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Research Article

High-throughput multiplex microsatellite marker assay for detection and quantification of adulteration in Basmati rice (*Oryza sativa*)

Basmati rice is a very special type of aromatic rice known world-wide for its extra long grains and pleasant and distinct aroma. Traditional Basmati rice cultivars, confined to Indo-Gangetic regions of the Indian subcontinent, are often reported to be adulterated with crossbred Basmati varieties and long-grain non-Basmati varieties in the export market. At present, there is no commercial scale technology to reliably detect adulteration. We report here a CE-based multiplex microsatellite marker assay for detection as well as quantification of adulteration in Basmati rice samples. The single-tube assay multiplexes eight microsatellite loci to generate variety-specific allele profiles that can detect adulteration from 1% upwards. The protocol also incorporates a quantitative-competitive PCR-based analysis for quantification of adulteration. Accuracy of quantification has been shown to be ±1.5%. The experiments used to develop and validate the methodology are described.

Keywords:
Adulteration / Basmati rice / CE / Microsatellite markers / Multiplex assay / PCR
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1 Introduction

Basmati (meaning fragrant in Hindi language) rice (*Oryza sativa*) commands a special place among all aromatic rice cultivars on account of extra long slender grain, soft and fluffy texture of the cooked rice, and pleasant distinct aroma. Authentic Basmati rice cultivation is confined to Indo-Gangetic regions of the Indian subcontinent and examples of genuine traditional Basmati (TB) varieties are Taraori Basmati and Dehradun Basmati. Very limited phenotypic [1] and genotypic [2] differences exist among the TB varieties. TB rice is not only in great demand in the domestic markets, but is also seen in the menu of connoisseurs worldwide. Globally, annual Basmati export market alone is valued at US$1 billion and is reported to be on the rise (Agricultural and Processed Food Products Export Development Authority (India), www.apeda.com; Trade Development Authority of Pakistan, www.epb.gov.pk). However, unsuitability of TB for intensive cultivation prompted the breeders to develop evolved Basmati (EB) varieties (that fall short of the quality traits of TB), leading to the presence of both TB and EB varieties in the market in addition to a relatively inferior non-aromatic non-Basmati (NB) long-grain rice varieties [2].

TB label not only brings along high returns but also attracts duty reduction in some markets [3]. Since it is difficult to differentiate between TB and EB or NB grains based on visual test or physicochemical tests, fraudulent traders make a sizeable profit by adulterating TB samples with cheaper EB or NB grains and exploit consumers. Such practices have been reported to be prevalent (survey conducted by the Food Standards Agency of the United Kingdom, http://www.food.gov.uk/science/surveillance/fsis2004branch/fsis4704Basmati). The adulteration of TB grains also affects the exporting countries in terms of diminished interest in the brands and consequently the Basmati trade. Hence, to protect the interests of consumers and trade, precise identification of genuine Basmati rice samples and devaluation of adulterated samples is vital. Although TB varieties can be potentially differentiated from other long grain rice varieties based on the intrinsic properties of the grain such as presence or absence of aroma, chemical composition, and grain elongation quotient, their applicability for routine and large-scale use are yet to be demonstrated. That brings us to the employment of DNA-based approaches. A similar but more

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Abbreviations: EB, evolved Basmati; **5′-FAM,** 5-carboxyfluorescein; **GM,** genetically modified; **JOE,** 6-carboxy-4′-5′-dichloro-2′-7′-dimethoxyfluorescein; **NB,** non-Basmati; **TAMRA,** carboxytetramethyl rhodamine; **TB**, traditional Basmati

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stringent traceability requirement in case of genetically modified (GM) food has been routinely and comprehensively met by the use of real-time PCR-based and array-based protocols [4, 5]. However, in case of GM food, detection and quantification of transgenes have been relatively straightforward because they are distinct "foreign" DNA elements in the host genome. The foreign sequences, therefore, can be specifically amplified with high sensitivity (detection) and accuracy (quantification) by real-time PCR. On the other hand, in case of Basmati adulteration, the so-called "adulterant" is another long-grain rice (often a recently bred Basmati-derivative). Thus far, no specific sequence (genic or otherwise) has been identified that can, upon amplification, generate private alleles specific to various Basmati cultivars. The demand of a signature sequence, that can exploit techniques like real-time PCR, has made researchers to look for polymorphisms using microsatellite markers. Microsatellite DNA markers have been extensively used in mapping, breeding, population genetics, disease diagnosis, and forensic genetics [6]. Microsatellite-based markers have been used in rice for construction of linkage maps [7], phylogenetic analysis [8], DUS testing [9], and diversity analysis [10]. However, there has been no instance of genotyping application akin to forensic DNA fingerprinting except for an initial report on the use microsatellites for detection of adulteration in Basmati rice [11], and subsequent demonstration of the potential of specific microsatellite profiles for unambiguous identification of TB rice varieties [2]. The multiplex panel of microsatellites has been employed for surveying intra- and intersubspecies variability in rice [12, 13]. Yet, there is no established high-throughput protocol for the detection and quantification of adulteration although standard protocols are available to detect GM and other rendered materials in food, animal feed, and plant seeds [14–18].

Consumer preference brings higher returns for TB varieties, leading to generation of brand equity. Therefore, exporting firms and countries would be cautious to maintain purity of the samples and importing countries would be keen on protecting consumer interests. Consequently, testing the samples for purity becomes important. Considering the trade volume, a high-throughput and accurate identification method is necessary. In fact, some Basmati importers like the European Union demand that all Basmati imports carry a mandatory certificate of purity based on DNA test [19]. Here, we report the development of a high-throughput "single-tube assay" method based on multiplexing microsatellite markers for the unambiguous identification of different Basmati cultivars and detection of adulteration. We also demonstrate the utility of microsatellite-based assay for the quantification of adulteration. The study also provides evidence for the accuracy and consistency with which the methodology can be applied on large scale to certify genuineness of Basmati rice samples. To our knowledge, this is the first report of a microsatellite marker-based multiplex genotyping assay employed in qualitative as well as quantitative detection of adulteration protecting consumer and trade interests.

2 Materials and methods

2.1 Basmati rice varieties

Although the term "Basmati" actually denotes only a small number of aromatic rice cultivars, vernacular names in the cultivation area have resulted in a longer list. Additionally, recent breeding attempts toward agronomic improvements have resulted in a number of entries as Basmati varieties. However, government agencies recognize only a few as notified varieties. For instance, India and Pakistan have notified 12 and 5 varieties, respectively. In the present study, we used all the notified varieties that included six TB varieties and nine EB varieties, along with two long-grain nonaromatic varieties and a short-grain variety to establish the utility of the protocol (Table 1).

2.2 Sampling procedure and DNA extraction

Each rice sample consisted of at least 50 g of powdered grain, from which subsamples of 1 g were drawn. Three subsamples were randomly selected and bulked, from which 100 mg of grain powder was collected for DNA extraction. Backup cum reference samples are maintained at all the stages. DNA was isolated using Qiagen DNeasy plant mini kit and DNA concentration was fluorometrically estimated with VersaFluor (BioRad) using Hoechst 33258 (Sigma) dye with 300–490 excitation/460–320 emission filters. Average values of two readings were taken for each sample. Quality of the DNA (260/280 absorbance) was verified using UV1 UV-Vis spectrophotometer (Thermo Scientific).

2.3 Microsatellite markers

Three-hundred-fifty microsatellite loci (sequence source: www.gramene.org) were screened using six TB (Basmati370, Dehradun Basmati, Taraori Basmati, Basmati386, Ranbir Basmati, Basmati217), three EB (Pusa Basmati, Super Basmati, Haryana Basmati), two NB long-grain rice varieties (Sharbati, IR64), and a short-grain variety (Jaya). Loci were selected for (i) amplification of a single allele and (ii) high discrimination power to differentiate the varieties under study.

2.4 PCR amplification

The PCR mixture (10 μL) contained 10 ng of DNA template, 80 μM dNTPs, 2 mM $MgCl_2$, 0.5 U Amplitaq Gold DNA polymerase (Applied Biosystems), and 0.1 μM each of forward and reverse primers. The 5′-end of forward primers (Sigma) was labeled with any one of the following fluorescent fluorophores: carboxytetramethyl rhodamine (TAMRA), 6-carboxy-4′-5′-dichloro-2′-7′-dimethoxyfluorescein (JOE), or 5′-carboxyfluorescein (5′-FAM) (Table 2). After an initial denaturation at 94° for 10 min, the PCR mix was cycled 25 times at 94, 55, and 72°C for 45, 90, and 60 s, respectively

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Table 1. Rice varieties included in the multiplex analysis

Name of the variety	Source	Type	Pedigree	Traits
Basmati370	I, P	TB	Selection	Aromatic, long grain
Dehradun Basmati	I	TB	Selection (Type-3)	Aromatic, long grain
Ranbir Basmati	I	TB	Selection (IET11348)	Aromatic, long grain
Taraori Basmati	I	TB	Selection (Karnal Local, HBC-19)	Aromatic, long grain
Basmati386	I	TB	Selection	Aromatic, long grain
Basmati217	I	TB	Selection	Aromatic, long grain
Kernel Basmati	P	EB	CM7/Basmati370, 1968	Aromatic, long grain
Basmati385	P	EB	TN1/Basmati370, 1985	Aromatic, long grain
Super Basmati	P, I	EB	Basmati320/IR661, 1996	Aromatic, long grain
Basmati198	P	EB	Basmati370/TN1, 1972	Aromatic, long grain
Pusa Basmati	I	EB	Pusa150/Karnal Local, 1989	Aromatic, long grain
Punjab Basmati	I	EB	Sona/Basmati370, 1982	Aromatic, long grain
Kasturi	I	EB	Basmati370/CR88-17-1-5, 1989	Aromatic, long grain
Mahi Sugandha	I	EB	BK79/Basmati370, 1995	Aromatic, long grain
Haryana Basmati	I	EB	Sona/Basmati370, 1991	Aromatic, long grain
Sharbati	–	NB	Selection from a UP landrace	Nonaromatic, long grain
IR64	–	NB	IR5657-33-2-1/IR2061-465-1-5-5, 1985	Nonaromatic, long grain
Jaya	–	NB	TN1/T141, 1967	Nonaromatic, medium grain

I, Basmati variety approved originally by India; P, Basmati variety approved originally by Pakistan.

Table 2. Microsatellite marker panel employed for multiplex assay

Locus	Repeat motif	Chr	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Allele pool (in base pairs)	Fluorophore
RM1	$(GA)_{26}$	1	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC	73, 100, 106, 108	5′-FAM
RM72	$(TAT)_5C(ATT)_{15}$	8	CCGGCGATAAAACAATGAG	GCATCGGTCCTAACTAAGGG	148, 158, 164, 173	5′-FAM
RM171	$(GATG)_5$	10	AACGCGAGGACACGTACTTAC	ACGAGATACGTACGCCTTTG	322, 336, 344, 348	5′-FAM
RM202	$(GA)_{30}$	11	CAGATTGGAGATGAAGTCCTCC	CCAGCAAGCATGTCAATGTA	160, 182, 186	JOE
RM241	$(GA)_{31}$	4	GAGCCAAATAAGATCGCTGA	TGCAAGCAGCAGATTTAGTG	128, 140, 143	JOE
RM44	$(GA)_{16}$	8	ACGGGCAATCCGAACAACC	TCGGGAAAACCTACCCTACC	103, 109, 113	TAMRA
RM55	$(GA)_{17}$	3	CCGTCGCCGTAGTAGAGAAG	TCCCGGTTATTTTAAGGCG	220, 230, 235, 237	TAMRA
RM348	$(CAG)_7$	4	CCGCTACTAATAGCAGAGAG	GGAGCTTTGTTCTTGCGAAC	131, 140	TAMRA

(GeneAmp PCR System 9700, Applied Biosystems). This was followed by a final extension step at 60°C for 30 min. Amplification was confirmed on a 1.5% agarose gel.

2.5 Genotyping and sequencing

Genotyping assays were carried out on a capillary-based ABI PRISM 3100 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. The PCR product (0.2 μL) was mixed with 0.3 μL of carboxy-x-rhodamine (ROX)-500 size standard and 12 μL of Hi-dye (both from Applied Biosystems), before CE. Subsequently, fluorescent DNA fragments were resolved using the GeneScan version 3.7, and allele size and peak-area of the true peaks were determined by Genotyper version 3.7. Bidirectional sequencing of PCR products was carried out on an ABI PRISM 3100 sequencer according to the manufacturer's instructions. Sequencing was repeated thrice to obtain accurate sequences of the repeat regions. Comparison of allele sequences among Basmati varieties was carried out for each of the loci by multiple sequence alignment using ClustalX 1.8 program [20] and manual editing using GeneDoc Version 2.6.002 [21].

2.6 Preparation of standard samples

To verify the sensitivity and accuracy levels of the methodology in detection and quantification of adulteration, standard samples of Basmati370 were prepared by mixing the grains of a common adulterant, Sharbati, at progressive ratio of 1, 3, 5, 7, 10, 15, 30, 50, and 60% to generate data at nine score points. Subsequent to genotyping, peak heights (relative fluorescence units, rfu) were recorded at each score point for evaluation, while peak areas (rfu) were determined and were plotted against the percent adulterant to develop a standard curve based on logistic model ($y = a/1 + be^{-cx}$) by using CurveExpert version 1.37 (http://curveexpert.webhop.biz).

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2.7 Real-time PCR

DNA of adulterant in the standard samples was quantified by real-time PCR using primers amplifying a short stretch of putative betaine-aldehyde dehydrogenase gene [22] that shows an 8 bp deletion in aromatic rices including Basmati in comparison to nonaromatic rices. Amplification with a forward primer (CATGGTTTATGTTTTCTGTTAGGTTG) and a reverse primer (TAGGAGCAGCTGAAGCCATAAT), with the latter binding to the seven of the eight bases deleted in Basmati varieties (GATTATGG), produces an 80 bp amplicon only in nonaromatic rice including Sharbati (Supporting Information Fig. 1). The qPCR Mastermix Plus for SYBR Green I kit was used according to the manufacturer's directions (Eurogentec). A 50 μL reaction buffer contained 2 × reaction buffer containing dNTP, HotGoldStar, $MgCl_2$, 0.5 μM primers, SYBR Green I, stabilizers and ROX passive reference. The cycling parameters were an initial DNA denaturation step at 95°C for 10 min followed by 40 cycles of PCR with DNA denaturation at 95°C for 15 s, primer annealing at 60°C for 1 min and extension at 72°C for 1 min. Fluorescence data were recorded with an ABI Prism 7000 sequence detection system (Applied Biosystems) and analyzed with Sequence Detection Software 1.2.3. Optimization of real-time PCR was carried out by comparing amplifications at *fgr* and RM348 loci (Supporting Information Fig. 2).

3 Results and discussion

3.1 Microsatellite marker panel for adulteration detection

Earlier reports on DNA-based detection of adulteration in food/feed material have employed specific sequences to amplify fragment length polymorphisms to reveal the existence of an adulterant. The specific loci included 12S rRNA [23], 5S rRNA [24], trnT-trnL spacer [25], and chloroplastic-SNP [26]. However, genuine TB samples are adulterated with either EB or NB that are genetically quite similar to TB and hence offer no leeway to employ routinely used genomic sequences for differentiation. Under such circumstances, microsatellite loci, that exhibit greater allelic polymorphism, have been employed for the detection of adulterants [15].

A. Identity codes

Variety	Allele code							
	RM171	RM55	RM202	RM72	RM348	RM241	RM44	RM1
Basmati370	B	C	B	D	B	B	B	A
Dehradun Basmati	B	C	B	D	B	B	B	A
Ranbir Basmati	B	C	B	D	B	C	B	A
Taraori Basmati	B	A	B	D	B	A	C	A
Basmati386	B	A	B	D	B	A	C	A
Basmati217	C	B	B	B	A	A	A	B
Kernel Basmati	C	A	B	D	B	B	B	A
Basmati385	B	C	A	D	B	B	C	A
Super Basmati	C	A	B	D	B	B	B	C
Basmati198	B	C	A	D	B	B	B	C
Pusa Basmati	C	B	B	B	A	A	C	A
Punjab Basmati	C	A	A	A	B	B	A	A
Kasturi	C	B	B	B	A	A	A	A
Mahi Sugandha	C	B	A	A	B	B	A	B
Haryana Basmati	C	B	A	B	A	A	A	D
Sharbati	A	B	A	B	A	A	A	C
IR64	D	B	C	C	A	A	A	C
Jaya	A	D	A	B	A	A	A	C

B. Code key

Allele code	Allele size in base pairs							
	RM171	RM55	RM202	RM72	RM348	RM241	RM44	RM1
A	322	220	160	148	131	128	103	73
B	336	230	182	158	140	140	109	100
C	344	235	186	164		143	113	106
D	348	237		173				108

Figure 1. Specific allele profiles of Basmati rice varieties and putative adulterants at eight microsatellite loci.

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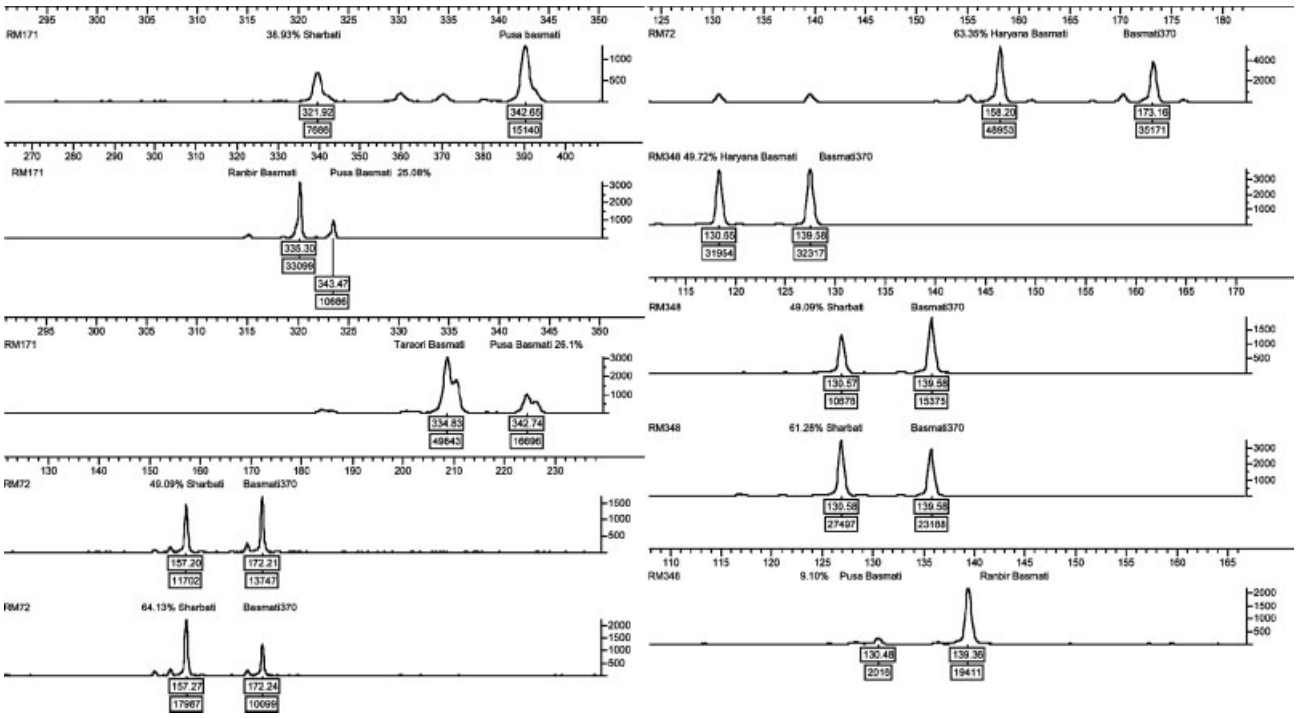

Figure 2. Quantification of adulteration using microsatellite-based CE. Each electropherogram represents a quantification assay of various Basmati rice samples at a given locus (any of RM348, RM72, and RM171). Peaks are annotated by allele size (top box) and peak area (bottom box). Main variety and adulterant are resolved, identified and adulterant is quantified (expressed as per cent) peak area ratios. Alleles are identified within one base pair bin compared to the sizes given in Fig. 1.

Therefore, primary step in detection of an adulterant in Basmati samples is to generate variety-specific microsatellite profiles. This was achieved by screening for polymorphic microsatellite loci so that upon PCR, a genuine TB variety, at a given microsatellite locus yields a single allele of known size. Any admixture of TB with either EB or NB would be detected readily because of distinct allele sizes. Eight out of 350 microsatellite loci consistently amplified single allele per variety and distinguished, in combination, all the Basmati varieties notified by India and Pakistan among themselves and from some putative adulterants (Table 2). The panel comprised of microsatellites with five di-, one tri-, one tetranucleotide repeats and one compound repeat. The panel generated an average of 3.3 alleles per locus in a size range of 73–348 bp. Success of a microsatellite-based genotyping application depends upon the informativeness of the loci. This means that although some of the microsatellite loci (dinucleotide repeats) exhibited great discriminatory power (*e.g.*, RM252, 22 alleles and allele frequency 11.6%, ref. [8]), they produced a series of nonspecific amplification products even under the best of conditions, precluding their inclusion in the genotyping assay. To avoid erroneous allele sizing, which is not acceptable for sensitive assays such as genotyping and determination of adulterants, we confirmed the allele sizes in 12 varieties by sequencing the alleles and counting the number of repeat units in each allele at all the loci (Supporting Information Fig. 3). Further, diversity analysis of Basmati varieties [7] showed that the microsatellites included in our panel exhibit moderate allele frequency such as RM171 (34.8%), RM55 (55%), RM44 (39%), and RM1 (50%). This, as evident from the present study, brings adequate polymorphism to differentiate between TB, EB, and NB varieties. In spite of the fact that Basmati is threatened by possible adulteration with any or a combination of several long grain rice varieties, methodology reported here relies on unambiguous identification of all the authentic Basmati varieties traded in the international market and trace adulterants based on deviation from the designated profile.

3.2 Stability of marker profile

Rice is highly homozygous due to absolute autogamy. Annual, selfing and/or early successional taxa allocate more of their genetic variability among populations and there exists little within population variation. Therefore, if at all any variation is observed in rice, it is expected between populations. However, unlike NB rice varieties that are cultivated as a staple crop, Basmati rice is grown as a commercial crop and hence has been maintained with little variability across populations. Further, SSR- and FISSR-based analyses have shown that there are no significant differences among the TB varieties as well, supporting the theory that TB varie-

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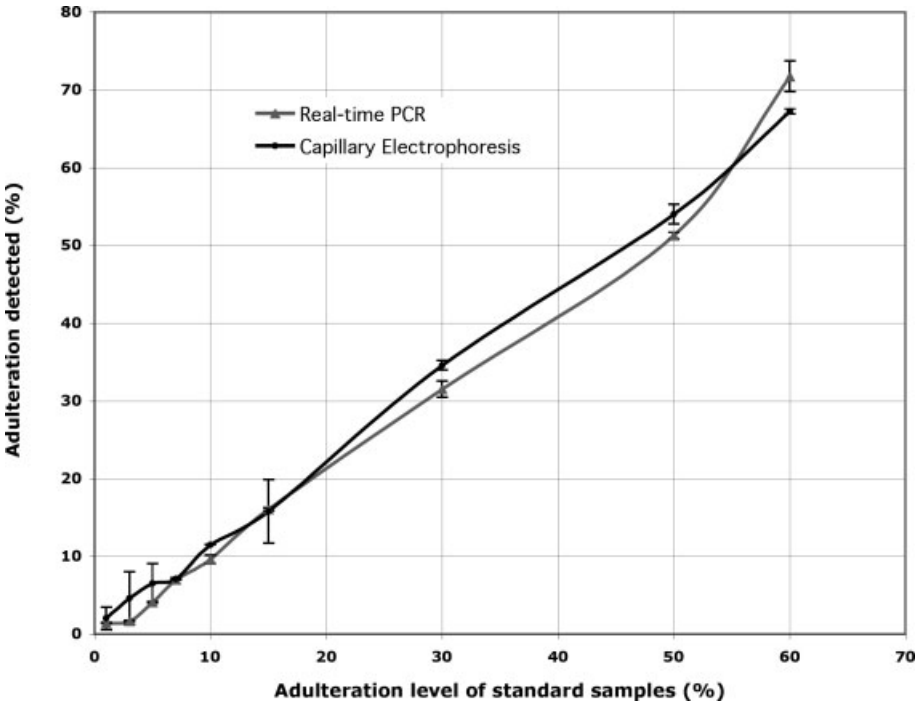

Figure 3. Corroboration of quantification of adulterant based on microsatellite marker assay by real-time PCR assay.

ties are possibly the descendants of a single land race, and the minor genetic variation is maintained as a result of the selection and preference imposed by farmers for several years [2]. Varietal homogeneity in TB was further established when a previous study has demonstrated the absence of within variety polymorphism in some Basmati varieties [9]. In the present study, Basmati varieties were sampled from multiple sources and specificity of the designated alleles in a given Basmati variety was confirmed by genotyping at least ten individual plants from each of the varieties. There was no intravarietal polymorphism among populations (Supporting Information Table 1). Varietal homozygosity and lack of interpopulation variation among Basmati varieties coupled with moderate allele frequency of the selected microsatellite loci ensure stability of the genotyping assay described in this report.

3.3 Multiplex assay and varietal specific profiles

Multiplexing all the informative microsatellite loci into a single-tube assay facilitates high throughput and reduces the cost of assay. Eight microsatellite loci of the panel were multiplexed into three groups after confirming that allele sizes did not overlap (Table 2) and that all the primers showed compatibility when cross-checked against one another (Supporting Information Table 2). The primers were multiplexed by using three different fluorophores – 5′FAM (RM1, RM171, RM72), JOE (RM202, RM241), and TAMRA (RM55, RM44, RM348). Microsatellite allele profiles of the varieties are given in Fig. 4 of Supporting Information and the identity codes of the varieties are presented as Fig. 1. All the TB varieties except Basmati217 generated identical allele profiles at each of the five loci – RM1, RM72, RM171, RM202, and RM348, whereas EB and NB varieties showed a diverse allele pattern. The TB varieties were distinguished readily and indisputably from EB and NB varieties using a combination of the five loci. Three loci, RM55, RM241, and RM44, were useful in detecting differences among TB varieties. Taraori/Basmati386 and Basmati370/Dehradun Basmati pairs had the same allele profiles and could not be distinguished. Otherwise, the eight-letter code was different for all the varieties analyzed in this study and specificity of the assay was beyond doubt (Fig. 1). Most common adulterants Basmati385 and Sharbati as well as EB varieties were assigned unique codes of identity.

3.4 Validation of the methodology and test of accuracy

Validation of a newly designed protocol is an integral process before it is adopted by other laboratories or for commercial purpose. DNA was extracted from aged rice grains, since traded grains are usually aged for one year. Care was taken to extract pure DNA since inhibitory components could be coextracted from rice grains that are particularly rich in starch and also contain small amount of lipids. Four procedures – cTAB method [27], modified cTAB method [28], Nucleon Phytopure DNA extraction kit (Amersham Biosciences), and Qiagen DNeasy plant mini kit – were tested for DNA extraction. The Qiagen kit was found to yield good

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quality DNA (260/280>1.7) and exhibited the least sample-to-sample variation in PCR amplification [29]. Experiments were carried out (i) to determine the optimum primer concentrations and the minimum number of PCR cycles, (ii) to assess the LOD, and (iii) to confirm the accuracy of quantification with real-time PCR.

3.4.1 Primer concentration

Varying the concentration of primers of one locus may affect the amplification at the other seven loci in the multiplex. Differences in the DNA sequences of the loci can lead to variation in the efficiency of primer binding. Therefore, it becomes essential to tune the primer concentrations to obtain not only best signal intensities but also balanced peak heights for a multiplex set. Equal concentrations of the primers resulted in intense allelic products at RM1 and RM348, leading to dwarfing of other peaks. Optimal primer concentrations for the multiplex were determined to be 0.1 μM (RM171, RM55, RM44), 25 nM (RM202, RM72, RM241), and 6.25 nm (RM1, RM348).

3.4.2 Number of PCR cycles

Improvement in sensitivity of PCR assay can be achieved by using an increased number of cycles. Forensic anthropologists have routinely used increased PCR cycle numbers (38–43) to obtain profiles from ancient DNA [31]. With increased template DNA, however, effect of cycle number is not that apparent; instead it could lead to artifact production and compromise on peak balance. Therefore, increased number of PCR cycles would adversely affect quantification of the adulterant. This is effectively achieved only in the linear phase of the PCR, where differences in the template DNA concentrations are best represented [32]. To optimize the cycle number keeping sensitivity as well as quantification of the adulterant in mind, we amplified template DNA at 20, 22, 24, 25, 26, 28, and 30 cycles. Allele dropout in 1–5% adulterated samples was observed at 20, 22, and 24 cycles frequently for the adulterant allele. Multiplex loci gave good signal intensities from 25 cycles onwards. We chose the minimum number of cycles (25) for quantification of adulterant using multiplex assay.

3.4.3 Peak balance and quantification

Amplification of polymorphic alleles may be imbalanced due to stochastic effects in the PCR reaction. Additionally, one allele can be preferentially amplified over the other due to unequal sampling of polymorphic alleles during the early stages of the PCR reaction [30]. Since an important requirement for accurate quantification is to produce balanced allele peaks, we calculated peak balance ratio at various ratios of adulteration. Only polymorphic loci were included in the calculations. Complete dropout of one allele was assigned a zero peak balance ratio. Good intralocus balance (≥0.7 ratio) was obtained at all the multiplexed loci except RM1 (Supporting Information Fig. 4). RM72 and RM348 exhibited excellent peak balance (≥0.80), confirmed over two sets of adulterated standard samples, Basmati370:Sharbati and Basmati370:IR64 (the latter is given as Fig. 5, Supporting Information). Therefore, RM348, RM72, and RM171 were used for the quantification of the adulterant in that order of preference (Fig. 2).

3.4.4 LOD and LOQ

LOD and LOQ were determined by an empirical approach consisting of measuring progressively more dilute concentrations of the adulterant. The LOQ (the adulteration levels at which quantitative results can be reported with a high degree of confidence) was also empirically determined. At 25 cycles PCR, all the eight loci were not equally efficient in detecting 1% adulteration level. Multiple runs show increasing SD, in that order, for RM348, RM72, RM171 and followed by dinucleotide repeat loci in detecting 1% adulterant in the otherwise pure Basmati sample or *vice versa*. LOQ was also found to be 1% in our analyses particularly for RM348 and RM72. Number of PCR cycles influenced both LOD and accuracy of quantification. Detection efficiencies were drastically improved at 30 cycles for all the eight loci in terms of consistent detection of 1% adulteration, and reduction of allele dropouts. Conversely, the error limits of quantification expressed as SD, calculated over replicated experiments substantially increased from +1.11% (25 cycles) to ±3.3% (30 cycles) for adulteration up to 15%.

3.4.5 Concordance studies with real-time PCR analysis

Quantification based on competitive-quantitative PCR coupled with the use of standard curve, essentially relies upon the accuracy of measuring the template DNA concentration in the PCR mixtures. Hence, it was considered critical to confirm the template DNA concentrations by employing real-time PCR. However, the challenge was to find a specific sequence (genic or otherwise) that can, upon amplification, generate private alleles specific to Basmati varieties and adulterants. We could identify an 80 bp amplicon that is specific to nonaromatic rices (Supporting Information Fig. 1). Using this sequence, standard samples of Basmati370 containing Sharbati (nonaromatic) as adulterant were analyzed by real-time PCR to quantify the Sharbati DNA. C_t values of standard samples were converted to the actual adulteration levels and the values were then compared to the adulteration quantified by the CE-based microsatellite assay. The values indicated reasonable agreement between quantification based on real-time and electrophoresis methods (Fig. 3), implying that the standard samples were reliable enough to construct standard curves and subsequently employ those standard curves for quantification of adulteration.

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3.5 Quantification of adulteration

It is possible that some Basmati rice samples contain adventitious mixture because of inadvertent mixing in the field/storage. If we can measure the actual amount of the adulterant, such samples having admixture within limits allowed by the importing countries could be certified as practically genuine. For instance, Basmati Code of Practice recommends 7% as the ceiling for inadvertent mixtures in Basmati imports in the UK (www.riceassociation.org.uk**)**. Hence, it is essential that the methodology incorporate accurate quantification of adulteration.

The protocol assumes (i) equivalent contribution of DNA from the grain mixtures and (ii) faithful representation of the samples. Therefore, adulterant is quantified based on the relative quantities of the amplified allelic products at a common locus between competing DNA templates (those of the Basmati and the adulterant) in the PCR mixture [33]. In this way, adulteration was computed in the standard samples (premixed grains of the genuine Basmati and a putative adulterant in a progressive ratio). The results were represented by plotting percentages of adulterant on the abscissa and the corresponding peak-area ratios (expressed in percentage) on the ordinate. A smooth curve was obtained using logistic model (Fig. 4). Using both RM72 and RM348-based standard curves; adulterants were marginally overestimated at lower levels of adulteration (up to 15%) and were generally overestimated at higher levels of adulteration (>15%). The estimation of adulterant for adulteration up to 15% was within +1.11% of the actual adulteration. However, accuracy of estimation was lower for adulteration beyond 15%, which was +2.5% (Fig. 4). Post DNA-test regime, legal ceiling of adventitious adulteration is proposed to be 7% for the lowest grade Basmati (www.food.gov.uk/multimedia/pdfs/fsis4704Basmati.pdf). Therefore, samples having higher adulteration could be rejected, and hence accuracy of estimation beyond 15% of the adulteration becomes practically redundant.

Detection of adulteration was relatively error-free once the representative samples are ensured. However, quantification (and subsequent acceptance/rejection of a sample) could be susceptible to minor experimental errors. When three blind samples with 4, 8 and 12% adulteration were genotyped and the peak-area ratios were plotted on the standard curves, the adulteration was estimated with an error of± 1.94, ±0.17 and ±0.93%, respectively. Possible sources of experimental error in the protocol were identified to be (i) DNA extraction, (ii) amplification of the microsatellite alleles by PCR, and (iii) CE. An experiment was laid down to quantify any possible deviation from actual adulteration value. Each of these steps was triplicated in series. For instance, 15% adulterated Basmati370 samples were assayed at RM72 indicated that deviation from the 15% was obtained with an overall error of ±1.23%. The same value at another locus RM348 was ±1.45%. This consistency level has been found to be adequate for all practical purposes of Basmati certification.

3.6 Limitations of the multiplex microsatellite marker assay

Taraori/Basmati386 and Basmati370/Dehradun Basmati pairs had the same allele profiles in the 8-loci multiplex and could not be distinguished. Nonetheless, this has no effect

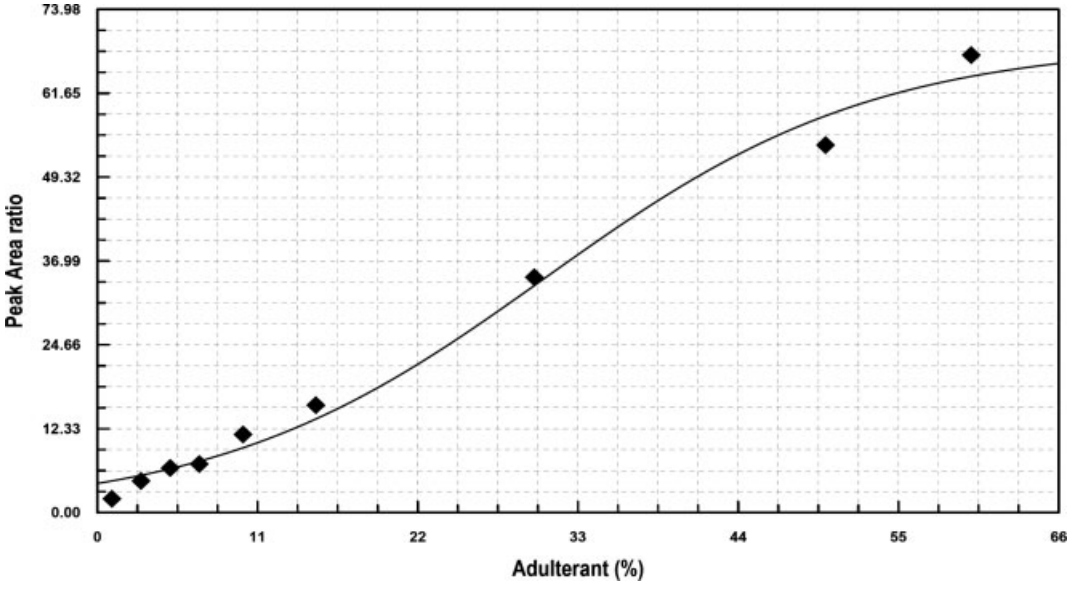

Figure 4. Standard curve generated based on Basmati370:Sharbati standard samples. Pure Basmati370 was mixed with Sharbati in progressive proportions (1, 3, 5, 7, 10, 15, 30, 50, and 60%). Peak area ratios obtained from microsatellite-based CE at RM348 locus were plotted against per cent adulteration at nine score points. The standard curve is developed based on logistic model.

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on the effectiveness of the protocol, as these varietal pairs are treated as practically the same variety in the market. Further, in our ongoing search for better loci, we have found few more potential candidate loci (RM440, RM525, JL14, RM16, and RM85) that are being developed as reserve set markers in case new adulterants in future challenge the existing multiplex. Among them, we have discovered that JL14 could distinguish between Taraori/Basmati386.

The gap between real-time PCR in GM detection (LOQ 0.06%, www.food.gov.uk/multimedia/pdfs/fsis0506.pdf) and CE-based microsatellite marker assay in the estimation of Basmati adulteration (LOQ 1%, present study) in terms of error is significant and probably reflects the efficiency of the techniques rather than the problem in hand or standardization of protocol. For instance, quantification of pork adulteration using SINE-specific sequences could be achieved at 1% limit [34] and a much-sophisticated approach (lab-on-chip capillary-based electrophoresis) could also detect at a limit of 5% contamination on Arabica coffee samples [26]. Opportunely, the stringency levels involved in detecting GM (legal limit of 0.9%) and detecting adulteration in case of Basmati trade (proposed legal limit of 7%) are as much disparate, and that allows us to employ the protocol presented here effectively for all practical purposes even if errors in the estimation of adulteration ranges at $\pm 1.5\%$.

4 Concluding remarks

Traceability is increasingly becoming standard across the agri-produce and agri-food market, largely driven by the experience with GM food as well as availability of a range of DNA-based concepts and technologies adapted to different industry needs. Traceability in the form of detection and quantification of adulteration forms an essential component of Basmati trade management as well as post-marketing surveillance. Given the unfeasibility of traceability through otherwise accurate and high-throughput technology like real-time PCR at the moment, a microsatellite-based multiplex assay is designed and tested for accuracy and practicability. Basmati trade is moving toward a uniform code of practice with a long-term goal to peg the adulteration of pure Basmati samples at 7%. The CE-based microsatellite multiplex assay presented here has high throughput and can effectively carry out quantification of adulteration with sensitivity and accuracy levels adequate for Basmati trade. The methodology is amenable to the development of a ready-to-use kit that can facilitate adherence to a common certification protocol by Basmati exporters, importers and regulators. The protocol has been authorized by the export development and regulatory agencies of the government of India for issuance of certificate of purity for Basmati export samples. Based on the multiplex assay, we have certified more than 500 referral samples of Basmati rice destined to international market. Patents are pending for the protocol reported here (USPTO 10/357, 488 and 11/406, 257; PCT/IN06/00254). A ready-to-use kit has been developed by M/S Labindia (partners of Applied Biosystems in India) under license transfer agreement.

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