A., Singh, P.K., Singh, R., Pandit, A., Mahato, A.K., Gupta, D.K., Tyagi, K., Singh, A.K., Singh, N.K., and Sharma, T.R. 2010. SNP haplotypes of the BADH1 gene and their association with aroma in rice (*Oryza sativa* L.). *Molecular Breeding* **26** : 325-338.

Sultan, S.M. and Subba Rao, V.L. 2013. Germplasm collection from last remnants of rice landrace genetic diversity in high altitude areas of Kashmir Himalayas. *International Journal of Conservation Science* **4**(4): 467-476.

Sun, S.X., Gao, F.Y., Lu, X.J. and Wu, X.D. 2008. Genetic analysis and gene fine mapping of aroma in rice (*Oryza sativa* L. Cyperales, Poaceae).*Genetics and Molecular Biology* **31**: 532-538.

Tang, W., Sun, J., Liu, J., Liu, F., Yan, J., Gou, X., Lu, B. and Liu, Y. 2014. RNAi‑directed downregulation of *betaine aldehyde dehydrogenase 1* (*OsBADH1*) results in decreased stress tolerance and increased oxidative markers without affecting glycine betaine biosynthesis in rice (*Oryza sativa*). *Plant Molecular Biology* DOI 10.1007/s11103-014-0239-0.

Vanavichit, A., Tragoonrung, S., Theerayut, T., Wanchana, S. and Kamolsukyunyong, W. 2005. Transgenic rice plants with reduced expression of Os2AP and elevated levels of 2-acetyl-1-pyrroline. United States Patent, Patent No. US 7,319,181 B2.

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

Wettewa, W. and Kottearahchi, N.S. 2014. Sequence analysis of the mutation in the 7th exon of BADH2 gene in traditional aromatic rice varieties in Sri Lanka. *The Journal of Agricultural Science* **9**:1.

Yano, M., Shimosaka, E., Sato, A. and Nakagahra, M. 1991. Linkage analysis of a gene for scent in indica rice variety, Surjamkhi, using restriction fragment length polymorphism markers (in Japanese). *Japan Journal of Breeding* **41**: 338-339.

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CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner Dr. Khalid Fazili, Prof. Department of Biotechnology, University of Kashmir, Srinagar during Viva-Voce examination held on January 14-2016 have been incorporated in the manuscript entitled **“Isolation, Sequencing, *In silico* analysis of *badh* homologues/alleles and validation of DNA Markers for MAS in Aromatic Rice (*Oryza sativa L.*)”** submitted by **Ms. Aafreen Sakina (Regd. No. 2013-A-981-M).**

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