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Full Length Article

Molecular Detection and Quantification of Non-Basmati Adulterants in Basmati Rice using BADH2 Gene Marker

Safeena Inam[1], Muhammad Ramzan Khan[1,2*], Nazia Rehman[1], Zaheer Abbas[1], Sabahat Noor[1] and Ghulam Muhammad Ali[*]

[1]*National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre (NARC), Park Road, Islamabad, Pakistan*

[2]*National Centre for Bioinformatics, Quaid-i-Azam University, Islamabad, Pakistan*

[*]For correspondence: mrkhan@qau.edu.pk; drgmali@yahoo.ca

Abstract

Basmati rice is the major export commodity of Pakistan and the most appealing aromatic rice in international market. Because of premium price and unique qualities it is subjected heavily to adulteration with other long grain non-Basmati rice. Traditional methods for the detection and quantification of adulterants in this exportable commodity remained obscure. In present study, real time PCR system was developed to check and quantify the adulterants present in Basmati rice varieties from Pakistan. A total of 37 rice varieties, including Basmati and non-Basmati rice were selected. Betaine aldehyde dehydrogenase (BADH2) gene specific primers were used to detect adulteration in Basmati rice varieties. Initial screening was done with conventional PCR. The results revealed that amplification of BADH2 was detectable only in non-Basmati rice. Real time PCR analysis was done for quantitative detection of mixing present in Basmati rice and results were recorded in the form of amplification plot and standard curve. Non-Basmati showed amplification of BADH2 gene, being expressed only in non-Basmati rice. However, BADH2 is barely detectable in Basmati rice. The standard curve exhibited a linear relationship. The slope of the curve recorded was 1.035 and R_2 as 1. The values determined for various mixed samples were very close to original mixing. This study posits that expression of BADH2 gene is reliable to quantify non-Basmati adulterants in Basmati rice.

Keywords: Rice; Real time PCR; Betaine aldehyde dehydrogenase; Basmati; Adulteration

Introduction

Rice is one of the main cereal crops for almost 50% of the world's population. There are two types of rice i.e., aromatic and non-aromatic rice. Non-aromatic rice includes *Indica* and *Japonica* type. *Indica* consists of long and medium grained rice while *Japonica* type consists of short grained rice. Non-aromatic rice counts for 79% of the world trade. Basmati rice also called fragrant or aromatic rice is very common in the international market (Bhattacharjee *et al*., 2002; Prodhan *et al.,* 2017). Himalaya's foothill of India and Pakistan are the major growing areas of Basmati rice. The name "Basmati" is due to its geographical location. "Bas" means smell and "mat" means soil (Sarreal *et al*., 1997). It is stated that strong aroma is due to genetic factors and environmental condition of Punjab climate and soil. If Basmati is grown outside the Punjab of India and Pakistan it will not produce aroma (Efferson, 1985).

The importance of Basmati rice is due to its unique fragrance in both cooked and uncooked form. The shape of grain is distinctive and its length increases two fold upon cooking and width remain as such. Basmati rice has good nutritional qualities and it is a good source of slow releasing carbohydrates. Physical appearance, distinct aroma and taste are important characteristics of Basmati rice. Some other distinct features are their delicate grains, soft texture and extreme grain elongation upon cooking. Best growing conditions for Basmati rice are warm, moist and valley or hill like environment (Giraud *et al*., 2009).

There is great economic importance of Basmati rice and is generally grown in Pakistan and India and these countries are the main exporters of Basmati rice (Bligh, 2000). Pakistan is one of the leading exporters of Basmati rice in international markets. Pakistan's annual rice export is about 28,615 m/tons of Basmati and 152,735 m/tons of non-Basmati rice (Anonymous, 2016) Rice commodity contributes 3.15% in value added in agriculture and 0.6% in GDP (Pakistan Economy Survey, 2016). The price of Basmati rice is four times more than non-Basmati rice. United States and Europe are the main importers of Basmati rice. Due to its premium price and high demand in global market, Basmati rice is exposed to substantial adulteration with other long grain non-Basmati rice. Now a day, adulteration rate is very high and important to check the

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adulterants present in Basmati rice. To fulfill the quality regulations of importing countries it is necessary to guarantee the purity of the product (Lopez, 2008).

Many chemical and physical methods have been used to measure adulteration. Physical methods include checking the physical appearance of rice grains. Chemical methods include boiling of leaf tissue and grains in water and then check it by smelling. Other chemical methods include the smelling of grain after treatment with KOH or I_2-KI (Sood and Siddiq, 1978). Also aromatic compounds were analyzed through chromatography technique (Lorieux *et al.*, 1996; Widjaja *et al.*, 1996). However these methods have not provided satisfactory results to control adulteration.

The above mentioned methods for detection of adulteration have certain limitations and cannot detect adulteration properly. Chemical methods like boiling the grain in water or treating it with some chemical are not reliable methods. Also these chemical methods can cause damage to the nasal passages. The chromatography method is expensive and time consuming (Kottearachchi *et al.*, 2010). Nagaraju *et al.* (2002) reported the SSR markers to check the adulteration in Basmati rice. But this method is very expensive (Lopez, 2008). Based upon the limitations of previous methods, new and alternative methods are desired. One of the methods of the detection of adulteration is through PCR. The method can be further refined and absolute quantity of adulterants can be determined by using real time PCR system.

It has been reported that there are 114 volatile compounds present in the aromatic rice (Yajima *et al.*, 1978). Out of these volatile compounds 2-acetyl-1-pyroline (2AP) is the important aromatic compound responsible for the unique fragrance in aromatic rice (Buttery and Ling, 1982). 2AP is present in all parts of aromatic rice plant excluding roots (Buttery *et al.*, 1983). It has been reported that reduced level of 2AP is present in non-Basmati rice while Basmati rice has higher level of 2AP (Widjaja *et al.*, 1996). Betaine aldehyde dehydrogenase (BADH) enzyme is associated with conversion of 4-amiobutyralaldehyde (AB-ald) to 4-aminobutyric acid (GABA) which is responsible for 2AP production. The 3 SNPs, 8bp deletions and a stop codon in gene of fragrant rice result in the truncated BADH2 protein of 251 residues. This mutation is responsible for the loss of function of the protein and evidences also showed that fragrance is a recessive trait (Bradbury *et al.*, 2005; Chen *et al.*, 2008).

Recent studies illustrated that the amount of 2AP is regulated by a recessive gene called betaine aldehyde dehydrogenase2 (BADH2) gene. BADH2 gene encodes betaine aldehyde dehydrogenase and present on chromosome number 8 of Basmati rice. The 8 base pair deletions and three SNPs are found on BADH2 gene. This deletion and SNPs present on the gene is associated with fragrance in Basmati rice (Bradbury *et al.*, 2005). In non-Basmati rice functional BADH2 is present and gene is associated with reduced level of 2AP production. The deletion in recessive gene of Basmati rice results in loss of function of BADH2, which deviates the direction of the biochemical pathway of BADH2 enzyme and results in the accumulation of 2AP (Fitzgerald *et al.*, 2008). BADH2 gene has 15 exons and 14 introns. In the exon 7 of Basmati rice there is an 8 bp deletion, 3SNPs and also a stop codon (Bradbury *et al.*, 2005). As BADH2 gene is present in non-Basmati rice therefore, this marker might have used to detect non-Basmati adulterants in Basmati with the advent of real time PCR system. The objectives of this study were to detect the adulterants in Basmati rice by using BADH2 gene marker and to validate and quantify the non-Basmati adulterants in Basmati rice through real time PCR system.

Materials and Methods

Plant Material

Basmati and non-Basmati rice grains of Pakistani origin were obtained from Gene Bank of Plant Genetic Resource Preservation Institute (PGRI), National Agricultural Research Center (NARC), Islamabad. A total of 37 rice samples including 13 Basmati and 24 non-Basmati rice were selected for this study (Table 1). Genomic DNA was extracted from rice seeds using CTAB method. The genomic DNA was run on the 1% agarose gel to check quality and quantity of DNA.

Sequences Retrieval

The homologs of BADH gene from various species such as *Hordeum vulgare, Sorghum bicolor, Zoysia tenuifolia, Oryza sativa, Brachypodium distachyon, Pyrus betulifolia, Glycine max, Medicago truncatula, Helianthus annuus, Carthamus tinctorius, Sesuvium portulacastrum, Spinacia oleracea, Haloxylonam modendron* and *Oncorhynchus mykiss* were retrieved using NCBI (http://www.ncbi.nlm.nih.gov/) data base.

Phylogenetic Analysis of BADH Gene

In order to infer the evolutionary relationship of BADH gene in different species phylogenetic analysis was performed. For this purpose coding sequences of BADH from different species including *H. vulgare, S. bicolor, Z. tenuifolia, O. sativa, B. distachyon, P. betulifolia, G. max, M. truncatula, H. annuus, C. tinctorius, S. portulacastrum, S. oleracea, H. modendron* and *O. mykiss* were collected. The sequences were edited using MacVector and a neighbor joining tree in MacVector™ 7.2.3 (Accelrys Inc.) gcg/Wisconsin Package University of Wisconsin) software was constructed. To validate the reliability of tree 1000 replications of bootstraps were calculated.

Table 1: List of basmati and non-basmati rice samples used for the detection of BADH2 genes through using conventional PCR amplification

Sr. No	Name	Basmati/ Non-basmati	PCR Results
1	Basmati-385	Basmati	+
2	Basmati-370	Basmati	+
3	Basmati-Pak	Basmati	+
4	Shaheen-Basmati	Basmati	+,-
5	Punjab- Basmati-1	Basmati	+
6	Kashmir- Basmati	Basmati	+
7	Dehradun-Basmati	Basmati	+
8	Super- Basmati	Basmati	+
9	Basmati-2000	Basmati	+
10	Basmati-198	Basmati	+
11	Rachna Basmati	Basmati	+,-
12	Dokri Basmati	Basmati	+,-
13	Basmati C-622	Basmati	+
14	DR-83	NB	-
15	Pakhal	NB	-
16	Swat-1	NB	-
17	Swat-2	NB	-
18	Jp-5	NB	-
19	Shadab	NB	-
20	Khushbu-95	NB	-
21	KS-282	NB	-
22	Pak-177	NB	-
23	IR-8	NB	-
24	Malhar-346	NB	-
25	Sarshar	NB	-
26	Purple Marker	NB	-
27	Dilrosh	NB	-
28	FakhareMalakand	NB	-
29	IR-36	NB	-
30	Nippon Bare	NB	-
31	Jajai-77	NB	-
32	DR-82	NB	-
33	NIAB IR-9	NB	-
34	Kanwal-95	NB	-
35	Shua-92	NB	-
36	DR-92	NB	-
37	Sada Hayat	NB	-

+, Basmati; -, non- Basmati; +, - mixture of Basmati and non-Basmati

Sequence Alignment

In order to show diversity of BADH2 gene in non-Basmati rice ClustalW alignment of exon-7 were generated. Consensus sequence was also selected.

Primer Designing

Betaine aldehyde dehydrogenase 2 (BADH2) gene present on chromosome 8 of rice was selected as a marker for this study. This gene is associated with fragrance in rice (Bradbury *et al.*, 2005). Alignments of alleles of Basmati and non-Basmati rice were performed using ClustalW to determine the suitable position for primer design. BADH2 has deletion in exon 7 of Basmati rice therefore the idea was to design the primer in this region, because this is the perfect position to differentiate Basmati from non-Basmati rice. The forward primer sequence was 5' GGAGCTTGCTGATGTGTGTAAAGAG 3' and reverse primer sequence was 5' CAGCTGAAGCCATAATCTTTTTACCAG 3'.

Standard PCR Amplification

As a first step, screening of Basmati and non-Basmati rice samples was done using forward and reverse gene specific primers through conventional PCR. Genomic DNA was used as a template. PCR profile was set at 95ºC for 5 min, followed by 40 cycles of 94ºC for 1 min, 52ºC for 1 min and 72ºC for 1 min. Final extension was carried at 72ºC for 10 min. Amplified products were run on 2% agarose gel and visualized through gel documentation system.

Real time PCR

Real time PCR quantification of non-Basmati adulterants in Basmati rice was performed using StepOne plus real time PCR system (Applied Biosystem). For this purpose genomic DNA was used as template and gene specific primers of BADH2 that amplify shorter fragment were designed. The standard curve method was applied for quantification of adulterants. In order to validate results obtained from conventional PCR first of all a few samples were selected. Fluorescent dye used was SYBR Green. The target gene was BADH2 and three types of samples i.e., Basmati, non-Basmati and mix were used. To build standard curve serial dilution of 1:2 was employed. Five points were selected on standard curve. The experiment was repeated three times for each of the biological as well as technical replicates. The reaction volume used was 20 µL containing 10 µL of SYBR Green. The PCR profile was set at 94ºC for 1 min and 60ºC for 1 min and a final melt curve analysis of 60 to 94ºC for 60 min. The data was recorded in the form of ΔCT values, standard curve, amplification plot and melt curve plot. The data were analyzed with StepOnePlus Software (ABI). In order to determine the absolute quantity of non-Basmati rice grains in Basmati rice samples a known quantity of non-Basmati and Basmati rice were mixed and analyzed.

Results

Monocot and Rice BADH Genes are Clustered together

In order to infer the phylogenetic relationship of BADH gene among various species a neighborjoining (NJ) tree was constructed (Fig. 1). Species can be differentiated into different clusters on the basis of BADH gene phylogeny. One group encompasses all the groups consisting of *H. vulgare* BADH1 and BADH2. The second cluster is the small group consisting of *S. bicolor*, *Z. tenufolia*, *O. sativa*, *H. vulgare* and *B. distachyon*. The big group that can be further divided into three small groups include, *P. betulifolia*, *G. max*, *M. truncatula*, *H. annuus*, *C. tinctorius*, *S. portulacastrum*, *S. olerace* and *O. mykiss*. Interestingly rice BADH is sister to *H. vulgare.* As these are cereal and monocots so their clustering might be a natural phenomenon. The high bootstrap values support the reliability of the distance based tree.

Basmati Rice has a Deletion of 8 Nucleotides in Exon 7 of BADH2 Gene

In order to detect the nucleotide diversity of BADH2 gene in Basmati and non-Basmati rice the partial gene sequences of the two samples were aligned using ClustalW program in MULAN package. For this purpose only exon 7 of the BADH2 gene was aligned (Fig. 2). However a dramatic difference exists in the form of 8 bp deletion in the exon 7 of BADH2 gene isolated from Basmati rice. Besides, there is a single nucleotide polymorphism and a premature stop codon in Basmati rice. This premature stop codon results in the truncation of this protein. But the remarkable feature of Basmati BADH2 gene is the 8 bp deletion. This deletion is associated with fragrance in Basmati rice (Bradbury *et al*., 2005). The functional BADH2 gene is present in non-Basmati rice. The region can be used to differentiate from non-Basmati rice. Therefore, for further studies primer were designed from this region.

Conventional PCR can Differentiate Basmati from Non-Basmati Rice

Basmati and non-Basmati samples were analyzed using conventional PCR. BADH2 gene specific forward and reverse primers were used for amplification. Out of 37 rice, 11 samples were Basmati, 3 were mixture of both Basmati and non-Basmati and 24 samples were non-Basmati.

As primers were designed for functional BADH2 gene therefore amplification is present only in non-Basmati rice (Fig. 3). While, no amplification is present in Basmati rice as it contain mutant BADH2 gene and the light band indicate the mixture of both Basmati and non-Basmati rice.

These results revealed that conventional PCR can differentiate Basmati from non-Basmati rice (Table 1). Conventional PCR can only detect the adulteration present in Basmati rice therefore to quantify the adulterants real time PCR is needed. The mutant BADH2 gene is present in Basmati rice, which is responsible for fragrance. The functional BADH2 present in non-Basmati rice is associated with reduced production of 2AcP a compound associated with fragrance (Bradbury *et al.*, 2005).

Real time PCR can Quantify the Adulterants Present in Basmati Rice

Detection of non-Basmati rice mixed with Basmati rice through PCR of active BADH2 gene cannot serve the purpose of this study. Therefore, the validation as well as the quantification of adulterants in Basmati rice was inevitable. For this purpose a SYBR Green based real time analysis was employed. The amplification plot of BADH2 gene for non-Basmati rice samples is shown in Fig. 4. Although, there is no perfect correlation between the serial dilutions and amplification of target gene but still appreciable correlation necessary for the validation of experiment exist. For example line 1 is at 18 cycles with 100 ng of DNA, line 2 is at 20 cycles with 20 ng of DNA, line 3 is at 22 cycles with 5 ng of DNA, line 4 is at 24 cycles with 2 ng of DNA and line 5 is at 28 cycle with 1 ng of DNA. The standard curve showed a linear relationship for at least 3 samples (Fig. 5). The other samples were also closed to this linear relationship. The relationship was between non-Basmati DNA concentration and C_T value. The slope of the curve recorded was 1.035 and R^2 was 1. The next important step was to quantify the mixing of non-Basmati adulterants in Basmati. For this purpose 3 Basmati and 3 non-Basmati rice samples were mixed together in known quantity and subjected to real time

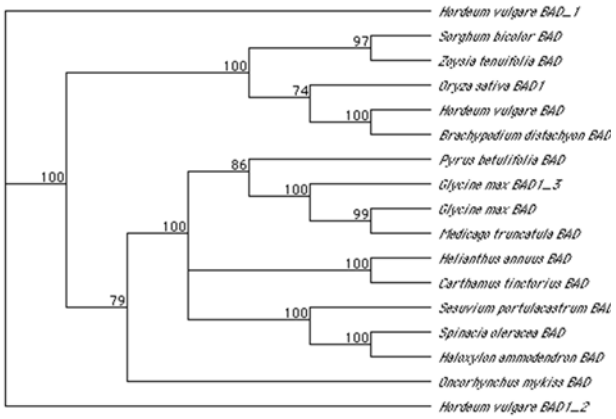

Fig. 1: Phylogenetic relationship of different species based on BADH genes phylogeny

A neighbor joining tree showing relationship of BADH genes from various species is shown. P un-corrected distance was used as default. Numbers on the branches show the bootstraps values for 1000 replications. *Oryza sativa* is sister to *Hordeum vulgare* and *Bradypodium distachyon*. Multiple copies of the genes are also indicated

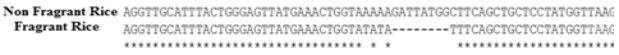

Fig. 2: BADH2 exon 7 alignments of Basmati and non-Basmati with Mulanprogram Sequence alignment of exon 7 of BADH2 genes of both fragrant and non-fragrant rice is shown. Deletion is shown in dashes. The (TAA) shows the stop codon. Conserved sequences are shown by asterisk as consensus sequences are at the bottom

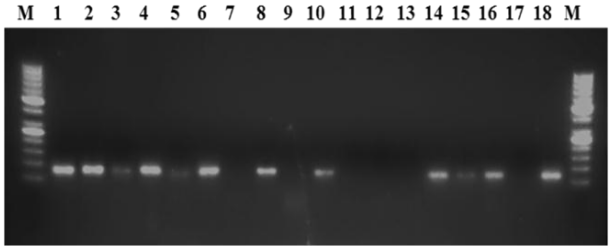

Fig. 3: Conventional PCR amplification of BADH2 gene

Lane 1, 2, 4, 6, 8, 10, 14, 16, 18 are non-Basmati rice samples. Lane 7, 9, 11, 12, 13, 17 are Basmati samples. Lane 3, 5 and 15 are mixture of non-Basmati and Basmati rice. A 50bp marker is also indicated

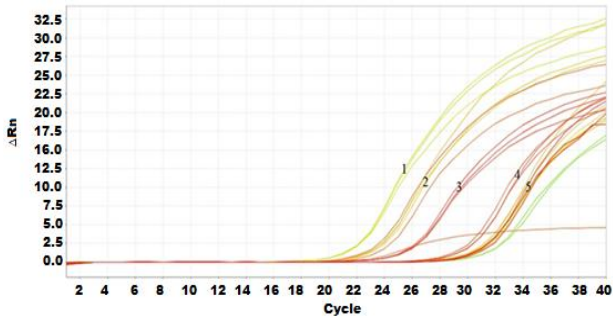

Fig. 4: Amplification plot of non-Basmati samples
The amplification plot of non-Basmati samples with BADH2 as target is shown. Number of PCR cycles is shown on x-axis and magnitude of fluorescence is shown on y-axis. ΔRn is the magnitude of fluorescence

present in 100 g. The values determined were very close to original mixing. These results show that real time PCR seems to be a reliable, quick and sophisticated method for the quantification of adulteration in Basmati rice.

Discussion

Best varieties of Basmati rice are mainly grown in India and Pakistan and are the leading exporters of Basmati rice (Bligh, 2000). Basmati rice cost more than the non-Basmati rice because of its premium qualities. As there is high demand of Basmati rice in local as well as in international market and its price is also on rise therefore it is mixed with some long grained non-Basmati rice. Adulteration rate is increasing day by day. To fulfill the quality regulation of

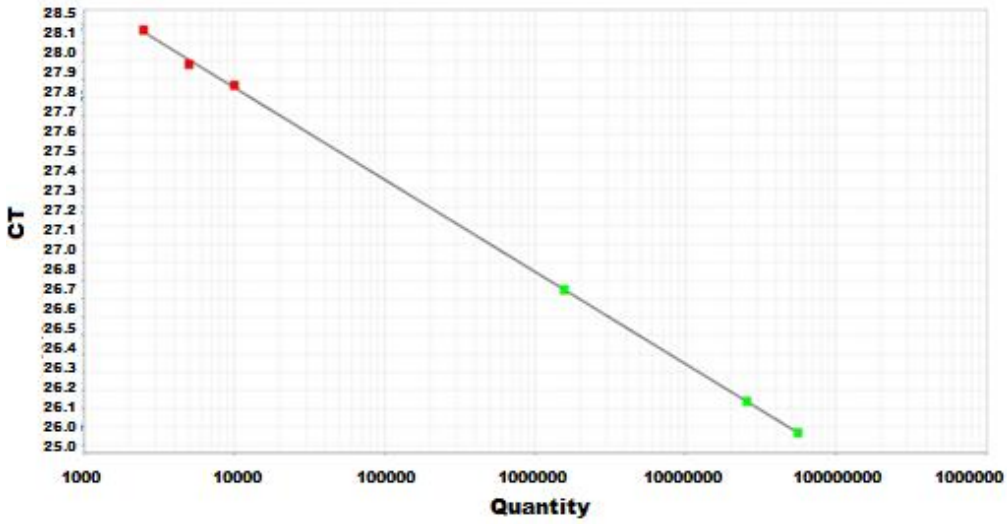

Fig. 5: Standard curve obtained from non-Basmati samples
Quantity of samples is shown on x-axis and CT value is shown on y-axis. CT value represent the threshold cycle

Table 2: Quantification of mixing by comparing ΔCt value and standard curve

S. No.	Mixture of rice Varieties analyzed	Actual concentration1/100 g	Observed non-basmati concentration 1/100g
1	IR-8	30	33.03 ± 2.19
	Basmati-385	70	
2	Pak-177	48	54.77 ± 3.30
	Basmati-370	52	
3	Pak-177	5	9.20 ± 0.57
	IR-36	5	
	Super-basmati	90	

±represents standard deviation of the mean

PCR. The C_T values of validation samples as plotted against standard curve. The quantity of non-Basmati in Basmati rice was calculated. Besides statistical analysis was employed and standard deviation of each result was calculated. Table 2 shows that in mixture 1 whose original ratio of mixing was 30:70. The real time PCR revealed that 33.03±2.19 non-Basmati rice in total of 100 gram sample was present. Similarly, in the second sample, the mixing was 48:52 and the results shows that 54.77±3.30 non-Basmati rice was

importing countries it is important to check mixing present in Basmati rice (Lopez, 2008).

Several methods have been developed to detect mixing and to differentiate Basmati from non-Basmati rice. These methods include physical, chemical and molecular methods. But it was found that these methods cannot detect the adulteration satisfactory (Lopez, 2008). The present study is conducted to detect the mixing precisely. Keeping the limitations of the previously established methods in

mind the present study was designed to quantify the adulteration through advanced molecular technique such as real time PCR.

Genes are being used as markers for the detection of adulteration. BADH2 present in Basmati rice is mutant gene and its functional copy is present in non-Basmati rice. Previous studies revealed that the deletion present in BADH2 is responsible for fragrance in rice (Bradbury *et al*., 2005). In this regard the remarkable tools that can be used for the detection is a marker BADH2 gene. There are two types of BADH2 genes one is the mutant and other is the active form of gene. The mutant gene has a deletion of 8 bp in Basmati rice. Therefore the primers were designed in this region, which can differentiate Basmati from non-Basmati. To detect the genetic diversity of BADH gene it was important to do the phylogenetic analysis of BADH gene. The neighbor joining shows the BADH genes homologs from different species. Rice BADH is sister to *H. vulgare*.

The present study also aims to differentiate Basmati from non-Basmati through conventional PCR. Amplification was present only in non-Basmati rice while no amplification was present in Basmati rice. The SSLPs markers were used to detect adulteration in Basmati rice. A total of 12 primers sets differentiate Basmati from non-Basmati rice (Bligh, 2000). Saini *et al.* (2004) used AFLP, ISSR and SSR markers to check the genetic diversity in Basmati and non-Basmati rice. Considerable level of genetic diversity was observed in Basmati and non-Basmati rice. Rabbani *et al.* (2010) reported that microsatellite markers are the efficient tool for the assessment of genetic variability in Basmati and non-Basmati rice. BADH gene specific primers were used to differentiate Basmati rice from non-Basmati through conventional PCR. Amplification was present in non-Basmati rice only (Lopez, 2008). Our results are in corroboration with Lopez (2008).

Real time PCR system was used for the validation and quantification of adulterants present in Basmati rice. Data were recorded in the form of amplification plot, melt curve and standard curve. Active BADH gene present in non-Basmati rice was amplified by real time PCR. Samples with more quantity of DNA showed amplification earlier. Basmati rice DNA showed no amplification through real time PCR. To check the quantification of adulterants Basmati and non-Basmati rice was mixed in different proportions and were subjected to real time PCR. From the C_T values the quantity of non-Basmati in Basmati rice was calculated. The quantity of non-Basmati was calculated through plotting the C_T values of validation samples against standard curve. Standard deviation was also recorded for statistical analysis.

Conclusion

Based on findings it is concluded that real time PCR is the rapid and reliable method to check the adulteration properly in Basmati rice. Real time PCR cannot only differentiate Basmati from non-Basmati but can also quantify the non-Basmati adulterants present in Basmati rice. This method can be adopted to check the mixing in export Basmati rice. It is concluded that real time PCR is the fast, sophisticated and authentic method.

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