



## DNA fingerprinting and phylogenetic study of some indica rice varieties using SSR markers

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### ABSTRACT

The genetic relationship and DNA fingerprinting of commercially available seven indica rice varieties were investigated by using 28 Simple Sequence Repeats (SSR) primers. The seven recombinant inbred lines (RILs) used in this investigation have been categorized under three distinct groups depending on their seed morphological similarities which are not differentiable to the naked eye. Thus, the objective was to develop a genetic fingerprint for the seven varieties basically in order to differentiate these three groups of rice varieties which exhibit similar morphological characters and to elicit the genetic relationship between the RILs. The results revealed that 20 SSR primers out of 28 primers showed polymorphism indicating the robust nature of SSR in eliciting the polymorphism. The information obtained from the DNA fingerprinting studies help to distinctly identify and characterize seven varieties. The cluster analysis grouped the seven varieties into four classes in which the varieties having similar morphological characters were clustered together, thus revealed the close genetic relationship among them.

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**Keywords:** SSR primers, DNA fingerprinting, Genetic Relationship, Polymorphism, Monomorphism, Dendrogram.

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### INTRODUCTION

Rice is a major food staple for the world's population and serves as a model species in cereal genome research and its finished sequence represents a major milestone. Six years of research work conducted by The International Rice Genome Sequencing Project, which includes The Institute for Genomic Research (TIGR), has found that the completed sequence for the genome consists of around 400 million DNA bases holding 37,544 genes on rice's 12 chromosomes (Pitman, 2005).

Rice varieties developed in Sri Lanka cover 97% of the cultivated extent in Sri Lanka and help in producing 90% of the national rice needs. At present at least 10 of

these varieties are grown on commercial scale in 17 Asian, African and South American countries. Sri Lanka commands the highest national average rice yield, 3.7 t/ha, among South and Southeast Asian countries (Research achievements of the Department of Agriculture, 2008).

Rice Research and Development Institute (RRDI) Bathalegoda, and Rice Research Station Ambalantota, Sri Lanka have developed nearly 50 high yielding recombinant inbred lines (RIL) with the aim of increasing the national rice production, during the past few decades. Some of these RILs show quite similar seed morphological characters including grain length, grain width, color of glumes, etc., as revealed by descriptor

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of cereals (2005), hence, cannot be differentiated to the naked eye thus leading to an underestimation in “other distinguishable varieties” category when the seed samples are undergoing the seed certification service before releasing them as certified seed paddy. The seven selected rice RILs are of such varieties which commonly undergo this problematic situation during the seed certification process. Depending on the similarity of seed characters, these seven varieties have been categorized into three distinct groups, namely, BG300 and BG305; BG359/BG379-2 and BG403 (rice varieties produced at RRDI Bathalegoda, Sri Lanka); AT303 and AT353 (rice varieties produced at rice research station, Ambalantota, Sri Lanka). Table 1 shows the pedigree and some phenotypic characters expressing variation between the selected varieties.

To solve the problem of differentiating these rice varieties with similar seed morphology during the seed certification, the molecular markers could be used. Virtually no investigation has been conducted in Sri Lanka up to date regarding this aspect. With the recent advancements of biotechnological tools many countries use molecular techniques specially to confirm the hybridity and assess the purity of rice hybrids (Ye-yun et al., 2005), characterization, fingerprinting and phylogenetic analysis, etc. (McCouch et al., 1996; Chung et al., 2002; Yashitola et al., 2002; Hariprasad et al., 2005).

Random Amplified Polymorphic DNA (RAPDs), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Single Nucleotide Polymorphism (SNP) and Simple Sequence Repeats (SSR) are some of the molecular markers which are available for the assessment of the genetic variation and for the elucidation of genetic relationships within and among species (Tautz, 1989). Yet, for this study SSR markers were used due to certain advantageous features that they possess over other marker systems such as high reproducibility, co-dominant inheritance, high precision, high abundance and considerably higher level of polymorphism in rice genome. Therefore, in this investigation SSR markers were used with the primary objectives of developing a fingerprint which enables the differentiation of selected commercially available seven indica rice varieties and secondarily to develop a dendrogram in order to disclose the genetic relationship among them.

## MATERIALS AND METHODS

### Extraction, confirmation, purification and quantification of genomic DNA

The CTAB protocol (Mayer et al., 1995) was used for the genomic DNA extraction from three week old rice seedlings, raised separately on nursery trays containing puddled mud as the growth medium without

**Table 1:** Pedigree and some phenotypic characters expressing variation between the selected varieties.

Group	Varieties	Pedigree	Phenotypic characters
1	BG 300 BG 305	Bg 367-7//IR 841/Bg 276-5 Bg 1203/Bg 1492	Time laps from sowing to 50% flowering, Panicle type, Axis appearance, Secondary branching within the panicle.
2	BG 359 BG 379-2 BG 403	Bg 12-1/Bg 1492 Bg 96-3*2/Ptb33 83-1026/Bg 379-2	Number of tillers per plant, Number of grains per panicle and Number of filled grains per panicle.
3	AT 303 AT 353	At 66-2/Bg 276-5 Bg 94-1 (R)/Bg 400-1//Bg 94-1	Time laps from sowing to 50% flowering stage, Number of grains per panicle, Leaf colour and nature of the leaf blade.

adding any fertilizer. The DNA extracted using the above procedure was stored at -20 °C. Confirmation of the presence of the genomic DNA was done by performing a gel electrophoresis by using a 0.8% agarose gel. Spectrophotometric method was used for the quantification of the genomic DNA. Each DNA sample was treated with 5 µL of RNase A in order to purify the extracted genomic DNA samples. The original DNA samples were diluted and 200 µL of 50 ng/µL template DNA samples were prepared.

#### PCR amplification of SSR regions

The PCR amplification was carried out for all the seven varieties using 28 SSR primers RM595, RM3614, RM3269, RM11679, RM11715, RM11690, RM8274, RM8096, RM3817, RM1067, RM6569, RM3648, RM319, RM8232, RM6696, RM6703, RM3362, RM8137, RM325A, RM185, RM334, RM270, RM184, RM107, RM101, RM160, RM338 and RM108. The following were contained in the reaction mixture used for PCR amplification; 1 × PCR buffer, 2.5 mM Magnesium chloride, 200 µM of dNTPs mixture, 10 pmol primer mixture (forward and reverse primer mixed into a ratio of 1:1), 2U of Taq polymerase and 50 ng template DNA in the final volume of 15 µL. The followed PCR amplification programme included an initial denaturing step of 5 min at 94 °C, which was followed by 35 cycles comprising 1 min at 94 °C, an annealing temperature of 60 °C for 1 min and 72 °C for 2 min. Eventually, an extension step at 72 °C for 2 min was followed prior to aborting the amplification. The final PCR product was brought down to 4 °C for storage for the future use.

#### Agarose and Polyacrylamide gel electrophoresis

A 3.0% agarose gel was used for the confirmation of the amplification of SSR regions targeted by different SSR primers. PCR products with amplified SSR regions were then subjected to 10% denaturing polyacrylamide gel electrophoresis, followed by silver staining for the visualization of bands to disclose the polymorphism. A modified silver staining protocol was used for staining (Samarasinghe *et al.*, 2001). Instead of the standard developing solution (60 g of

Sodium Carbonate in 2000 ml double distilled H<sub>2</sub>O), a solution of 1.5 g of Sodium hydroxide and 3.75 ml Formaldehyde (37%) in 500 ml of double distilled H<sub>2</sub>O was used.

#### Cluster analysis

Cluster analysis for selected rice varieties was done by using the "POPGENE" software (Yeh *et al.*, 1997).

## RESULTS AND DISCUSSION

### Polymorphism of SSR loci

Table 2 shows that out of total 28 SSR primers used for the study, 20 resulted in polymorphism when the selected seven indica rice varieties are considered as a single population. The bands generated by SSR primers RM319, RM3362, RM3817, RM6569, RM185, RM11715, RM8096 and RM8274 were monomorphic in all three groups. This suggests that in all seven varieties, there is no variation in those eight loci with respect to the number of tandem repeats in their respective repeat motifs. This might be due to highly conserved nature of those alleles found in the particular loci. However, according to Table 3, out of 28 SSR primers, six primers could be used to differentiate four combinations out of five (BG300/BG305, AT303/AT353, BG359/BG379-2, BG359/BG403 and BG379-2/BG403). Thus, SSR regions targeted by these six primers can be considered as the regions with the highest variation in terms of the number of tandem repeats in their respective alleles (repeat motifs). Intra allelic polymerase slippage during the replication, point mutation and the recombination could be the possible reasons for the variation of the allele length which has ultimately led to the polymorphism among the varieties (Ingram *et al.*, 2001).

According to the fingerprint (Figure 1), SSR primer RM107 showed polymorphic bands for BG300 and BG305, thus it can be used to differentiate BG300 from BG305. AT303 and AT353 also showed polymorphic bands. Therefore RM107 can also be used to differentiate AT303 from AT353. BG359 and BG379-2 were monomorphic thus RM107 cannot be used to differentiate those two varieties from each other. However BG403 was polymorphic to both BG359 and BG379-2, thus RM107 can be used to differentiate

**Table 2:** Number of SSR primers which showed polymorphic and monomorphic bands for the three groups of rice varieties.

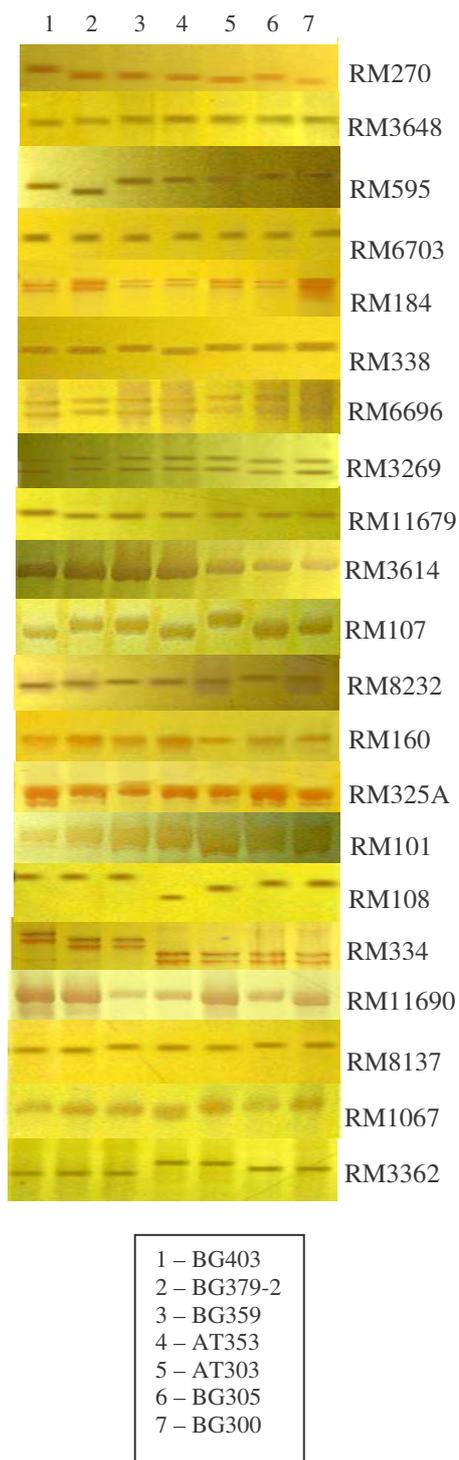
No.	Group	No. of SSR primers	
		Polymorphic	Monomorphic
1	BG300/BG305	6	22
2	AT303/AT353	11	17
3	BG359/BG379-2 and BG403		
	BG359/BG379-2	10	18
	BG359/BG403	17	11
	BG379-2/BG403	12	16

**Table 3:** SSR loci which resulted highest polymorphism among varieties.

Primer name	Differentiable combinations	Repeat motif	Chrom. No.
RM270	BG300/BG305, AT303/AT353, BG359/BG403, BG379-2/BG403	(GA)13	12
RM3614	BG300/BG305, AT303/AT353, BG359/BG379-2, BG359/BG403	(GA)13	01
RM11690	BG300/BG305, AT303/AT353, BG359/BG379-2, BG359/BG403	(GATC)5	01
RM107	BG300/BG305, AT303/AT353, BG359/BG403, BG379-2/BG403	(GA)7	09
RM184	AT303/AT353, BG359/BG379-2, BG359/BG403, BG379-2/BG403	(CA)7	10
RM325A	AT303/AT353, BG359/BG379-2, BG359/BG403, BG379-2/BG403	(CAT)4	08

BG403 from BG359 and BG379-2. RM270 also showed the same result as RM107. Similarly RM8232, RM3648 and RM595; RM160 and RM6703; RM325A and RM184; RM334, RM3269 and RM11679; RM11690 and RM3614; RM101 and RM338; RM1067 and RM6696; RM3362, RM319, RM3817, RM6569, RM8096, RM8274, RM185 and RM11715 showed similar banding patterns. Thereby, differentiation of BG300 from BG305 was possible by using primers, RM160, RM270, RM3614, RM6703, RM11690 and RM107 and AT303 was differentiated from AT353 by using primers, RM184, RM270, RM325A, RM338,

RM3614, RM6696, RM101, RM107, RM108, RM11690 and RM1067. Differentiation of BG359 from BG379-2 was possible by using primers, RM184, RM325A, RM3648, RM338, RM3614, RM8137, RM8232, RM595, RM11690 and RM101, while BG359 was differentiated from BG403 by using primers, RM184, RM270, RM325A, RM334, RM3648, RM338, RM3614, RM6696, RM8137, RM8232, RM595, RM3269, RM11679, RM11690, RM101, RM107 and RM1067. Differentiation of BG379-2 from BG403 was possible by using primers, RM184, RM270, RM325A, RM334, RM3648, RM6696, RM8232, RM595,



**Figure 1:** Genetic fingerprint of the seven rice varieties on 10% polyacrylamide gel.

RM3269, RM11679, RM107 and RM1067.

**Cluster analysis**

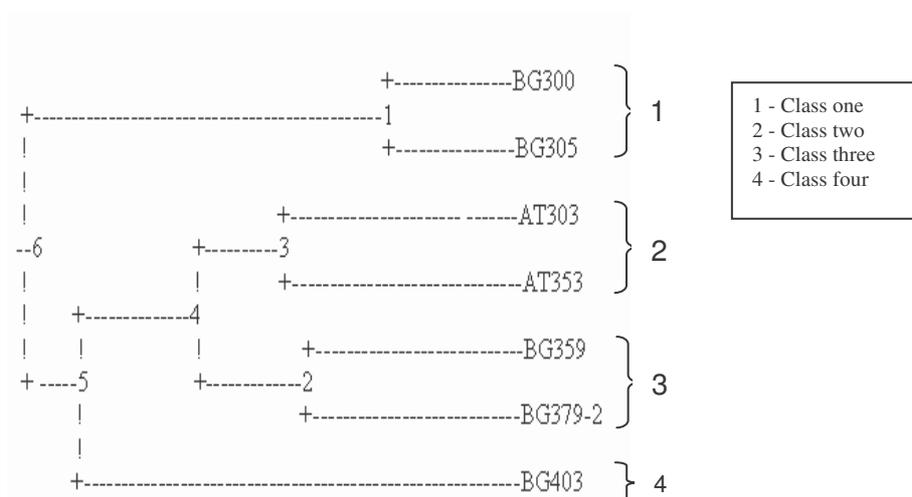
According to the dendrogram shown in Figure 2, all the varieties showed a clear separation which reveals the presence of the unique genetic makeup. This is obvious because most of the morphological characters are different among these varieties (Descriptor of cereals, 2005). Thus the genetic make up should be different for each variety, which indicates the presence of polymorphic genes within the genomes of rice varieties. The presence of morphological similarities of the used three rice groups is confirmed by the grouping pattern of the dendrogram. The grouping is purely based on the genetic similarities. Therefore, it is possible to expect similar morphological characters from the rice varieties which are grouped together. Thus, the findings of this study can effectively be used in seed certification service in identifying the seeds belonging to Other Distinguishable Varieties (ODV) category. On the other hand, the fingerprint of the rice varieties can be used to confirm the varietal identity thus will be important in selecting the pure lines and in hybridization programs. Furthermore, the results of this investigation provide sound evidence to elucidate the presence of similar morphological characters by using the phylogenetic relationships among the tested rice varieties.

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**Figure 2:** Dendrogram based on SSR data.

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