JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Capillary Electrophoresis Is Essential for Microsatellite Marker Based Detection and Quantification of Adulteration of Basmati Rice (*Oryza sativa*)

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Microsatellite markers are employed for genotyping of Basmati varieties and assaying purity of market samples. However, employment of diverse electrophoresis techniques across laboratories has resulted in inconsistent allele sizes, creating doubts about the suitability of the assay. This study evaluated agarose gel electrophoresis, slab gel electrophoresis, and capillary electrophoresis techniques for their efficiency in the detection and quantification of adulteration in Basmati samples. Comparative analysis across 8 microsatellite loci in 12 rice varieties demonstrated that the capillary electrophoresis method showed less error (±0.73 bp) in the estimation of allele sizes compared to slab gel (±1.59 bp) and agarose gel (±8.03 bp) electrophoretic methods. Capillary electrophoresis showed greater reproducibility (<0.5 bp deviation) compared to slab gel (1bp) and agarose (>3 bp) based methods. Capillary electrophoresis was significantly superior in quantification of the adulterant, with a mean error of ±3.91% in comparison to slab gel (±6.09%). Lack of accuracy and consistency of the slab gel and agarose electrophoretic methods warrants the employment of capillary electrophoresis for Basmati rice purity assays.

KEYWORDS: Adulteration; agarose gel electrophoresis; Basmati rice; capillary electrophoresis; microsatellite markers; *Oryza sativa*; slab gel electrophoresis

INTRODUCTION

Microsatellite markers are widely used in forensic DNA analysis, construction of linkage maps, gene tagging, population studies, etc. (*1–4*). With the advent of PCR technique and concomitant technological advancements in DNA fragment separation and detection methodologies, the past decade witnessed an enormous increase in the number of genetic analysis reports based on microsatellite markers. The initial use of microsatellite markers was based on agarose gel assays, and even today construction of linkage maps and diversity analysis in plants are usually based on agarose gel assays. However, forensic genetic analysis requires higher resolution fragment separation and greater accuracy in allele sizing because diagnostic markers often possess alleles with as little as 2 bp size difference. Thus, polyacrylamide gel electrophoresis (PAGE) was recruited to achieve better fragment separation, and agarose gel electrophoresis was soon replaced by sophisticated and semiautomated PAGE platforms such as ABI373 and ABI377 (Applied Biosystems) and ALF express DNA sequencer (Pharmacia Biotech). Fluorescent dye chemistry (e.g., TAMARA, JOE, FAM, etc.) amenable to laser-based detection improved the resolution and accuracy of the electrophoresis (*5, 6*). However, a major upward shift in the accuracy of microsatellite marker based forensic analysis took place with the introduction of capillary electrophoresis technology by various manufacturers, for example, Applied Biosystems (ABI310, ABI3100, ABI3700, ABI3730), Amersham Biosciences (MegaBASE500, 1000, 4000), Beckman Coulter (CEQ8800), and SpectruMedix Corporation (SCE2410, 9610, 19210) (*4*). Capillary electrophoresis heralded accurate and consistent allele sizing with minimal manual intervention, thereby reducing the sizing error (*7*). Furthermore, the higher throughput of capillary electrophoresis was found to be unmatched by any of the previous methods. Application of capillary-based microsatellite analysis was initially confined to human forensic DNA fingerprinting (*8*). The technique was subsequently extended to animal (*9*) and plant (*10*) variety identification and high-density genetic mapping projects (*11*).

Basmati is an aromatic long-grain type of rice (*Oryza sativa*) cultivated exclusively in the foothills of the Himalayas stretching across the Haryana and Uttar Pradesh regions of India and the Punjab province of India and Pakistan (*5, 12*). Among the Basmati varieties traded in the international market, traditional

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10.1021/jf0714517 CCC: $37.00
Published on Web 09/15/2007

Basmati (TB) varieties such as Basmati370, Dehradun, and Taraori command higher price (U.S. $850/MT) compared to evolved Basmati (EB) varieties such as Pusa Basmati, Mahi Sugandha (U.S. $480/MT) from India and Super Basmati (U.S. $500–520/MT) from India and Pakistan (www.basmati.com, www.oryza.com), whereas long-grain non-Basmati (NB) varieties such as PR106 command only U.S. $167/MT. Because it is difficult to differentiate between TB, EB, and NB rice grains on the basis of visual observation or physicochemical tests, unscrupulous traders adulterate TB samples with EB or NB grains to earn profits. For instance, by mixing 30% of evolved Basmati varieties with traditional Basmati, traders can earn $111/MT profit, whereas non-Basmati varieties with similar degrees of adulteration can earn $204/MT additional profit. Hence, distinguishing traditional Basmati rice varieties from evolved Basmati and look-alike long-grain non-Basmati varieties has become an important aspect of the Basmati trade. The suitability of microsatellite markers for Basmati genotyping and adulteration detection has been demonstrated earlier (*5, 13*). These studies have enabled Basmati importers and exporters to move toward a uniform and strict regime of DNA-based testing of the export lots. A certificate of purity based on a DNA test is considered to be value addition (for instance, Basmati imports into the European Union). Although there has been a consensus on employing a panel of microsatellite markers to solve the identity problem, the actual protocol accredited across laboratories to generate profiles is not finalized as yet. Laboratories working on mapping and diversity studies of Basmati have been using different fragment separation techniques and have reported contradictory allele profiles of various Basmati varieties. For instance, Jain et al. (*14*) used fluorescent-labeled primers coupled with slab gel, whereas Navinder et al. (*15*) and Singh et al. (*16*) used PAGE and agarose to study the genetic relationship of different Basmati cultivars. These published results have generated ambiguity among stakeholders in the Basmati trade and have led to delays in establishing a Basmati code of practice describing diagnostic DNA markers for a Basmati purity test.

The differences in the skill, cost, and infrastructure requirement among various techniques are so enormous that a simple decision is far from possible about the necessity and sufficiency criteria of fragment separation and detection techniques. The Centre for DNA Fingerprinting and Diagnostics houses the Centre for Basmati DNA Analysis, commissioned by Indian government agencies, and hence establishment of minimum standards for fragment separation employed during purity testing was taken up. Here, we describe the results of the comparison of various fragment separation techniques and conclude that capillary electrophoresis is essential to obtain accurate and consistent results.

MATERIALS AND METHODS

DNA Extraction and PCR Amplification. Twelve rice cultivars including six traditional Basmati, three evolved Basmati, and three non-Basmati varieties (**Table 1**) were genotyped in agarose, slab gel, and capillary electrophoresis using eight microsatellite loci (**Table 2**) described previously (*5, 17*). DNA was extracted from brown rice grains (*18*). Ten microliters of PCR mixture contained 10 ng of DNA template, 80 μM dNTPs, 2 mM $MgCl_2$, 0.5 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 0.1 μM each of forward and reverse primers (Sigma). The 5′ end of each forward primer was fluorescently labeled with either TAMRA, JOE, or 5FAM (**Table 2**). Thermal cycling conditions were as follows: initial denaturation of 15 min at 94 °C; 35 cycles of 45 s at 94 °C; 45 s at 55 °C; and 1 min at 72 °C; and final extension of 10 min at 72 °C. The PCR was performed on a GeneAmp PCR System 9700 (Applied Biosystems).

Table 1. Rice Varieties Included in the Comparative Analysis of Electrophoresis Techniques

variety	type	pedigree
Basmati370	traditional Basmati	selection
Dehradun Basmati (Type-3)	traditional Basmati	selection
Taraori Basmati (Karnal Local, HBC-19)	traditional Basmati	selection
Basmati386	traditional Basmati	selection
Ranbir Basmati (IET11348)	traditional Basmati	selection
Basmati217	traditional Basmati	selection
Haryana Basmati	evolved Basmati	Sona/Basmati370
Pusa Basmati	evolved Basmati	Pusa150/Karnal Local
Super Basmati (Pakistan)	evolved Basmati	Basmati320/IR661
Sharbati	non-Basmati	selection from a UP landrace
IR64	non-Basmati	IR5657-33-2-1/IR2061-465-1-5-5
Jaya	non-Basmati	TN1/T141

Agarose Gel Electrophoresis. PCR samples were mixed with bromo-phenol blue and loaded in 3% agarose gel (Pharmacia model EPS-200) containing ethidium bromide and run at 5.3 V/cm (Bio-Rad PowerPac 300) for an hour. Gels were photographed using a gel documentation system (Bio-Rad Molecular Imager Gel Doc XR System). Allele sizes were estimated in comparison with a 50 bp ladder (MBI Fermentas). Three researchers from our laboratory, who have been carrying out PCR, agarose gel electrophoresis, and genotyping work on a routine basis, were asked to size the alleles. Average allele sizes were determined on the basis of triplicate measurements obtained manually as well as by using software (Quantity One version 4.1.1). As it is difficult to quantify the amplified products manually in agarose gels, we did not carry out tests for quantification of adulteration in Basmati based on agarose gel assays.

Slab Gel Electrophoresis. One microliter of PCR product was mixed with 1.5 μL of 6× loading buffer (1:4 mixture of loading buffer and formamide; Sigma), and 0.3 μL of GeneScan-500 ROX-labeled molecular weight standard (Applied Biosystems) was included in the loading samples. The samples were denatured at 95 °C for 3 min before loading onto an ABI 377 automated sequencer (Applied Biosystems) and electrophoresed on 5% polyacrylamide gel (Long Ranger, FMC) containing 7 M urea, in 1× TBE buffer (90 mM Tris borate, pH 8.3, and 2 mM EDTA). Allele sizes were estimated using GeneScan 3.1 and Genotyper 2.1 software (Applied Biosystems) following the manufacturer's instructions.

Capillary Electrophoresis. The fluorescently labeled PCR products were mixed with 0.3 μL of GeneScan-500 ROX size standard (Applied Biosystems) and 12 μL of Hi-Di Formamide (Applied Biosystems), electrophoresed by capillary electrophoresis on an ABI PRISM 3100, and viewed with GeneScan 3.7 and Genotyper 3.7 software (Applied Biosystems). Each experiment was replicated at least three times to verify the reproducibility of markers.

Sequencing. Bidirectional sequencing of PCR products was carried out on an ABI PRISM 3100 sequencer according to the manufacturer's instructions. The sequencing was repeated three times to obtain accurate sequences of the repeat regions.

Calculation of Accuracy and Consistency in Estimation of Allele Sizes. Allele sizes estimated on the basis of sequencing results were considered to be *actual allele sizes* (*19*) and were compared with *observed allele sizes* or *called allele sizes* obtained from agarose, slab gel, and capillary electrophoresis instruments. Although different versions of the software were used to estimate the allele sizes in slab gel and capillary electrophoresis, as mentioned above, we did not find any noticeable size differences as the basic algorithm is common and had no influence on our analysis. Allele size differences were averaged over all 12 rice varieties to obtain accuracy estimated at each locus. The accuracy of allele size estimation was expressed as the difference (in base pairs) between the actual allele size and the observed allele

Table 2. Allele Size Estimation Accuracies of Agarose, Slab Gel, and Capillary Electrophoresis Methods

		mean difference (± bp)			
locus	size range (bp)	agarose (manual)	agarose (software)	slab gel electrophoresis	capillary electrophoresis
RM171	323–347	6.50	6.88	0.08	1.80
RM55	218–235	5.42	8.08	3.05	0.44
RM202	161–182	4.22	2.59	0.40	0.45
RM72	148–173	14.61	13.02	2.15	0.08
RM348	130–139	6.03	9.64	1.30	0.58
RM241	128–144	12.53	9.15	2.40	0.28
RM44	103–113	5.92	7.32	0.03	0.21
RM1	73–108	9.00	8.69	3.31	2.00
av		8.03	8.17	1.59	0.73

size. Consistency in allele size estimation was computed from standard deviations obtained in triplicated assays involving electrophoresis and size determination of 12 rice varieties at eight microsatellite loci.

Calculation of Accuracy in Quantification of Adulteration. In slab gel and capillary electrophoresis methods, the quantity of an amplified PCR product is represented by peak area (measured in relative fluorescent units, rfu). Accuracy in quantification of adulteration was calculated by mixing of Basmati370 grains with a common adulterant, Haryana Basmati, in progressive proportions from 10 to 90% with a 10% interval at two informative loci, RM72 and RM348. Adulteration was expressed as a percent fraction as

$$\text{adulteration} = \left[\frac{A}{(A+B)}\right] \times 100$$

where A = rfu of the adulterant and B = rfu of the main variety. Accuracy of quantification was expressed as the difference (in percent adulterant) between the actual adulteration and the observed adulteration.

Comparison of Capillary Electrophoresis with Real Time PCR. Real time PCR is acknowledged as an accurate protocol for DNA quantification and is routinely employed for GM detection assays. We evaluated the quantification accuracies of capillary electrophoresis vis-à-vis real time PCR essentially as described earlier (*17*). A polymorphic deletion of 8 bp specific to aromatic rices in the betaine-aldehyde dehydrogenase-2 gene (*bad*2) (*20*) was exploited to amplify an 80 bp product exclusively in a non-Basmati variety (i.e., adulterant) in a mixture. Real time PCR and capillary electrophoresis (at two loci, RM348 and RM72) were carried out on standard samples prepared by mixing grains of Basmati370 with Sharbati in different proportions of 1, 5, 7, 10, 15, 30, and 50%. PCR amplification and capillary electrophoresis were performed as described earlier. Real time PCR was carried out by employing qPCR Mastermix Plus for SYBR Green I kit (Eurogentec). A 50 μL reaction contained 2× reaction buffer containing dNTP, HotGoldStar, $MgCl_2$, 0.5 μM primers (forward CATGGTTTATGTTTTCTGTTAGGTTG and reverse TAGGAGCAGCTGAAGCCATAAT), SYBR Green I, stabilizers, and ROX passive reference. The PCR conditions included 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. Relative fluorescence units at various points of the amplification cycle were recorded with an ABI Prism 7000 sequence detection system (Applied Biosystems) and C_t values were obtained using Sequence Detection Software 1.2.3. ΔC_t values (reflecting the changed adulteration content) were determined from averaged C_t values of duplicated runs. Template DNA (expressed as percent quantity of adulterant in the sample) was computed by taking 7% score-point as the reference. These estimates were then compared to the adulteration quantified by the capillary electrophoresis (averaged over two loci, RM348 and RM72).

RESULTS AND DISCUSSION

There are three essential aspects for an electrophoresis technique to be successfully employed in genotyping assays: (i) accuracy (allele sizing), (ii) consistency (reproducibility), and (iii) sensitivity (quantification). Because the demand on accuracy in genotyping assays having legal (as in forensic DNA fingerprinting) or commercial (as in Basmati purity tests) connotations is much more stringent than required for typical electrophoretic applications, it is imperative that the best available technique is employed for the purpose. Multiple electrophoretic techniques are employed for Basmati genotyping, although it is apparent that capillary-based genotyping methodology is a superior choice (*13–16*). In this paper, we demonstrate, in statistical terms, that capillary electrophoresis shows greater accuracy and replicate concordance. Furthermore, we also show that capillary electrophoresis is up to the mark for quantitative assay by comparison with real time PCR analysis.

Accuracy and Consistency of Adulterant Detection. To evaluate the accuracies of agarose gel, slab gel, and capillary electrophoresis, a total of 864 independent microsatellite-based genotyping assays were carried out (Supplementary Table 1). To exclude the variations that may arise due to diverse DNA sources measured at different genomic loci, each technique employed 12 rice varieties (**Table 1**) genotyped at 8 microsatellite loci (**Table 2**). Each assay was run in triplicate, and average values of allele sizes and deviations were computed across all varieties at each microsatellite locus.

Accuracy was expressed as the difference (in base pairs) between observed allele size (obtained by genotyping assay) and the actual allele size (obtained by sequencing). Allele sizing is carried out with co-electrophoresed or internal fragments of known sizes. Capillary electrophoresis returned the most precise allele sizes, twice as accurately as slab gel electrophoresis could produce and more than an order of magnitude more than agarose gel electrophoresis could achieve (**Table 2**). The observations on averaged mean differences suggested that if the actual size of an allele were 74 bp, capillary electrophoresis, with a mean deviation of 0.73 bp, would estimate the allele size to be in the range of 73.27–74.73 bp. This would effectively create an error bin of 3 bp (73–75 bp). Slab gel electrophoresis, on the other hand, would create a 5 bp error bin (72–76 bp), and the agarose gel electrophoresis technique would have a 17 bp range (66–82 bp). The latter values illustrate why previous studies on Basmati genotyping based on agarose gel electrophoresis technique generated contradictory results (*13–16*).

When the average deviations were further scrutinized for the actual distribution of allele sizes, it was observed that nearly 58% of the alleles estimated by capillary electrophoresis showed exact values (zero deviation, **Figure 1**). In all, >90% of the capillary electrophoresis assays resulted in allele sizing within a base-pair bin, whereas the corresponding estimate was <50% for slab gel electrophoresis and a dismal 7 and 2%, respectively, for software-based and manually measured agarose gel elec-

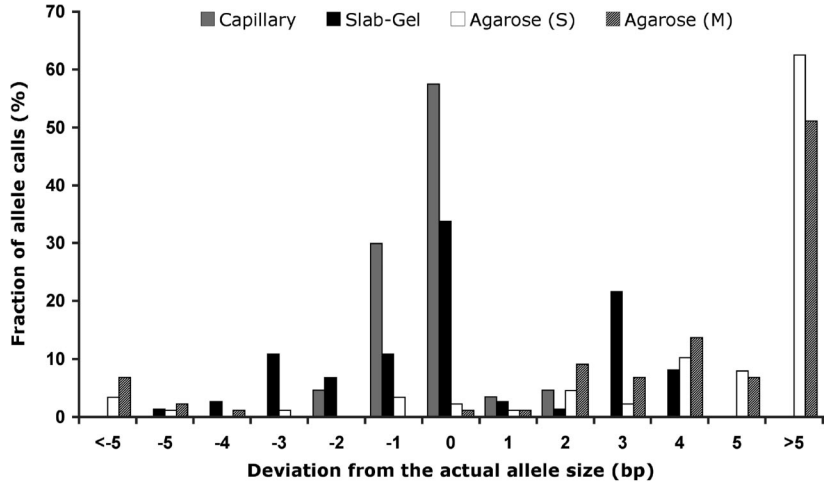

Figure 1. Allele size estimation accuracies of agarose, slab gel, and capillary electrophoresis methods. *Y*-axis presents fraction of total alleles (expressed in percentage) showing deviations from the actual allele sizes. *X*-axis shows the deviation (in bp) of the observed allele sizes from the actual allele sizes obtained by bidirectional sequencing. Agarose gel allele sizes were estimated separately by software (S) and manual (M) methods.

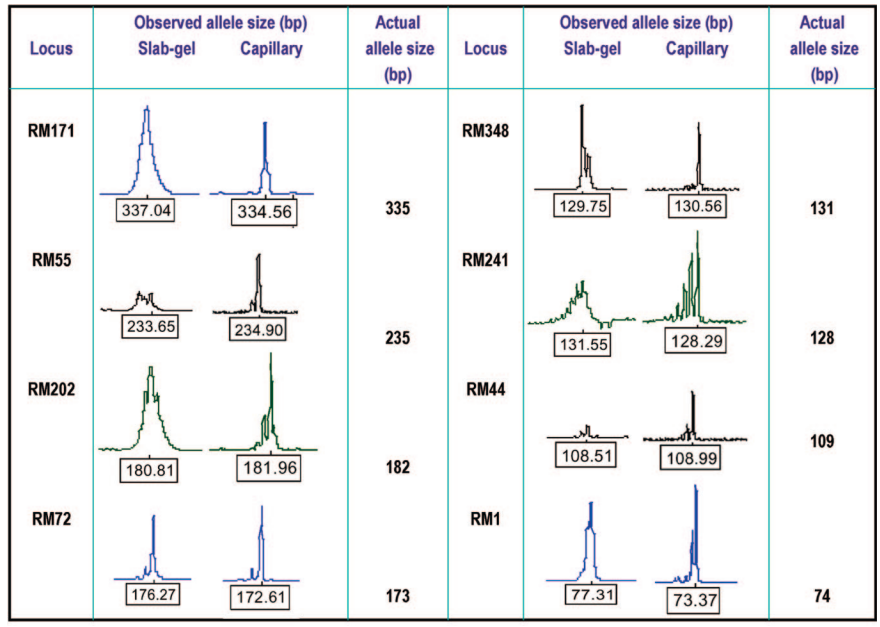

Figure 2. Extent of allele size differences between slab gel and capillary electrophoresis at eight rice microsatellite loci. Each peak represents a single allele; its size (in bp) is given below the peak. Please see **Table 2** for allele size range; only a representative profile is shown here. The actual allele sizes, determined by bidirectional sequencing, are also provided in the column for comparison.

trophoresis (**Figure 1**). It was observed that agarose gel electrophoresis returned overestimated allele sizes, whereas departure from the actual allele sizes in the case of slab gel electrophoresis spread like a normal distribution (**Figure 1**).

Although similar algorithms were used for allele calling and allele sizing, characteristics of slab gel and capillary electrophoresis peaks differ noticeably (**Figure 2**). The broader peaks observed in slab gel meant greater chance of errors in sizing. Only microsatellite loci having trinucleotide repeats (RM348 and RM72) appear to produce sharp peaks as good as capillary electrophoresis. Broader peaks at other loci (dinucleotide repeats) restricted the bin size and ended up in differential allele sizing over repeated runs and across template DNA samples. The reproducibility problem was clearly reflected in 1 bp deviation observed in the case of slab gel electrophoresis (**Table 3**). On the other hand, capillary electrophoresis showed <0.5 bp. Agarose-based methods obviously exhibited far greater inconsistencies (~6 and ~3 bp in manual and software methods, respectively). Interestingly though, deviations across all of the loci did not show any significant relationship with allele size range in all three separation methods. This is in contrast to locus-specific sizing bias observed in capillary electrophoresis based genotyping reported earlier (*21*).

Accuracy of Adulterant Quantification. It is common for Basmati rice, a farm product, to contain adventitious mixtures because of inadvertent mixing during postharvest operations in the field or during storage. Taking this practical problem into consideration, each importing country allows a certain limit of adulteration (e.g., up to 15%) in Basmati imports, beyond which consignments are rejected. This calls for accurate quantification of adulterants in the Basmati export samples. Adulteration in Basmati lots was accurately quantified on the basis of peak areas of additional peaks in relation to the peak area corresponding to Basmati in the electropherogram (*17*). The mean difference in quantification of adulteration estimated from slab gel and capillary electrophoretic instruments assayed using premixed standard samples revealed that slab gel shows high mean difference (±6.09%) compared to the capillary electrophoresis

Table 3. Consistency in Estimation of Allele Sizes Obtained from Agarose, Slab Gel, and Capillary Electrophoresis Methods

	standard deviations (± bp)			
locus	agarose (manual method)	agarose (software method)	slab gel electrophoresis	capillary electrophoresis
RM171	10.68	3.28	0.16	0.26
RM55	7.15	0.78	2.76	0.47
RM202	5.52	1.64	0.67	0.22
RM72	7.74	3.52	0.68	0.26
RM348	4.74	4.43	0.47	0.22
RM241	2.15	3.69	1.38	0.66
RM44	4.47	3.61	0.19	0.11
RM1	5.77	2.03	1.13	0.41
av SD	6.03	2.87	0.93	0.33

method (±3.91) (**Figure 3**). Although neither assay exhibited a smooth curve, the slab gel based assay showed a greater amplitude of variations. More significantly, in the range of adulteration at which samples are accepted or rejected (<15%), slab gel electrophoresis showed unacceptably erroneous results (**Figure 3**). On the other hand, capillary electrophoresis showed greater accuracy in that critical zone (<1%).

The quality of the peaks, as in detection accuracy, was also observed to affect the sensitivity of the assay to quantify the adulterant. **Figure 4** illustrates comparison of the quantification accuracy between slab gel and capillary methods at two loci, RM348 and RM72, in a mixture of Basmati370 and Sharbati. It was obvious that the difference in the accuracies is mainly due to the peak quality, sharper peaks of capillary electrophoresis being more accurate. These observations strongly point out the indispensability of capillary electrophoresis for quantification assay to protect the interests not only of consumers but also of farmers and traders.

Concordance Studies of Capillary Electrophoresis and Real Time PCR. Comparison with other electrophoresis methods showed that capillary electrophoresis is far superior in accuracy and consistency. However, accuracy of quantification needed further confirmation using a sensitive assay. A similar but more stringent quantification assay of genetically modified (GM) food samples has been comprehensively met by the use of real time PCR based protocols (*22, 23*). However, in the case of GM food, detection and quantification of transgenes have been relatively straightforward because transgenes are distinct "foreign" DNA elements in the host genome, which can be specifically amplified with high accuracy (detection) and sensitivity (quantification) by real time PCR. On the other hand, in the case of Basmati adulteration, the so-called "adulterant" is another long-grain rice (often a recently bred Basmati derivative). So far, no Basmati-specific sequence (genic or otherwise) has been identified that, upon amplification, can generate varietal-specific private alleles. For want of signature sequences, real time PCR has not been useful in adulteration detection. Although it was possible to distinguish genuine Basmati (Basmati370) from non-Basmati (Sharbati) by real time PCR at the betaine-aldehyde dehydrogenase-2 (*bad*2) gene (*17*), this assay is not suitable for practical applications as many adulterants also carry the same genuine Basmati allele at this locus. In the present paper, however, we employed this assay only to assess the quantification accuracy of capillary electrophoresis. Capillary electrophoresis showed a mean deviation of ±1.76% compared to real time assay (Supplementary Table 2), indicating that the estimation of adulteration based on the capillary electrophoresis method is realistically accurate for practical applications.

In the present study, we have demonstrated the necessity of capillary electrophoresis for a microsatellite marker based assay for the accurate and consistent detection and quantification of adulteration of Basmati samples. Even if routinely used agarose

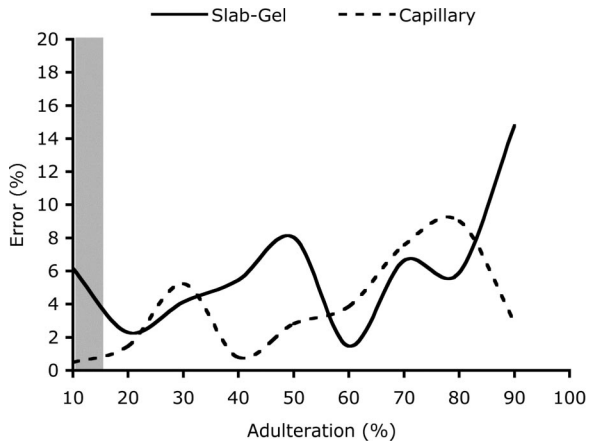

Figure 3. Accuracy in quantification of adulteration by slab gel and capillary electrophoresis. Quantification assays were carried out in standard samples of Basmati370 mixed with Haryana Basmati, in progressive proportions from 10 to 90% with a 10% interval at two informative loci, RM72 and RM348. The range of adulteration in which samples are accepted or rejected (<15%) is depicted in gray.

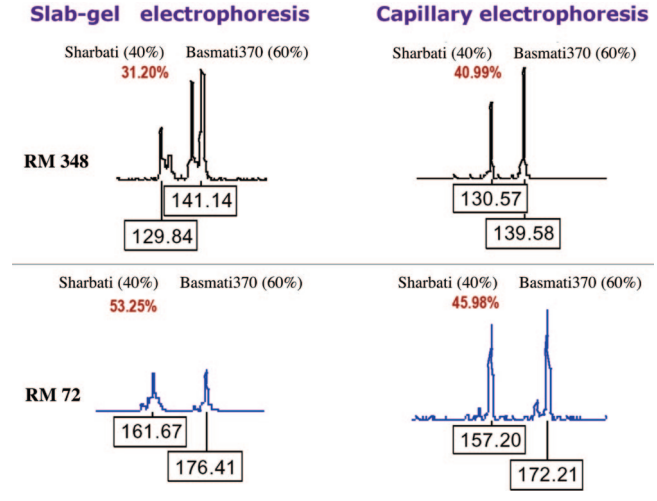

Figure 4. Accuracy of slab gel and capillary electrophoresis techniques in quantification of Basmati rice adulteration. A representative profile of Basmati370 mixed with Sharbati (another common adulterant) in a 60:40 ratio is shown. Adulteration was quantified at two microsatellite loci, RM348 and RM72. Sharbati is detected by the shorter of the amplicons (left). Peaks are labeled with allele sizes (bp) as determined by Genotyper software; estimated adulteration values (percent) are given in boldface.

and polyacrylamide gel electrophoresis methods can distinguish Basmati rice varieties (provided microsatellite makers have huge differences among allele sizes, at least 10–15 bp), they are not suitable for the accurate quantification of adulterants. Although advanced versions of PAGE or slab gel electrophoresis coupled with fluorescent labeled primers allow the detection and quantification of adulteration in Basmati rice, they often give inaccurate estimation of allele sizes. Capillary electrophoresis, on the other hand, produces accurate and consistent results. Hence, it is necessary that a microsatellite-based genotyping assay to detect and quantify adulteration in Basmati rice employ capillary electrophoresis.

ACKNOWLEDGMENT

We thank the Directorate of Rice Research, Hyderabad, Nandini Seshadri, and R. S. Seshadri for providing seed material.

Supporting Information Available: Tables showing allele sizes based on sequence analysis, capillary electrophoresis, slab gel electrophoresis, and agarose methods and concordance studies of capillary electrophoresis and real-time PCR. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review May 16, 2007. Revised manuscript received August 3, 2007. Accepted August 6, 2007. L.R.V. acknowledges the Council of Scientific and Industrial Research for a senior research fellowship. S.A. acknowledges the Indian Council of Agricultural Research for support in the form of a study leave. The work was supported by the APEDA-CDFD Centre for Basmati DNA Analysis by a grant to J.N.

JF0714517