Rice Authentication using SSR and InDel markers

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Katherine Steele
k.a.steele@bangor.ac.uk
CAZS Natural Resources, Bangor University

Summary

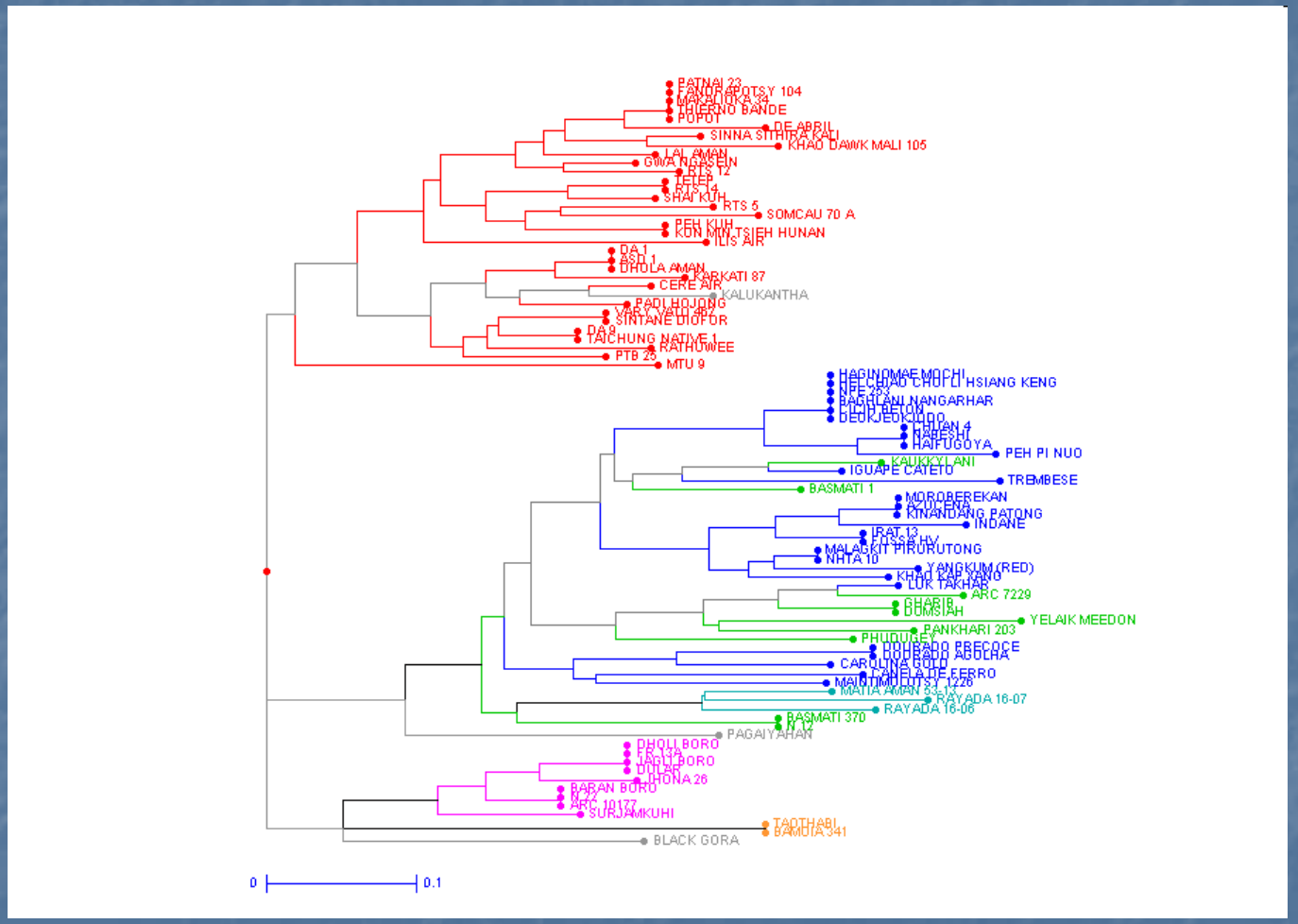
- Genetic variation in rice
- Why do we need to authenticate Basmati rice?
- Development and validation of Standard operating procedures
- Current situation

Genetic variation in rice

Ecogeographic differentiation

- Within *Oryza sativa* there are two broad sub-species:
  - Indica – adapted to tropics
  - Japonica – adapted to temperate and tropical uplands
- 'Traditional' Basmatis are morphologically most similar to Indicas
- Markers show that they are genetically distinct from either group

Isozymes B. Courtios, CIRAD, Montpellier



1 = indica

2 = aus & boro

3 = aman

4 = rayada

5 = basmati & sadri

6 = japonica

7 = intermediates

Rice genomic variation

- The complete rice genome is 388.82 Mbp
- 37,544 protein coding sequences
- 35% is repetitive (transposable elements)
  1,703,176 SNPs
- 479,406 InDels: 1 every 953 bp.
- 48,351 Simple Sequence Repeats:
  1 every 8000 bp
  - (di- ≥8 repeat units, tri- ≥5, tetra- ≥4)

Microsatellites
Simple Sequence Repeats

- Microsatellite markers are repeat sequences in non-coding DNA, di-, tri or tetra- bp repeats

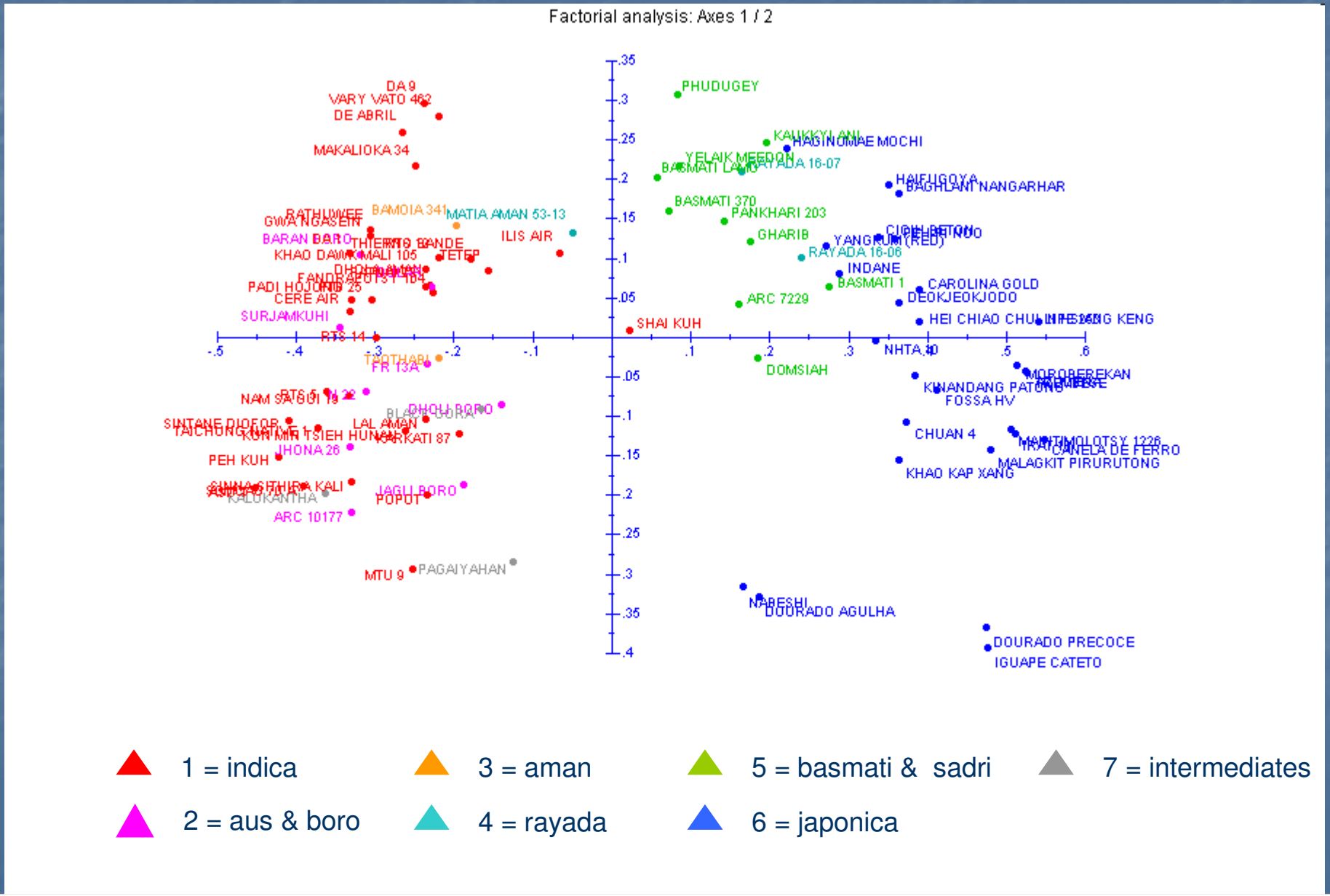
  e.g.

  ATATATATATATATATATATATATAT
  ATATATATATATATATATATAT

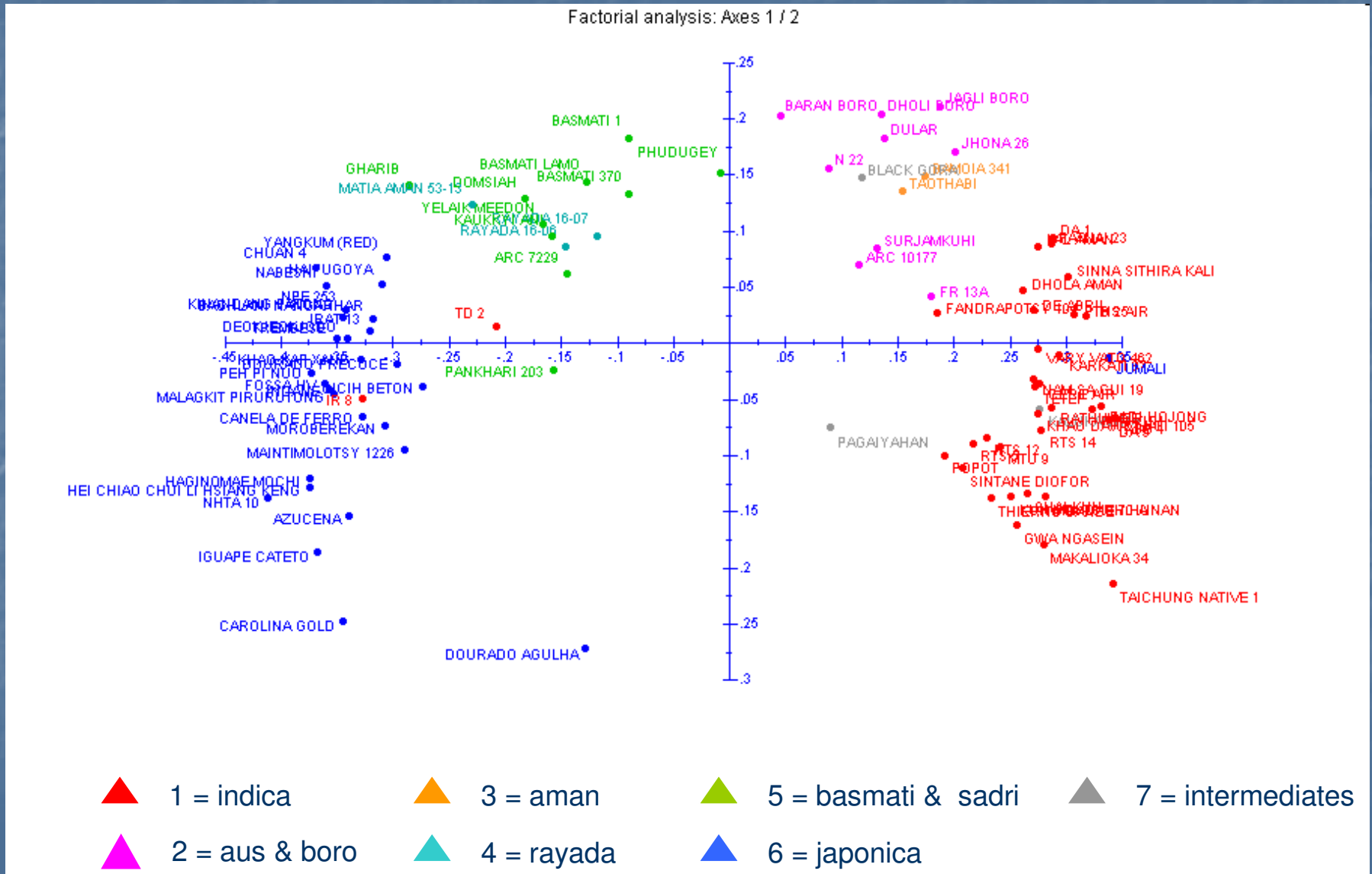
- Different varieties have different numbers of repeats

- Can distinguish them with PCR and gel analysis.

SSRs B. Courtios, CIRAD, Montpellier



DArT B. Courtios, CIRAD, Montpellier



Why do we need to authenticate Basmati rice?

Basmati Growing Area

north and north western part of Indian sub-continent

- Indo-Gangetic plains
- N. Punjab
- Haryana
- Jammu
- Uttaranchal
- Himachal Pradesh
- Rajasthan
- Uttar Pradesh

A definition?

- Aroma and grain elongation on cooking.
- 'Traditional' Basmatis are tall and low yielding compared to other varieties.
- Pusa Basmati 1 is a higher yielding semi-dwarf (from Basmati 370 crossed to IR8).

Basmati rice in the UK

- The UK market for Basmati rice is increasing
  - 120,000 – 160,000 tonnes imported annually to UK
  - 269,000 – 360,000 tonnes imported annually to EU
- Code of Practice for Rice only allows the term 'Basmati rice' to be applied to certain long grain aromatic rice varieties grown in India and Pakistan (Rice Association, 2005)
- The Code of Practice states that the maximum adulterant levels in basmati rice varieties can not exceed 7%.

List of Approved Basmatis

Basmati rice varieties eligible for a zero import duty under Regulation (EC) 1549/2004

Basmati 217 (I) *[not grown]*
Ranbir basmati (IET 11348) (I)
Basmati 370 (I, P)
Super basmati (P)
Basmati 386 (I)
Taraori basmati (HBC-19,
Karnal Local) (I)
Kernel basmati (Basmati Pakistan) (P)
Type –3 (Dehradun) (I)
Pusa basmati (IET 10364) (I) *[semi-dwarf]*

Other Basmati rice varieties approved by India and Pakistan

Basmati 198 (P) Kasturi (IET 8580) (I)
Basmati 385 (P) Mahi Suganda (I)
Haryana Basmati
(HKR 228/IET 10367) (I)
Punjab Basmati (Bauni Basmati) (I)

I - Originally approved by India
P - Originally approved by Pakistan

Non-fragrant non-approved varieties

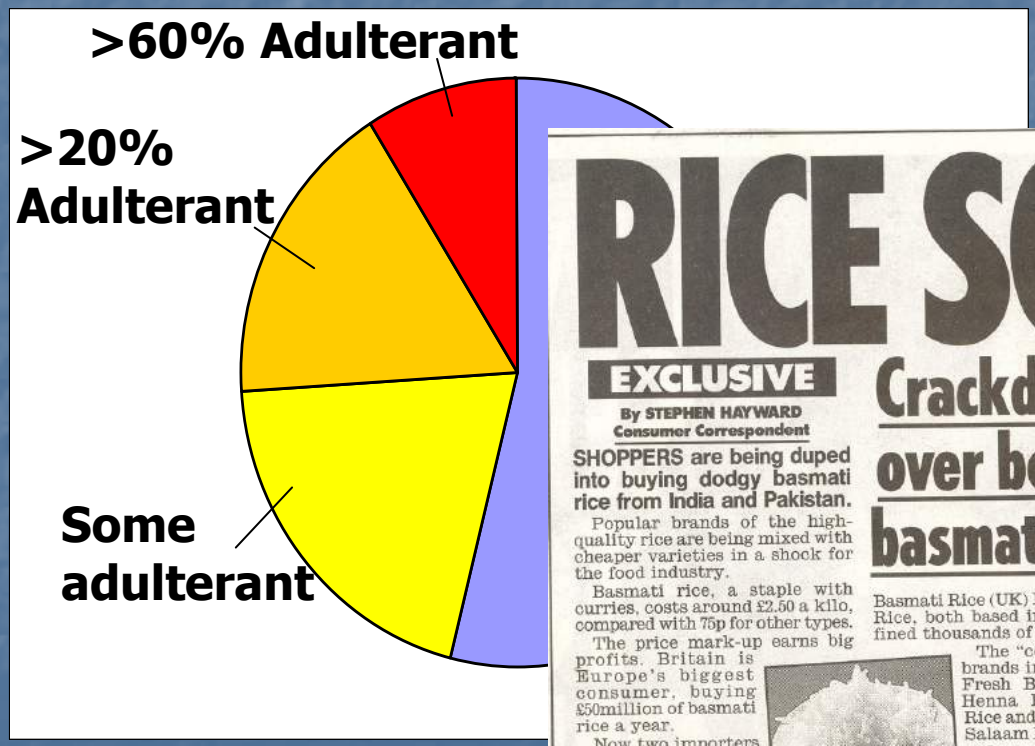
- Sherbati (India)
- Pak 386 (Pakistan)

Fragrant non-approved varieties

Basmati 2000	India	
Shaheen Basmati	Pakistan	Salt tolerant
Mugad Sugandha	India	
Superfine		
Pusa Sugandha (1, 2 & 3)	India (IARI)	
Yamini (CSR 30)	India	Semi dwarf Salt tolerant
Supra		
Pusa 1121 (Pusa sugandha 4)	India (IARI)	Semi-dwarf

2003 survey

- 382 UK commercial samples were tested
- 46 % were adulterated (detected with RM201)



RICE SQUAD

EXCLUSIVE

By STEPHEN HAYWARD
Consumer Correspondent

Crackdown over bogus basmati con

SHOPPERS are being duped into buying dodgy basmati rice from India and Pakistan.

Popular brands of the high-quality rice are being mixed with cheaper varieties in a shock for the food industry.

Basmati rice, a staple with curries, costs around £2.50 a kilo, compared with 75p for other types.

The price mark-up earns big profits. Britain is Europe's biggest consumer, buying £50million of basmati rice a year.

Now two importers have admitted supplying basmati containing "inferior" rice destined for shops and supermarkets.

Basmati Rice (UK) Ltd and Surya Rice, both based in Essex, were fined thousands of pounds.

The "contaminated" brands included Daily Fresh Basmati Rice, Henna Pak Basmati Rice and Golden Foods Salaam Basmati Rice.

One sample had 75 per cent of non-basmati grains, another 55 per cent.

Now a new code of practice means non-basmati rice must not exceed seven per cent of a batch, rather than the previous 20 per cent.

It comes after a survey by the Food Standards Agency found nearly half of 363 basmati samples from across the country showed high levels of "contamination".

Damien Tester, of the UK Rice Association, said: "There is concern that consumers are being ripped off.

"It's possible there are minor elements of the trade where the message hasn't got through."

Roger Walters, spokesman for Essex trading standards which brought the two prosecutions, said: "We want to ensure consumers get the real deal."

Basmati Rice (UK) was fined £2,700 and ordered to pay £6,376 costs by magistrates. Surya Rice was fined £2,500 and £6,294 costs.

Both companies blamed an innocent mix-up for the problem.

Real thing... basmati

s.hayward@mgn.co.uk

Development and validation of SOPs

Development of SOPs 2003 - 2007

- Variety testing with SSRs – analysis on sequencer using panel of markers
- Quantitative analysis with SSR – with analysis of RM201 on sequencer
- Variety testing with InDels – analysis on Agilent BioAnalyser using panel of markers
- Quantitative analysis (RM201 & InDel) on Agilent BioAnalyser

Visualising DNA fragments

- Gel electrophoresis is used for DNA fragment separation
- DNA is negatively charged, so it migrates toward the positive pole in an electric field
- Gels are matrices of agarose or polyacrylamide
- Fully automated gel electrophoresis systems are available

Fragment separation for variety testing

	SSR	**InDel**
Size range	2-60 bp	25-150 bp
Number of alleles	multiple	2
Agarose gel	X	✓
Agilent BioAnalyser	x	✓
DNA sequencer	✓	✓

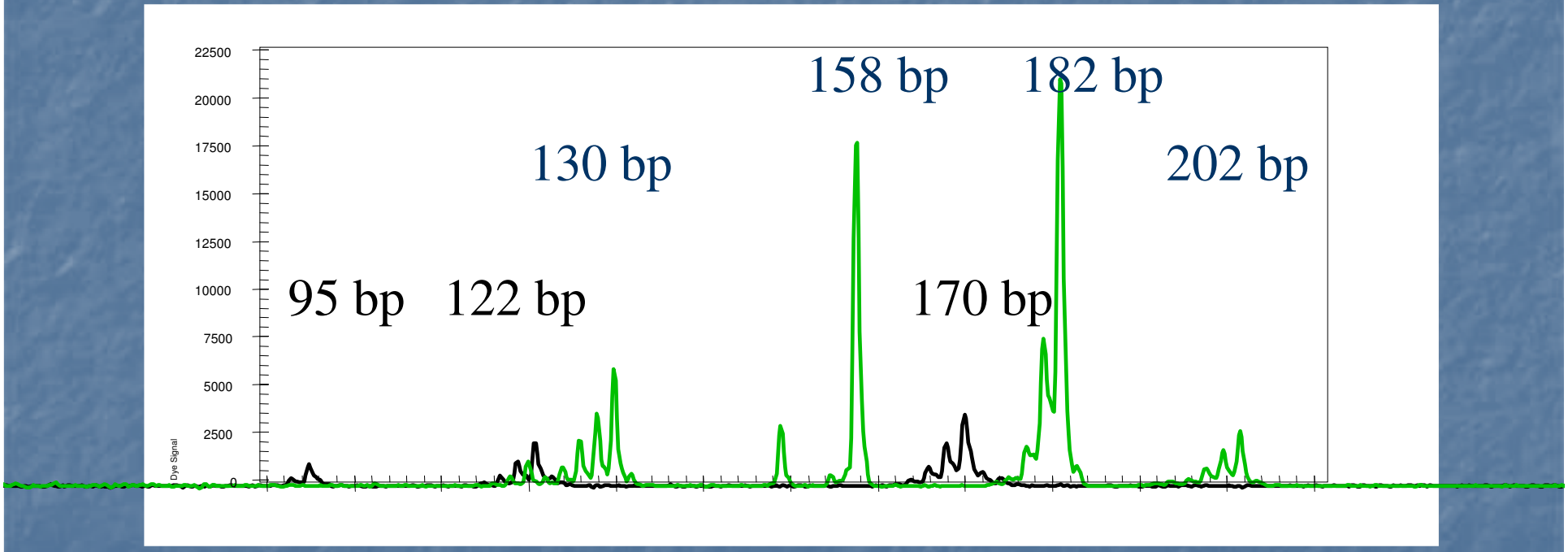
Variety testing

- Need a database of fragment sizes in known standards and known adulterants
- Test unknown sample with a set of markers and compare the result with the database
- DNA sequencers allow PCR products to be pooled for multiplex analysis
- Can identify pure varieties and mixtures

Development of SSR database

- Sources standards of known varieties, including adulterants, from India and Pakistan
- Data for 130 SSR markers
- Identify most the informative markers

Multiplex analysis with a panel of SSR markers on DNA sequencer

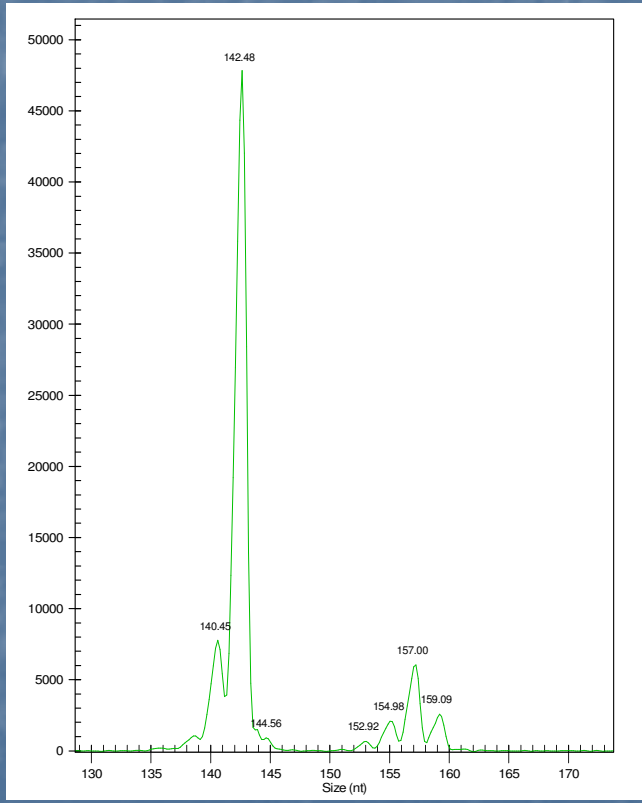


SSR database for variety test

Approved varieties			RM212	RM282	RM72	RM263	RM222		RM44	RM201	RM339	RM55	RM171
Basmati 370 group	T	I/P	116	131	177	159	209		107	143	184	234	336
Dehra Dun (Type 3)	T	I	116	131	177;162	163	209;219		107	143	184	234	336
Basmati 217	T	I	116	131	177	159	209		107	143	184	234	336
Ranbir Basmati	T	I	116	131	177	159	209		107	143	184	234	336
Taraori	T	I	116	131	177	163	211		111	143	184	219	336
Basmati 386	T	I	116	131	177	163	211		111	143	184	219	336
Kernel	T	P	116	131	177	163	211		111	143	184	219	336
Super Basmati	E	P	116	131	177	163	211		107	143	187	219	344
Pusa Basmati	E	I	116	139	162	163	209		111	143	184	229	344
Basmati 198	E	P	134	131	177	159	209		107	143	184	234	336
Basmati 385	E	P	134	131	177; 158	163	209		111	143	184	234	336
Kasturi	E	I	114	131	152	202	221		101	143	149	229	344
Haryana Basmati	E	I	134	131	162	159	219		101	143	149	229	344
Mahi Sugandha	E	I	134	137	152	159	219		101	157	149	229	344
Punjab Basmati	T	I	134	137	177	159	209;205		107	143	184	234	336
Non-approved varieties													
Basmati 2000	A	P	134	131	177	163	211		111	143	187	219	344
Shaheen Basmati	A	P	134	139	177	163	211?		111	143	184	219	336
Sherbati short	A	I	112	137	152	184	219		101	159	149	229	?
Sherbati	A	I	112	142	162	184	221		101	159	149	229	324
Sherbati awns	A	I	112	137	162	184	221		101	159	149	229	324
Mugad Sugandha	A	I	114	139	162	184	219;221		101	159	149	229	324
Pak 386	A	P	112	137	152	202	221		101	159	149	229	344
Superfine	A	P	114	139	162	184	219		111	159	149	229	344
Pusa Sugandha	A	I	114	139	162	163	211;219		101	143	162	229	344
Pusa Sugandha 2	A	I	114	139	162	163	211		101	143	162	229	344
Pusa Sugandha 3	A	I	114	139	162	163	211		101	143	162	229	344
Yamini	?	I	116	131	177	163	211		107	143	184	219	336
Supra	A	P	116	139		184	219			143	149		344
Pusa 1121	A	I	134	131		163	219		111	143	184	219	336
KDML 105	X	T	114	139		190				159	184	234	344

Qunantitative testing with SSR RM201 on sequencer

- This compares the area ratio of two peaks produced by one microsatellite marker.
- It can detect the level of 4 non-permitted varieties
  - Sherbati
  - Pak 386
  - Superfine
  - Mugad Sugandha
- Requires a standard curve



2005 – Inter-laboratory trial of the variety test

- Test used 8 microsatellite markers (2 pools)
- 11 laboratories
- Different DNA sequencers were used
- Good agreement where sample was predominantly one variety
- Recommended use of internal reference

2005 – Quantitative ring trial

- 11 laboratories
- Labs received:
  - standard mixes for standard curves
  - blind admixtures
- The mean results for admixtures were within 0.7%
- Expanded measurement uncertainty 6%
- Method is fit for purpose

2005 Ring trial participants

- CAZS-NR, Bangor UK
- CCFRA, Chipping Campden, UK
- CCL, Switzerland
- Centre for DNA Fingerprinting and Diagnostics, India
- CSL, York, UK
- Dutch Customs, NL
- EC Joint Research Centre, IRRM, Belgium
- Eurofins Ltd, Germany
- LGC, Teddington, UK
- NIAB, Cambridge, UK
- RSSL, Reading, UK
- United Riceland, India

2006 - 2007 – "Lab-on-a-Chip" 2100 Bioanalyzer Agilent Technolgies

- FSA recommended the Agilent Bioanalyser as a more user-friendly system for public analyst labs.

- Two novel marker systems were evaluated for analysis Basmati rice without a DNA sequencer.

Available online at www.sciencedirect.com
ScienceDirect
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Field Crops Research
www.elsevier.com/locate/fcr

InDel markers distinguish Basmatis from other fragrant rice varieties

Katherine A. Steele [a,b,*], Rob Ogden [a], Ross McEwing [a], Helen Briggs [a], John Gorham [a,b]

[a] *Food DNA Services, Bangor, Gwynedd LL57 2UW, UK*
[b] *CAZS Natural Resources, University of Wales, Bangor, Gwynedd LL57 2UW, UK*

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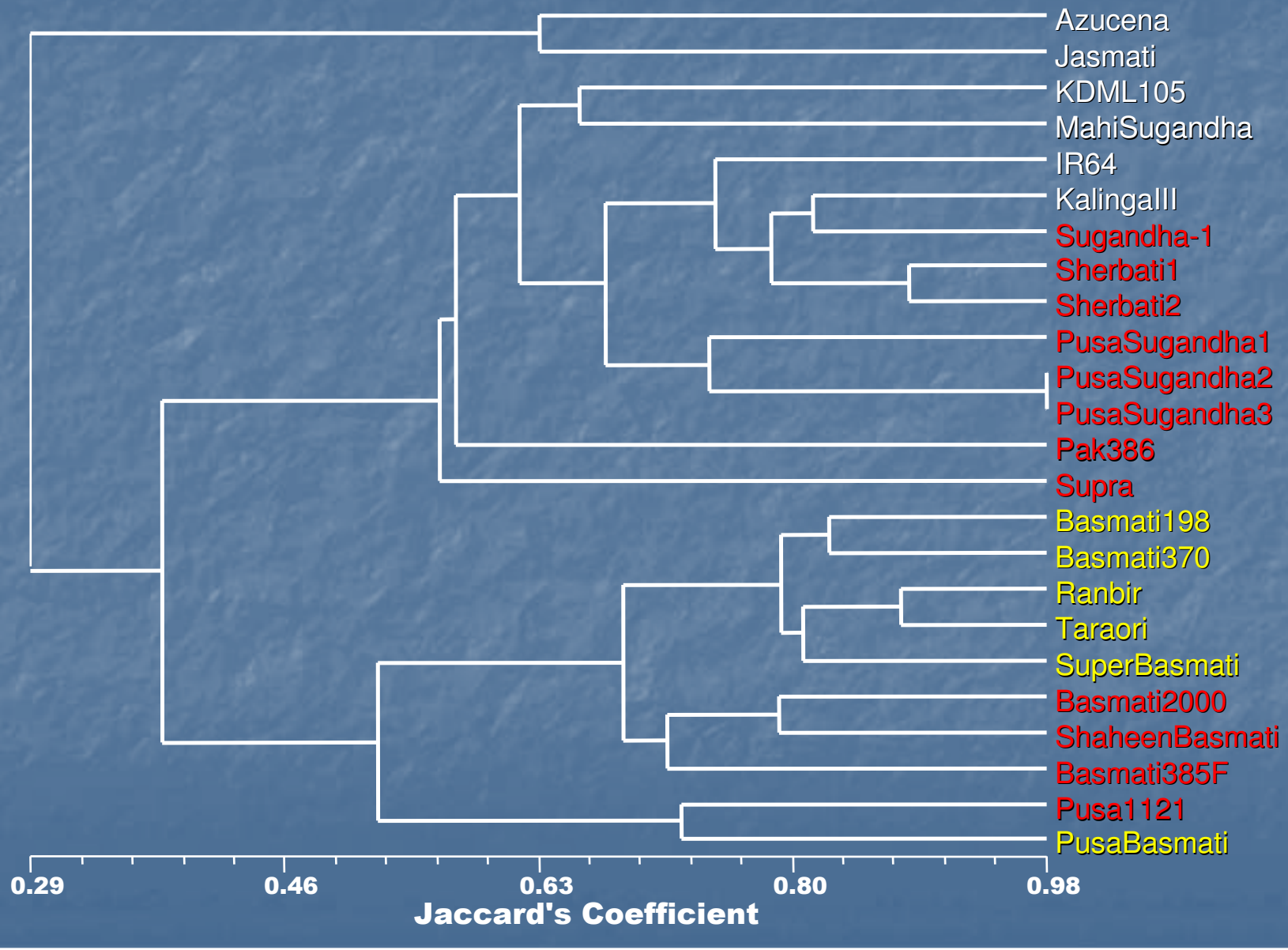
Abstract

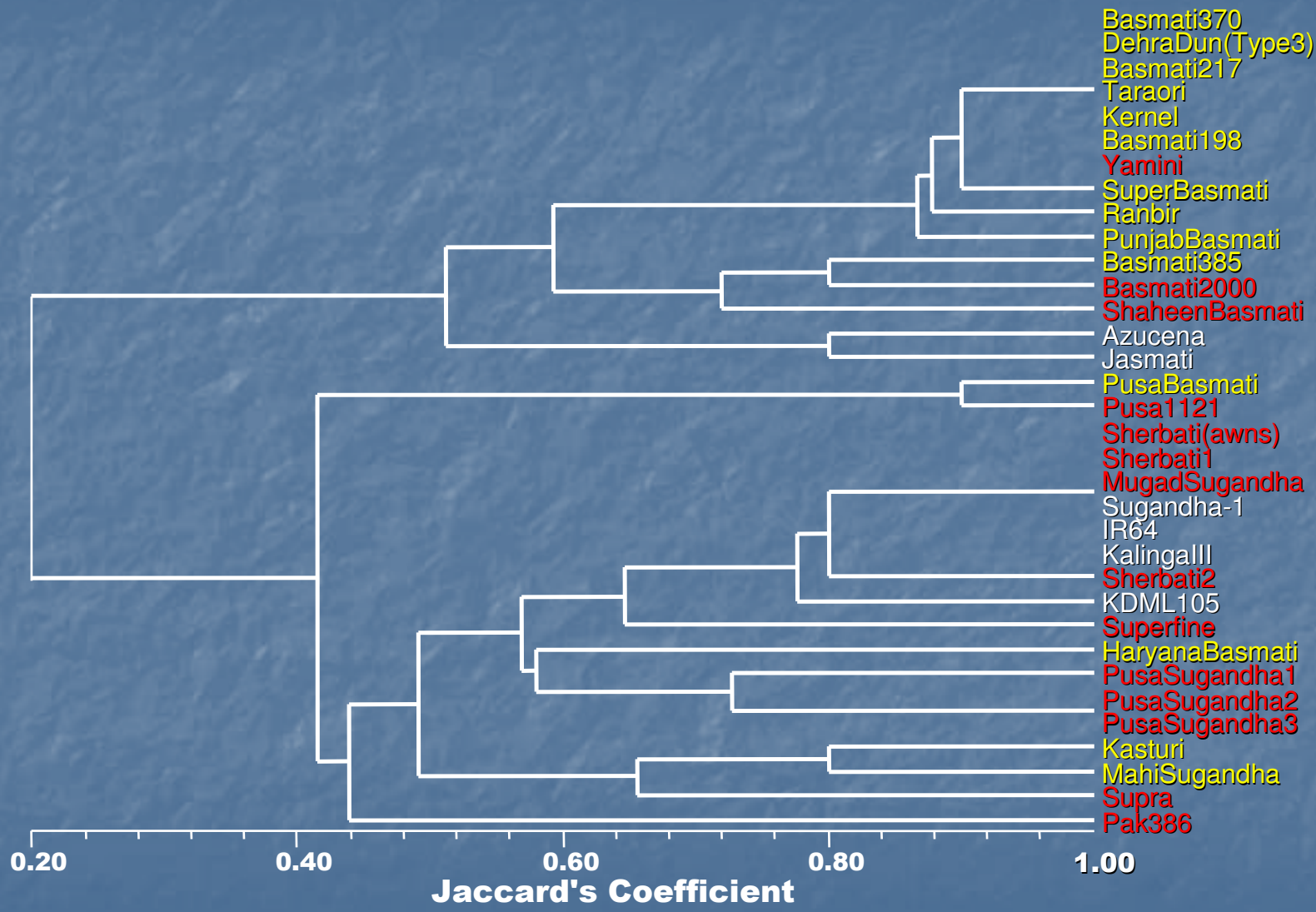
Two alternative PCR-based marker systems were tested for polymorphism in a set of fragrant and non-fragrant rice (*Oryza sativa* L.) varieties. Markers based on InDels (sequences with an insertion/deletion) gave reliable polymorphisms that split Basmatis and other varieties into different groups, however, markers based on *Rim2/Hipa* transposons were less reliable or informative. Of 42 InDels tested, 71% showed polymorphisms between Basmati varieties and non-Basmati *indicas*. A sub-set of nine InDel markers was selected as a reliable test to distinguish between Basmatis and other fragrant rice varieties using high-resolution agarose gels. Both marker systems tested are suitable for rice breeding and genetic analysis in laboratories where polyacrylamide gels or sequencers are not available.

Keywords: Fragrant rice; Marker; Polymorphism; Transposon

41 Indels


Azucena
Jasmati
KDML105
MahiSugandha
IR64
KalingaIII
Sugandha-1
Sherbati1
Sherbati2
PusaSugandha1
PusaSugandha2
PusaSugandha3
Pak386
Supra
Basmati198
Basmati370
Ranbir
Taraori
SuperBasmati
Basmati2000
ShaheenBasmati
Basmati385F
Pusa1121
PusaBasmati
0.29
0.46
0.63
0.80
0.98
Jaccard's Coefficient

Sub-set of 9 Indels



Agilent SOPs for PA labs

- Variety test (4 + 2 INDELs) and 2 alternative quantitative tests were developed.
- Public Analysts were trained in variety and quantitative tests in 2007
- SOPs for variety test and RM201 quantitative test were validated by 10 PA laboratories in 2008

SOP 1 Detection of Non-Permitted Rice varieties by INDEL-PCR Analysis using the Agilent 2100 BioAnalyser

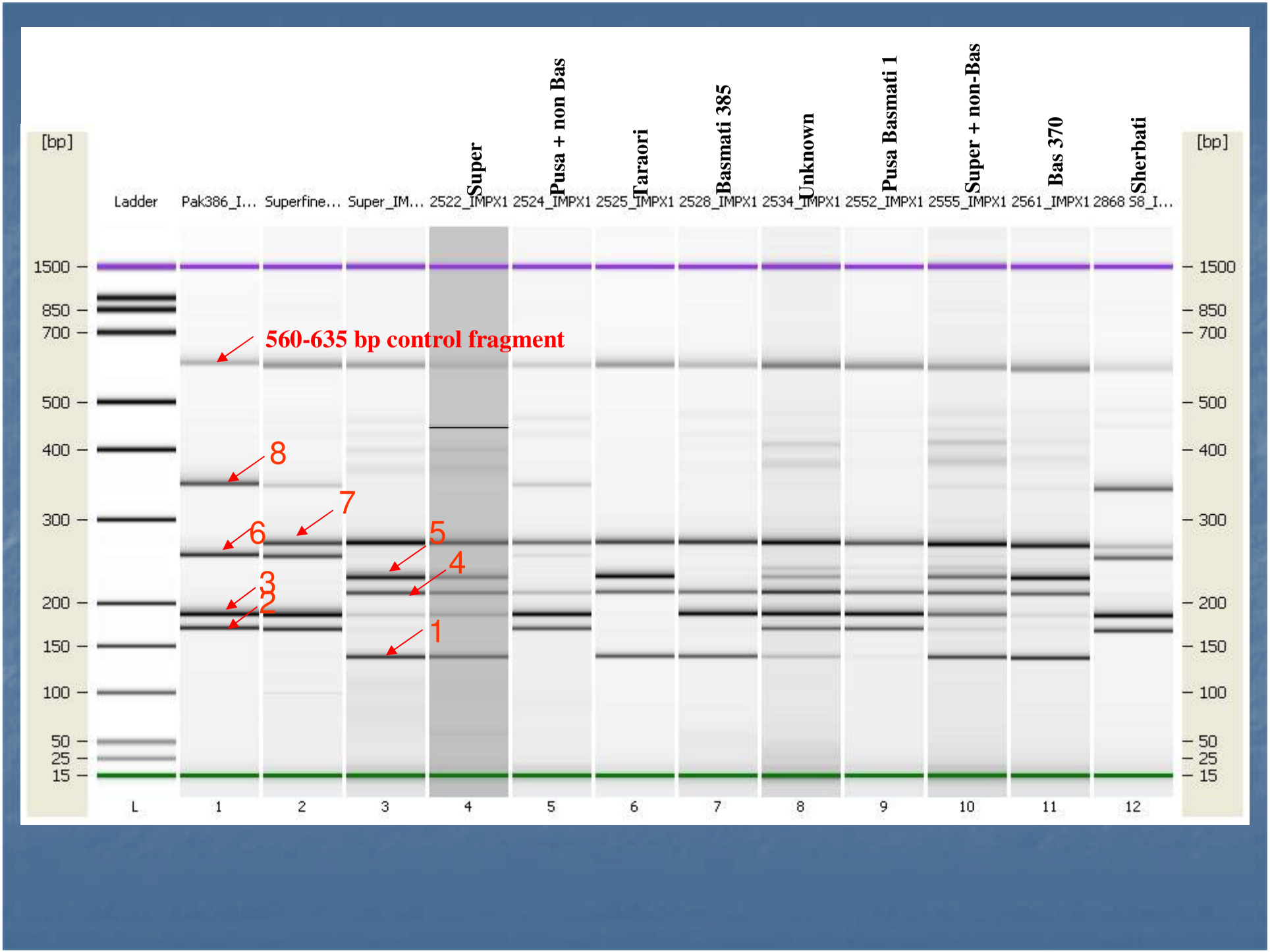
- A single Multiplex PCR with 10 primers using Qiagen Multiplex PCR master mix
- The reaction gives 9 possible fragments, including a control present in all rice
- Two optional follow-on PCRs can be done if necessary, to identify Pusa 1121, Basmati 2000 and Shaheen Basmati.
- Outcome: determines whether sample of rice grains is one pure permitted variety or a mixture of permitted and non-permitted varieties

Multiplex INDEL test for Agilent

Scoring

9	596 (550-600)	control (present in all)
8	354 (330-370)	present in non-permitted varieties
7	273 (270-280)	present in all approved Basmatis
6	256 (252-260)	*
5	230 (224-235)	
4	214 (208-218)	present in all approved Basmatis
3	186 (181-187)	*
2	174 (170-175)	*
1	142 (134-144)	

* if these three bands are all present, the sample contains a non-permitted variety


[bp]
Ladder
Pak386_I...
Superfine...
Super_IM...
2522_IMPX1
Super
2524_IMPX1
Pusa + non Bas
2525_IMPX1
Taraori
2528_IMPX1
Basmati 385
2534_IMPX1
Unknown
2552_IMPX1
Pusa Basmati 1
2555_IMPX1
Super + non-Bas
2561_IMPX1
Bas 370
2868 S8_I...
Sherbati
[bp]
1500
850
700
500
400
300
200
150
100
50
25
15
560-635 bp control fragment
8
7
6
5
4
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2
1
L
1
2
3
4
5
6
7
8
9
10
11
12

Possible test outcomes

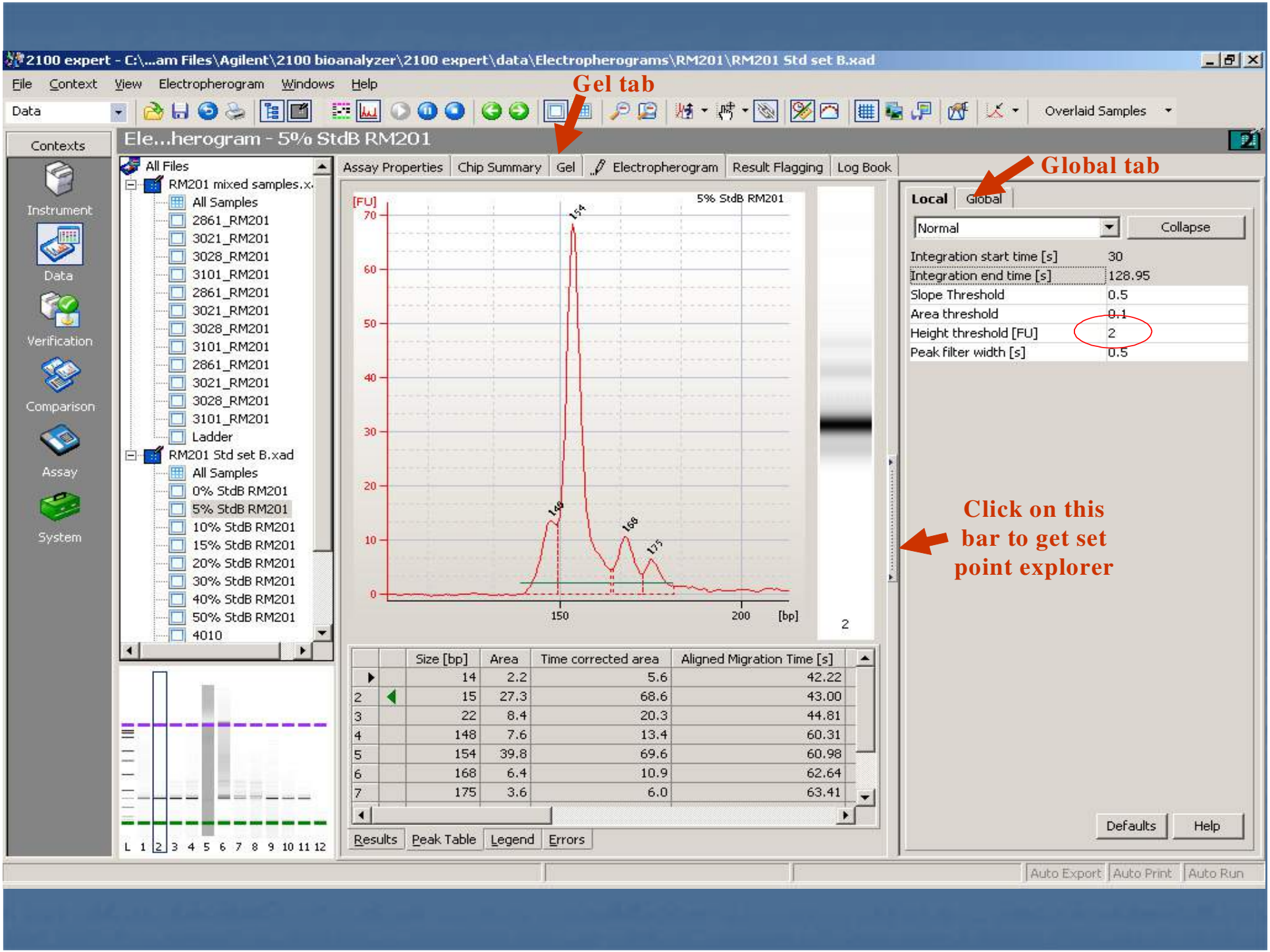
Outcome	Action or Result
Bands present in blanks	Repeat with fresh reagents
B9 not visible	Repeat PCR with increased sample DNA conc.
B8 band visible	Non-permitted adulterant variety present
B2, B3 and B6 all visible	Non-permitted adulterant variety present
Profile matches Group 1	Approved Basmatis: Basmati 370, Basmati 198, Type 3, Basmati 217, Ranbir, Kernal, Super Non-permitted Yamini
Profile matches Group 2	Follow on test with I5 to separate Pusa 1121 from Pusa Basmati
Profile matches Group 3	Follow on test with I10 to separate Basmati 2000 and Shaheen Basmati from Basmati 385
Profile matches Group 4, 5 or 6	Most likely to be an approved Basmati: Basmati 385; Kasturi Haryana Basmati or Mahi Sugandha

Summary of Ring Trial results from 10 PA labs

Sample	Variety	Expected result	Number of labs who correctly identified sample
1	Pusa Basmati	Group 2 Pusa Basmati	9
2	Super	Group 1	6
3	7% Pak 386 in Taraori	Group 1 + adulterant	8
4	25% Pak 386 in Taraori	Group 1 + adulterant	10
5	Unknown	Group 1	6
6	Pusa 1121	Pusa 1121	9

SOP 2 Quantitative Analysis of Adulteration with the varieties Sherbati, Mugad Sugandha, Pak 386 or Superfine using the Agilent 2100 BioAnalyser

- Adapted SSR RM201 marker for use on BioAnalyser
- Detects the level of four non-permitted varieties present in a sample
- The relative proportion of the adulterant can be estimated by comparing the areas under the peaks for the permitted and non-permitted variety fragments


2100 expert - C:\...am Files\Agilent\2100 bioanalyzer\2100 expert\data\Electropherograms\RM201\RM201 Std set B.xad
File Context View Electropherogram Windows Help
Gel tab
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Overlaid Samples
Contexts
Instrument
Data
Verification
Comparison
Assay
System
Ele...herogram - 5% StdB RM201
All Files
RM201 mixed samples.x
All Samples
2861_RM201
3021_RM201
3028_RM201
3101_RM201
2861_RM201
3021_RM201
3028_RM201
3101_RM201
2861_RM201
3021_RM201
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50% StdB RM201
4010
L 1 2 3 4 5 6 7 8 9 10 11 12
Assay Properties
Chip Summary
Gel
Electropherogram
Result Flagging
Log Book
[FU]
5% StdB RM201
70
60
50
40
30
20
10
0
154
148
168
175
150
200
[bp]
2
Size [bp] Area Time corrected area Aligned Migration Time [s]
14 2.2 5.6 42.22
2 15 27.3 68.6 43.00
3 22 8.4 20.3 44.81
4 148 7.6 13.4 60.31
5 154 39.8 69.6 60.98
6 168 6.4 10.9 62.64
7 175 3.6 6.0 63.41
Results
Peak Table
Legend
Errors
Global tab
Local
Global
Normal
Collapse
Integration start time [s] 30
Integration end time [s] 128.95
Slope Threshold 0.5
Area threshold 0.1
Height threshold [FU] 2
Peak filter width [s] 0.5
Click on this bar to get set point explorer
Defaults
Help
Auto Export
Auto Print
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Ring Trial result from PA labs

Quantitation with RM201 on Bioanalyser

			7%	25%		
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Lab 1	**8.25**		8.57	24.03	5.84	
Lab 2			8.46	19.41		
Lab 3			9.32	20.83		
Lab 4	**9.73**	4.03	7.19	19.13	5.28	3.65
Lab 5	**8.2**	**7.48**	7.35	22.91	5.07	
Lab 6			10.7	21.69	**9.63**	
Lab 7			4.38	14.53		
Lab 8			8.2	35.36		
Mean	8.73	5.76	8.02	22.24	6.46	3.65
SD	*0.71*	*1.73*	*1.72*	*5.65*	*1.85*	*0.00*
Sequencer	4.13	0.31	9.5	28.73	3.28	0.07

2 labs did not return quantitative results

SOP 3 Quantitative Analysis of Adulteration of Basmati Rice with the varieties Sherbati, or Pak 386 using the Agilent 2100 BioAnalyser and a PCR Marker based on an INDEL

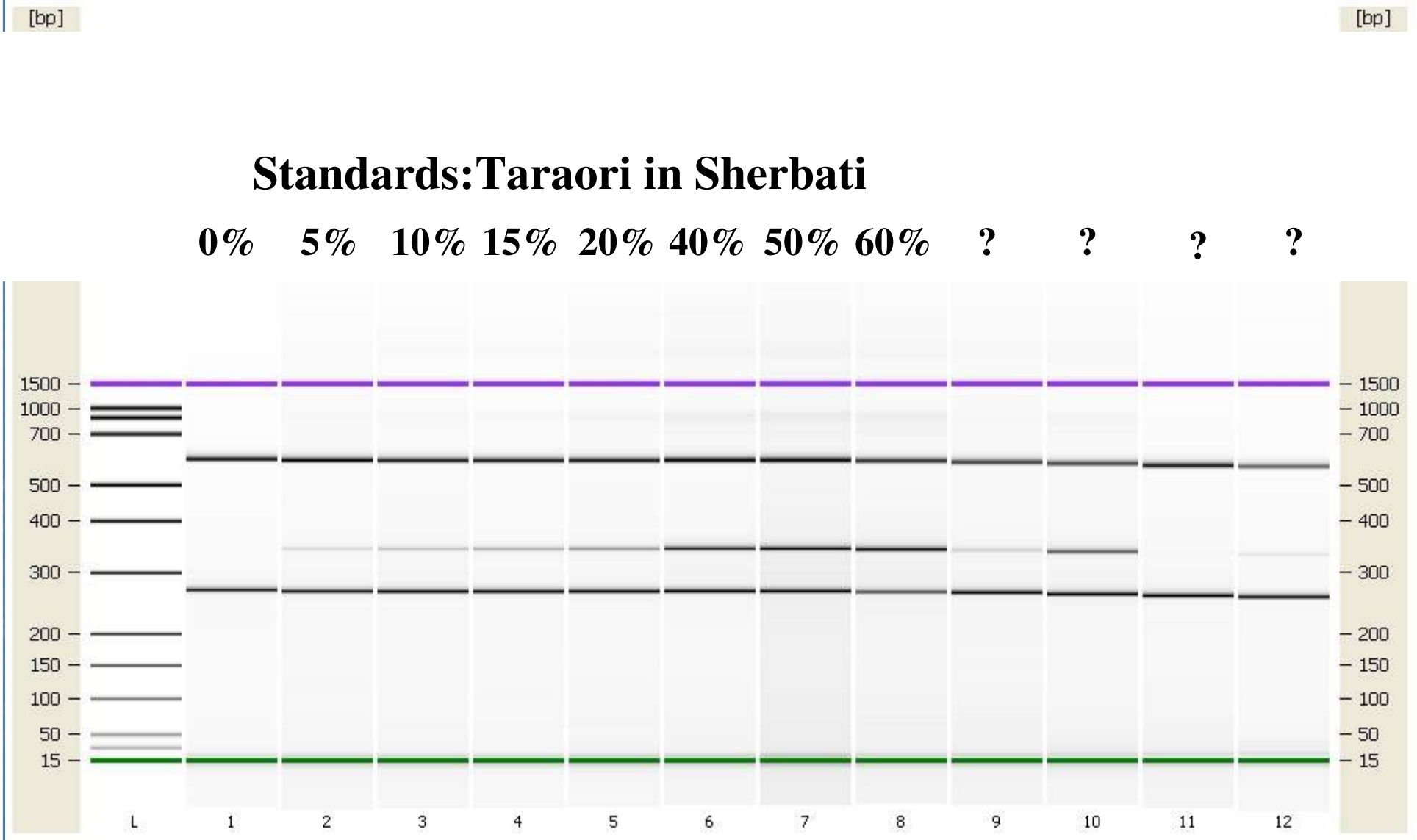
- Adapted *frg* gene marker for use on BioAnalyser

  This test only quantifies the adulteration with non-aromatic adulterant varieties Sherbati and Pak 386

- A control fragment present in rice

- The relative proportion of the adulterant can be estimated by comparing the areas under the peaks for the permitted and non-permitted variety fragments

INDEL Quantitative Test



Comparison and limitations of SOPs

Variety Tests

- For both SSRs it was difficult to compare fragments sizes between labs
  - An internal reference DNA fragment may help
- The SSR method is more informative than the 4+2 InDels
- Neither test can distinguish Yamini

Quantitative tests

- Limited set of adulterant varieties detected
- InDel detects fewer varieties than RM201
- Sequencer analysis of area under curve is fit for purpose.
- Agilent SOPs are not recommended for enforcement purposes.

- Major limitation of all SOPs: unknown new adulterants.

Current situation

Survey work since 2006

- 2006 approximately 16% samples adulterated (RA survey)
- 2007 < 10% samples adulterated (RA survey)

Levels of adulteration have decreased since 2003… but the problem still exists and adulteration could be on the increase again….

- 2009 Public analyst says there are more un-identifiable varieties/mixtures in recent batches.

Is this due to new varieties that are not in the database?

Potential new adulterants

- From India
  - Improved Pusa Basmati 1 (IET 18990) MAS
  - Bhogavari
  - Geetanjali
  - Malviya Basmati -1
  - Others?
- From Pakistan
  - ?
- Other countries
  - Sugandha 1 (Nepal)
  - ?

Standard samples have not yet been obtained

Yamini

- Derived from Pakistan Basmati x Bura Rata
- Salt tolerant
- 50 extra SSRs linked to salt tolerance traits were tested in 2007
- Still not yet distinguishable from approved Basmatis with available protocols

Recommendations

- Continued public surveillance
- Routine sourcing of standards (work with India and Pakistan)
- Add many more loci to database (>200)
  - Target InDels and SNPs located near key genes (e.g. grain elongation, salt tolerance)
- Develop Invader Assays for quantitation without PCR (detect using Mass Spectrometer)
- Analysis of complex mixtures with next generation sequencing.

Thanks

- Mark Woolfe (formerly FSA)
- Jacqui Coutts (Gen-Probe)
- Sophie Rollinson (FSA)
- Rachel New (Hampshire Scientific Services)
- John Gorham, Rob Ogden, Ross McEwing
- Helen Briggs, Liz Heap, Gwen Edwards