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Assessment of genetic purity in rice using polymorphic SSR markers

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Abstract

Genetic purity is conventionally performed through Grow-Out-Test (GOT) with morphological characters. Simple sequence repeat (SSR) markers are independent of G X E interaction. Herewith, 16 high yielding varieties of rice were analyzed using 55 SSR markers for DNA fingerprinting and identification of genetic impurities: 14 were found to be polypmorphic and amplified 48 alleles with an average of 3.43 alleles *per* each primer pair. The number of alleles amplified ranged from 2 to 6 and the size of the PCR products amplified from these 14 primer pairs ranged from 80–450 bp with polymorphic information content (PIC) from 0.14 (RM 346) to 0.99 (RM 5900). PICs at 0.5 or higher are highly informative SSR markers for genetic studies, DNA fingerprinting and scoring polymorphism rate of SSR markers pertinent to specific locus. The 14 polymorphic SSR markers can be used for DUS testing, genetic purity and DNA finger printing of rice varieties.

1. Introduction

Rice (*Oryza sativa* L.) is an important cereal crop feeding more than one third of the global population (Sarangi et al., 2019). It supplies 23% of the world's dietary energy needs with 16% of the per capita protein requirement (Moorthy et al., 2011). In Asia alone, 90% of rice is produced and consumed (Sarangi et al., 2019). India ranks first in consumption, whereas in the world it is the second most consumed cereal next to maize (Chandusingh et al., 2017a; Tiwari et al., 2018). With the advent of Green Revolution, rice production in the country has not only doubled but also enabled self-sufficiency (Chandusingh et al., 2017b; Singh et al., 2019). However, the demand for rice is still anticipated to escalate unequivocally in the coming future due to steady increase in population.

Several reports suggested that the rice production in Asian countries and India must be doubled by 2025 in order to encounter the hunger needs of exploding population (Hossain, 1996; Paroda, 1998). This implies that more than half of the population across the globe relies on the production and supply of rice, which ensures food and nutritional security (Bora et al., 2016; Bandumula, 2017; Singh et al., 2019). Since the land is scarce and expansion is not likely, achieving higher levels of production and productivity in rice is only feasible through adoption of high yielding and improved varieties. Nevertheless, the success of these high yielding and improved varieties largely depends on the availability of quality seed with greater genetic purity standards is critical to realize the genetic gains accumulated through efforts of plant breeding (Agarwal et al., 1999). Further, high demand and market price fluctuations for highly preferred quality rice varieties often promote with certain level of adulteration in the seed supply chain. Hence, ensuring the genetic purity of crop varieties is a pre-requisite and immensely critical for realizing their full yield potential.

In India, seed lots often pass through multi-level generation system during the process of seed multiplication that increases the risk of genetic contamination through pollen shedders and by physical admixtures during post-harvest handling. Impurities stemmed from these routes would remain undetected morphologically and cause deterioration of quality seed pertinent to variety, which ultimately

leads to a drastic reduction in crop yields (Liu and Wang, 2000). Moreover, the characterization of genetic stocks and varieties is mandatory for unambiguous identification of varieties that are genetically very close, protection of intellectual rights and for grant of Plant Breeder's Rights besides preventing unauthorized commercial use of seeds (Faccioli et al., 1995; Bora et al., 2016). Thus, conservation of seed purity continues to be an important aspect of crop improvement for both seed production and multiplication.

The genetic purity of commercial seed lots is traditionally assayed by performing Grow Out Tests (GOT) based on morphological characters. Morphological characterization based on physical traits descriptors for quality seed production is not only time taking and laborious but also environment responsive, hence less reliable and often provide impetuous results due to hindering effect of G X E interaction (Li et al., 2002). Although methods based on biochemical approaches such as isozyme analysis through protein separation using electrophoresis is available but their ability to distinguish closely related genotypes is meagre due to finite polymorphism and influenced by environment similar to GOT's (Ainsworth and Sharp, 1989). This makes the process of varietal identification and cultivar protection less empirical and sometimes more elusive using conventional approaches.

In contrast, genetic purity assessment using molecular markers based on DNA sequence polymorphism has been proven to be an unbiased means of identifying cultivar purity in several crop species (Karkousis et al., 2003). Among the various DNA based markers available, simple sequence repeats (SSR) also called microsatellites are widely deployed for rapid genetic purity assessment of both varieties and hybrids in rice (Bora et al., 2016; Sundaram et al., 2008; Joshi and Behera, 2006; Nandakumar et al., 2004; Singh et al., 2004; Yashitola et al., 2002) and in several other crop species (Mingsheng et al., 2006; Dongre et al., 2011; Nagawade et al., 2016). The specific advantage of these microsatellite markers include their reproducibility, abundance, uniform genetic distribution, co-dominant nature and ease in handling (McCouch et al., 1997; Akagi et al., 1996) that remarkably enhanced their utilization in genetic purity assessment in several crop species of agricultural value. Owing to its potential features than the other markers, the study under investigation has been carried out for assessment of genetic purity in rice using several SSR markers.

2. Materials And Methods

2.1 Plant materials

The seeds of sixteen popular rice cultivars (Supplementary Table 1) used in the study were collected from various centres across India.

2.2 Methods

2.2.1 Genomic DNA extraction

Genomic DNA from paddy seeds was extracted using DNeasy Plant Mini Kit as per the manufacturer's instructions. The extracted DNA samples concentrations and quality were determined using ultra-microspectrophotometer ND-2000 (Nanodrop, USA). Thereafter, all the varietal genomic DNA samples were diluted with 1X TE buffer (10 mM Tris-HC1, pH 8.0; 1 mM EDTA) to make a final concentration of 20 ng μL^{-1} and stored at -20 ^{0}C for further use.

2.2.2 PCR amplification

A total of fifty five rice SSR primers (Supplementary Table 2) were selected in this study for the purpose of molecular fingerprinting. Primers were obtained from the Gramene database (http://www.gramene.org/markers/microsat/ssr.html). Polymerase chain reaction (PCR) was performed with a reaction volume of 25 µL containing 1X PCR assay buffer (50 mM KCl, 10 mM Tris-Cl, 1.5 mM $MgCl_2$), 200 µM each of dNTP's, 0.2 µM each of forward and reverse primers, 0.6 U *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd., Bengaluru, India) and genomic DNA (25 ng). The PCR protocol comprised of an initial denaturation at 94 ^{O}C for 5 min, followed by 33 cycles with denaturation for 1 min at 94 ^{O}C, annealing for 1 min at 55 ^{O}C and primer extension at 72 ^{O}C for 2 min and a final extension step of 72 ^{O}C for 7 min. All PCR reactions were performed in a thermal cycler AG 22331 (Eppendorf, Hamburg, Germany). The amplified PCR products were separated using electrophoresis on 3% Metaphor™ agarose gel (Lonza, USA), stained with ethidium bromide (1 mg/ml). USA) and visualized under UV light using Image Lab™ software version 2.0.1 (Bio-Rad, USA).The size of the amplified products was estimated through deploying standards size DNA ladder (Low range DNA ladder, MBI Fermentas, Lithuania).

2.2.3 Data analysis

Scoring of band profiles was done only for reproducible and distinct bands either as present (1) or absent (0) for each SSR primer pair. To identify the informative SSR marker, polymorphism information content (PIC) value for each SSR marker was calculated as per Anderson et al., 1993.

$$PIC = 1-\sum_{i=1}^{k} P_i^2$$

Where, k denotes the total number of alleles (bands) detected for each SSR locus and p is the proportion of the varieties consists of allele (band) in all the analyzed samples.

3. Results

A total of 16 high yielding varieties of rice were analyzed using 55 rice specific microsatellite markers for DNA fingerprinting and identification of genetic impurities in the seed lot. On the basis of polymorphism, 14 SSR markers were found to be informative (polypmorphic) and a total of 48 alleles were amplified from these 14 primer pairs with an average of 3.43 alleles per each primer pair. The number of alleles amplified by each primer pair ranged from 2 to 6. The overall size of the PCR products amplified from

these 14 primer pairs ranged from 80–450 bp and the polymorphic information content (PIC) for these 14 SSR markers ranged from 0.14 (RM 346) to 0.99 (RM 5900). Table 1 illustrates the amplification of alleles by each polymorphic SSR markers coupled with their PIC values.

Table 1
Allelic status and polymorphic information content (PIC) of varieties used in the study

Primer	Number of amplified bands	Number of polymorphic bands	Allele size		Highest frequency allelle		PIC
			Range	Difference	Size (bp)	Frequency (%)	
RM-452	5	5	230–400	170	230, 250	50.0	0.16
RM-247	3	2	230–300	70	230	50.0	0.61
RM-253	3	3	200–230	30	230	80.0	0.32
RM-316	6	6	230–300	70	250	70.0	0.14
RM-27	4	3	100–270	170	100, 150	50.0	0.26
RM-243	2	1	100	-	100	43.7	0.81
RM-106	2	1	230	-	230	62.5	0.61
RM-495	4	3	100–270	170	270	43.7	0.67
RM-6933	2	1	150	-	150	18.7	0.96
RM-510	5	2	100–230	130	230	81.2	0.28
RM-1812	3	2	100–230	130	100	31.2	0.90
RM-5900	2	1	150	-	150	6.2	0.99
RM-3	5	5	80–450	370	250	62.5	0.32
RM-408	2	2	100–230	130	100	68.7	0.29
Total	48	37	100–450	-	-	-	-
Average	3.43	2.64	-	-	-	51.31	0.52

Out of the 55 SSR markers used in the present study, a set of 14 markers (Table 2) exhibited unique alleles and could distinguish one variety from the other by amplification of alleles specific for that variety. SSR marker RM-452 amplified alleles ranging from 230–400 bp and distinguishes 15 out of the 16 varieties used in the current study (Fig. 1). Within these 15 varieties Pusa-1612, Rajendra Bhagawati had similar amplification pattern with alleles at 250 and 300 bp, respectively. Similarly, Rajendra Shweta and Samba Sub-1 showed similar amplification pattern with allele at 300 bp whereas varieties Pusa-1612, Shiats Dhan-1 amplified at 350 bp. Marker RM-27 amplified three allelic variants of size ranging from 100–270 bp that could precisely identify four varieties *i.e.*, Co-51, HUR-917, Samba Sub-1 and PB-1509 each with unique amplification pattern.

Table 2
SSR markers displaying variety-specific amplification of allele(s) in rice varieties evaluated in the study

SSR Marker	Variety	Size of the allele (bp)
RM-452	Co-51	230, 250
	PB-1	300
	PB-1121	230, 250, 300
	Pusa-1612, Rajendra Bhagawati	250, 300
	KN-3	280, 350
	PS-5	250, 280
	MTU-7029	280
	PB-1509, SHiats Dhan-1	350
	BPT-5204	250, 280, 350
	HUR-917	250, 280
	Rajendra Shweta, Samba Sub-1	300
	Swarna Sub-1	250, 350
RM-27	Co-51	100, 150
	HUR-917	150, 230
	Samba Sub-1	230
	PB-1509	150
RM-3	Co-51	300
	BPT-5204	250 and 300
	HUR-917	450
	Rajendra Shweta	250
	PB-1	450, 300 and 250
RM-247	PB-1121	300
	PS-5	230 and 300
	MTU-7029	230
RM-408	HUR-917	100
	Rajendra Shweta	230

SSR Marker	Variety	Size of the allele (bp)
RM-1812	Rajendra Bhagawati	270
RM-5900	Rajendra Shweta	150
RM-495	Rajendra Shweta	100 and 270
	PB-1509	100
	Shiats Dhan-1	230
RM-253	Samba Sub-1	200
	PB-1509	200 and 230
	PB-1	230
RM-6933	Pusa 1612, MTU-7029	150
RM-243	PB-1	50 and 100
RM-316	Shiats Dhan-1	300
	Co-51	250 and 300
	BPT-5204	230
RM-510	HUR-105	230
RM-106	Co-51, Swarna Sub-1	230

Another SSR marker RM-3 amplified alleles of size ranging from 80–450 bp and these allelic variants are visually distinguishable for differentiating five varieties *i.e.*, Co-51, BPT-5204, HUR-917, Rajendra Shweta and PB-1 (Fig. 2). SSR marker RM-247 resulted with two polymorphic alleles of size 230 and 300 bp useful for distinguishing three varieties PB-1121, PS-5 and MTU-7029 with discrete amplification profile (Fig. 3). Moreover, SSR marker RM-408 also amplified two allelic variants of size 100 and 230 bp specific for the identification of two varieties *viz.*, HUR 917 and Rajendra Shweta (Fig. 4), while SSR marker RM-495 amplified three polymorphic alleles of size ranging from 100–270 bp that could precisely discriminate three varieties Rajendra Shweta, PB-1509 and Shiats Dhan-1.

Four markers *viz.*, RM-1812, RM-5900, RM-243 and RM-510 had amplified one allele each specific to single variety and these markers could inadvertently distinguish four varieties Rajendra Bhagawati, Rajendra Shweta, PB-1and HUR-105, respectively. SSR marker RM-6933 amplified only one polymorphic allele at 150 bp specific for the identification of two different varieties *viz.*, Pusa-1612 and MTU-7029. SSR marker RM-316 amplified three polymorphic alleles of size ranging from 230–300 bp that could distinguish three varieties Shiats Dhan-1, Co-51 and BPT-5204, respectively. SSR marker RM-253 had amplified two polymorphic alleles of size 200 bp, 230 bp and these allelic variants could discriminate three different varieties Samba sub-1, PB-1509, PB-1 of rice based on their amplification pattern (Fig. 5).

SSR marker RM -106 amplified only one allele of size 230 bp unique for the identification of two different varieties Co-51 and Swarna Sub-1.

4. Discussion

In the present study, 55 evenly distributed SSR markers of 12 chromosomes were deployed for identification of polymorphic markers. Among these 55 SSR markers, 14 SSR markers showed hyper variable polymorphic markers that are able to identify 16 varieties used widely in seed production. In addition to polymorphic nature, these 14 polymorphic markers showed an average PIC value of 0.52.

Conventionally, SSR markers with PIC values of either 0.5 or higher are considered as highly polymorphic in nature that can be deployed for genetic purity assessment, DNA fingerprinting and hybridity determination (DeWoody et al. 1995, Ashikawa et al., 1999). Further all the 14 primer pairs exhibited amplification of alleles ‘specific’ or ‘unique’ to different varieties of rice used in this study. On the basis of PIC values it can be considered that seven SSR markers RM-247, RM-243, RM-106, RM-495, RM-6933, RM-1812 and RM-5900 were highly polymorphic markers that have potential for unambiguous identification of these selected rice varieties.

Hence, these set of SSR markers serve as molecular descriptors could be utilized for genetic purity of rice varieties, DUS testing and hybridity determination. Similar results have been reported in many studies wherein SSR markers were found to be essentially useful for the assessment of genetic purity of both varieties and hybrids of rice (Verma et al., 2017; Bora et al., 2016; Kumar et al., 2015; Sundaram et al. 2007; Yun et al. 2005; Yashitola et al. 2002).

5. Conclusions

Unlike morphological and biochemical methods, DNA based SSR markers have distinguishing ability from one variety/hybrid with other varieties and hybrids, which can be used as an essential tool for identification of off-types and ensures genetic purity of seed lot. Besides, this technique is reproducible, rapid and performs at lesser time. Therefore, this study implies that the genetic purity of seed lot/seed production can be done efficiently using SSR markers (microsatellite). Besides, these SSR markers can be deployed for seed certification, hybridity determination and DUS testing processes that ultimately ensures food and nutritional security.

Declarations

Ethics approval and consent to participate:

Not applicable.

Consent for publication:

Not applicable.

Availability of data and material:

Available at Research Square.

Competing interests:

No competing interests.

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Authors' contributions:

All authors conceive, design, experiment, write and revise the paper.

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Figures

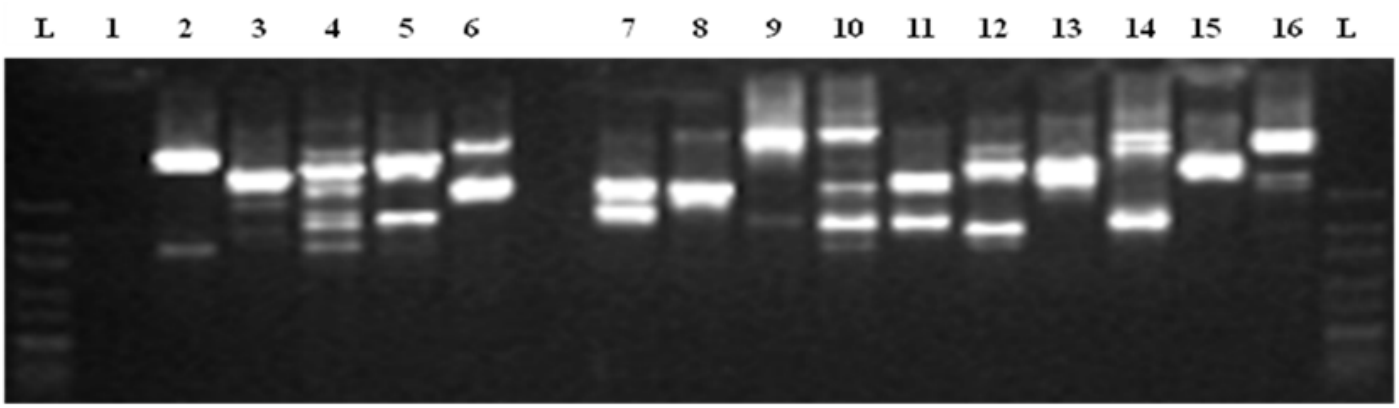


Figure 1

Amplification profile of primer pair RM 452 from 16 rice cultivars. L- DNA ladder (50 bp). The numbers of lanes 1-16 correspond to the rice cultivars 1.HUR-105 2.Co-51 3.PB-1 4.PB-1121 5.Pusa 1612 6.KN-3 7.PS-5 8.MTU-7029 9.PB-1509 10.BPT-5204 11.HUR-917 12.Rajendra Bagawati 13.Rajendra Shweta 14.Swarna Sub-1 15.Samba Sub-1 16.Shiats Dhan-1

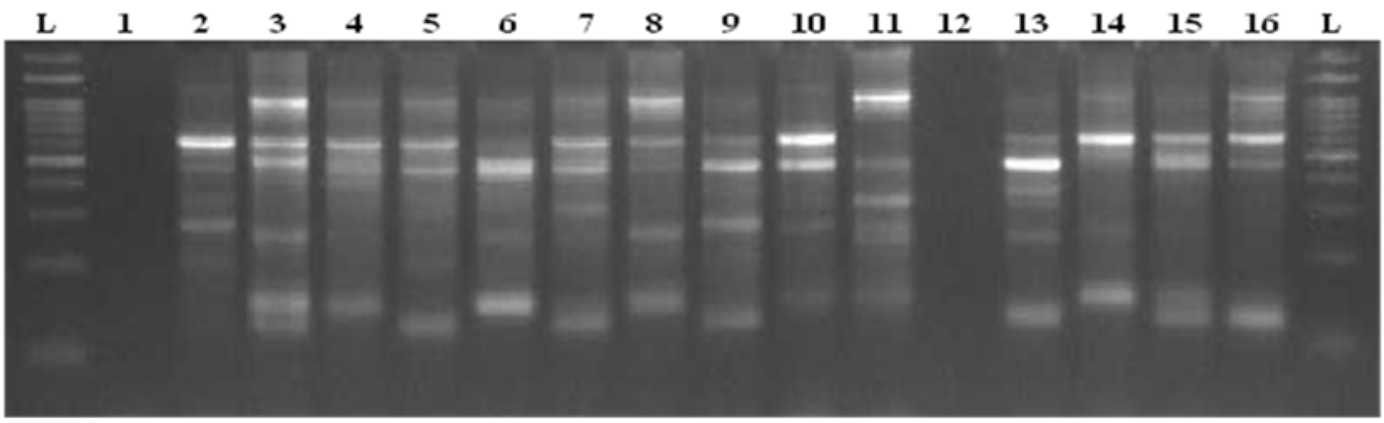


Figure 2

Amplification profile of primer pair RM 452 from 16 rice cultivars. L- DNA ladder (50 bp). The numbers of lanes 1-16 correspond to the rice cultivars 1.HUR-105 2.Co-51 3.PB-1 4.PB-1121 5.Pusa 1612 6.KN-3 7.PS-5 8.MTU-7029 9.PB-1509 10.BPT-5204 11.HUR-917 12.Rajendra Bagawati 13.Rajendra Shweta 14.Swarna Sub-1 15.Samba Sub-1 16.Shiats Dhan-1

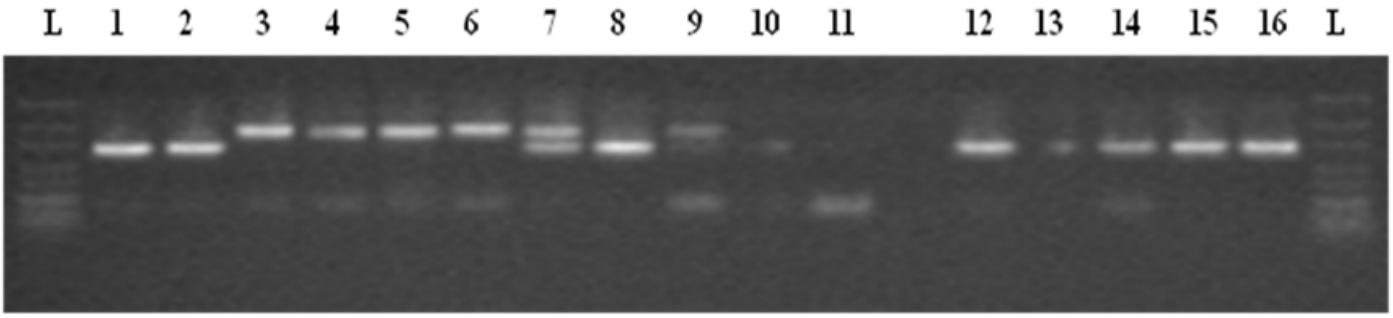


Figure 3

Amplification profile of primer pair RM 247 for 16 rice cultivars. L- DNA ladder (50 bp). The numbers of lanes 1 to 16 correspond to the rice cultivars: 1.HUR-105 2.Co-51 3.PB-1 4.PB-1121 5.Pusa-1612 6.KN-3 7.PS-5 8.MTU-7029 9.PB-1509 10.BPT-5204 11.HUR-917 12.Rajendra Bagawati 13.Rajendra Shweta 14.Swarna Sub-1 15.Samba Sub-1 16.Shiats Dhan-1

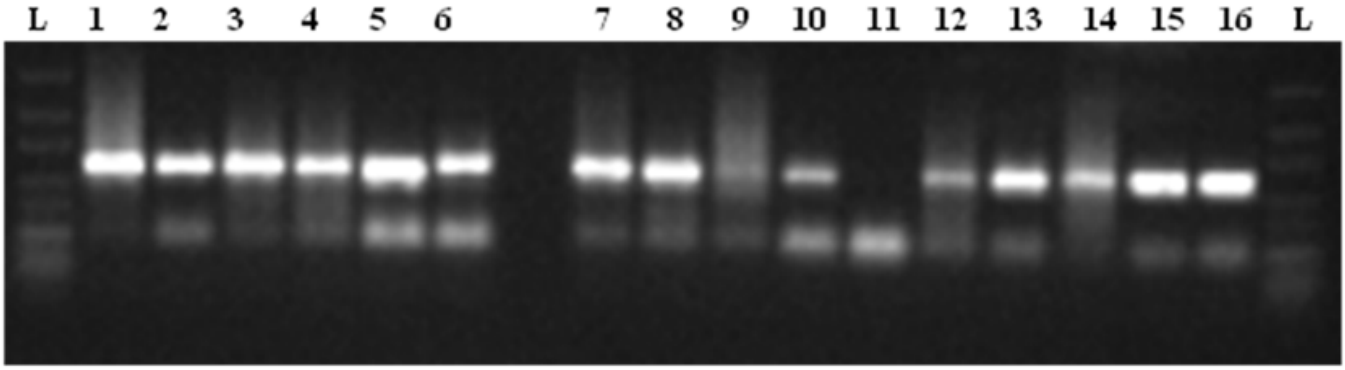


Figure 4

Amplification profile of primer pair RM 408 from 16 rice cultivars. L- DNA ladder (50 bp). The numbers of lanes 1 to 16 correspond to the rice cultivars: 1.HUR-105 2.Co-51 3.PB-1 4.PB-1121 5.Pusa 1612 6.KN-3 7.PS-5 8.MTU-7029 9.PB-1509 10.BPT-5204 11.HUR-917 12.Rajendra Bagawati 13.Rajendra Shweta 14.Swarna Sub-1 15.Samba Sub-1 16.Shiats Dhan-1

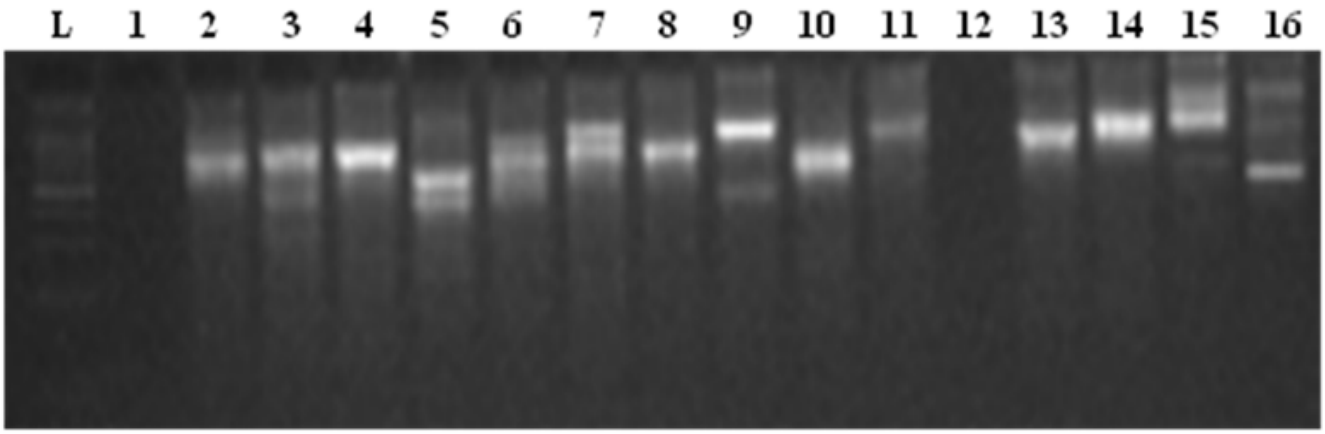


Figure 5

Amplification profile of primer pair RM 253 for 16 rice cultivars L- DNA ladder (50 bp). The numbers of lanes 1 to 16 correspond to the rice cultivars: 1.HUR-105 2.Co-51 3.PB-1 4.PB-1121 5.Pusa 1612 6.KN-3 7.PS-5 8.MTU-7029 9.PB-1509 10.BPT-5204 11.HUR-917 12.Rajendra Bagawati 13.Rajendra Shweta 14.Swarna Sub-1 15.Samba Sub-1 16.Shiats Dhan-1

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