

Detection of adulteration of Basmati rice with non-premium long-grain rice

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Summary Basmati rice is, on average, sold at 2–3 times the price of other rice available on the world market. As such, there is a requirement for a method that would allow the detection of non-Basmati long-grain rice within samples of Basmati. This paper reports the use of fluorescent simple sequence length polymorphisms (SSLPs) between known Basmati rice cultivars and likely adulterants, to detect the presence of any adulterant. The competitive nature of the fluorescent detection method also enabled crude estimations of the amount of adulteration present in blind test samples. Further, the difficulty in quantifying the level of adulteration is discussed.

Keywords Quantitative polymerase chain reaction (PCR), short sequence length polymorphism.

Introduction

Basmati rice is traditionally grown in the Himalayan foothill regions of India and Pakistan, and the name is traditionally associated with this geographical origin. The grain is characterized by its long slender shape, the 1.5- to twofold extension of the grain upon cooking, and the strong natural aroma. As a result of these properties, Basmati rice commands a premium price on the world market, and much of the crop is sold for export. Because of the premium price, methods for the detection of adulteration of Basmati by other long-grain cultivars are desirable to ensure that the authenticity of this product is maintained. Additionally, with the advent on the market of United States cultivars which have been protected as Basmati type rice, and also have typical Basmati characteristics (Sarreal *et al.*, 1997), methods which can distinguish genuine Indian/Pakistani Basmati cultivars from their US counterparts are desirable (Juliano, 1998). Use of short sequence repeat polymorphisms (SSLPs) to distinguish closely related US long-grain cultivars, utilizing only white milled

or brown rice as the analyzed material, has recently been demonstrated (Bligh *et al.*, 1999). As such, DNA-based methods can distinguish between closely related cultivars and allow the use of white milled grain, often the only material available. Methods for the authentication of Basmati as well as other aromatic cultivars have previously been investigated using physical characteristics such as image analysis (Whitworth *et al.*, 1996) and chemical analysis of aromatic compounds from rice cooking water (Petrov *et al.*, 1996). These methods rely on complex analyses of the results and have a large uncertainty as a result of their complexity and time-consuming nature. In contrast, analysis using polymerase chain reaction (PCR) based methods enables a large number of samples to be tested simultaneously using equipment that is becoming routinely available in many analytical laboratories. The latter refers to the increase in PCR-based testing methods, e.g. for genetically manipulated organisms. In addition, PCR is now widely used in quantitative analysis, although there are problems associated with the truly quantitative analysis as even with several internal controls it is difficult to standardize (Shaw *et al.*, 1998).

Short sequence repeat polymorphisms for rice have been widely studied both as a means of mapping the rice genome (e.g. Chen *et al.*, 1997),

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and as a tool for the identification of rice accessions (Yang *et al.*, 1994). As a result, a large number of primer sets are available (Panaud *et al.*, 1996; Chen *et al.*, 1997), many of which can be obtained commercially. This makes the routine testing of cultivars for polymorphic markers a relatively cheap and simple task. The use of PCR for the analysis of multiple populations or samples has previously been established for use in estimating relative allele frequencies (Pacek *et al.*, 1993; Khatib *et al.*, 1994), making this technology also a candidate for use in the detection of adulterants, quantitatively as well as qualitatively. The use of this technology for the identification of Basmati rice cultivars and the detection of adulteration of Basmati rice samples is reported in this paper.

Materials and methods

Sample collection

The rice cultivars chosen included: a selection of authentic Basmati cultivars, a selection of Indian and Pakistani long-grain cultivars that were considered to be likely adulterants, and also some US long-grain cultivars, including commercially obtained Texmati, a US cultivar with Basmati characteristics.

Authentic samples of both Basmati and non-Basmati rice were obtained from a variety of sources. Samples from the US were supplied by Dr A. McClung USDA-ARS, with the exception of Texmati, which was bought in a US supermarket. Indian and Pakistani samples were supplied by the Ministry of Agriculture, Fisheries and Food (MAFF) authenticity programme. Most samples consisted of white rice grains, although the IR6 was supplied as paddy, no milled sample being available, and the US Basmati 385 was supplied as brown rice. A small number of samples were ground to flour using a coffee grinder at CSL, Norwich, however, in most cases a 2 g sample was ground by hand in a pestle and mortar at Nottingham University prior to DNA extraction. Only those samples available as white milled grain were used for either the standard curve construction or blind trial samples.

DNA extraction

DNA was extracted from 0.2 g (manufacturer's

recommendation for dried material) of rice flour as prepared above, using Nucleon Phytopure™ (Nucleon Biosciences, Coatbridge, UK) according to the manufacturer's instructions for dried plant material. The yield per sample was quantified spectrophotometrically and ranged between 18 and 100 µg, although most samples gave a yield of 30–40 µg. Samples were dissolved in sterile distilled water at a concentration of approximately 0.2–0.3 mg mL⁻¹.

Oligonucleotide primers

Twenty-nine primer sets, as used previously (Bligh *et al.*, 1999), were selected from the large number of commercially available primers and primer sets developed at Nottingham University. Primers sequences are available from Panaud *et al.*, 1996, Chen *et al.*, 1997, and Bligh *et al.*, 1999.

Commercial rice marker (RM) primer sets were purchased from Research Genetics Inc. (Huntsville, AL, USA), fluorescent primers were custom-synthesized by Cruachem (Glasgow, UK) and other custom primers were synthesized at the Biopolymer Synthesis and Analysis Unit at the University of Nottingham.

Short sequence repeat polymorphisms amplifications were performed using an Omnigene™ Thermocycler (Hybaid, Hampton, UK) and *Taq* Supreme enzyme (Helena Bioscience, Sunderland, UK). Reactions contained 1–2 µL (300 ng) of template and 20 pmol of each primer in a 50 µL reaction. Reactions were cycled through 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. Reaction products were visualized on either 2% agarose gels or 4% Metaphor™ (Flowgen, Lichfield, UK) gels. After confirming successful amplification, products were then run on an ABI310 DNA sequencer using the Genescan™ (Applied Biosystems Inc., Foster City, CA, USA) software.

Construction of calibration curves

To create calibration curves for the estimation of contamination of test samples, seed mixes of two cultivars, one Basmati and one non-Basmati, were used. Where possible, if sample quantity allowed, cultivars were chosen in probable Basmati/adulterant combinations. Cultivars to be used in curves were selected on the basis of their polymorphism between the chosen primer sets. In addition, only certain

combinations could be chosen owing to only limited amounts of samples being available. One hundred (approximately 1.7–2 g) seeds per individual curve point were counted, using a range of percentage ratios of Basmati : non-Basmati seeds (except in one case where two US long-grain cultivars were chosen to test the M7 primer set). The DNA was then extracted from these samples as described above, and the DNA used as a template for the PCR. The PCR for each percentage-point sample was then analyzed on the ABI 310 DNA sequencer using the GenescanTM software and the ratio of peak area for the two allele peaks was calculated. The ratio was then plotted against percentage and the best curve drawn through these points using the Curve Expert Program v1.32 (Hyams, 1995). This curve was then used to calculate the estimated percentage of the test samples based on the ratio calculated from the peak areas generated by PCR conducted with the same primer set on the test sample.

Results

A range of rice cultivars from India, Pakistan and the US were initially screened with a selection of SSLP primers. From the commercial primer sets plus the sets already available, 12 sets that all distinguished between most Basmaties and non-Basmaties were selected (Table 1). These were selected based on two criteria: the ability to distinguish Basmati from non-Basmati cultivars, and the large difference (> 10bp) between allele sizes to try to eliminate 'shadow' bands causing an overlap between the two alleles to be used. Very few primer sets actually distinguished all Basmati cultivars from all non-Basmaties, but using a range of primer sets, it was possible to distinguish all the Basmati cultivars from the non-Basmaties. One factor of note is that duplicate accessions from different geographical locations did not all have the same SSLP profile, a factor that has previously been reported for other groups of duplicate accessions (Olufwote *et al.*, 1997). For example, the two samples of Tericot were polymorphic with respect to each other at four out of 12 loci, although this was less of a problem with authentic Basmati cultivars such as Basmati 370 and Basmati 385, where all 12 loci were identical within duplicates for an accession (Table 1).

From the subset of 12 SSLP primer sets, RM1,

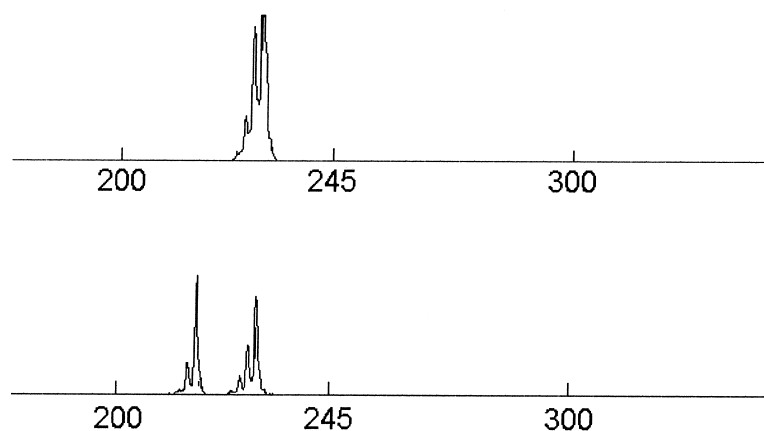
RM55, M7, M16, RM225, RM229 and RM9 were selected for fluorescent analysis. Calibration curves consisting of between 10% and 90% of admixture with a Basmati cultivar were constructed and these were used as templates for PCR with the appropriate primer sets. The test calibration curve mixes were: Basmati 370 and Sonasali, Basmati 385 and Tericot, Basmati Pakistan and Gulfmont, Super Basmati and Texmati, and Maybelle and Kaybonnet. Mixtures of rice were made up by counting grains, and the proportion of grains of each type were weighed. However, percentages calculated by weight were found to only vary by 1–3% in comparison with those calculated by grain number. The one exception to this was Basmati 370, which had a smaller grain than other cultivars, and was found to vary by as much as 6% between grain and weight percentages. However, because of the lack of quantity of samples available, grain number was used for the rest of the study. The PCR reactions performed on these samples were then analyzed on an ABI 310 DNA sequencer to determine whether peaks corresponding to the two differently sized DNA products could be distinguished (Fig. 1). As can be seen from Fig. 1, all peaks showed some 'shadow' banding, however in most cases, this did not result in any overlap of the two individual alleles. The level of amplification after 35 cycles was sufficient for visualization on an agarose gel (Fig. 2) although it was difficult to get any clear idea as to the level of adulteration from such gels. Amplification for under 30 cycles showed no detectable product using either agarose gels or the ABI, and subsequent experiments using 30, 32 and 35 cycles suggested that at 35 cycles, the reaction was still at the exponential phase (data not shown). As the peak height and area from the GenescanTM data for these reactions corresponds to the percentage of each allele within the template sample (Shaw *et al.*, 1998), the ratios of the major peak areas for each PCR were calculated and plotted against the known percentage of the allele mix. The peak area was chosen for ratio calculation, as for some alleles it was observed that the tallest peak was not always that associated with the main expected allele. By comparison, peaks generated from fluorescent PCR on pure templates routinely only showed a single major peak (Fig. 1), indicating that this had the potential to be a useful method for identification of adulterated rice samples. Analyses were carried out for several primer sets either in triplicate on a single

Table 1 The table shows all rice samples used in the study, the country of origin and type. Classification according to each of the 12 SSLP alleles is also given as size of the PCR product as sized using the ABI310 sequencer internal markers

Cultivar	Country of origin	Rice type	RM1	RM9	RM19	RM208	RM55	RM201	RM219	RM225	RM229	RM202	M7	M16
Basmati 198	Pakistan	Basmati	108	112	211	164	230	139	192	120	116	183	155	136
Basmati 370	Pakistan	Basmati	74	112	211	164	230	139	192	120	116	183	155	136
Basmati 370	US	Basmati	74	112	211	164	230	139	192	120	116	183	155	136
Basmati 385	India	Basmati	108	112	211	164	230	139	192	120	116	160	155	136
Basmati 385	Pakistan	Basmati	108	112	211	164	230	139	192	120	116	160	155	136
Basmati 385	US	Basmati	108	112	211	164	230	139	192	120	116	160	155	136
Basmati PAK	Pakistan	Basmati	74	112	211	164	215	139	192	120	116	183	155	136
Dehradun Basmati	India	Basmati	74	112	211	164	230	139	192	120	116	183	155	136
Line 4048	Pakistan	Basmati	74	112	211	164	215	139	192	120	116	183	155	136
Basmati PAK	India	Basmati	74	112	211	164	215	139	192	120	116	183	155	136
Pusa Basmati 1	India	Basmati	74	128	211	164	225	139	192	120	111	183	155	127
Pusa Basmati 1	India	Basmati	74	128	211	164	225	139	192	120	111	183	155	127
Super Basmati	India	Basmati	74	112	211	164	215	139	192	120	116	183	155	136
Tarori Basmati	India	Basmati	74	112	211	164	215	139	192	120	116	183	155	127
Guarav	India	non-Basmati	H	128	241	179	225	155	217	138	116	183	155	127
IR6	US	non-Basmati	H	128	241	179	225	155	217	138	111	160	155	127
Kasturi	India	non-Basmati	74	176	211	179	225	139	192	120	116	183	155	127
PR106	India	non-Basmati	108	128	241	179	225	155	198	120	111	183	155	127
PR109	India	non-Basmati	108	128	241	179	230	155	192	120	116	160	155	127
PR110	India	non-Basmati	108	H	241	179	225	155	217	138	116	183	176	127
Ratna	India	non-Basmati	108	H	219	179	225	155	217	120	111	183	155	127
Sonasali	India	non-Basmati	108	182	234	179	225	155	217	138	111	160	155	127
Tericot	India 1	non-Basmati	108	H	241	179	225	139	217	138	111	183	176	127
Tericot	India 2	non-Basmati	108	128	241	179	225	155	217	138	116	183	155	127
Texmati	US	US Basmati type	84	176	211	164	H	139	192	130	126	160	176	136
Cypress	US	US long-grain	84	176	211	179	225	139	189	138	126	160	176	136
Gulfrmont	US	US long-grain	84	176	211	179	225	139	192	138	126	160	179	127
LaGrue	US	US long-grain	84	176	211	179	230	139	192	138	126	160	179	127
Kaybonnet	US	US long-grain	84	176	211	179	230	139	192	138	126	160	176	136
Maybelle	US	US long-grain	84	176	211	179	225	139	189	130	126	160	133	127

H refers to samples showing more than one band for that locus indicating heterozygosity.

Figure 1 Chromatogram readout for the RM55 polymerase chain reaction (PCR), performed on pure Basmati 385 (top) and Basmati 385 adulterated with 20% Terricot (bottom). The base pair sizes given on the horizontal axis correspond to the internal molecular markers (peaks not shown).



curve, or on three separate curves. Curves could be plotted through the points using either equation

$$y = a + b / (1 + c * x) \quad (1), \text{ or}$$

$$y = a * ((100/x) - 1) + b \quad (2)$$

where x is the per cent adulterant and y is the ratio of peak heights. Equation 1 is a more generalized form of equation 2 that allows for the presence of an additional constant caused, for example, by the presence of impurities, while equation 2 assumes y is directly proportional to x . Standard deviations for triplicate repeats on a single calibration set were considerably lower than those calculated between several different mixes (see Fig 3 for examples of curves). As the highest standard deviations were usually found at the lower percentage points for adulteration (10%), which are permitted levels in export standards, it was decided to investigate to what extent such standard curves could be used to predict quantitative adulteration in a series of blind samples.

Blind samples were provided by Dr Tom Hartman

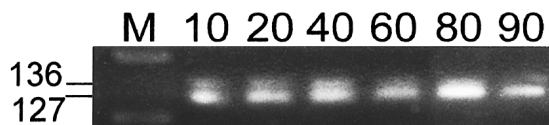


Figure 2 Two per cent gel showing the PCR amplification products of Basmati 385 adulterated with Terricot using the primer set M16. M = size markers, 10–80 refers to the percentage number of Terricot grains in each sample. The sizes given at the side are the sizes of the amplified products in the base pairs.

in the Division of Plant Science, University of Nottingham, from any combination of the available samples of Basmati and non-Basmati rice, and labelled A–D. The only condition stipulated was that the combination must be of a Basmati rice with a non-Basmati. In each case, samples were prepared by counting the grains, which were then weighed. Each sample was used to extract the DNA, and then this was used as a template for seven PCR reactions using the fluorescent primers sets, RM55, RM9, RM1, RM225, RM229, M7 and M16. As was expected, not all samples showed two peaks for all reactions when the PCR peaks were analyzed, and sample D showed only one peak in all seven PCR reactions, indicating that this sample was pure Basmati. From the PCR product sizes, it was concluded that samples A, B and C were all showing two peaks for at least three alleles, which were known to distinguish between Basmati and non-Basmati cultivars. Using these results, the PCRs were then repeated with two calibration curves and the calibration curves used to calculate the level of adulteration in the blind samples. Calculated experimental levels of adulteration, both for individual loci and overall calculated average, and actual values are shown in Table 2. The breakdown of the constituents of the blind samples are given in Table 3.

Discussion

The aim of this study was to develop a method to detect the adulteration of Basmati rice. The use of SSLPs has already been demonstrated for the identification of individual samples (Yang *et al.*, 1994; Olufwote *et al.*, 1997), and this study has taken

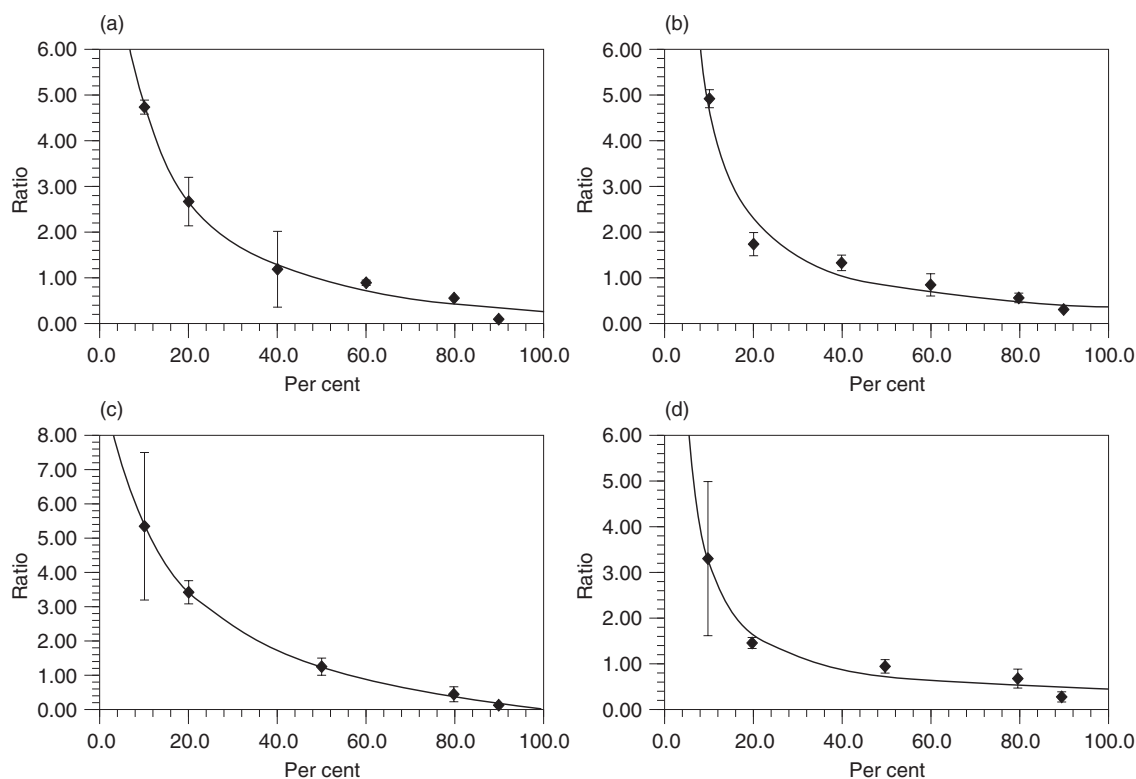


Figure 3 Results of the calibration curve mix PCRs performed using the RM55 (a and c) and the M16 (b and d) PCR primers. The Basmati 385 was adulterated with the Terricot calibration curve mix (a and b). The average of samples were performed in triplicate. The average of the three separate calibration curve mixes (c and d): Basmati 385 adulterated with Terricot, Basmati 370 adulterated with Sonasali and Kaybonnet adulterated with Maybelle. Error bars in all graphs represent the standard deviation. The per cent refers to the percentage of the adulteration cultivar in the total mix.

this further by using the detection of multiple alleles as markers for the presence of an adulterant. By using fluorescent primers and the detection of PCR products on a laser sequencer, such as an ABI 310 DNA sequencer, the presence of a second peak can be detected down to 10% of adulteration, a level which cannot be detected using ordinary agarose or acrylamide gel systems (H. F. J. Bligh, unpublished observations).

The standard curves constructed for a variety of different mixes of Basmati and adulterant rice show that, while the method is reproducible within a single curve, comparison of several different combinations of cultivars, with each pair polymorphic at the same locus, does result in a higher standard deviation for each point. Plots of ratio against grain weight calculated percentages showed only small differences with respect to the comparable plots for seed number,

Table 2 Individual, average and actual (in grain number) estimates of each of the four blind trial samples A–D

Trial samples	RM55	RM229	RM9	RM1	RM225	Average experimental adulteration (%)	SD	Actual adulteration (%)
A	NP	34.2	32.6	34.6	NP	33.8	±1.06	39
B	57.8	56.5	NP	NP	57.9	57.4	±0.77	44
C	20	38.2	34.1	40.2	47.1	35.9	±10.07	25
D	NP	NP	NP	NP	NP	0		0

NP = Not polymorphic. Primers that were not polymorphic could not be used to determine adulteration for that sample.

Table 3 Comparison of percentages for grain weight and number in the blind trial samples

Trial samples	Cultivar Basmati	Adulterant	Grain number		Grain weight (g)		Percentage weight	
			Basmati	Adulterant	Basmati	Adulterant	Basmati	Adulterant
A	Basmati 370 (Pakistan)	Gulfmont	61	39	0.73	0.52	58.4	41.6
B	Basmati 370 (USA)	Tericot (India 2)	56	44	0.61	1.21	33.5	66.5
C	Dehradun Basmati	Cypress	75	25	1.19	0.42	73.9	26.1
D	Basmati 385 (Pakistan)		100	0	1.86	0	100	0

even when the most variant weight curve Basmati 370 with Sonasali was included (data not shown).

The variability encountered between the standard curves is likely to be due to factors such as the amount of DNA extractable from each grain, which can be affected by such factors as age of grain and harshness of milling, as most of the DNA is found in the embryo, on the outside of the grain. This in turn would affect the ratio of DNA for each cultivar in a standard curve, and so also lead to the variability between a standard curve and any test sample. The fact that equation 1 was often used in preference to equation 2 (see Results) to obtain a better fitting curve, shows that factors such as those suggested above were having an effect on the overall relationship between ratio and percentage. Other factors which could also affect the reproducibility of these results include the average DNA yield for each sample. While most samples gave fairly consistent DNA levels, the total DNA extracted from some samples could be as low as 18 μg (e.g. Sonasali), suggesting that at least some of the samples used could be giving skewed ratios if a high yielding sample was mixed with a lower yielding one. Because of the limited availability of some of the samples in this study, it was not possible to determine whether this was caused by the age of the sample, or a more intrinsic factor of the grain such as embryo size, which would affect the total amount of extractable DNA. The small sample size of grains available for construction of standard curves may also have played a role in the inaccuracy of the results obtained, especially at the lower levels of adulteration. The quality of some samples could well have resulted in the loss of the embryo from some grains, which in a samples containing, for example,

only 10 grains of an adulterant, could have caused a significant shift away from the expected result. Any inaccuracies in weighing and counting of grains could also have had a significant effect with such small samples as well, and further investigation into this method using larger samples would be highly desirable. Therefore, while the method appears very effective in detecting adulteration, and for detection of pure samples, the numerical results may well be inaccurate by up to 15%, as shown by the data from the blind trial.

The results of the blind trial show varying inaccuracies, with sample B showing the apparent highest discrepancy. However, in the case of sample B, there seems to be a considerable difference between adulteration levels as calculated by weight as opposed to grain number. As the two cultivars used do not normally vary so much in grain weight from one another (Table 3), it must be concluded that some other factor such as the use of slightly broken grains may have influenced this result. This was most likely due to the availability of such a small amount of sample rice, and would be less of a problem when assaying normal commercial samples for which a larger amount would be available, as mentioned earlier.

For longer term use, it would be better to use standard curves based on weight rather than grain number, as adulteration at the levels to be considered deliberate would be carried out on a weight basis. While this would probably not affect the overall result by more than 1–3%, using weight and larger amounts of grain per individual point on a standard curve would allow factors such as broken grains to be taken into account, as this has obviously influenced the result obtained in this trial. However, use of

weight would not overcome any problems such as DNA yield from different varieties or susceptibility to loss of embryo, both of which could cause inaccurate results. By comparison, samples A and C were both incorrect by less than 10% at the grain count level, with sample D being obviously pure. Overall this suggests that this method would be comparable with previous methods developed (Whitworth *et al.*, 1996) as well as working equally well for all the rice types tested, unlike the image analysis method, which has limitations with certain cultivars of long-grain rice. In addition, this method would be easier to use as there is no reliance on specialized image analysis software, and the type of equipment and software required are similar to those available in many analytical laboratories routinely performing DNA, PCR and sequencing analysis. Another factor which may have caused the high variations between the individual points for different cultivar combinations is the fact that samples had been collected over a period of several years from a variety of different sources. Differences in milling of the samples could have affected the amount of DNA available for extraction, as the majority of DNA is found in the embryo, on the outside of the grain. Long-term storage of some of the samples could also have resulted in greater degradation of the DNA in some samples, which would have also affected the peak area ratios. Such factors could be eliminated by repeating standard curves using rice collected from the same year's crop, and milled under controlled conditions. However, as most commercial rice is from the previous year's crop, this problem would be eliminated for routine use of this method as long as standard curves were constructed from rice from the same year.

Shaw *et al.* (1998) have also reported that the use of dinucleotide repeats gives greater variation around the true value, an observation which also proved marker dependent, indicating that the use of triplet and tetranucleotide repeat SSLPs would also improve the quality of the data for this test. Although a few trinucleotide repeat markers were available during this study (M7, M16 and RM19), these were not used in the blind trial as, in most cases, there was no polymorphism between the two mixed cultivars. Currently, most of the commercially available SLP markers for rice are dinucleotide repeats, but the development of tri- and tetranucleotide based markers in the future should also prove beneficial to this

type of analysis. The use of dinucleotide repeats in this study may also have contributed to some of the inaccurate estimations, as many of the repeats chosen showed levels of 'shadow' banding which could have affected the calculation of the ratios as suggested by Pacek *et al.*, 1994. Unlike methods developed that rely on the detection of aroma (Petrov *et al.*, 1996), which would be unable to distinguish Basmati from other aromatic rice such as Thai aromatic rice or US Texmati type rice, this method can distinguish these quite easily as Texmati has a considerably different genetic profile from Indian or Pakistani Basmati rice. Initial work on Thai fragrant rice suggests that this too would be easily distinguishable by this method (H. F. J. Bligh, unpublished observations).

It would be hoped that further analysis of this method, using larger samples of grain, could result in greater accuracy of this method in detecting adulteration, although at present, while this method would be useful for the detection of the presence of an adulterant, an accurate measure of quantification is less realistic. The genetic basis of this method also implies that, with further analysis of cultivars, such a method also has the potential to be used for testing for adulteration of other premium cultivars such as Thai Fragrant rice, for which morphological tests are less possible.

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