

InDel markers distinguish Basmati from other fragrant rice varieties

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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 Basmati 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 Basmati 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.

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1. Introduction

Basmati rice is cultivated in India and Pakistan and many other aromatic varieties are produced in other rice growing countries. The world market price of aromatic rices is rising and that of Basmati rose by up to 50% between January 2006 and 2007 (Rice Association, 2007). Most Basmati rice varieties are tall and low yielding although higher yielding aromatic varieties, including semi-dwarfs (e.g., Pusa Basmati 1), have been bred since 1960s. Many modern varieties share the characteristic traits of Basmati, including fragrance and grain elongation on cooking (Bhattacharjee et al., 2002). Traditional Basmati varieties are neither *indicas* nor *japonicas*, but cluster as a genetically distinct group when tested with isozymes (Glaszmann, 1987) and microsatellites (Garris et al., 2005). Marker systems are used to classify rice germplasm collections, however, more and more modern cultivars are being bred that do not fit into these traditional groups because they are derived from crosses between varieties from different groups. The UK Food Standards Agency has listed varieties that can be labelled in the EU as Basmati or approved for sale as Basmati. Any other

fragrant rices that have the words “Basmati” or “Sughadha” (both mean fragrant) in their name are not approved for sale as Basmati in the EU.

Microsatellite (SSR, simple sequence repeat or SSLP, short sequence repeat polymorphisms) markers are currently used to test for varietal differences between modern varieties of aromatic and non-aromatic rice (Bligh, 2000; FSA, 2004; Jain et al., 2004). Bradbury et al. (2005) have developed a PCR test based on the sequence surrounding a small 8 bp InDel and two single nucleotide polymorphisms that occur within the major gene determining fragrance, *fgr* (*BAD II*) on chromosome 8. This test is ideal to distinguish between fragrant and non-fragrant varieties, but it cannot be used alone to distinguish between different fragrant varieties. Other PCR marker systems have been used to differentiate between fragrant rice genotypes, including RAPD (Ray Choudhury et al., 2001), AFLP (Aggarwal et al., 2002) and inter-SSR-PCR (Nagaraju et al., 2002). Saini et al. (2004) compared three marker systems on the same set of genotypes and found different grouping patterns with different marker types. For accurate variety testing microsatellites require fragment separation on polyacrylamide gels, often with fluorescently labelled primers on DNA sequencers. Most PCR-based marker methods currently in use cannot distinguish between different fragrant rice varieties using the cheaper agarose gel systems.

A genome-wide rice polymorphism database developed by Shen et al. (2004) contains >400,000 insertion/deletion

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polymorphisms (InDels). Fifty of these were converted to PCR markers by Shen et al. (2004) that could distinguish between the *indica* variety 93-11 and the *japonica* variety Nipponbare. They are co-dominant markers that give two possible alleles according to presence or absence of the insertion sequence, situated between the primers. They give PCR products of 120–250 bp with fragment size differences in the range 25–50 bp that are suitable for detection with ‘low-tech’ agarose gels.

Almost 35% of the rice genome is transposon sequences of which approximately 3% are Class II CACTA-type elements (Nature 436: 793–800). *RIM2/hipa* elements fall into this category and are unique to the *Oryza* genome. They are approximately 5.8 kb, highly heritable and have a high level of variation within their internal sequences (Wang et al., 2003). Transposon display methods based on the internal variable regions of *RIM2/hipa* transposons have been developed that

reveal polymorphism on polyacrylamide gels (Kwon et al., 2005) and agarose gels (Tian et al., 2006).

We tested whether the *RIM2/hipa* primers of Tian et al. (2006) and the InDel primers of Shen et al. (2004) could be used to reveal polymorphism between rice varieties that could be resolved in agarose gels with a ‘low-tech’ protocol.

2. Materials and methods

2.1. DNA extraction from rice varieties

DNA was extracted from de-husked rice grains of 24 authenticated rice varieties, including approved Basmati, and non-approved fragrant and non-fragrant varieties, from the collection held at CAZS-NR (Table 1). Bulk grain samples were ground to a fine powder using a coffee grinder. Nucleon

Table 1
Authenticated rice samples sourced by the FSA or CAZS-NR, indicating varieties approved in the EU for labelling as Basmati (Rice Association, 2005)

	Origin	Noteworthy parents	Other details
EU listed Basmati varieties			
Basmati 370	India	Selection from landrace	
Dehra Dun (Type 3)	India	Selection from landrace	
Basmati 217 (IRGC 53637)	India		
Ranbir	India	Basmati 370	Early Basmati Anantnag
Taraori (HBC 19, Karnal local)	India	Selection from landrace	
Kernel	Pakistan	Selection from landrace	
Pusa Basmati 1 (IET 10364)	India (IARI)	Ratna, IR8 and Basmati 370 ^a	Semi-dwarf, high-yielding
Super Basmati	Pakistan (RRI-KSK)	Basmati 320/IR661	Shabnam in India
Other approved Basmati varieties			
Basmati 198	Pakistan (RRI-KSK)	T(N)1/Basmati 370	
Basmati 385	Pakistan (RRI-KSK)	T(N)1/Basmati 370	
Kasturi (IET 8580)	India (DRR, Hyderabad)	Basmati 370/CR88-17-1-5	High-yielding
Haryana Basmati (IET 10367)	India (RRS, Kaul)	Sona/Basmati 370	Dwarf
Mahi Sugandha	India	BK79/Basmati 370	
Punjab Basmati (Bauni Basmati)	India (RRS Kapurthala)	Sona/Basmati 370	
Non-approved fragrant varieties			
Basmati 2000	India		
Shaheen Basmati	Pakistan		Salt tolerant
Mugad Sugandha	India		
Superfine			
Pusa Sugandha 1	India (IARI)		Potential adulterant
Pusa Sugandha 2	India (IARI)		Potential adulterant
Pusa Sugandha 3	India (IARI)		Potential adulterant
Yamini (CSR 30)	India	Possibly Pusa Basmati 1	Semi dwarf, salt tolerant
Supra			
Pusa 1121 (Pusa Sugandha 4)	India (IARI)	Sister lines of Pusa Basmati 1	Semi-dwarf
Sugandha-1	Nepal (LI-BIRD/CAZS-NR/NARC)	Pusa Basmati 1	
Azucena	Phillippines	Selection from landrace	Upland tropical <i>japonica</i>
KDML 105	Thailand	Khao Dawk Mali (landrace)	
Jasmati	RiceTec (USA)	(see discussion)	
Non-fragrant varieties			
Sherbati 1	India		Potential adulterant
Sherbati 2	India		Potential adulterant
Sherbati (awns)	India		Potential adulterant
Pak 386	Pakistan		Potential adulterant
IR64 ^b	Phillippines (IRRI)	Multiple lines	High-yielding semi-dwarf
Kalinga III (CR 237-1)	India	AC 540 and Ratna	Upland rice, early

DDR, Directorate of Rice Research; IARI, Indian Agricultural Research Institute; IRRI, International Rice Research Institute, RRI-KSK, Rice Research Institute, Kala Shaha Kaku; RRS, Rice Research Station.

^a See p. 87 in Singh et al. (2000) for full ancestry of Pusa Basmati 1.

^b See Hargrove et al. (1988).

Phytopure Genomic DNA extraction kits (Tepnel Life Sciences Plc, Manchester, UK) were used to extract DNA. 1 g of the fine rice powder was mixed with 4.0 ml of Nucleon Phytopure Reagent 1, incubated at 65 °C for 10 min, then chilled on ice. The clear surface liquid (1.8 ml) was removed for centrifugation at $13,000 \times g$ for 15 min. The resulting supernatant (0.5 ml) was chilled on ice before 0.5 ml of ice-cold chloroform and 0.1 ml Nucleon Phytopure DNA extraction resin were mixed with it at room temperature. After centrifugation ($16,000 \times g$ for 15 min) the upper phase was recovered and mixed with 0.5 ml ice-cold propan-2-ol to precipitate the DNA. The DNA was pelleted by centrifugation and air-dried before re-suspending in 0.5 ml TE. The DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer.

2.2. Transposon display *RIM2/hipa* markers

Four primer pairs (A–D given by Tian et al., 2006) based on the *RIM2/hipa* coding/pseudogene region were synthesised by MWG-biotech (www.mwg-biotech.com). PCR was conducted in a PTC-100 thermocycler (MJ Research) with 30 cycles of: 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min; followed by 72 °C for 10 min. PCR products were separated on 1.2% agarose gels with ethidium bromide staining. Twenty-five varieties were tested and all PCRs were repeated three times to check reproducibility.

2.3. InDel PCR and fragment analysis

Primer pairs for 42 InDels were synthesised by MWG-biotech using the sequences given by Shen et al. (2004). PCR was carried out in 10 μ l volume containing ThermoStart PCR mastermix at 1.5 mM MgCl₂ (Abgene). The PCR profile was:

95 °C for 15 min followed by 40 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 5 min. Fragments were analysed on 3% Amresco high-resolution (super-fine resolution, SFR) agarose (NBS Biologicals Ltd., Huntindon, Cambs., UK) for 3–4 h at 90 V and visualised under UV with ethidium bromide staining. A 100 bp ladder was used for sizing. Twenty-four varieties were tested with 41 InDel primer pairs, and 10 additional varieties were tested with the nine most discriminative InDel primer pairs (Table 1).

2.4. Data analysis

Binary data (presence or absence of fragments) were recorded as data matrices. Jaccard's similarity coefficient for each pair of varieties was found from the binary data matrix. Cluster analysis with UPGMA (unweighted pair group method with arithmetical means) clustering was carried out using NTSYS-pc (Exeter Software).

3. Results

The *Rim2/Hipa* primer sets gave multiple polymorphic alleles, with up to 15 fragments amplified. The A primer-set were the most reliable while the B primer set revealed the most fragments. Of 41 possible fragments detected with five primer pairs, approximately 50% were repeatable across runs. A dendrogram was constructed based on Jaccard's similarity of 21 consistently repeatable *Rim2/Hipa* fragments (Fig. 1). Unique profiles were obtained for 16 varieties, but the Basmati varieties could not be readily separated from non-Basmatis.

The InDel markers were highly repeatable with most revealing one of two possible alleles in each variety (Table 2).

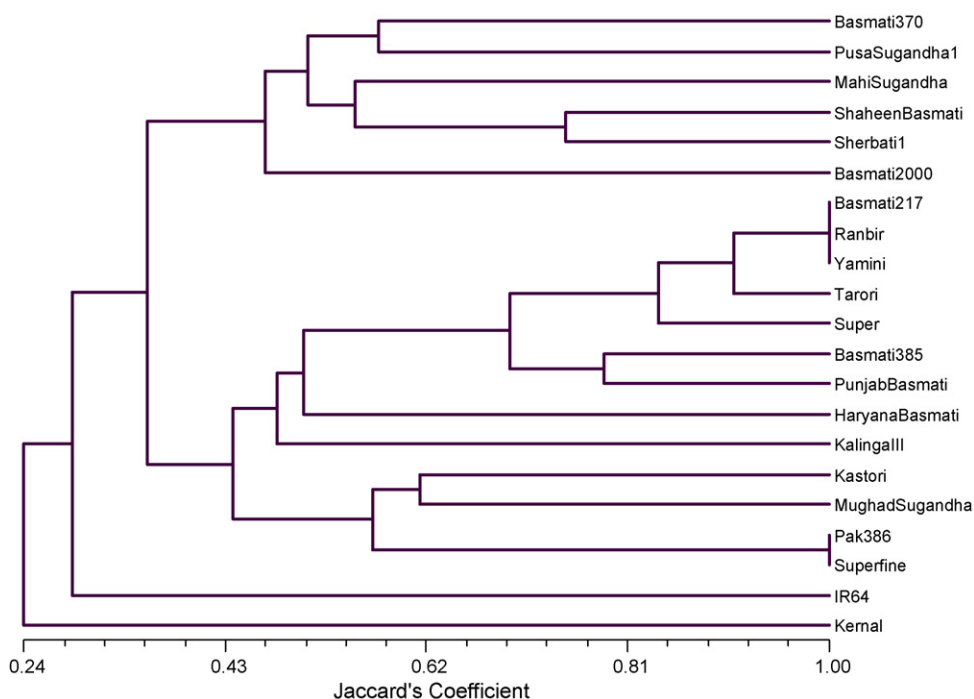


Fig. 1. Dendrogram showing groupings (UPGMA) of 21 rice varieties with 21 fragments amplified by *Rim2/Hipa* markers.

One marker (R1M20) amplified nine possible bands that were not repeatable so it was dropped. R8M23 was monomorphic for this set of varieties. R3M37 gave two repeatable alleles: a triplex fragment around 250–300 bp (that sometimes resembled a smear) and a duplex band of approximately 186 and 166 bp, the upper fragment being brightest. R9M30 gave three different fragments, the middle band being present in all Basmatitis and modern *indica* fragrant varieties except Pusa Sugandha and Mahi Sugandha. Nine markers (R1M7, R1M37, R2M10, R5M43, R6M30, R7M30, R9M42, R12M10, R12M43) could separate Azucena and Jasmati from the others, but they were monomorphic for all *indica* and Basmati varieties tested with a low polymorphism information contents (PIC) of 0.16. Cluster analysis of all alleles generated with 41 InDel markers revealed that Azucena and Jasmati were more similar to each other than

they were to all other varieties (Fig. 2A). At the second node two groups were separated: one group contained all the non-aromatic varieties studied plus two aromatic, non-Basmati varieties (KDML 105 and Mahi Sugandha); the other group contained all of the permitted Basmatitis, along with modern varieties derived from Basmatitis. Two of these (Pusa Basmati 1 and Pusa 1121) varieties were separated at the third node. They may be related to or derived from varieties in both of the second level groups. Two of the non-Basmati lines, Pusa Sugandha 2 and Pusa Sugandha 3 were indistinguishable from each other (Jaccard's similarity is 0.98), and are likely to be the same variety, they had 0.8 similarity with Pusa Sugandha 1. The latter was the only line tested that showed a high level of heterozygous alleles, it had two fragments with a quarter of the InDels tested. It is noteworthy that with the 41 InDels,

Table 2
InDel markers and allele sizes (approximate base pairs) in the fragrant varieties Taraori and Azucena

Locus	Chromosome	Azucena allele	Taraori allele	Other allele (if Azucena and Taraori are monomorphic)	Example of variety with other allele
R1M7	1	191	154		
R1M37	1	159	212		
R1M47	1	159	159	108	Basmati 385
R2M10	2	134	182		
R2M26	2	143	143	181	KDML 105
R2M50	2	No-amp	212	254	KDML 105
R3M23	3	185	149		
R3M37	3	186	186	250	Pusa Basmati 1
R3M53	3	204	204	169	Sherbati
R4M13	4	169	201		
R4M17 ^a	4	169	169	220	KDML 105
R4M30	4	173	173	133	Basmati 385
R4M43 ^a	4	160	160	194	KDML 105
R4M50 ^a	4	132	132	165	Sherbati
R5M13 ^a	5	207	175		
R5M20	5	214	214	175	KDML 105
R5M30 ^a	5	224	224	178	Basmati 385
R5M43	5	109	78		
R6M14 ^a	6	251	251	217	KDML 105
R6M30	6	181	147		
R6M44	6	156	156	122	Sherbati
R7M20	7	200	266		
R7M30	7	187	187	219	Jasmati
R8M10	8	122	122	174	KDML 105
R8M23	8	157	157		Monomorphic
R8M33	8	205	205	167	Sherbati
R8M46	8	113	113	83	KDML 105
R9M10	9	135	135	178	KDML 105
R9M20	9	182	142		
R9M30	9	153	185	200	KDML 105
R9M42	9	164	212		
R10M10	10	170	170	132	Mahi Sugandha
R10M30 ^a	10	152	152	133	Sherbati
R10M40	10	133	133		Monomorphic
R11M17	11	183	131		
R11M23 ^a	11	212	254		
R11M40	11	178	137		
R12M10	12	214	261		
R12M27 ^a	12	155	155	188	Pak 386
R12M33	12	208	208	250	KDML 105
R12M43	12	193	163		

Bold values indicate that the Azucena had a different allele to that reported in Nipponbare by (Shen et al., 2004).

^a Used in nine-marker variety test.

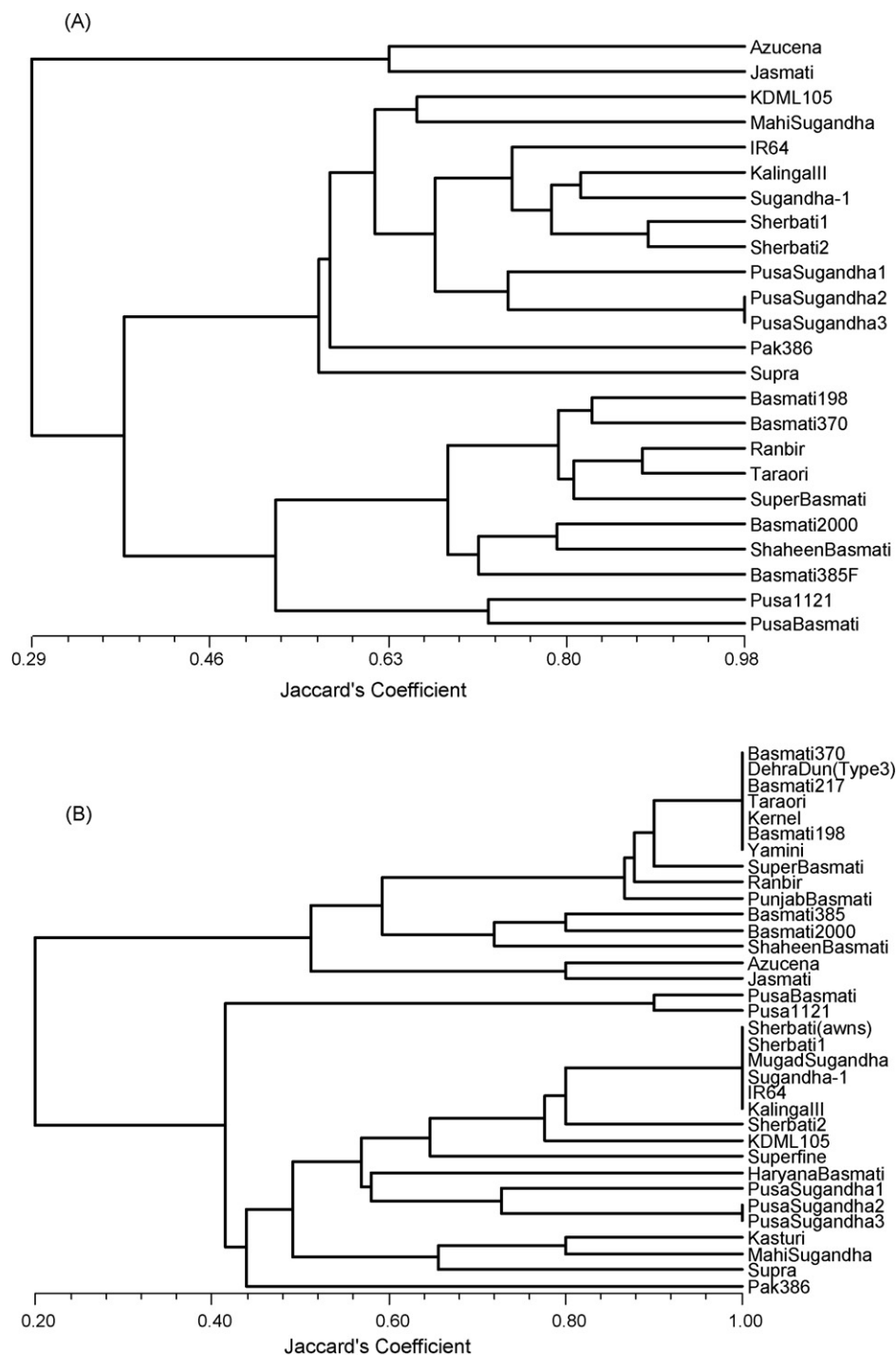


Fig. 2. Dendrogram showing groupings (UPGMA) of 24 rice varieties with 41 InDel markers (A) and 34 rice varieties with a sub-set of nine InDel markers (B).

Basmati 198 can be separated from the EU listed Basmati varieties.

A set of nine InDel markers that were polymorphic between Basmati and non-Basmati *indicas* were tested on 10 additional varieties. The 18 possible alleles were analysed with cluster analysis and the results showed that this set of markers can distinguish fragrant non-approved varieties (KDML 105, Azucena, Jasmati, Shaheen Basmati, Pusa 1121, Basmati 2000, Superfine, Sugandha-1 and Pusa Sugandha) and non-fragrant varieties (including possible adulterants Pak 386 and

Sherbati) from permitted Basmati varieties (Fig. 2B). Only the modern variety Yamini could not be separated from BasmatIs with this set of markers. Although Haryana Basmati and Kasturi clustered with non-Basmatis, they could be distinguished by their marker profiles. The groupings with this sub-set of markers were slightly different; notably the modern varieties Pusa Basmati 1 and Pusa 1121 clustered with non-Basmatis and Azucena and Jasmati clustered with Basmatis. With the full set, Pusa 1121 and Pusa Basmati 1 had a Jaccard's similarity of 0.73 and they shared a mean Jaccard's similarity of 0.6 ± 0.03 with the permitted

Basmati. Their shared parent line IR8 is likely to donate alleles corresponding to the *indica* cluster.

4. Discussion

The InDel markers we tested were highly reliable and informative, in contrast only 50% of the fragments produced with the *Rim2/Hipa* markers were consistent. Cluster analysis of the fragments amplified with the full set of InDel markers clearly separated Azucena and Jasmati from the Basmati and *indica* varieties and most of the BasmatIs fell into a different group from the non-Basmati *indic*as. These results show that InDels can be used for more purposes than merely separating *japonicas* from *indic*as, as suggested by Shen et al. (2004). It must be noted that the choice of a sub-set of markers used has a great deal of influence on clustering, therefore, the different groupings revealed by InDels and *Rim2/Hipa* may not be entirely due to the effect of different marker systems. In comparison to microsatellites, InDels revealed fewer alleles but were equally reliable and they have the advantage of being separated on agarose gels, hence they are more cost-effective and suitable for use in small laboratories.

An InDel-based marker situated within the fragrance gene (*fgr*) has been developed by Bradbury et al. (2005). Although it can separate fragrant and non-fragrant rice varieties, it is not useful for distinguishing between Basmati and other fragrant varieties. For example, we found that the following non-approved varieties: Basmati 2000, Pusa 1121, Pusa Suganda 1–3, Shaheen Basmati and Sugandha-1, all have the same allele as all the approved BasmatIs (data not shown) with this marker. It can be used in addition to other InDels, although it does not substantially improve the discrimination. Therefore, we recommend the set of nine InDel markers to distinguish between these groups of varieties (Fig. 2B), supplemented with additional InDel markers to separate within the approved group (e.g., to separate Basmati 198 from Basmati 370; Fig. 2A).

The *Rim2/Hipa* markers were not easy to score because some of the bands were less reproducible than others. Variation in PCR product reproducibility between labs has been seen with these primers by Zuhua He, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences (personal communication). Despite these problems at least half of the *Rim2/Hipa* fragments are reproducible and some can be useful in breeding programmes. We confirmed that one 1163 bp PCR product from primer set A was reproducible and heritable. It segregated in the expected frequency for a co-dominant allele in the F₂ progeny of Kalinga III/IR64 (unpublished). It was present in both Azucena and IR64 but absent in most Basmati varieties. We mapped this fragment to chromosome 8 (between RFLP loci R902 and G1010b) in the Azucena/Bala population (Price et al., 2000). It is approximately 34 cM above *fgr*, therefore, these two loci are unlikely to be in linkage disequilibrium with each other.

Although the origins of most InDels are unknown, some are created through the transposition of transposons and retro-elements (Edwards et al., 2004). Different types of polymorphisms, including those created by different types of

transposon, may have arisen at discrete stages in the history or rice evolution. The more recent types are likely to be of most practical use for revealing variation between Basmati and *indica* varieties because they are more likely to have arisen after these groups split.

Jasmati is a modern variety from Rice Tec (Texas, USA) although its pedigree has not been disclosed (Bhattacharjee et al., 2002). It is reputedly derived from a cross between KDML 105 with a Basmati variety. The mean Jaccard's similarity (based on 41 InDels) between Jasmati and seven Basmati varieties was 0.4 ± 0.02 . It had the greatest similarity (0.63) to Azucena and only 0.23 similarity to KDML105. Although this supports the conclusion of Vanvijit, who used microsatellites and reported that KDML 105 is unlikely to be a parent of Jasmati (1998, cited by Tanasugarn, 1998), it must be noted that key genes for agronomic characteristics or quality traits may be donated by parents whose alleles are not always detected with molecular marker analysis because the majority of alleles are conserved from the better adapted variety. This was the case for the most successful upland varieties selected from the cross Kalinga III/IR64 in which few, if any, IR64 alleles were detected with microsatellites (Steele et al., 2004).

5. Conclusion

The rice marker systems developed from insertion/deletion and *Rim2/hipa* transposon sequences are both cost-effective alternatives that can be used instead of (or alongside) microsatellites for applications such as variety testing, diversity analysis and mapping. InDels reveal inherited variation that is present in modern varieties and that can distinguish them from more 'traditional' varieties. They constitute a reliable and informative set of PCR markers that can be used to unravel complex relationships between rice varieties.

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