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(54) SINGLE TUBE MULTIPLEX ASSAY FOR DETECTION OF ADULTERANTS IN BASMATI RICE SAMPLES

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(57) **ABSTRACT**

The present invention provides a single tube multiplex assay for distinguishing basmati from non-basmati rice varieties, and thereby identifying the adulteration of basmati rice varieties. The present invention further provides a method for quantifying adulteration in basmati rice varieties. The present invention also provides a kit for performing a muliplex assay for distinguishing basmati from non-basmati rice varieties. The kit may comprise a primer directed to an SSR loci, appropriate reagents for PCR, and optionally, a package insert for conducting the assay.

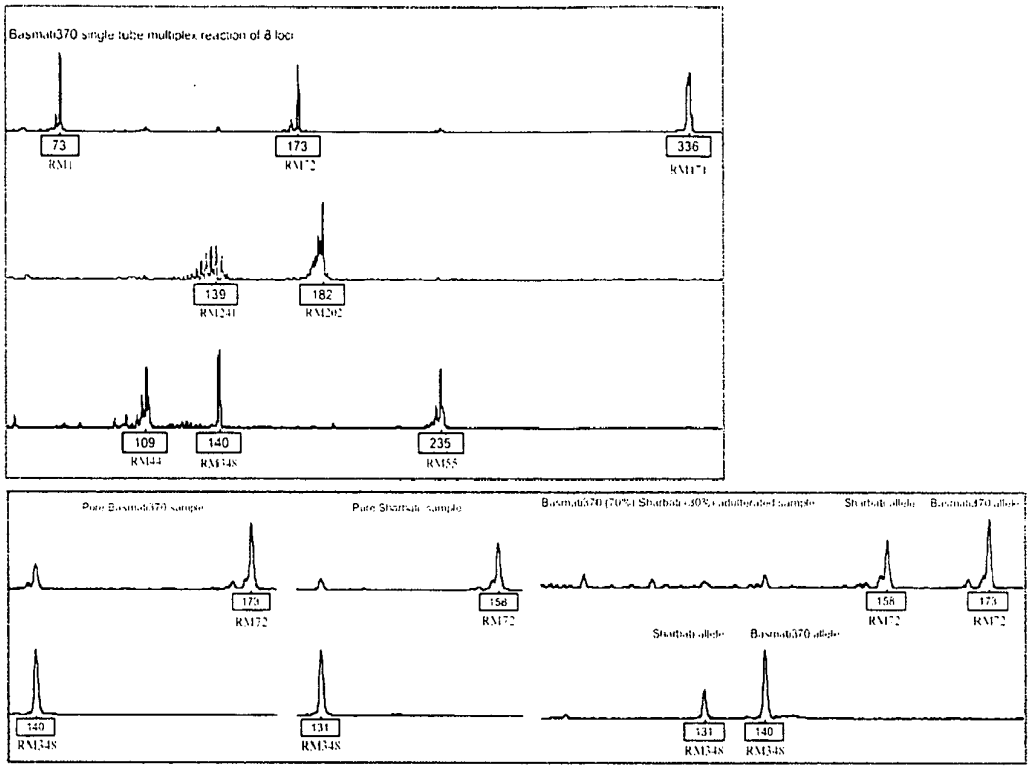


Figure 1 Top panel. Allele profiles of Basmati370 obtained by single assay multiplex reaction. Three colours represent three groups of primers labelled with specific fluorescent ligands (Blue is FAM, Green is JOE and Black is TAMRA). Locus name and allele size in base pairs are given below the peaks. Figure 1 Bottom panel. Allele profiles of pure Basmati370, pure adulterant Sharbati and an adulterated sample, by using only two primers from the multiplex panel.

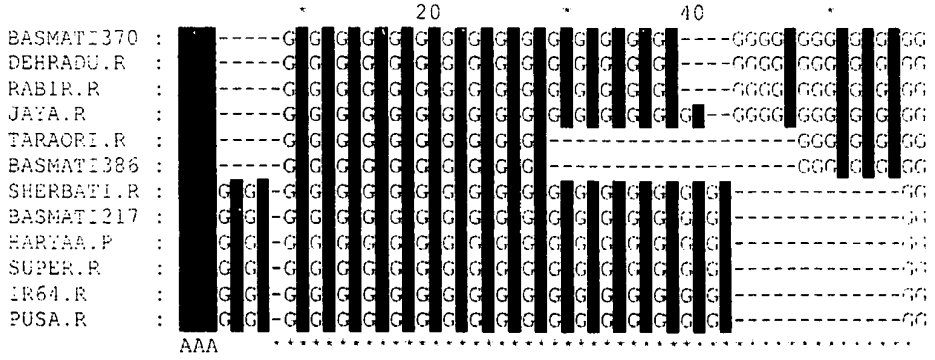


Figure 2. Sequence alignment of alleles of RM55 locus from different basmati rice varieties showing variation in length

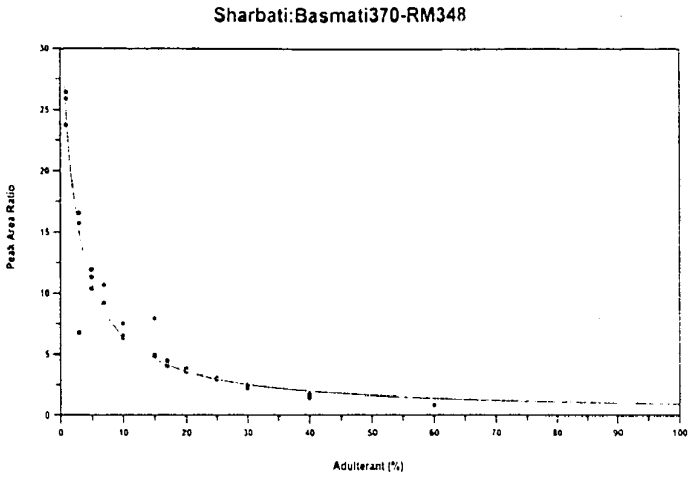
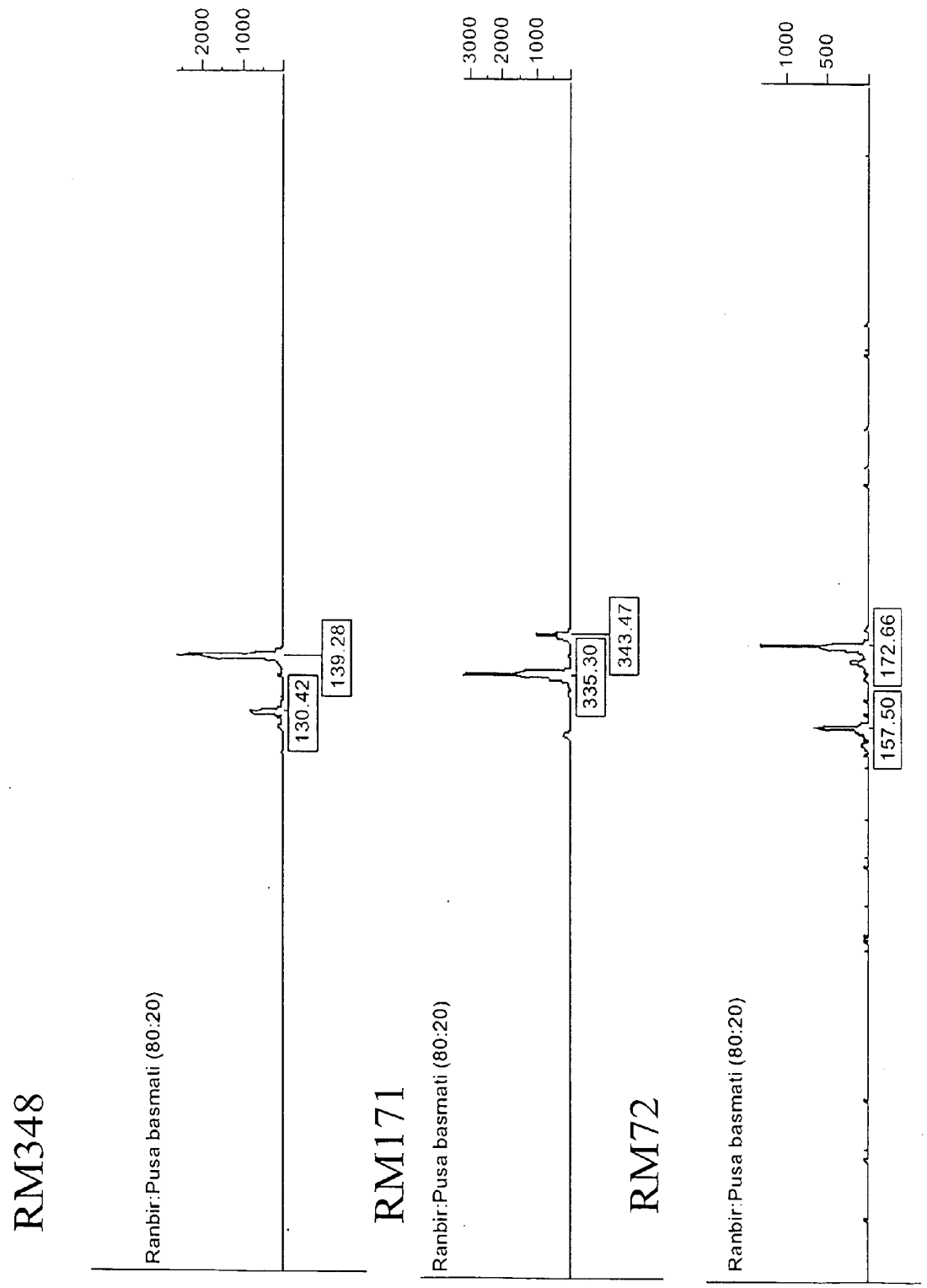


Figure 3. Standard curve generated for a combination of Basmati370 adulterated with Sharbati using allele differences at RM348 locus.

Fig. 4.


RM348
Ranbir:Pusa basmati (80:20)
2000
1000
130.42
139.28
RM171
Ranbir:Pusa basmati (80:20)
3000
2000
1000
335.30
343.47
RM72
Ranbir:Pusa basmati (80:20)
1000
500
157.50
172.66

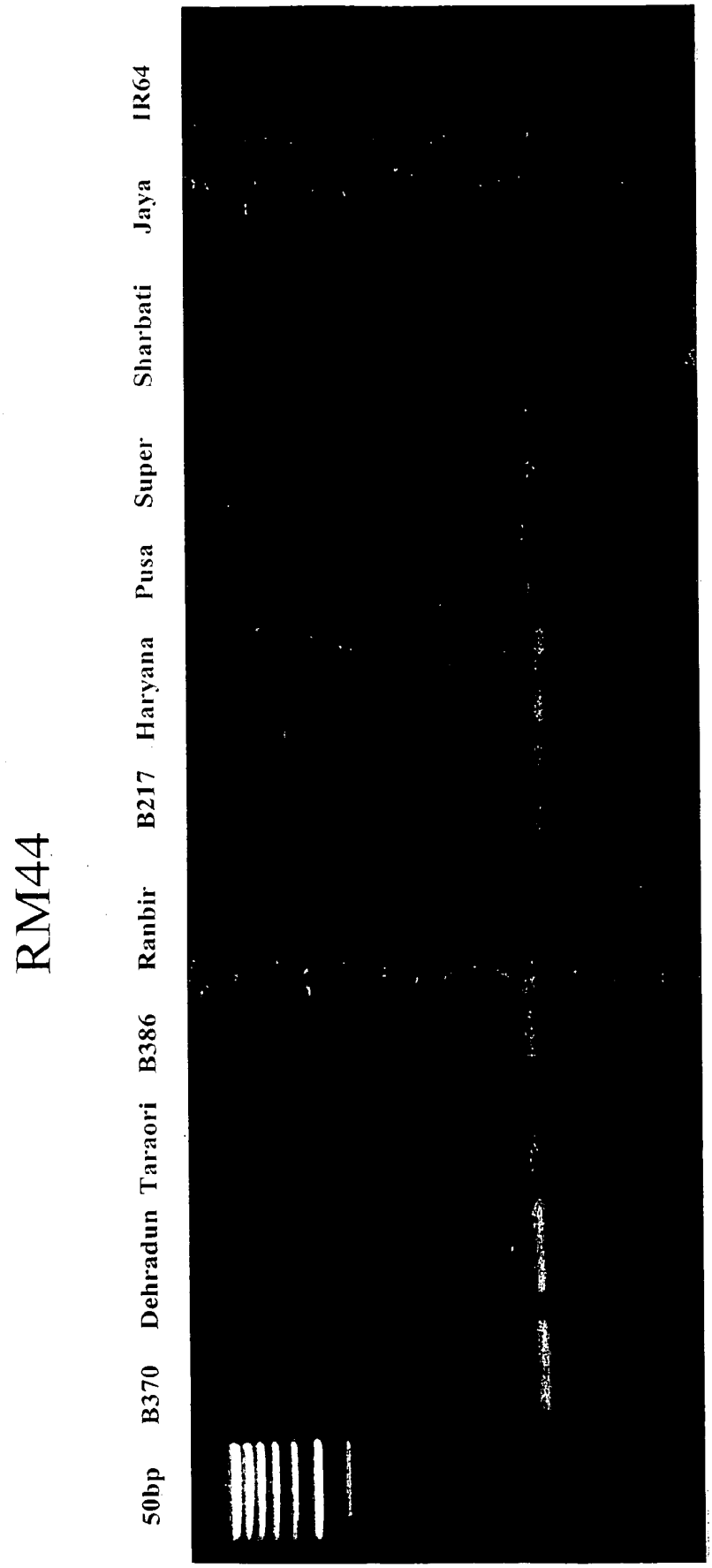

RM44
50bp
B370
Dehradun
Taraori
B386
Ranbir
B217
Haryana
Pusa
Super
Sharbati
Jaya
IR64

Fig. 5

RM1

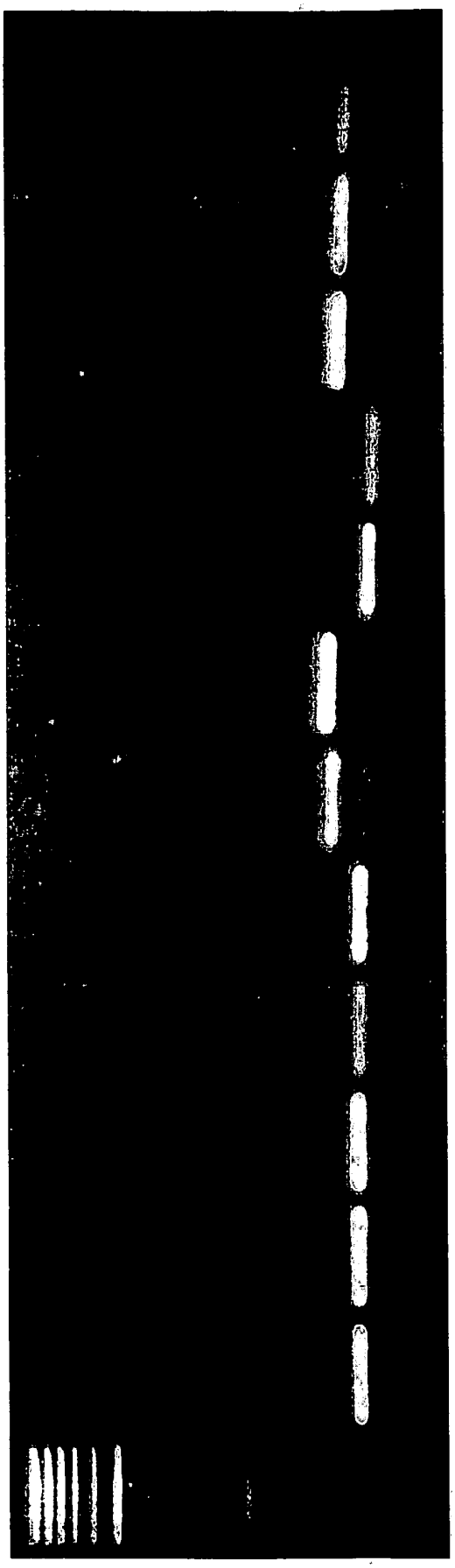

50bp
B370
Dehradun
Taraori
B386
Ranbir
B217
Haryana
Pusa
Super
Sharbati
Jaya
IR64

Fig. 5a

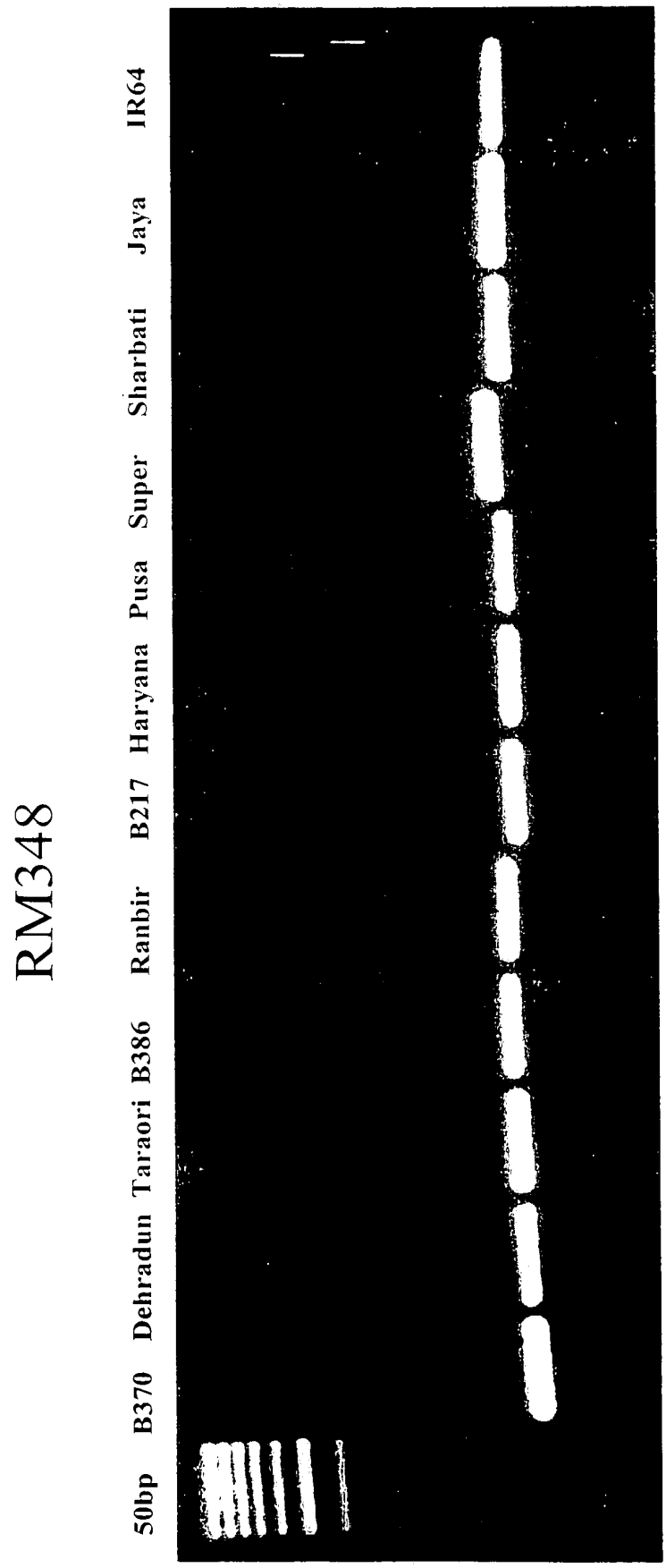

RM348
50bp
B370
Dehradun
Taraori
B386
Ranbir
B217
Haryana
Pusa
Super
Sharbati
Jaya
IR64

Fig. 5b

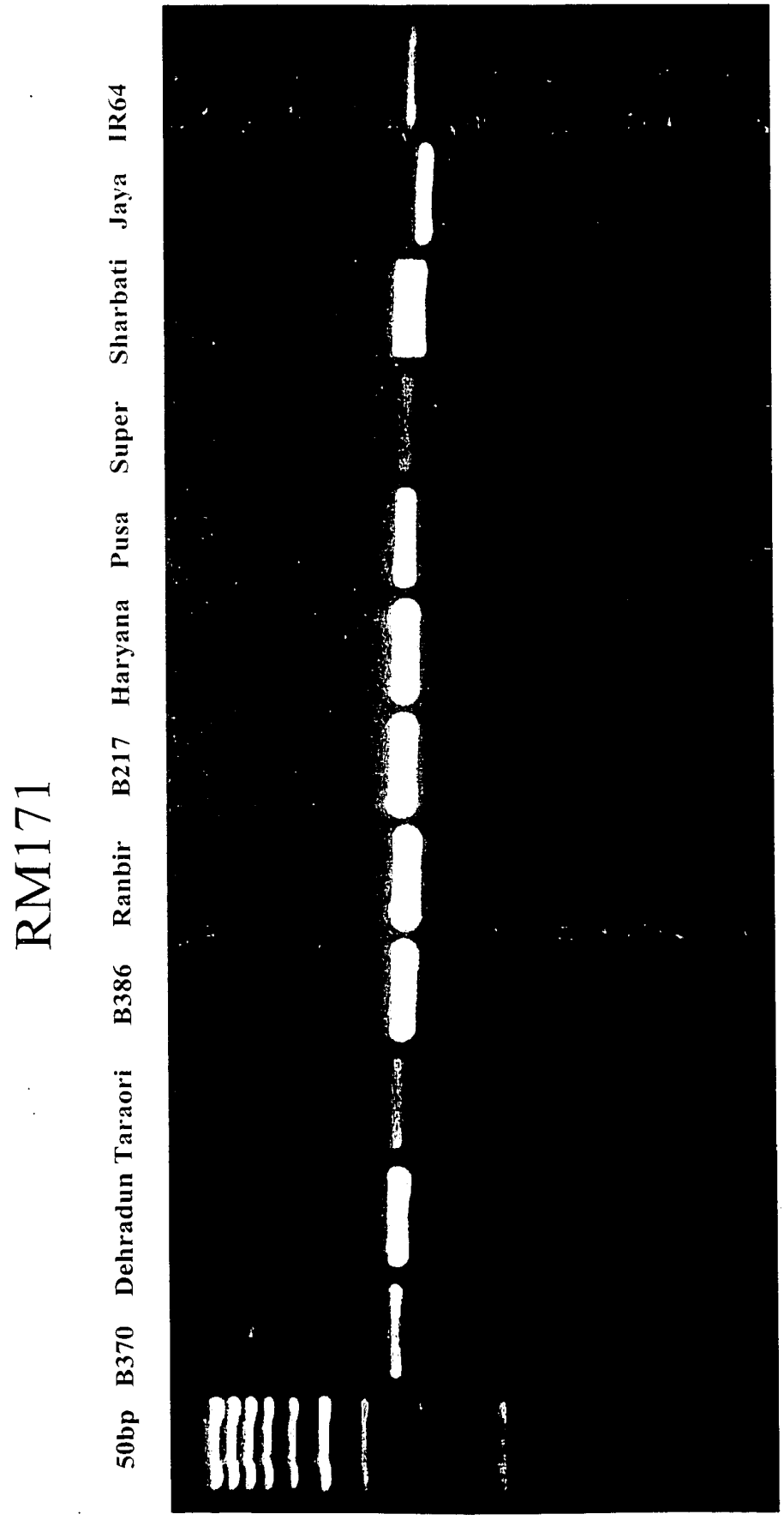

RM171
50bp
B370
Dehradun
Taraori
B386
Ranbir
B217
Haryana
Pusa
Super
Sharbati
Jaya
IR64

Fig. 5c

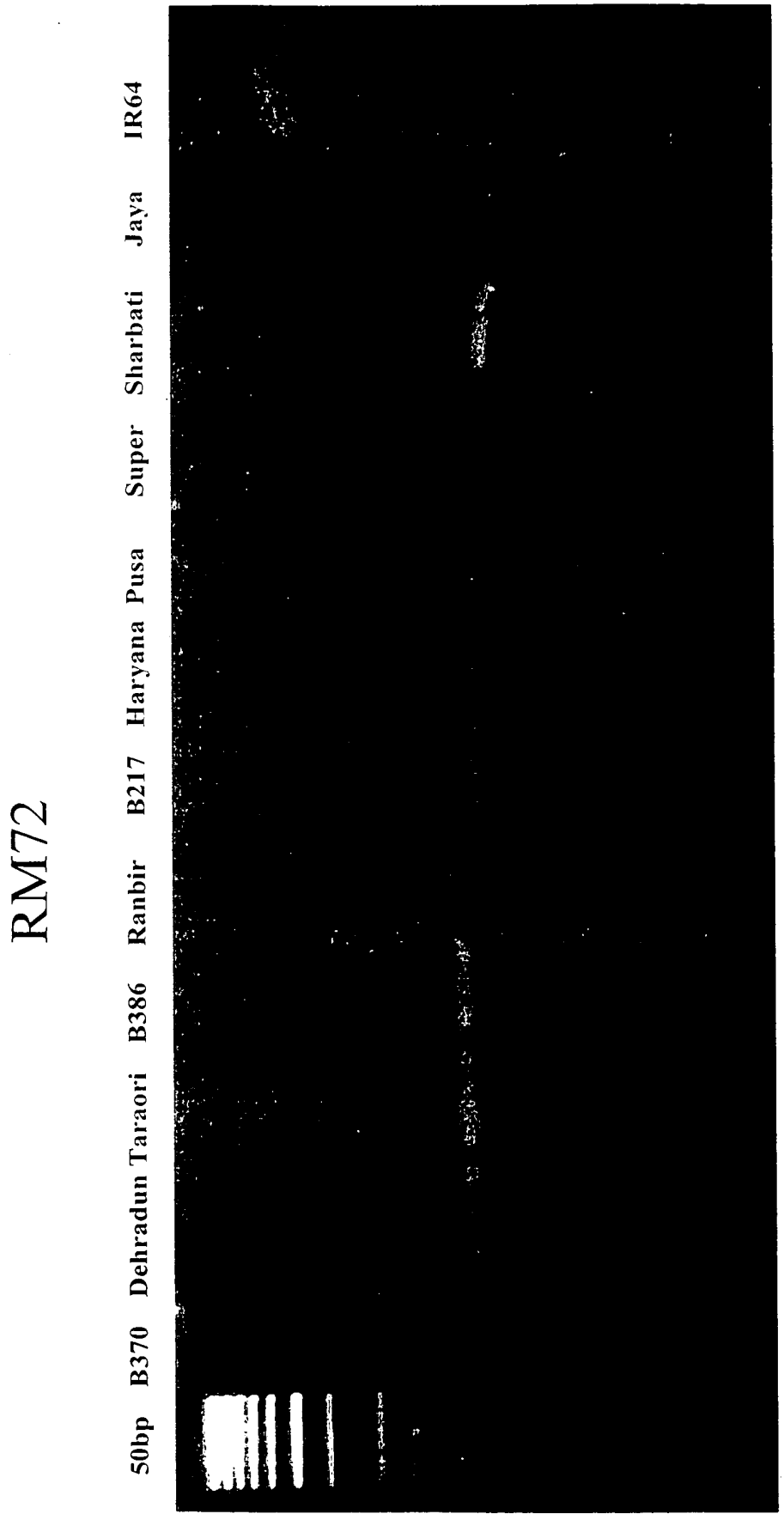

RM72
50bp
B370
Dehradun
Taraori
B386
Ranbir
B217
Haryana
Pusa
Super
Sharbati
Jaya
IR64

Fig. 5d

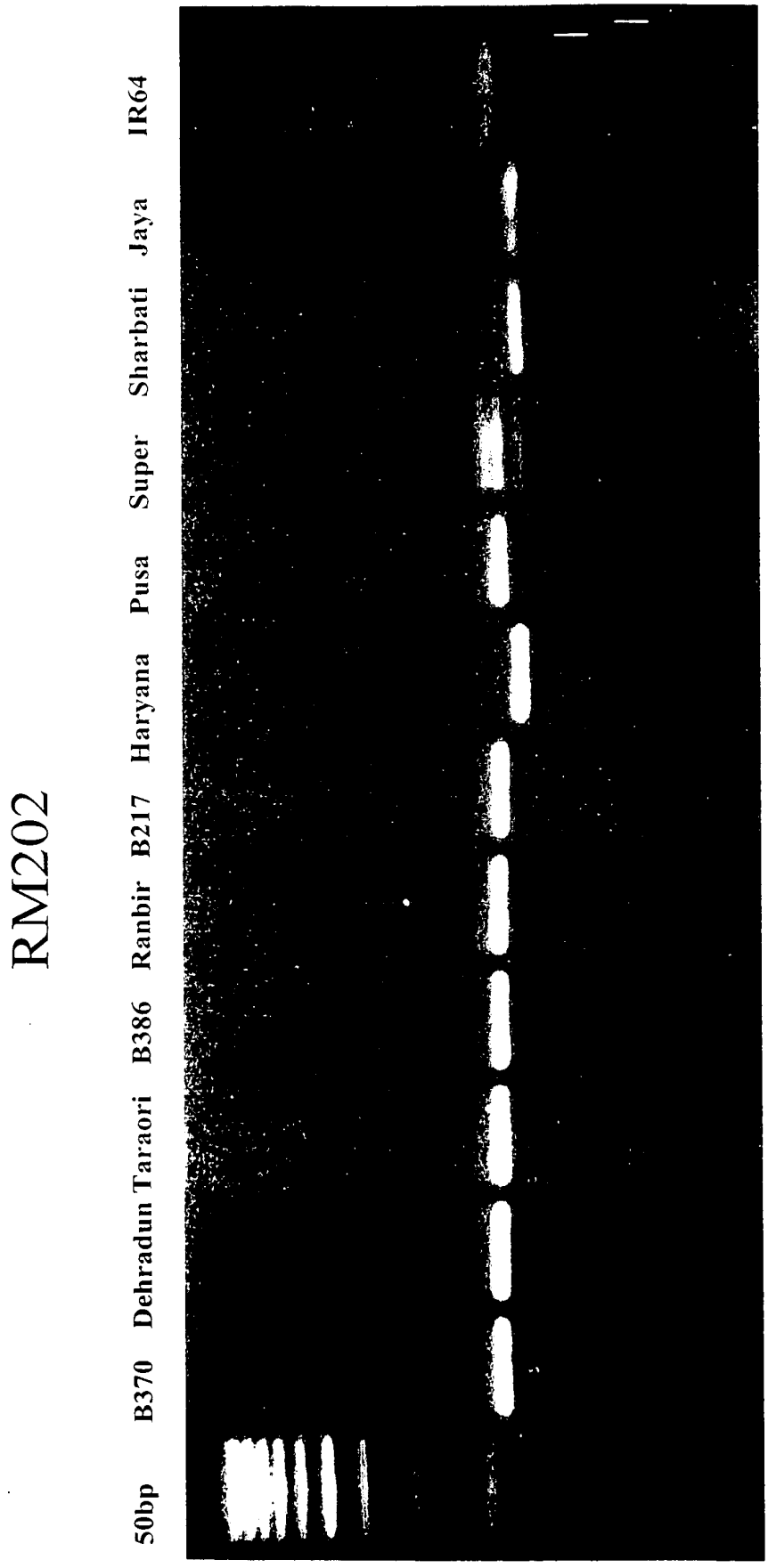

RM202
50bp
B370
Dehradun
Taraori
B386
Ranbir
B217
Haryana
Pusa
Super
Sharbati
Jaya
IR64

Fig. 5e

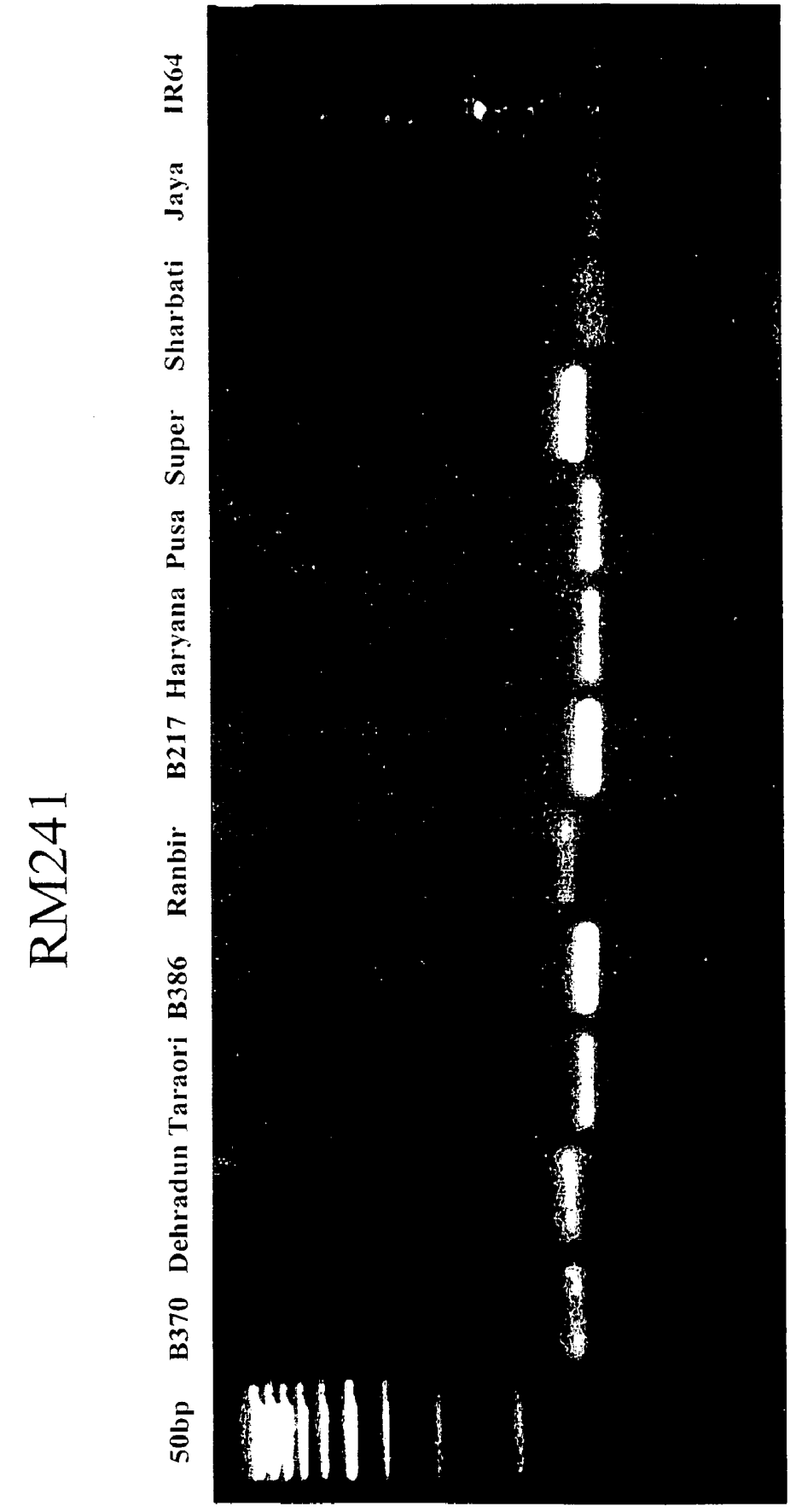

RM241
50bp
B370
Dehradun
Taraori
B386
Ranbir
B217
Haryana
Pusa
Super
Sharbati
Jaya
IR64

Fig. 5f

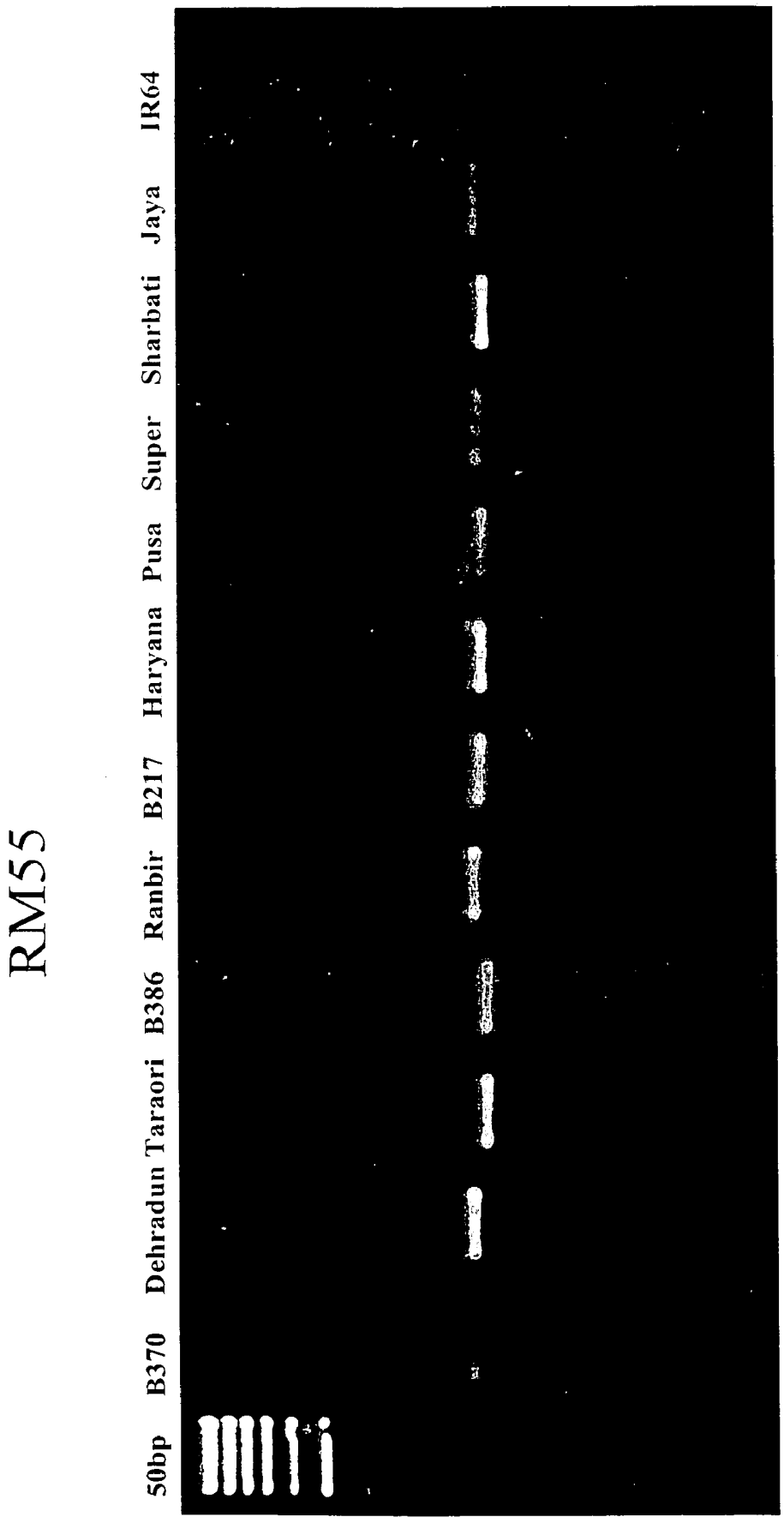

RM55
50bp
B370
Dehradun
Taraori
B386
Ranbir
B217
Haryana
Pusa
Super
Sharbati
Jaya
IR64

Fig. 5g

Sequence alignment of alleles from different basmati rice varieties showing variation in length

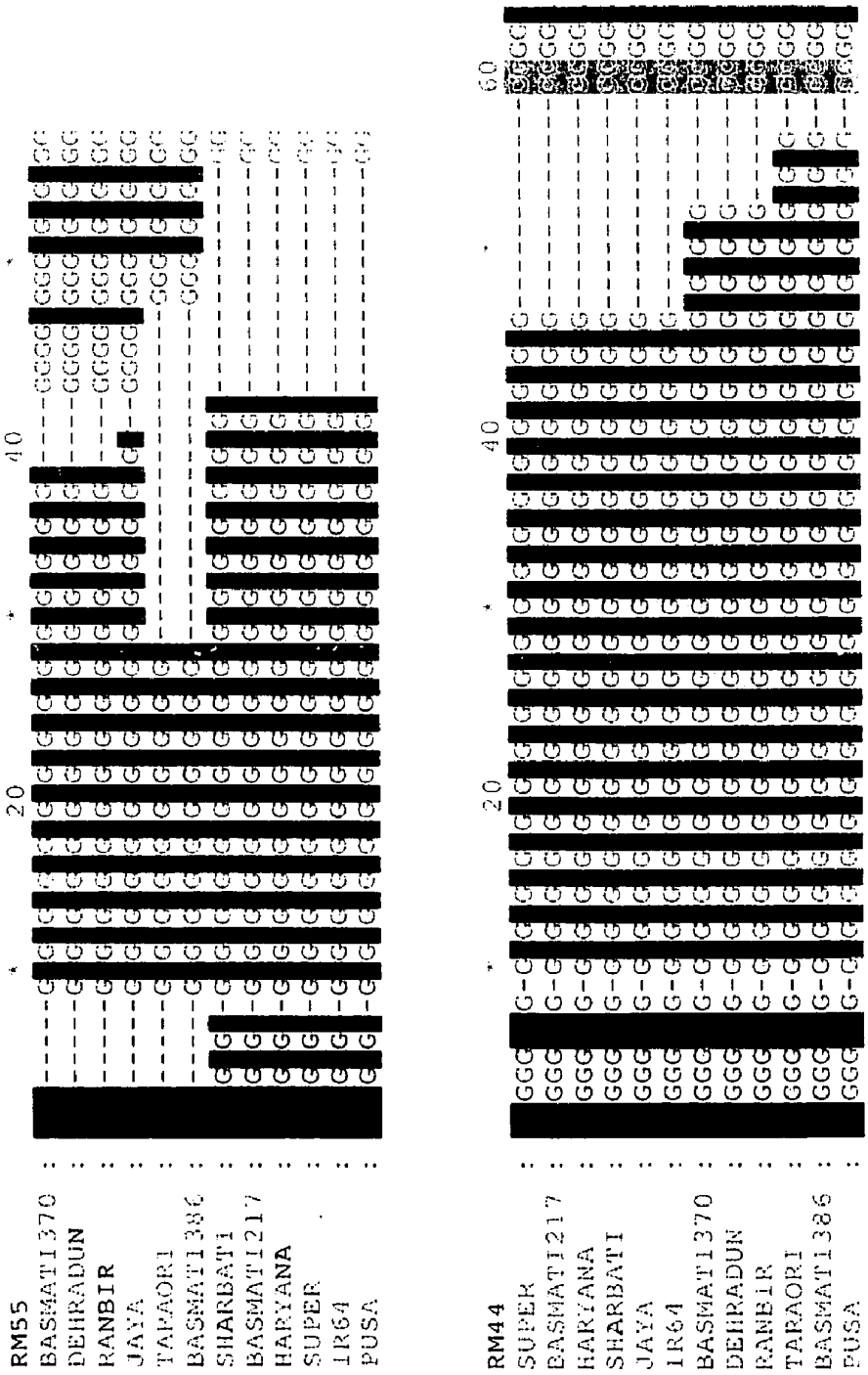


Fig. 6

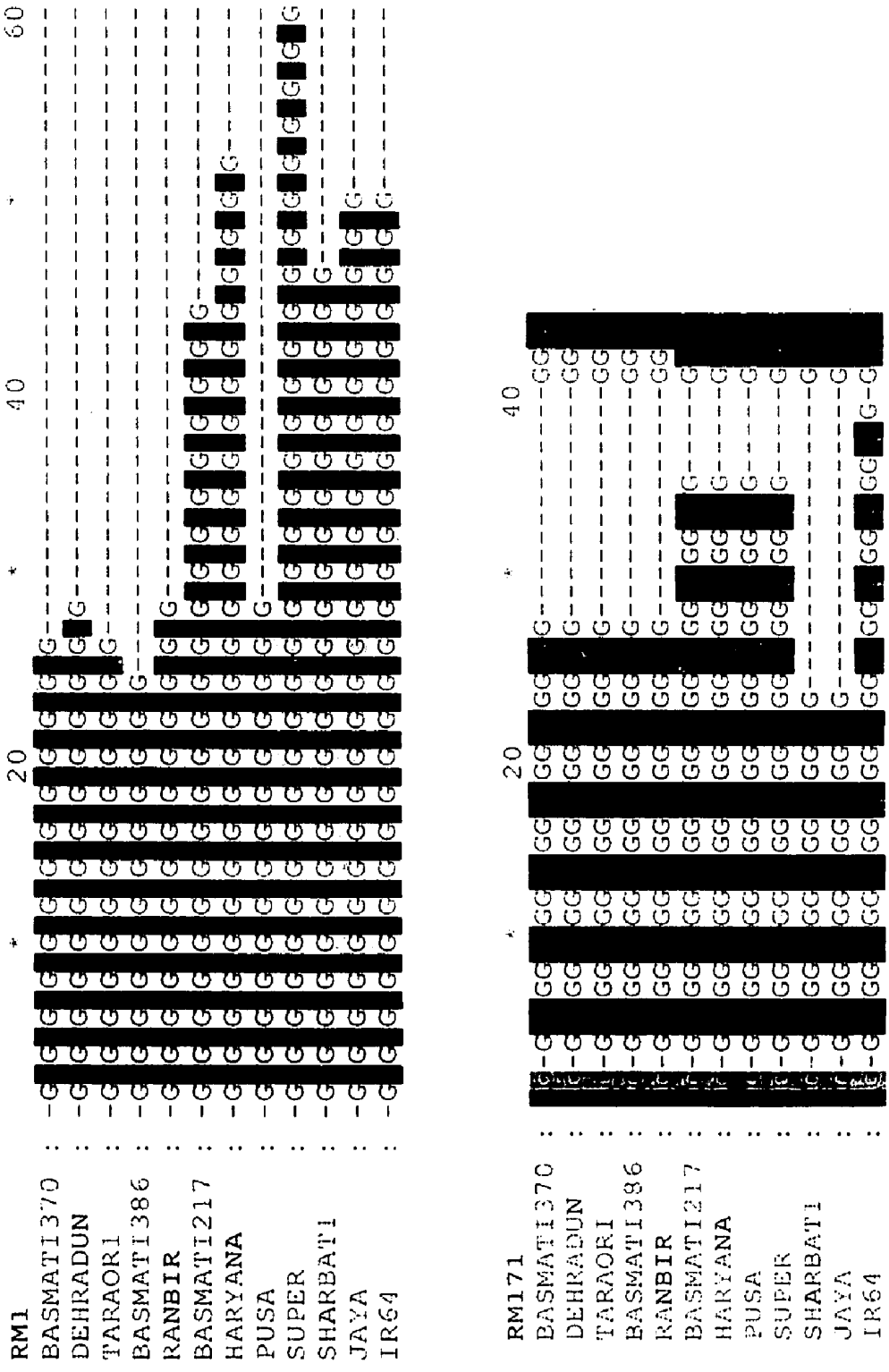

RM1
BASMATI370
DEHRADUN
TARAORI
BASMATI386
RANBIR
BASMATI217
HARYANA
PUSA
SUPER
SHARBATI
JAYA
IR64
20
40
60
RM171
BASMATI370
DEHRADUN
TARAORI
BASMATI386
RANBIR
BASMATI217
HARYANA
PUSA
SUPER
SHARBATI
JAYA
IR64
20
40

Fig. 6a

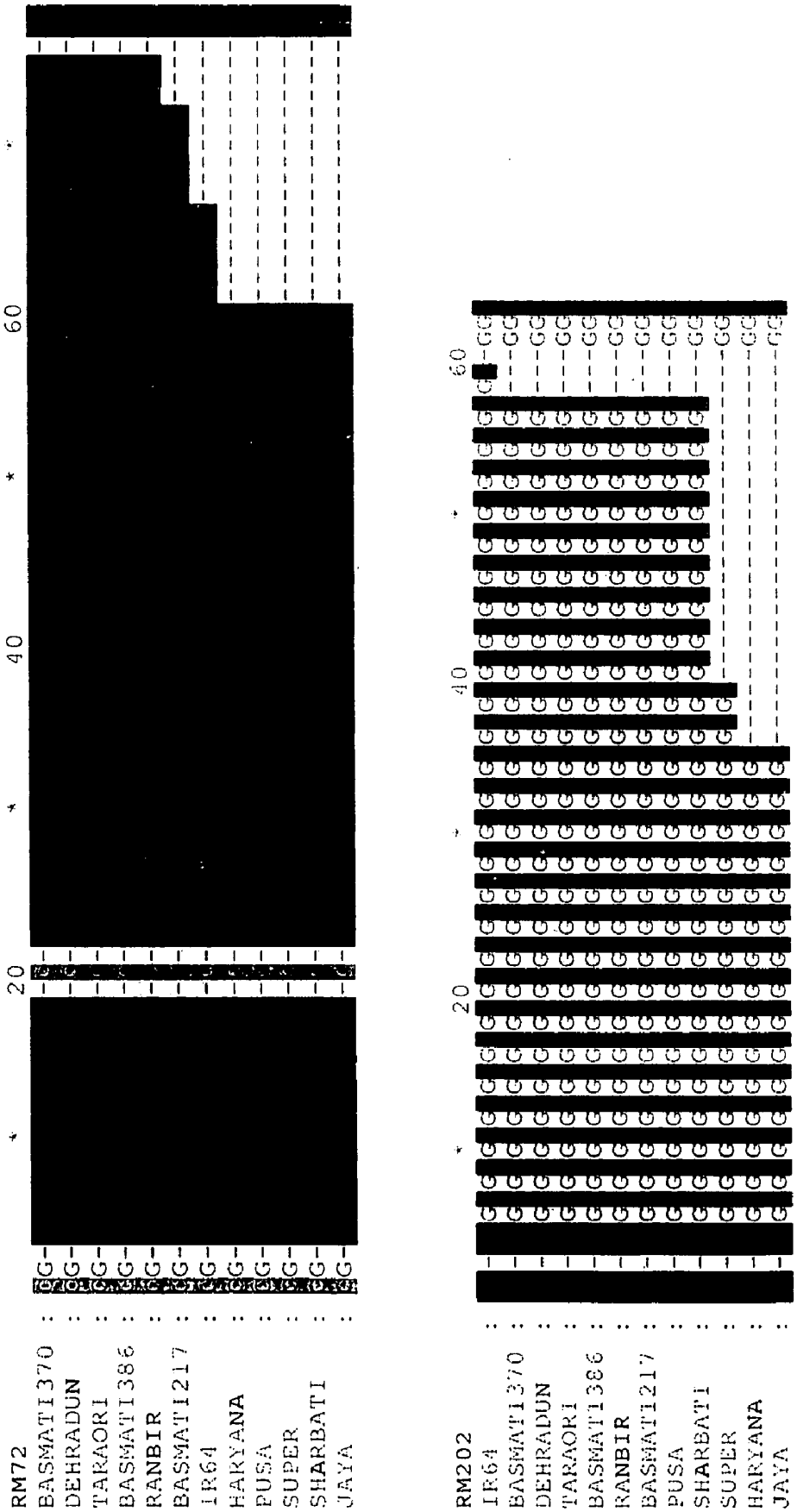

RM72
BASMATI370
DEHRADUN
TARAORI
BASMATI386
RANBIR
BASMATI217
IR64
HARYANA
PUSA
SUPER
SHARBATI
JAYA
20
40
60
RM202
IR64
BASMATI370
DEHRADUN
TARAORI
BASMATI386
RANBIR
BASMATI217
PUSA
SHARBATI
SUPER
HARYANA
JAYA
20
40
60

Fig. 6b

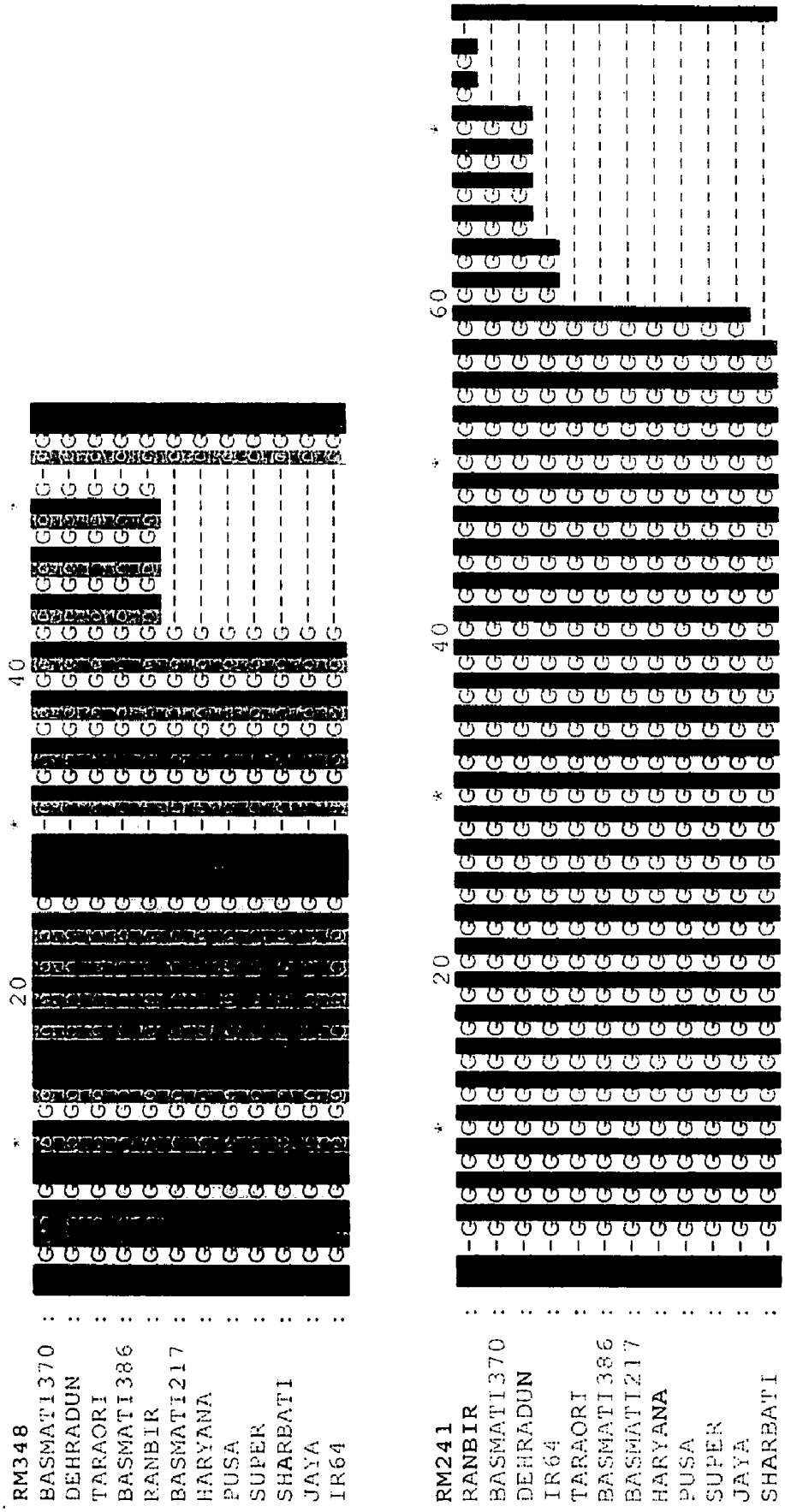

RM348
BASMATI370
DEHRADUN
TARAORI
BASMATI386
RANBIR
BASMATI217
HARYANA
PUSA
SUPER
SHARBATI
JAYA
IR64
20
40
RM241
RANBIR
BASMATI370
DEHRADUN
IR64
TARAORI
BASMATI386
BASMATI217
HARYANA
PUSA
SUPER
JAYA
SHARBATI
20
40
60

Fig. 6c

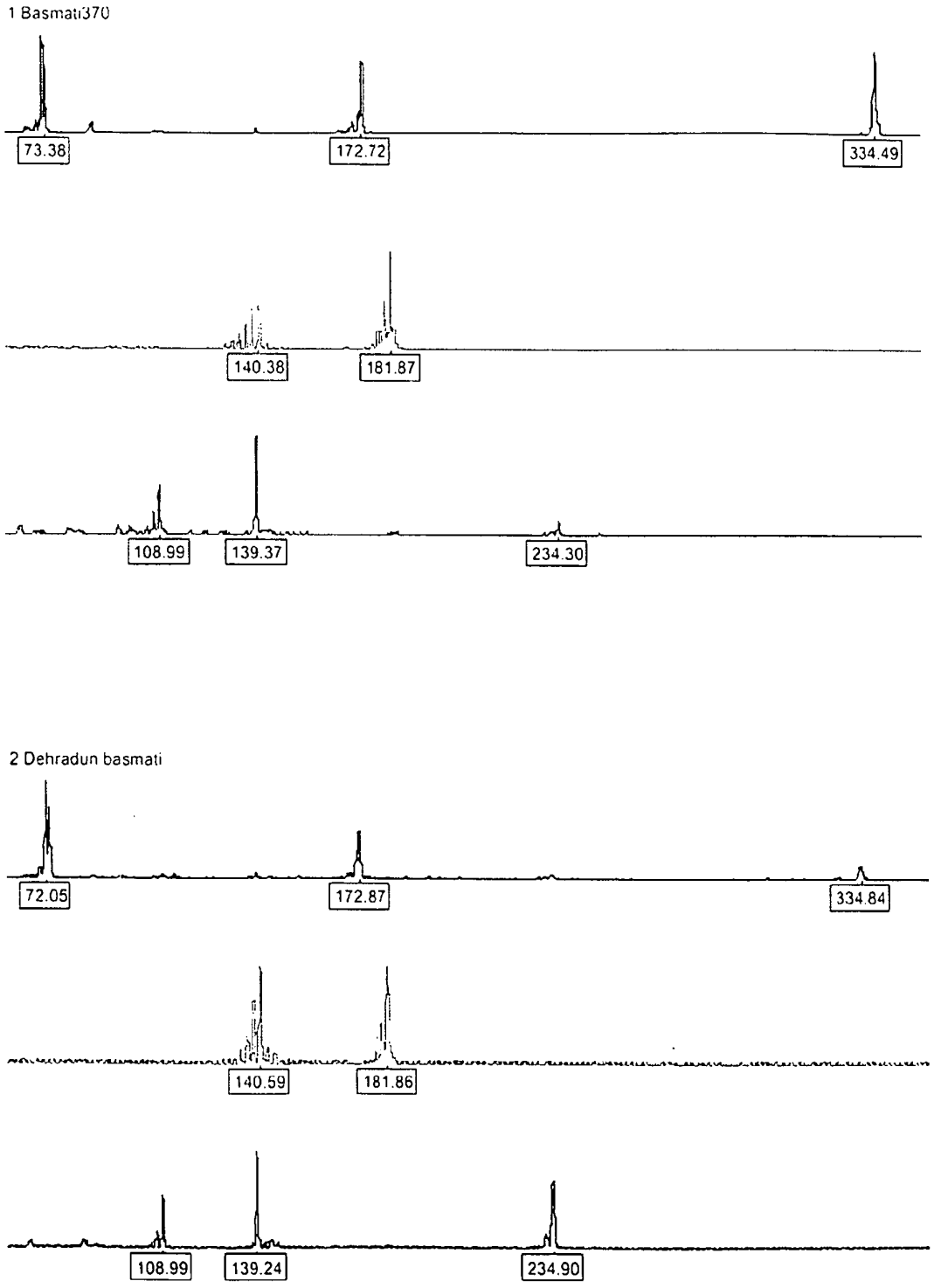

1 Basmati370
73.38
172.72
334.49
140.38
181.87
108.99
139.37
234.30
2 Dehradun basmati
72.05
172.87
334.84
140.59
181.86
108.99
139.24
234.90

Fig. 7

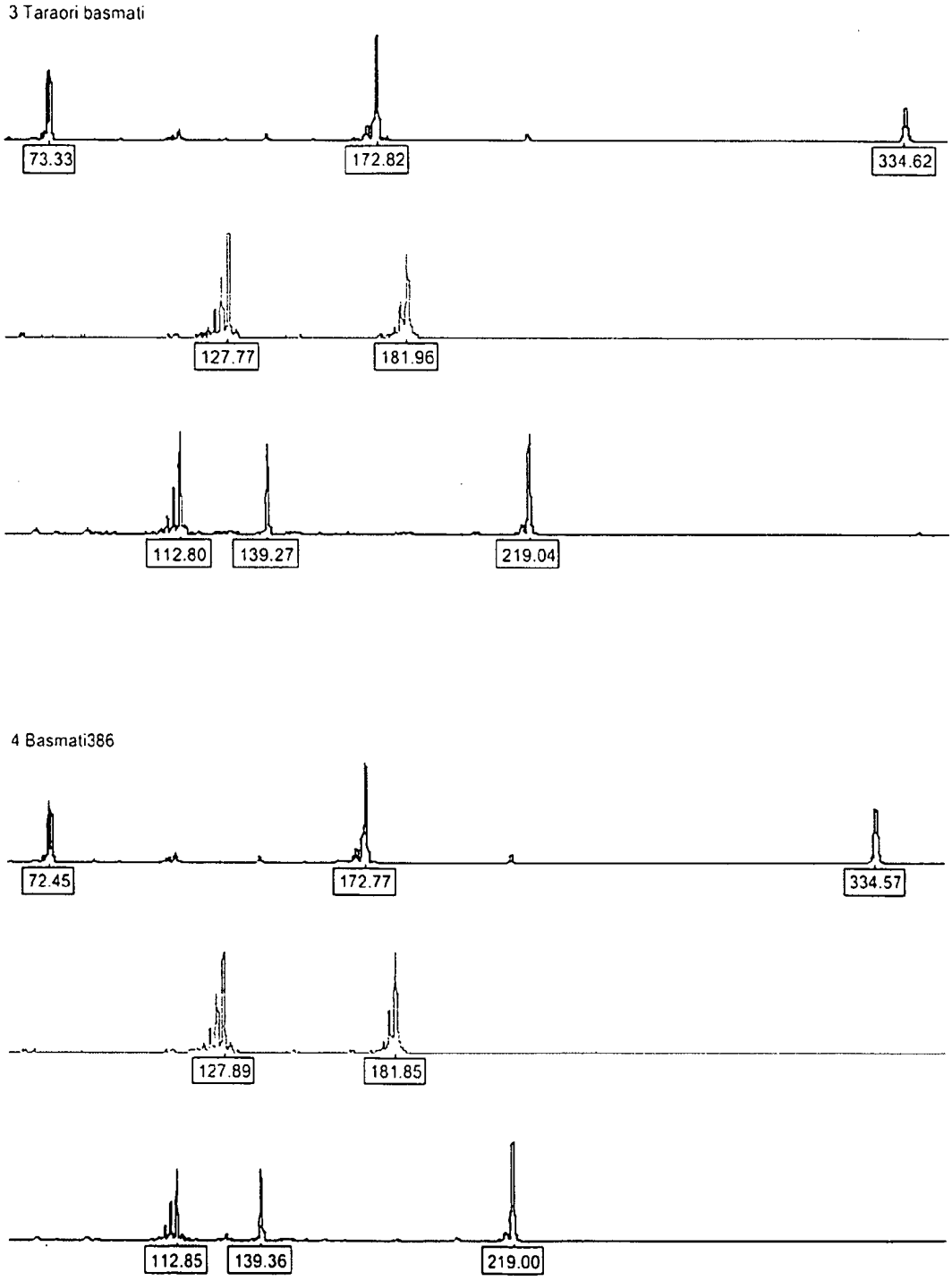

3 Taraori basmati
73.33
172.82
334.62
127.77
181.96
112.80
139.27
219.04
4 Basmati386
72.45
172.77
334.57
127.89
181.85
112.85
139.36
219.00

Fig. 7a

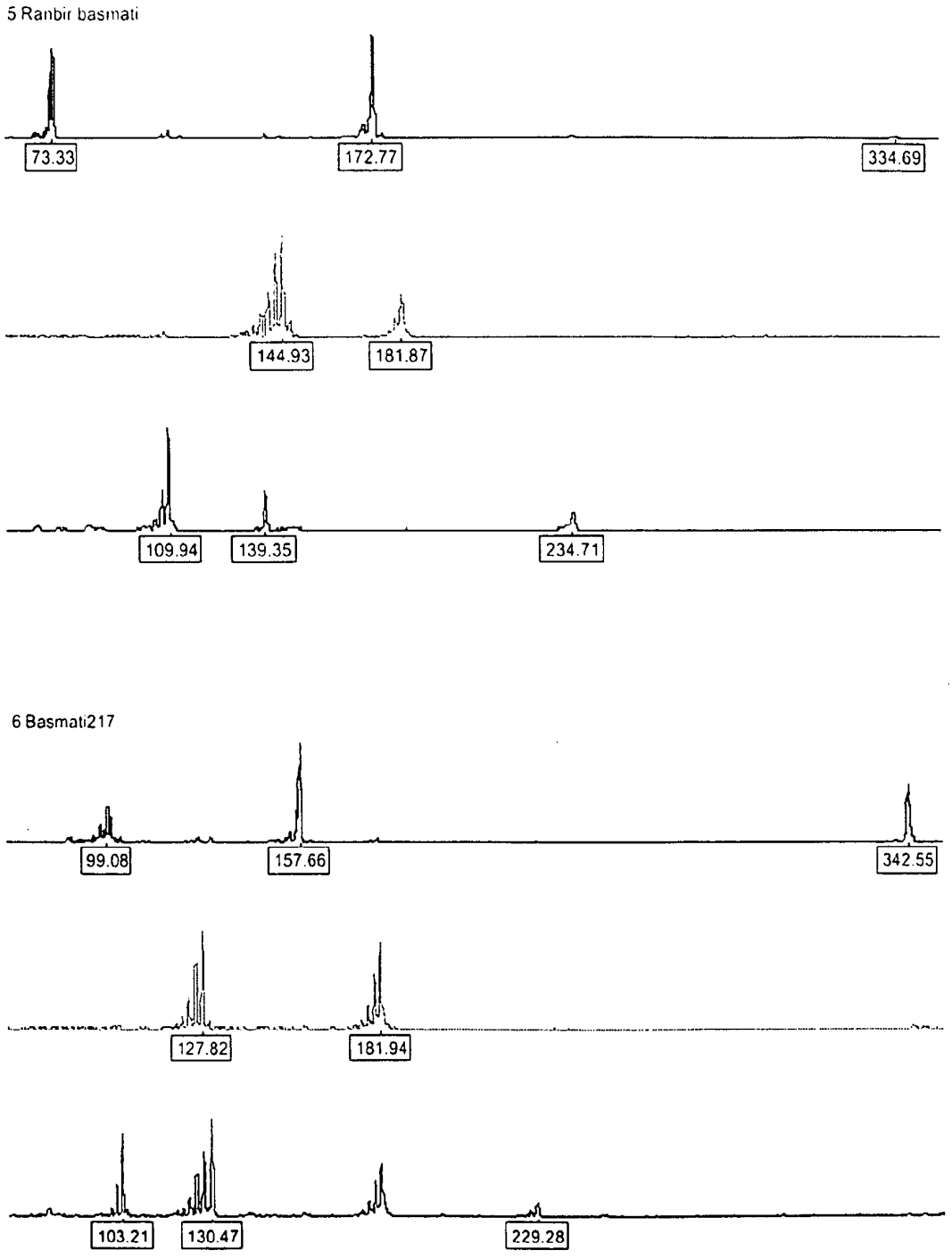

5 Ranbir basmati
73.33
172.77
334.69
144.93
181.87
109.94
139.35
234.71
6 Basmati217
99.08
157.66
342.55
127.82
181.94
103.21
130.47
229.28

Fig. 7b

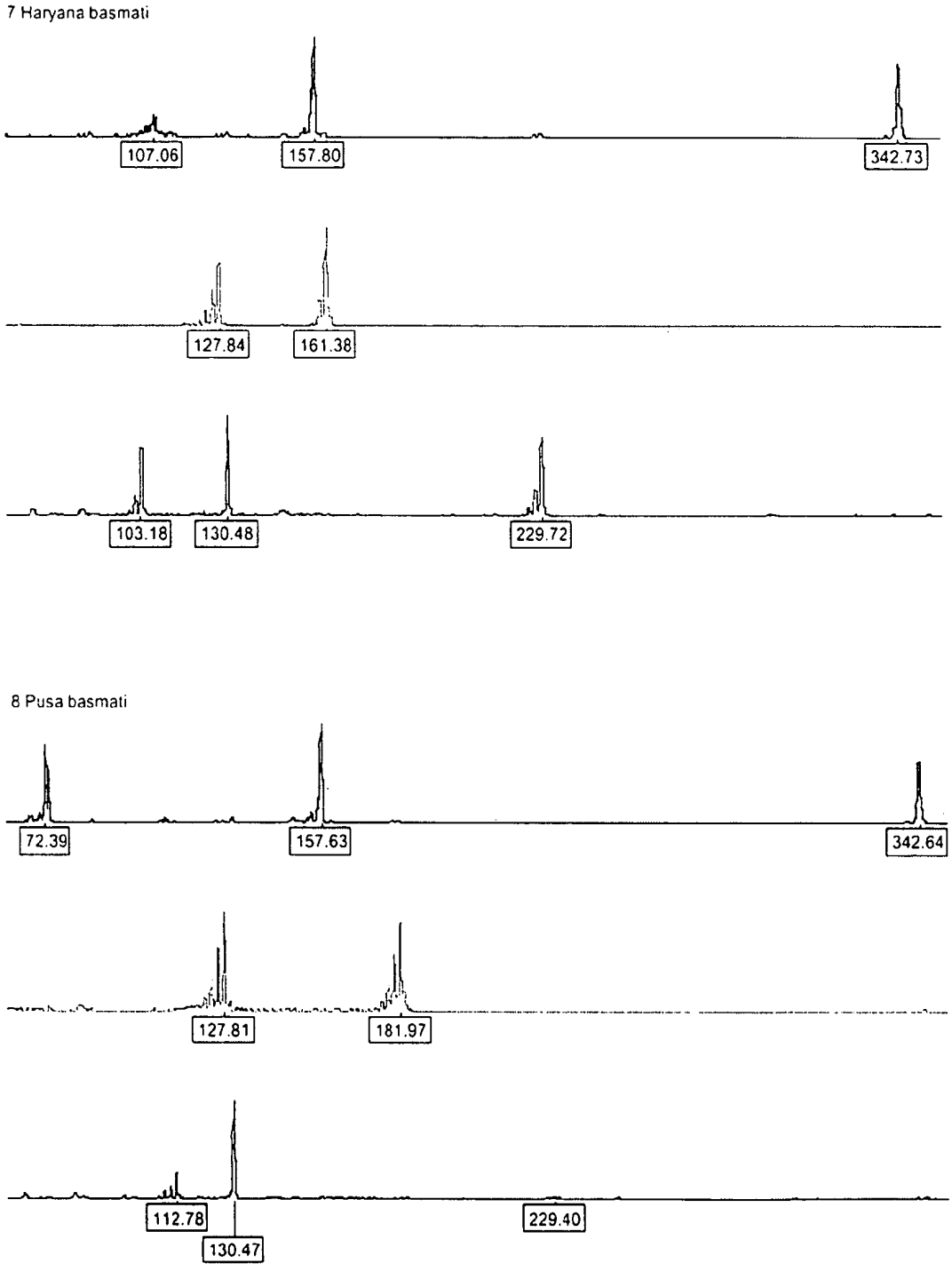

7 Haryana basmati
107.06
157.80
342.73
127.84
161.38
103.18
130.48
229.72
8 Pusa basmati
72.39
157.63
342.64
127.81
181.97
112.78
130.47
229.40

Fig. 8

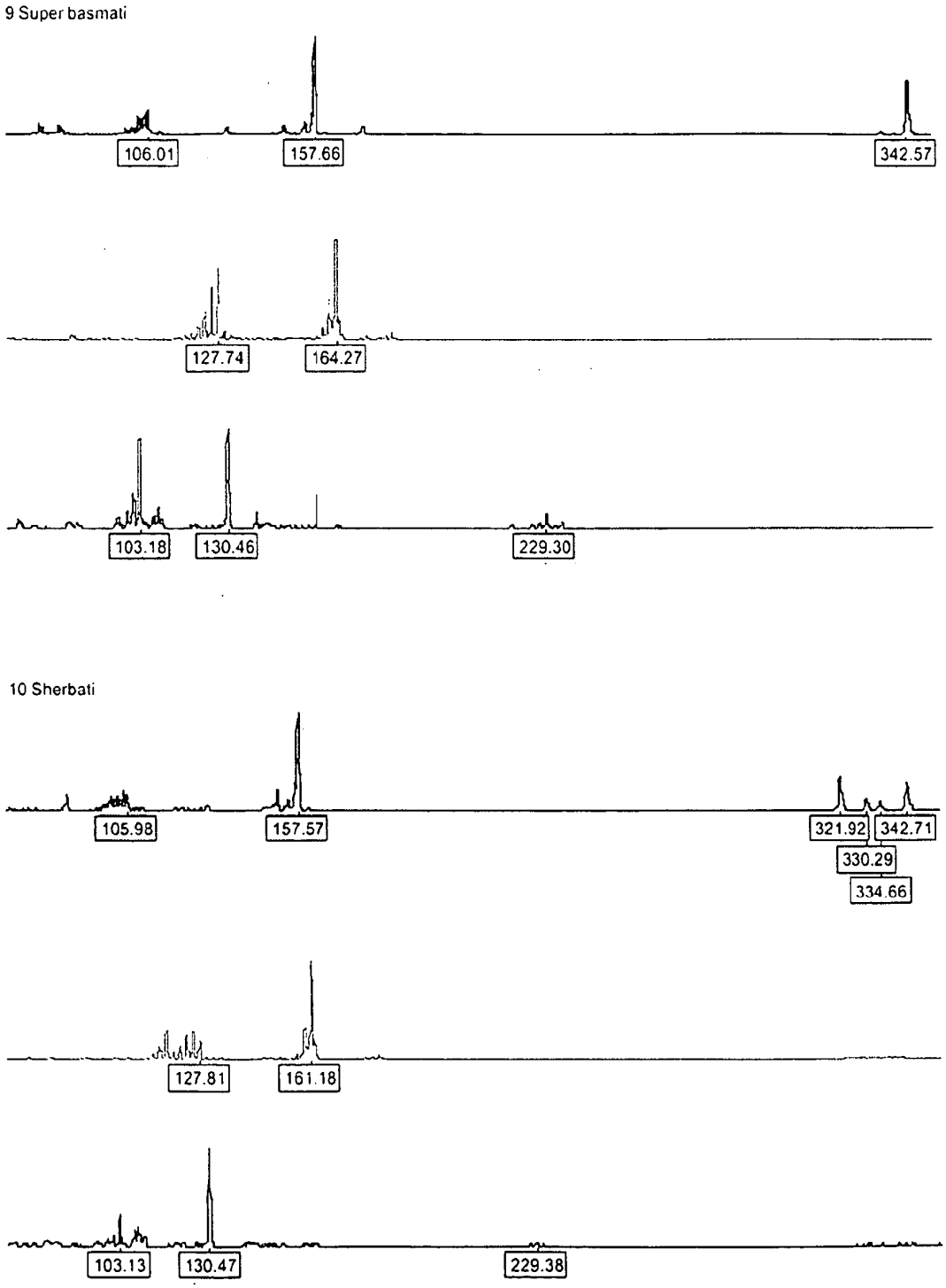

9 Super basmati
106.01
157.66
342.57
127.74
164.27
103.18
130.46
229.30
10 Sherbati
105.98
157.57
321.92
342.71
330.29
334.66
127.81
161.18
103.13
130.47
229.38

Fig. 8a

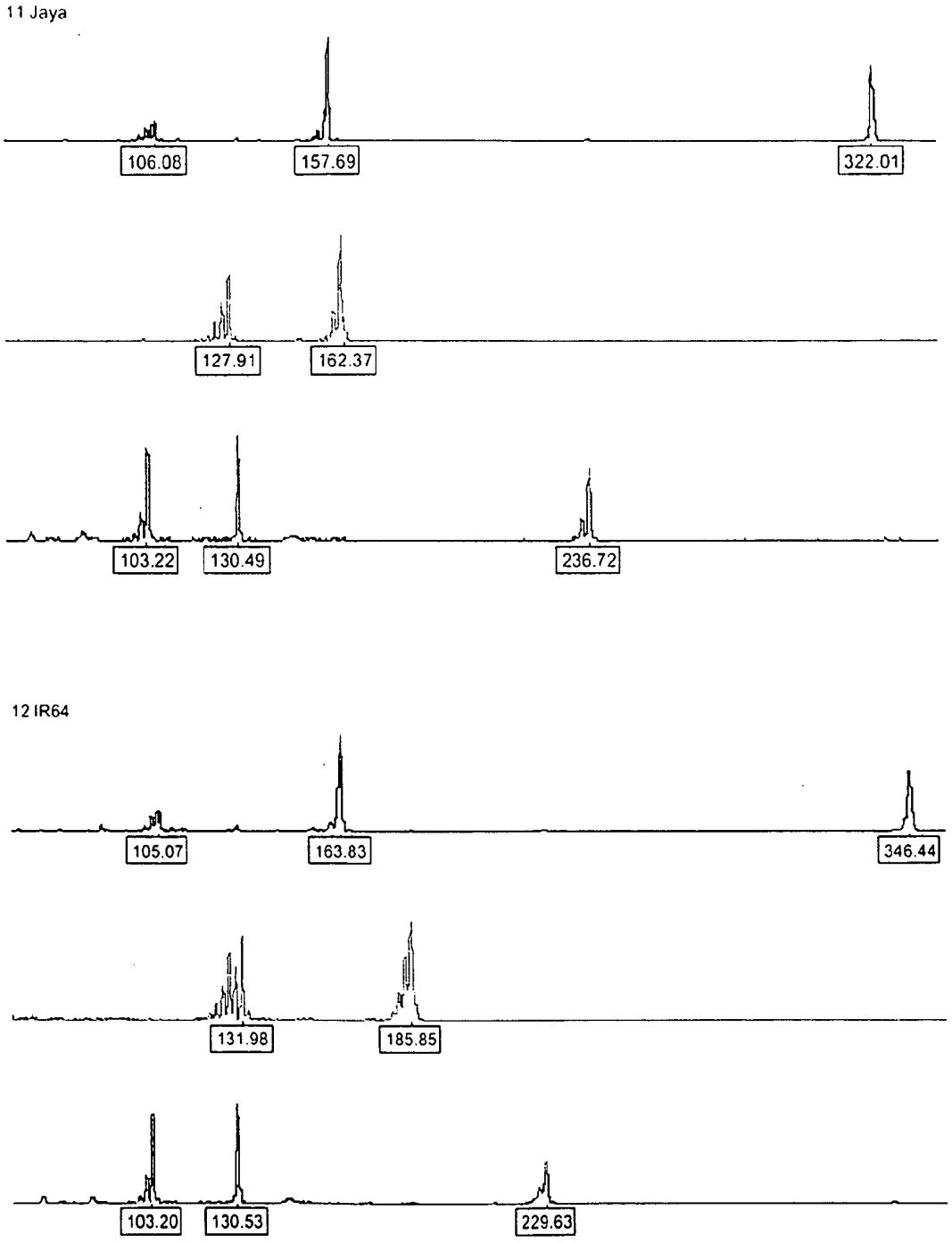

11 Jaya
106.08
157.69
322.01
127.91
162.37
103.22
130.49
236.72
12 IR64
105.07
163.83
346.44
131.98
185.85
103.20
130.53
229.63

Fig. 8b

SINGLE TUBE MULTIPLEX ASSAY FOR DETECTION OF ADULTERANTS IN BASMATI RICE SAMPLES

[0001] This application is a Continuation-in-Part (CIP) of the pending U.S. patent application Ser. No. 10/357,488.

FIELD OF THE PRESENT INVENTION

[0002] The present invention relates to the assays for detection and quantification of adulterants in basmati rice varieties.

BACKGROUND AND PRIOR ARTS OF THE PRESENT INVENTION

[0003] Traditional basmati varieties command a considerable price advantage in the international market over others. For instance, in European market, Indian traditional Basmati like Dehradun Basmati commands $850 per tonne where as evolved basmati cultivars like Pusa Basmati and Super Basmati get $480 and $500 per tonne respectively, and non-basmati long-grain rice fetch a meagre $160 per tonne. Additionally, some overseas markets encourage varieties that are more authentic by granting duty exemption. For example, in European market, a tariff of $78 per tonne is imposed on husked rice; whereas for nine Basmati varieties, the import duty is completely exempted (European Commission regulation 1549/2004).

[0004] Considering the price differences in the light of the total volume of international basmati rice trade (~1.5 million MT), it is obvious that unscrupulous practices such as adulteration of traditional basmati offer cost advantage to the traders. Since it is not quite easy to differentiate between traditional basmati and other long grain rice varieties, and a label of traditional basmati brings along duty advantage, fraudulent traders make a substantial profit by adulterating traditional basmati with either evolved basmati or non-basmati varieties and exploit the gullible consumer. Such practices have been shown to be existing and rampant by a food survey conducted by the Food Standards Agency of the United Kingdom (world wide web_food.gov.uk/science/surveillance/fsis2004branch/fsis4704basmati). The adulteration of traditional basmati grains affects the exporting countries too in terms of the tarnished image and diminished interest in the brands. Hence, to protect the interests of consumers and trade, identification of genuine basmati rice samples and devaluation of adulterated samples becomes vital.

[0005] Differentiation of traditional basmati varieties from other long grain varieties based on aroma, chemical composition and grain elongation arc impracticable for large-scale applications. Microsatellite profiles can be used for cultivar identification and detection of adulteration. We have already designated microsatellite profiles of traditional basmati, evolved basmati and non-basmati rice varieties (Nagaraju et al 2002). In fact, importers like European Union have now stipulated that all Basmati imports carry a certificate of purity based on a DNA test.

OBJECTS OF THE PRESENT INVENTION

[0006] The main object of the present invention relates to development of a single tube multiplex assay for distinguishing basmati from non-basmati rice varieties and thereby the adulteration.

[0007] Yet another object of the present invention is to develop a method of quantifying adulteration in basmati rice varieties.

SUMMARY OF THE PRESENT INVENTION

[0008] The present invention relates to a single tube multiplex assay for distinguishing basmati from non-basmati rice varieties and thereby the adulteration, said assay comprising steps of running multiplex PCR with sample using one or more loci of Table 3, and distinguishing the basmati from non-basmati rice varieties and thereby the adulteration on the basis of varietal specific multiplex allele profile; and also, a method of quantifying adulteration in basmati rice varieties, said method comprising steps of constructing a standard curve on the basis of ratio of quantity of amplified products of the alleles of adulterant and the basmati rice against the progressive proportion of adulteration, and quantifying the adulteration in basmati rice variety on the basis of peak area of the alleles corresponding to basmati and that of the adulterant.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0009] Accordingly, the present invention relates to a single tube multiplex assay for distinguishing basmati from non-basmati rice varieties and thereby the adulteration, said assay comprising steps of running multiplex PCR with sample using one or more loci of Table 3, and distinguishing the basmati from non-basmati rice varieties and thereby the adulteration on the basis of varietal specific multiplex allele profile; and also, a method of quantifying adulteration in basmati rice varieties, said method comprising steps of constructing a standard curve on the basis of ratio of quantity of amplified products of the alleles of adulterant and the basmati rice against the progressive proportion of adulteration, and quantifying the adulteration in basmati rice variety on the basis of peak area of the alleles corresponding to basmati and that of the adulterant.

[0010] A set of ten SSR loci has been identified and the competence its allele profiles to genotype various basmati varieties has been demonstrated. Further, a multiplex system to make use of allele size information for the identification of adulterants in commercial samples of basmati rice has been designed. It was also demonstrated that the multiplex system could be used to quantify the adulterant. Here, a high throughput "single tube assay" method based on multiplexing all or a combination of the ten microsatellite markers is described as a tool to certify genuineness of Basmati rice samples as shown in **FIG. 7**.

[0011] 1. Identification of the Adulterant

[0012] Primary step in the identification of an adulterant is to make unequivocal identification possible by generating variety-specific microsatellite profiles of the basmati varieties designated for trade and possible adulterants (Table 1). 350 primers were screened on the varieties (sequence source: world wide web.gramene.org). Sixteen primers were selected based on amplification of a single and clear band and discrimination power (Table 2). A panel of ten informative microsatellite loci was developed that differentiate various traditional basmati, evolved basmati varieties and others as well as amenable for multiplexing (Table 3). Upon PCR, a genuine sample of a traditional basmati variety

yields a single allele of the size listed in the panel. However, any admixture of traditional basmati with either evolved basmati or non-basmati would be detected at least at one of the microsatellite loci because of different allele sizes. Subsequently, we arranged these primers based on allele sizes in such a way that using 3 fluorescent ligands in the PCR primers we could run a single genotyping assay. The above two steps resulted in a methodology where, (a) Pure samples of all varieties could be unequivocally identified, and (b) Allele pattern could also identify the varietal mixtures.

[0013] 2. Construction of Standard Curve and Quantitation of Adulterant

[0014] It is possible that some basmati rice samples may contain adventitious mixture as a result 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 (for instance, 7% recommended by The Grain and Feed Trade Association, GAFTA Code of Practice for Rice) could be certified as practically genuine. Therefore, we went a step ahead in our effort and designed experiments to actually quantify the adulterant in basmati rice samples.

[0015] Given the differentiating alleles between the traditional basmati (major component) and evolved basmati or non-basmati (adulterant), the quantitation procedure was based on the premise that if we can quantify the amplified allelic products of a "common locus", the ratio between quantities of the amplicons can reveal the ratio of the quantities of competing DNA templates in a PCR mixture. The procedure involved preparation of a series of standards of traditional basmati rice samples with a progressive proportion of adulteration. The approach was to generate a "standard curve" by plotting the ratio of the quantity of amplified products of the alleles of adulterant and the traditional Basmati against the progressive proportion of adulteration. Quantity of the amplified allele was calculated based on the peak area of the allele obtained on the electropherogram.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

[0016] **FIG. 1** shows top panel with allelic profile of Basmati 370 obtained by single assay multiplex reaction. Three colours represent three groups of primers labelled with specific fluorescent ligands (blue is FAM, Green is JOE and black is TAMRA). Locus name and allelic size in base pairs are given below the peaks; bottom panel shows allelic profiles of pure basmati 370, pure adulterant sharbati and an adulterant sample, by using only two primers from the multiplex panel.

[0017] **FIG. 2** shows sequence alignment of alleles of RM55 locus from different basmati rice varieties showing variation in length. The sequences in **FIG. 2** are, from top to bottom, SEQ ID NOS: 38-49.

[0018] **FIG. 3** shows standard curve generated for a combination of basmati 370 adulterated with sharbati using allele differences at RM 348 locus.

[0019] **FIG. 4** shows mixing experiments in different combinations and the peaks obtained thereby at particular combinations.

[0020] **FIG. 5** shows photographs of Agarose Gel run to establish amplification for various PCRs.

[0021] **FIG. 6** shows sequence alignment data of various loci. The top panel of **FIG. 6** shows, from top to bottom, SEQ ID NOS: 38-49. The bottom panel of **FIG. 6** shows, from top to bottom, SEQ ID NOS: 50-61.

[0022] **FIG. 6*a***, top panel, shows from top to bottom SEQ ID NOS: 62-73. **FIG. 6*a***, bottom panel, shows SEQ ID NOS: 74-85.

[0023] **FIG. 6*b***, top panel, shows SEQ ID NOS: 86-97. **FIG. 6*b***, bottom panel, shows SEQ ID NOS: 98-109.

[0024] **FIG. 6*c***, top panel, shows SEQ ID NOS: 110-121. **FIG. 6*c***, bottom panel, shows SEQ ID NOS: 122-133.

[0025] **FIG. 7** shows multiplex mixing combinations.

BRIEF DESCRIPTION OF THE TABLES OF THE PRESENT INVENTION

[0026] Table 1 shows list of varieties used for standardisation of multiplex.

[0027] Table 2 shows SSR loci (including those added in the CIP) that are selected to distinguish basmati from non-basmati subsequent to large-scale screening.

[0028] Table 3 shows the panel of ten informative SSR loci selected for multiplex assay.

[0029] Table 4 shows allele sizes (in base pairs) of various basmati rice varieties obtained by multiplex single assay method.

[0030] Table 5 shows Genotype codes of various basmati rice varieties based on single assay multiplex method. The order of codes from left to right correspond to loci 1 to 8 given in Table 5.

[0031] Table 6 shows Allele sizes in base pairs for corresponding codes of Table 4.

[0032] Table 7 Shows how these 10 primers were arranged in a particular manner to facilitate single genotyping assay. It is clear from the table that loci were grouped so as to avoid overlapping allele sizes in the same fluorescence label (read as 'same coloured peaks in the electrophoresis') as shown in **FIG. 7**.

[0033] The loci could be employed to distinguish basmati and non-basmati in a 'single tube assay' is the result of the present research. The number of markers would vary from case to case and thus, the requirement can vary from 1 to all the 10 markers. The assay can differentiate any two known varieties using only one locus. However, a combination of the markers is employed in a multiplex single tube reaction to identify the main variety and any combination of adulterants in the genuine basmati grains.

[0034] The web link for the rice microsatellite primer list is world wide web_gramene.org/microsat/ssr.html. This site had only 350 loci when the study was initiated, but now contains nearly two thousand microsatellite loci.

Experimental Data on the Basis of which 10 Markers were Selected is Provided Below.

[0035] 1. Preliminary screening of the loci was done for the amplification of a clear and single amplicon. Those loci,

at which a) no amplification b) non-specific amplification c) stutter problem and, d) inconsistent amplification were obtained were eliminated.

[0036] 2. In the second step of screening only those loci for which primer pairs have annealing temperature of at least 55° C. were selected to ensure stringent PCR conditions in the assay.

[0037] 3. Ideally such loci were selected that generated more than two alleles and could be easily differentiated from stutters if any.

[0038] 4. Loci generating private alleles specific to particular variety were given preference.

[0039] 5. Among the most distinguishing loci, those with high reproducibility of the allele size were selected for further analysis.

[0040] 6. The loci were then tested for existence of polymorphism among and between basmati genotypes especially a set of the varieties that are commercially important.

[0041] Comprehensive details of the experimental data to arrive at the "Standard Curve" are provided as given below. In addition, shown are standard curve experiments for other combinations also, apart from Basmati 370 and Sharbati using locus RM348. Here, calculations are also provided to arrive at Peak Area and also, the percentage adulteration determined in such cases.

[0042] Construction of Standard Curve and Quantitation of the Adulterant

[0043] 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 (for instance, 7% recommended by The Grain and Feed Trade Association, GAFTA Code of Practice for Rice) could be certified as practically genuine. Therefore, we designed experiments to actually quantify the adulterant in basmati rice samples.

[0044] Given the differentiating alleles between the traditional basmati (major component) and evolved basmati or non-basmati (adulterant), the quantitation procedure is based on the premise that if we can quantify the amplified allelic products of a "common locus", the ratio between quantities of the amplicons can reveal the ratio of the quantities of competing DNA templates in the PCR mixture. The procedure involved preparation of a series of standards of traditional basmati rice samples with a progressive proportion of adulteration. The approach was to generate a "standard curve" by plotting the ratio of the quantities of amplified products of adulterant and the traditional Basmati alleles against the degree of adulteration. Quantity of the amplified allele was calculated based on the peak area of the allele obtained on the electropherogram.

[0045] Standard curves were constructed for a combination of Basmati370:Sharbati mixtures at two discriminating loci, RM72 and RM348. Standard samples were prepared by mixing the grains of the Basmati370 with Sharbati at progressive ratio of 1%, 3%, 5%, 7%, 10%, 15%, 17%, 20%, 25%, 30%, 40% and 60% to generate data at 12 score points. Subsequent to genotyping, peak areas were determined for each score point and were plotted against the percent adulterant to develop a standard curve based on logistic model ($y=a/1+be^{-cx}$) by using CurveExpert 1.38 (http://curveexpert.webhop.net). A standard curve was also generated by mixing DNA isolated from the milled grains of Sharbati, a common adulterant, in various ratio at 5%, 10%, 20%, 30%, 40%, 50% and 60% to Basmati370 DNA to generate seven score points on the curve. Systematic bias associated with the employment of standard curves was calculated. The differences were averaged over three independent runs to compute the bias (b) at each score point. Bias (B) introduced by using standard curve was computed as, $B=\sqrt{\Sigma b^2}$.

[0046] For illustrating mixing experiments in different combinations, peaks obtained at particular combinations are given as **FIG. 4**. Further, photographs of Agarose Gel run to establish amplification for various PCRs is provided as **FIG. 5**.

[0047] Bi-directional sequencing of PCR products was carried out thrice on ABI 3100 sequencer using ABI PRISM BigDye Primer Cycle Sequencing Kit according to the manufacturer's instructions. Sequence alignment data of various loci, as provided for locus RM 55 in **FIG. 2** is provided in as given as **FIG. 6**.

[0048] The invention is further elaborated with the help of following examples. However the examples should not be construed to limit the scope of the invention.

EXAMPLE 1

Multiplex PCR

[0049] PCR amplification was carried with the following reaction mixture composition. 10 ng of DNA template, 80 μM dNTPs, 2 mM $MgCl_2$, primer-mix providing 0.1 μM of each primer pair to the reaction, 0.5 unit Ampli Taq Gold DNA polymerase (Applied Biosystems), were mixed in a reaction volume of 10 μl. 5' ends of forward primers were labelled with any one of the following fluorescent ligands: TAMRA, JOE or FAM (Sigma). After an initial denaturation of 15 min at 95° C., the PCR mix was cycled 30 times at 94°, 55° and 72° C. for 30, 90 and 60 seconds respectively. This was followed by a final extension step at 60° C. at 30 min. Amplification was carried out on a PE9700 thermal cycler.

EXAMPLE 2

Genotyping

[0050] Amplification was confirmed on 1.5% agarose gel before running genotyping assays on the capillary-based ABI 3100 genetic analyser according to manufacturer's instructions. 0.2 μl PCR product was mixed with ROX-500 size standard and Hi-dye before loading. Subsequent to electrophoresis, lanes were extracted and analysed using GeneScan version 3.1 and allele sizes of the true peaks were determined by Genotyper version 2.1. Bi-directional sequencing of PCR products was carried out thrice on ABI 3100 sequencer to obtain accurate sequences of the repeat regions.

EXAMPLE 3

Quantification of Adulterant

[0051] Standard curves were constructed for a combination of Basmati370:Sharbati mixtures at two discriminating

loci, RM72 and RM348. Standard samples were prepared by mixing the grains of the Basmati370 with Sharbati at progressive ratio of 1, 3, 5, 7, 10, 15, 17, 20, 25, 30, 40 and 60% to generate data at 12 score points. Triplicate 1 g samples at each score point were used for DNA isolation. Subsequent to genotyping, peak areas were determined for each score point and were plotted against the percent adulterant to develop a standard curve based on logistic model ($y=a/1+be^{-cx}$). A standard curve was also generated by mixing DNA isolated from the milled grains of Sharbati in various ratio at 5%, 10%, 20%, 30%, 40%, 50% and 60% to Basmati370 DNA to generate seven score points on the curve. Systematic bias associated with the employment of standard curves was calculated. The differences were averaged over three independent runs to compute the bias (b) at each score point. Bias (B) introduced by using standard curve was computed as, $B=\sqrt{\Sigma b^2}$.

RESULTS

[0052] 1. Variety Specific Profiles and Identification

[0053] Excellent quality peaks were obtained in the single assay multiplex reactions to obtain allele sizes for all the rice varieties tested (Table 3). **FIG. 1** top panel shows the multiplex profile (8 loci) for Basmati370, **FIG. 1** bottom panel shows the allele profile (2 loci) of pure and adulterated Basmati370 samples. All varieties were assigned specific profiles (Table 4). The multiplex single assay can identify all the listed basmati varieties. RM171 alone can clearly separate traditional basmati from others.

[0054] Confirmation of Allele Sizes

[0055] Microsatellite alleles may produce stutters even under best of the conditions. Determination of the allele sizes can therefore be prone to errors, which is not acceptable for sensitive assays such as determination of adulterants. We confirmed the allele sizes in twelve varieties by Bi-directional sequencing of the alleles and actual counting the number of repeat units in each allele at all the loci. Sequencing also helps discover reasons for the size differences between alleles. Sequencing of PCR products was carried out thrice on ABI 3100 sequencer. In RM55, the size differences between alleles were due to disparate repeat numbers as well as indel events in the flanking sequences (**FIG. 2**). In all other loci, differences in the allele sizes were entirely due to differences in the number of repeat units. We therefore have confirmed sizes of all the alleles at all loci.

[0056] Quantification of the Adulterant

[0057] Sample standard curve obtained at RM348 is shown in **FIG. 3**. Systematic bias associated with the employment of standard curves was calculated to be ±4.95% for RM72 based curve and ±5.2% for RM348, based curve in the region of 1-15% adulteration. The standard curves were validated by quantifying the adulteration in blind samples. Three blind samples with 4%, 8% and 12% adulteration were genotyped and the peak-area ratios were plotted on the standard curves. The per cent adulteration was estimated with an error of ±2.6% and ±2.3% respectively for RM348 and RM72 based curves. Therefore our protocol quantifies the adulterant with an accuracy of at least ±3% adulteration.

TABLE 1

List of varieties used for standardisation of multiplex

Traditional Basmati Varieties	Evolved Basmati varieties	Non-Basmati long-grain varieties
Basmati 370	Haryana Basmati	Sharbati
Type-3 (Dehradun)	Pusa basmati	IR-64
Taraori basmati (HBC-I9)	Super basmati	
Basmati 386	Basmati385	
Ranbir basmati		
Basmati 217		

[0058]

TABLE 2

SSR loci that are selected to distinguish basmati from non-basmati subsequent to large-scale screening. Loci marked with asterisk are added in the CIP.

	Locus	Repeat Motif	
1.	RM 1	$(AG)_{26}$	SEQ ID NO: 1
2.	RM 110	$(GA)_{15}$	SEQ ID NO: 2
3.	RM 171	$(GATG)_5$	SEQ ID NO: 3
4.	RM 201	$(GA)_{17}$	SEQ ID NO: 4
5.	RM 202*	$(GA)_{30}$	SEQ ID NO: 5
6.	RM 212	$(GA)_{24}$	SEQ ID NO: 6
7.	RM 241*	$(GA)_{31}$	SEQ ID NO: 7
8.	RM 263	$(GA)_{34}$	SEQ ID NO: 8
9.	RM 282	$(GA)_{15}$	SEQ ID NO: 9
10.	RM 339	$(CTT)_8CCT(CTT)_5$	SEQ ID NO: 10
11.	RM 348*	$(CAG)_7$	SEQ ID NO: 11
12.	RM 44*	$(GA)_{16}$	SEQ ID NO: 12
13.	RM 440*	$(CTT)_{22}$	SEQ ID NO: 13
14.	RM 525*	$(AAG)_{12}$	SEQ ID NO: 14
15.	RM 55*	$(GA)_{17}$	SEQ ID NO: 15
16.	RM 72	$(TAT)_5C(ATT)_{15}$	SEQ ID NO: 16

[0059]

TABLE 3

The panel of ten informative SSR loci selected multiplex assay

Locus	Repeat motif	Chromosome no.	Forward primer	Reverse primer
RM171	$(GATG)_5$	10	AACGCGAGGACACGTACTTAC	ACGAGATACGTACGCCTTTG
RM55	$(GA)_{17}$	3	CCGTCGCCGTAGTAGAGAAG	TCCCGGTTATTTTAAGGCG
RM202	$(GA)_{30}$	11	CAGATTGGAGATGAAGTCCTCC	CCAGCAAGCATGTCAATGTA

TABLE 3-continued

The panel of ten informative SSR loci selected multiplex assay

Locus	Repeat motif	Chromosome no.	Forward primer	Reverse primer
RM72	$(TAT)_5C(ATT)_{15}$	8	CCGGCGATAAAACAATGAG	GCATCGGTCCTAACTAAGGG
RM348	$(CAG)_7$	4	CCGCTACTAATAGCAGAGAG	GGAGCTTTGTTCTTGCGAAC
RM241	$(GA)_{31}$	4	GAGCCAAATAAGATCGCTGA	TGCAAGCAGCAGATTTAGTG
RM44	$(GA)_{16}$	8	ACGGGCAATCCGAACAACC	TCGGGAAAACCTACCCTACC
RM1	$(GA)_{26}$	1	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC
RM440	$(CTT)_{22}$	5	CATGCAACAACGTCACCTTC	ATGGTTGGTAGGCACCAAAG
RM525	$(AAG)_{12}$	2	GGCCCGTCCAAGAAATATTG	CGGTGAGACAGAATCCTTACG

[0060] Repeat motif column discloses, from top to bottom, SEQ ID NOS: 3-5, 16, 11, 7, 12, 17 and 13-14. Forward primer column discloses, from top to bottom, 18-27. Reverse primer column discloses, from top to bottom, SEQ ID NOS 28-37.

TABLE 4

Allele sizes (in base pairs) of various basmati rice varieties obtained by multiplex single assay method

Locus	Basmati370	Dehradun Basmati	Taraori Basmati	Basmati386	Ranbir Basmati	Basmati217	Haryana Basmati	Pusa Basmati	Super Basmati	Basamati385	Sharbati	IR64
RM1	73	73	73	73	73	100	108	73	106	73	106	106
RM72	173	173	173	173	173	158	158	158	158	158	158	164
RM171	335	335	335	335	335	343	343	343	343	335	322, 343, 335	346
RM241	140	140	128	128	144	128	128	128	128	140	128	128
RM202	182	182	182	182	182	182	161	182	164	161	161	186
RM44	109	109	113	113	109	103	103	113	103	113	103	103
RM348	139	139	139	139	139	130	130	130	130	230	130	130
RM55	235	235	219	219	235	230	230	230	230	139	230	230
RM440	150	146	150	150	146	146	150	202	150, 202	150, 202	150	202
RM525	146	146	146	146	146	106	146	146	146	106	106	106

[0061]

TABLE 5

Genotype codes of various basmati rice varieties based on single assay multiplex method. The order of codes from left to right correspond to loci 1 to 8 given in Table 5. Shaded part to show traditional basmati varieties.

Locus	Code	Additional alleles
Basmati370	**ACB BC BCB**	
Dehradun Basmati	**ACB BC BCB**	
Taraori Basmati	**ACB AC CAB**	
Basmati386	**ACB AC CAB**	
Ranbir Basmati	**ACB CC BCB**	
Basmati217	**BAC AC ABA**	
Haryana Gasman	DAC AA ABA	

TABLE 5-continued

Genotype codes of various basmati rice varieties based on single assay multiplex method. The order of codes from left to right correspond to loci 1 to 8 given in Table 5. Shaded part to show traditional basmati varieties.

Locus	Code	Additional alleles
Pusa Basmati	AAC AC CBA	
Super Basmati	CAC AB ABA	
Basmati385	AAB BA CBB	
Sharbati	CAA AA ABA	*C, B
IR64	CBD AD ABA	

[0062]

TABLE 6

Allele sizes in base pairs for corresponding codes of Table 4.

#	Locus	A	B	C	D
1.	RM1	73	100	106	108
2.	RM72	158	164	173	
3.	RM171	322	335	343	346
4.	RM241	128	140	144	
5.	RM202	161	164	182	186
6.	RM44	103	109	113	
7.	RM55	219	230	235	
8.	RM348	130	139		

[0063]

TABLE 7

Arrangement of 10 primers in a particular manner to facilitate single genotyping assay. It is clear from the table that loci were grouped so as to avoid overlapping allele sizes in the same fluorescence label (read as 'same coloured peaks in the electrophoresis') as shown in FIG. 7.

	Allele pool (in base pairs)	Fluorophore
Locus		
RM1	73, 100, 106, 108	FAM
RM72	158, 164, 173	FAM
RM171	322, 335, 343, 346	FAM
RM202	161, 164, 182, 186	JOE
RM241	128, 140, 144	JOE
RM44	103, 109, 113	TAMRA
RM55	219, 230, 235	TAMRA
RM348	130, 139	TAMRA
Additional Loci		
RM440	146, 150, 202	As needed
RM525	106, 146	As needed

[0064]

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aaagagagag agagagagag agagggagag agg 33

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<400> SEQUENCE: 44

aaagagtgag agagagagag agagagagag agagagagag agg 43

<210> SEQ ID NO 45
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<400> SEQUENCE: 45

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<210> SEQ ID NO 46
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<212> TYPE: DNA
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<400> SEQUENCE: 46

aaagagtgag agagagagag agagagagag agagagagag agg 43

<210> SEQ ID NO 47
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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 47

aaagagtgag agagagagag agagagagag agagagagag agg 43

<210> SEQ ID NO 48
<211> LENGTH: 43
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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 48

aaagagtgag agagagagag agagagagag agagagagag agg 43

<210> SEQ ID NO 49
<211> LENGTH: 43
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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 49

aaagagtgag agagagagag agagagagag agagagagag agg 43

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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 50

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<210> SEQ ID NO 51
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<400> SEQUENCE: 51

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<210> SEQ ID NO 52
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<400> SEQUENCE: 52

atgggatgga gagagagaga gagagagaga gagagagaga gagagccgga 50

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<400> SEQUENCE: 53

atgggatgga gagagagaga gagagagaga gagagagaga gagagccgga 50

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atgggatgga gagagagaga gagagagaga gagagagaga gagagccgga 50

<210> SEQ ID NO 55
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<400> SEQUENCE: 55

atgggatgga gagagagaga gagagagaga gagagagaga gagagccgga 50

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atgggatgga gagagagaga gagagagaga gagagagaga gagagagaga gccgga 56

<210> SEQ ID NO 57
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atgggatgga gagagagaga gagagagaga gagagagaga gagagagaga gccgga 56

<210> SEQ ID NO 58
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<400> SEQUENCE: 58

atgggatgga gagagagaga gagagagaga gagagagaga gagagagaga gccgga 56

<210> SEQ ID NO 59
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<400> SEQUENCE: 60

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<400> SEQUENCE: 61

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<400> SEQUENCE: 62

gagagagaga gagagagaga gagag 25

<210> SEQ ID NO 63
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<400> SEQUENCE: 63

gagagagaga gagagagaga gagagag 27

<210> SEQ ID NO 64
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<400> SEQUENCE: 64

gagagagaga gagagagaga gagag 25

<210> SEQ ID NO 65
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gagagagaga gagagagaga gag 23

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gagagagaga gagagagaga gagagag 27

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<400> SEQUENCE: 67

gagagagaga gagagagaga gagagagaga gagagagaga gag 43

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gagagagaga gagagagaga gagagagaga gagagagaga gagagagaga g 51

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<210> SEQ ID NO 69
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<400> SEQUENCE: 69

gagagagaga gagagagaga gagagag 27

<210> SEQ ID NO 70
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<400> SEQUENCE: 70

gagagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagag 59

<210> SEQ ID NO 71
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<400> SEQUENCE: 71

gagagagaga gagagagaga gagagagaga gagagagaga gagag 45

<210> SEQ ID NO 72
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<400> SEQUENCE: 72

gagagagaga gagagagaga gagagagaga gagagagaga gagagagag 49

<210> SEQ ID NO 73
<211> LENGTH: 49
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<400> SEQUENCE: 73

gagagagaga gagagagaga gagagagaga gagagagaga gagagagag 49

<210> SEQ ID NO 74
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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 74

acgatggatg gatggatgga tggatgggtt 30

<210> SEQ ID NO 75
<211> LENGTH: 30
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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 75

acgatggatg gatggatgga tggatgggtt 30

<210> SEQ ID NO 76
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<400> SEQUENCE: 76

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acgatggatg gatggatgga tggatgggtt 30

<210> SEQ ID NO 77
<211> LENGTH: 30
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<400> SEQUENCE: 77

acgatggatg gatggatgga tggatgggtt 30

<210> SEQ ID NO 78
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<400> SEQUENCE: 78

acgatggatg gatggatgga tggatgggtt 30

<210> SEQ ID NO 79
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<400> SEQUENCE: 79

acgatggatg gatggatgga tggatggatg gatggatt 38

<210> SEQ ID NO 80
<211> LENGTH: 38
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<400> SEQUENCE: 80

acgatggatg gatggatgga tggatggatg gatggatt 38

<210> SEQ ID NO 81
<211> LENGTH: 38
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<400> SEQUENCE: 81

acgatggatg gatggatgga tggatggatg gatggatt 38

<210> SEQ ID NO 82
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<400> SEQUENCE: 82

acgatggatg gatggatgga tggatggatg gatggatt 38

<210> SEQ ID NO 83
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<400> SEQUENCE: 83

acgatggatg gatggatgga tggatt 26

<210> SEQ ID NO 84
<211> LENGTH: 26

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<212> TYPE: DNA
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<400> SEQUENCE: 84

acgatggatg gatggatgga tggatt 26

<210> SEQ ID NO 85
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<400> SEQUENCE: 85

acgatggatg gatggatgga tggatggatg gatggatgga tt 42

<210> SEQ ID NO 86
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<400> SEQUENCE: 86

cgtattatta ttattatcat tattattatt attattatta ttattattat tattattatt 60

attattatta ttat 74

<210> SEQ ID NO 87
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<212> TYPE: DNA
<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 87

cgtattatta ttattatcat tattattatt attattatta ttattattat tattattatt 60

attattatta ttat 74

<210> SEQ ID NO 88
<211> LENGTH: 74
<212> TYPE: DNA
<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 88

cgtattatta ttattatcat tattattatt attattatta ttattattat tattattatt 60

attattatta ttat 74

<210> SEQ ID NO 89
<211> LENGTH: 74
<212> TYPE: DNA
<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 89

cgtattatta ttattatcat tattattatt attattatta ttattattat tattattatt 60
attattatta ttat 74

<210> SEQ ID NO 90
<211> LENGTH: 74
<212> TYPE: DNA
<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 90

cgtattatta ttattatcat tattattatt attattatta ttattattat tattattatt 60

attattatta ttat 74

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<210> SEQ ID NO 91
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<212> TYPE: DNA
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<400> SEQUENCE: 91

cgtattatta ttattatcat tattattatt attattatta ttattattat tattattatt 60

attattatta t 71

<210> SEQ ID NO 92
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<400> SEQUENCE: 92

cgtattatta ttattatcat tattattatt attattatta ttattattat tattattatt 60

attat 65

<210> SEQ ID NO 93
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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 93

cgtattatta ttattatcat tattattatt attattatta ttattattat tattattat 59

<210> SEQ ID NO 94
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<400> SEQUENCE: 94

cgtattatta ttattatcat tattattatt attattatta ttattattat tattattat 59

<210> SEQ ID NO 95
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<400> SEQUENCE: 95

cgtattatta ttattatcat tattattatt attattatta ttattattat tattattat 59

<210> SEQ ID NO 96
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<400> SEQUENCE: 96

cgtattatta ttattatcat tattattatt attattatta ttattattat tattattat 59

<210> SEQ ID NO 97
<211> LENGTH: 59
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<400> SEQUENCE: 97

cgtattatta ttattatcat tattattatt attattatta ttattattat tattattat 59

<210> SEQ ID NO 98

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<211> LENGTH: 61
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<400> SEQUENCE: 98

ttaagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagagg 60

a 61

<210> SEQ ID NO 99
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<400> SEQUENCE: 99

ttaagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagga 59

<210> SEQ ID NO 100
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<400> SEQUENCE: 100

ttaagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagga 59

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<400> SEQUENCE: 101

ttaagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagga 59

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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 102

ttaagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagga 59

<210> SEQ ID NO 103
<211> LENGTH: 59
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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 103

ttaagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagga 59

<210> SEQ ID NO 104
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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 104

ttaagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagga 59

<210> SEQ ID NO 105
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<400> SEQUENCE: 105

ttaagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagga 59

<210> SEQ ID NO 106
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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 106

ttaagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagga 59

<210> SEQ ID NO 107
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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 107

ttaagagaga gagagagaga gagagagaga gagagagagg a 41

<210> SEQ ID NO 108
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<400> SEQUENCE: 108

ttaagagaga gagagagaga gagagagaga gagagga 37

<210> SEQ ID NO 109
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ttaagagaga gagagagaga gagagagaga gagagga 37

<210> SEQ ID NO 110
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<400> SEQUENCE: 110

aagccagaac agcaaacaca cacagattac agcagcagca gcagcagcag cgaa 54

<210> SEQ ID NO 111
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<400> SEQUENCE: 111

aagccagaac agcaaacaca cacagattac agcagcagca gcagcagcag cgaa 54

<210> SEQ ID NO 112
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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 112

aagccagaac agcaaacaca cacagattac agcagcagca gcagcagcag cgaa 54

<210> SEQ ID NO 113

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<211> LENGTH: 54
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<400> SEQUENCE: 113

aagccagaac agcaaacaca cacagattac agcagcagca gcagcagcag cgaa 54

<210> SEQ ID NO 114
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 114

aagccagaac agcaaacaca cacagattac agcagcagca gcagcagcag cgaa 54

<210> SEQ ID NO 115
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<400> SEQUENCE: 115

aagctagaac agcaaacaca cacagattac agcagcagca gcgaa 45

<210> SEQ ID NO 116
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<400> SEQUENCE: 116

aagctagaac agcaaacaca cacagattac agcagcagca gcgaa 45

<210> SEQ ID NO 117
<211> LENGTH: 45
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<400> SEQUENCE: 117

aagctagaac agcaaacaca cacagattac agcagcagca gcgaa 45

<210> SEQ ID NO 118
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<400> SEQUENCE: 118

aagctagaac agcaaacaca cacagattac agcagcagca gcgaa 45

<210> SEQ ID NO 119
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<400> SEQUENCE: 119

aagctagaac agcaaacaca cacagattac agcagcagca gcgaa 45

<210> SEQ ID NO 120
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aagctagaac agcaaacaca cacagattac agcagcagca gcgaa 45

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<400> SEQUENCE: 121

aagctagaac agcaaacaca cacagattac agcagcagca gcgaa 45

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aagagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagaga 60

gagagagaga gagat 75

<210> SEQ ID NO 123
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aagagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagaga 60

gagagagaga t 71

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aagagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagaga 60

gagagagaga t 71

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aagagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagaga 60

gat 63

<210> SEQ ID NO 126
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<400> SEQUENCE: 126

aagagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagat 59

<210> SEQ ID NO 127
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<400> SEQUENCE: 127

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<210> SEQ ID NO 128
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<400> SEQUENCE: 128

aagagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagat 59

<210> SEQ ID NO 129
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<400> SEQUENCE: 129

aagagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagat 59

<210> SEQ ID NO 130
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<400> SEQUENCE: 130

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<210> SEQ ID NO 131
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<400> SEQUENCE: 131

aagagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagat 59

<210> SEQ ID NO 132
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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 132

aagagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagat 59

<210> SEQ ID NO 133
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<213> ORGANISM: Oryza sativa

<400> SEQUENCE: 133

aagagagaga gagagagaga gagagagaga gagagagaga gagagagaga gagagat 57

What is claimed is:

1. A single tube multiplex assay for distinguishing basmati from non-basmati rice varieties and thereby the adulteration, said assay comprising steps of:

a) running multiplex PCR with sample using one or more loci of table 2,

b) distinguishing the basmati from non-basmati rice varieties and thereby the adulteration on the basis of varietal specific multiplex allele profile.

2. A method of quantifying adulteration in basmati rice varieties, said method comprising steps of:

a) constructing a standard curve on the basis of ratio of quantity of amplified products of the alleles of adulterant and the basmati rice against the progressive proportion of adulteration, and

b) quantifying the adulteration in basmati rice variety on the basis of peak area of the alleles corresponding to basmati and that of the adulterant.

3. A kit to perform a single tube multiplex assay for distinguishing basmati from non-basmati rice varieties and thereby the adulteration, said kit comprises:

a) primers of table 3;

b) appropriate reagents for PCR; and

c) optionally, package insert to conduct the assay.

* * * * *