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Identification of microsatellite markers for genetic differentiation and authentication of promising aerobic rice genotypes

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Abstract

A study was undertaken to evaluate the simple sequence length polymorphism in six aerobic rice genotypes in order to elucidate their genetic differentiation at molecular level using six microsatellite primer pairs. Additionally, the primer pairs were validated using twelve inter-genotypic mixtures for their utilization in genetic purity assessment of these aerobic rice genotypes. Statistical methods and parameters used for deriving inference were polymorphism percent, polymorphism information content, discrimination and non-discrimination coefficient, similarity coefficient and analysis of divergence. Polymorphism was recognized among the genotypes on the basis of presence or absence of bands, besides variation in number and position of bands. Considering the number of alleles generated in conjunction with the level of polymorphism detected and discrimination ability exhibited, the primers RM 263, RM 5359 and RM 337 appeared to be highly polymorphic and comparatively more informative for the purpose of genetic differentiation of entries under evaluation. Molecular profiling data based on these three primer pairs allowed easily recognizable differentiation of the inter-genotypic mixtures from referral genotypes at least with a single marker allele difference. The complementary banding patterns visualized in inter-genotypic mixtures served as discrete molecular tags for distinguishing the referral genotypes and inter-genotypic mixtures. These three microsatellite primers may be further utilized in genetic purity assessment and authentication of aerobic rice genotypes.

Keywords: aerobic rice, microsatellite, similarity coefficient, genetic distance, purity test

Introduction

Rice grown in non-flooded, non-puddle and unsaturated aerobic soils is regarded as aerobic rice. An alternative rice production system in the form of aerobic rice promises substantial water savings by minimizing seepage, percolation and greatly reducing evaporation (Parthasarathi *et al.*, 2012) [10]. However, high yielding rice varieties specifically suitable for aerobic farming of rice are currently not available. Therefore, research efforts are being directed towards the development of aerobic rice varieties that combine the drought-resistant characteristics of upland varieties with the high-yielding traits of lowland varieties to achieve higher and sustainable yields.

Assessment of the potential of available germplasm and the development of rice varieties specifically suitable for cultivation under aerobic condition are gaining much more importance for ensuring further increase in rice production. Using the available upland paddy and high-yielding rice germplasm, several aerobic rice genotypes have been developed by employing conventional breeding methods supplemented with marker assisted selection techniques for improving productivity under water limiting conditions. But most of the derivatives and varieties developed to-date fall short of yield potential and quality features of other commercially cultivated rice varieties. A number of morphological, physiological and biochemical traits have been reported to improve the performance of crops challenged by aerobic condition. Hence, use of different rice varieties with different adaptive traits and distinct genetic structures are not only important prerequisites for further genetic improvement, but vital for selection and breeding of high yielding aerobic rice varieties. Traditional breeding technologies used for development of aerobic varieties of rice have certain limitations. Alternatively, molecular marker techniques dependent marker aided selection approaches enhance selection efficiency and offer capability to reduce the time, space and cost of varietal development with higher precision. Therefore, use of molecular markers can greatly assist traditional breeding approaches used for development of aerobic varieties of rice.

Maintenance of genetic purity of the commercial seed lots is crucial because any impurities would reduce the expected yield. Owing to technical efficiency, chromosome specificity, greater level of allelic diversity, co-dominance, high power of resolution, abundance, operational ease

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and low cost, carefully chosen microsatellite markers are suitable and preferable for fingerprinting and testing of the genetic purity in rice (Nandkumar *et al.*, 2004; Hashemi *et al.,* 2009; Tamilkumar *et al.*, 2009; Kumar *et al.*, 2012; Kumar *et al.*, 2015) [5, 13, 6, 7]. Molecular profiles developed by the utilization of such genomic markers would be especially valuable to unambiguously discriminate and authenticate the genotypes. Keeping all above in view, the present investigation was carried out to generate molecular profiles of some aerobic rice genotypes using microsatellite markers for identification of polymorphic and informative markers and to investigate the usefulness of these markers based simple sequence length polymorphism for genetic purity assessment of aerobic rice genotypes.

Materials and Methods

The experimental materials of the present study comprised six aerobic rice genotypes and twelve inter-genotypic seed mixtures each containing pair-wise combinations of the six genotypes. Seeds of all together eighteen entries under evaluation comprising six aerobic rice genotypes, namely, AER-06 (IR 78875-207-8-1-8), AER-05 (IR 55423-1), AER-04 (IR 80312-6-8-3-2-8), IR 64, AER-02 (IR 81449-8-8-128-1) and Rasi along with their twelve inter-genotypic mixtures, such as, Mixture-1 (AER-06+AER-05), Mixture-2 (AER-06+AER-04), Mixture-3 (AER-06+IR 64), Mixture-4 (AER-06+AER-02), Mixture-5 (AER-05+AER-04), Mixture-6 (AER-05+IR 64), Mixture-7 (AER-05+AER-02), Mixture-8 (AER-04+IR 64), Mixture-9 (AER-04+AER-02), Mixture-10 (IR 64+AER-02), Mixture-11 (IR 64+Rasi) and Mixture-12 (AER-02+Rasi) were planted in plastic pots. Leaves were collected from the three weeks old seedlings of each entry and used for DNA isolation by using CTAB method (Ferdous *et al.*, 2012) with slight modifications. The purification of extracted DNA sample was performed by RNase treatment and DNA pellet was dissolved in TE buffer.

Adopting standard protocol of polymerase chain reaction adjusted to laboratory conditions, amplification of targeted genomic regions was carried with known six pairs (Table 1) of forward and reverse microsatellite primer pairs in 15 µl reaction mixture containing 2.8 µl water (Protease and Nuclease free), 3.0 µl 5X PCR buffer, 1.3 µl 10 mM MgCl$_2$, 3.0 µl 1 mM dNTPs mixture, 1.2 µl (5 µM) Primer F, 1.2 µl (5 µM) Primer R, 0.5 µl Taq Polymerase (1 unit) and 2.0 µl DNA template. The reaction condition was optimized in a thermal cycler (Eppendorf) using initial denaturation at 95^0 C for 5 min, 35 cycles of denaturation at 94^0 C for 1 min, primer annealing at 54 - 60^0 C (varied with different primers) for 1 min and extension at 72^0 C for 1 min followed by final extension at 72^0 C for 7 min and cooling at 4^0 C. After amplification, the amplified products were subjected to agarose (2%) gel

electrophoresis at 120 V for one and half hour and then visualized and documented under gel documentation system (Alpha Innotech, USA). The size of amplified fragment was estimated with the help of gel reader (Alpha View Gel Reader) and 50 bp ladder (Fermentas).

Allelic diversity of the markers was assessed by calculating the polymorphic information content (Anderson *et al.*, 1993) [1] of the primer pairs as follows.

$$PIC_i = 1 - \sum_{j=1}^{k} P^2{}_{ij}$$

Where, k is the total number of alleles detected for a marker; P_{ij} is the frequency of the jth allele for i^{th} marker and summation extends over k alleles.

Suitability of the marker based polymorphism for characterization and differentiation of the entries was evaluated by computing the polymorphism per cent (PP) as follows:

PP = (Number of unique alleles/Total number of alleles) x 100

Efficacy of individual primers in differentiation of the genotypes was assessed by computing the discrimination coefficient (Yadav *et al.*, 2003) [15] as follows:

DC=Number of pairs of genotypes discriminated/Total number of pairs of genotypes

Binary data generated as discrete variables on the basis of presence or absence of the bands in different entries was utilized for computation of genetic similarities. The genetic associations were analyzed by calculating the similarity coefficient (Dice, 1945) [3] for pair-wise comparisons based on the proportions of shared bands produced by primers as follows:

Similarity coefficient = 2a/(2a+b+c)

Where,

a = Number of shared bands between Jth and Kth genotypes

b = Number of bands present in Jth genotype but absent in Kth genotype

c = Number of bands absent in jth genotype but present in Kth genotype

The method employed for tree building in the cluster analysis involved sequential agglomerative hierarchical non-overlapping (SAHN) clustering based on similarity coefficients and the dendrogram was obtained by un-weighted pair-group method using arithmetic mean (UPGMA). Analysis was performed with the help of NTSYS-pc software (Rohlf, 1997) [11]. The nature of differentiation and divergence between entries under evaluation was investigated by a comparison of the clusters and neighbor joining tree. Principal coordinate analysis was conducted to obtain a two-dimensional ordination of the genetic profiles of the elite genotypes and inter-genotypic mixtures.

Table 1: List of six primers utilized for targeted amplification of genomic regions in six aerobic rice genotypes and twelve inter-genotypic mixtures

Sl. No	Primer	Chromosome number	Primer sequence (5'-3')	Repeat motif	Annealing temp. (^{0}C)
1.	RM 263	2	(F)CCCAGGCTAGCTCATGAACC (R)GCTACGTTTGAGCTACCACG	(CT)$_{34}$	60
2.	RM 3530	1	(F)GTAGATCCGGTCAGCTCCTC (R)CAAGGAGATTCCCTTCCATG	(CT)$_{39}$	60
3.	RM 5359	1	(F)CGTGATCTCGTGCATCCC (R)CCCTCAGGAGCTTCATGAAC	(TC)$_{13}$	54
4.	RM 337	8	(F)GTAGGAAAGGAAGGGCAGAG (R)CGATAGATAGCTAGATGTGGCC	(CTT)$_{4-19}$ (CTT)$_8$	57
5.	RM 538	5	(F) GGTCGTTGAAGCTTACCAGC (R) ACAAGCTCTCAAAACTCGCC	(GA)$_{14}$	58
6.	RM 558	12	(F)GAACTCCTCGAACTCGATGC (R)AGGCATTCAACCTGTTCGAC	(CT)$_{39}$	60

Result and Discussion

The amplification of targeted genomic regions using six microsatellite primers exhibited different levels of polymorphism among the eighteen entries including six genotypes and twelve inter-genotypic mixtures under evaluation in the present investigation. Molecular level genetic polymorphism among the entries was recognized on the basis of presence or absence and size of bands, in addition to variation in respect of number and relative position of bands (Figure 1). The primer pairs differed remarkably in their ability to reveal variability among the six genotypes (Table 2). All the primer pairs utilized in the present study generated unique alleles, but the number and proportion of unique alleles varied considerably among the primer pairs. While some of the primers generated several allelic variants due to variation in the length of simple sequence repeats among the genotypes, some generated only few. Altogether 30 allelic variants were detected among the six genotypes with an average of 5.0 alleles per primer. The number of alleles per primer pair ranged from four in the cases of RM 538 and RM 558 to six in the cases of RM 263 and RM 337.

Allelic variants identified among the six genotypes were classified into shared alleles; and unique alleles. A total of 10 shared and 20 unique allelic variants were generated in the form of amplified products. The number of unique alleles per primer pairs ranged from two alleles in the cases of RM 538 and RM 558 to five alleles in the case of RM 263 (Table 2). The primer pairs RM 263, RM 5359 and RM 337 generated considerably greater percentage of unique alleles and exhibited remarkably higher polymorphism amongst the genotypes (Mahajan *et al.*, 2011; Sandhu *et al.*, 2012; Kumar *et al.*, 2015) [12, 8]. Allele size differences recorded amongst the genotypes varied from 9 bp with RM 558 to 55 bp with RM 263. The primer pairs RM 263, RM 337, RM 3530 and RM 5359 generated more than one amplified product in combinations with some of the genotypes under evaluation, indicating most probably the existence of residual heterozygosity in the genotypes.

Polymorphic information content of each of the primer pairs revealing simple sequence length polymorphism based allele diversity and frequency among the genotypes (Anderson *et al.*, 1993) [1] varied from 0.417 the case of RM 3530 to 0.777 in the case of RM 5359 with an average of 0.652. Considerably greater numerical value was obtained in the cases of primer pairs RM 5359, RM 263, RM 558 and RM 538 in descending order of magnitude. Among the six primers, RM 263, RM 5359 and RM 337 had considerably greater number of allelic variants and polymorphism per cent in terms of percentage of unique alleles, reflecting their remarkably greater ability to discriminate the genotypes. The inference derived above was well supported by the data on discrimination coefficient (Yadav *et al.*, 2013) [15], clearly reflecting greater efficacy of these three primers in discrimination of the pair-wise combinations of genotypes.

Genetic similarities computed amongst the genotypes by a comparison on the basis of presence and absence of amplified products indicated ample genetic variation at the molecular level among the genotypes (Mahajan *et al.*, 2011; Sandhu *et al.*, 2012; Yadav *et al.,* 2013) [12, 15, 8]. The larger range of similarity coefficients provided greater confidence for the investigation on nature and magnitude of genetic differentiation, facilitating the test of genetic purity of the entries. A perusal of the data on similarity coefficients revealed that the highest similarity in respect of six microsatellite primers dependent regions of the genome existed between rice genotypes IR-64 and AER-06 (0.4000) amongst pair-wise combinations of genotypes. This was followed by similarity between IR-64 and AER-04 (0.3077), AER-04 and AER-05 (0.2667), IR-64 and AER-05 (0.2500), AER-05 and AER-06 (0.2353), Rasi and AER-04 (0.1667), Rasi and IR-64 (0.1538), AER-04 and AER-06 or AER-02 and AER-06 (0.1429) in descending order of magnitude. The primers used in the present study did not reveal any similarity between other pair-wise combinations. As revealed by dendrogram, the genotypes were basically classified into a mono-genotypic group containing AER-02 and a multi-genotypic group accommodating remaining five genotypes. Microsatellites based polymorphism revealed in the form of variation in the length of simple sequence repeats appeared to be an efficient tool for differentiation and genotyping of aerobic rice genotypes. Apparently therefore, the primers utilized in the present study were sufficient for complete discrimination and unambiguous identification of the six genotypes of aerobic rice.

Usefulness of these markers based simple sequence length polymorphism for genetic purity assessment and authentication of aerobic rice genotypes was established by the analysis of marker generated polymorphism between the aerobic rice genotypes and inter-genotypic mixtures. Principal coordinate analysis for displaying relationships among objects in terms of their positions along a set of axes (Fig. 2) clearly exhibited the genetic associations amongst the aerobic rice genotypes and inter-genotypic mixtures under evaluation. Spatial distribution pattern revealed by two dimensional ordinations of the genetic profiles of the genotypes and genotypic mixtures along the two principal axes indicated that the inter-genotypic mixtures were more or less invariably placed at nearly intermediate positions between the corresponding referral genotypes.

Experimental results obtained from principal coordinate analysis were almost in complete agreement with the genetic relationships reflected by the neighbor joining tree (Figure 3) and the dendrogram generated by unpaired group method using mathematical average (Figure 4). Aerobic rice genotypes were observed to be closely associated with the genotypic mixture(s) containing the genotype(s) in question, validating that the microsatellite markers used in the present study can be effectively and efficiently utilized for individualization and authentication of the aerobic rice entries used in the present study. Among the six primer pairs employed during molecular characterization of aerobic rice genotypes, three primer pairs, namely, RM 263, RM 337 and RM 5359, appeared to be relatively more polymorphic and comparatively more informative than rest of the primer pairs. These three primer pairs exhibited easily recognizable and clearly recordable polymorphism allowing genotypic authentication and unambiguous differentiation of the genotypes and genotypic mixtures. Molecular profile of the inter-genotypic mixtures based on these three primers showed a complementary banding pattern representing the bands existing or missing in referral genotypes, thereby completely distinguishing them from their inter-genotypic mixtures. Evidently therefore, molecular fingerprinting data based on these three primer pairs served as distinct molecular tags for distinguishing the genotypes and inter-genotypic mixtures and provided a sound basis for genetic purity evaluation of the genotypes at least with a single marker allele difference.

Use of morphological differences between genotypes for genotypic authentication and genetic purity evaluation is not always effective, accurate and efficient. Alternatively, molecular markers can be more accurately and efficiently employed in genotypic authentication and genetic purity

analysis (Nandkumar *et al*., 2004; Xin *et al*., 2005; Hashemi *et al*., 2009; Tamilkumar *et al*., 2009; Youssef *et al*. 2011; Kumar *et al*., 2012; Deshmukh *et al.,* 2013; Kumar *et al*., 2015) [5, 13, 6, 7]. Analysis of microsatellites based amplification profiles of aerobic rice genotypes and inter-genotypic mixtures in the present study finally led to identification and validation of three microsatellite primer pairs, which allowed unambiguous differentiation of referral genotypes from inter-genotypic and varietal mixtures. These three microsatellite primer pairs may be purposefully and effectively utilized for identification, authentication and genetic purity assessment of other promising genotypes of aerobic rice, provided the genetic polymorphism at molecular level is established with these markers.
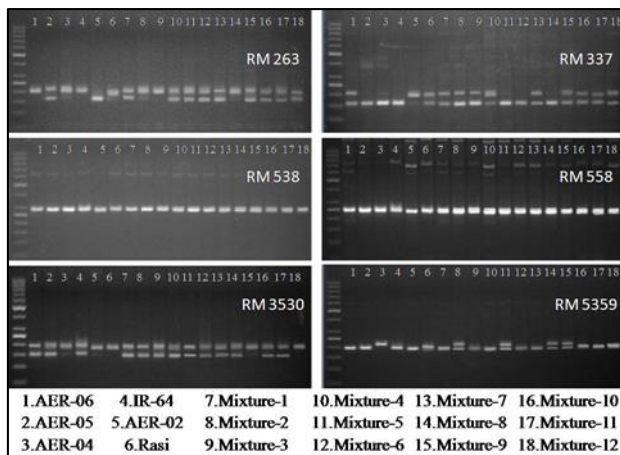

Fig 1: Amplification patterns of the six primer based genomic regions in six aerobic rice genotypes and twelve inter-genotypic mixtures
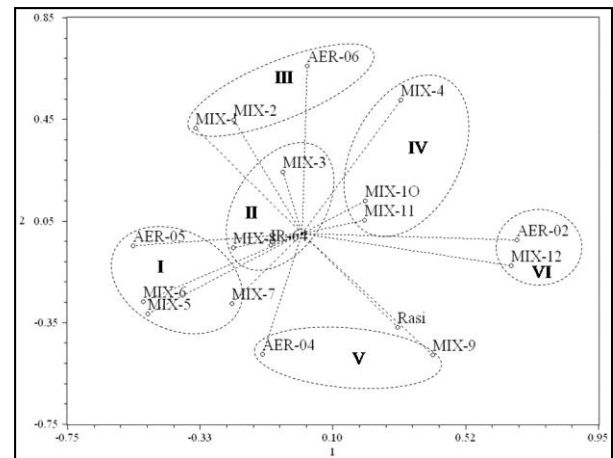

Fig 2: Two-dimensional ordinations of the genetic profiles of six aerobic rice genotypes and twelve inter-genotypic mixtures
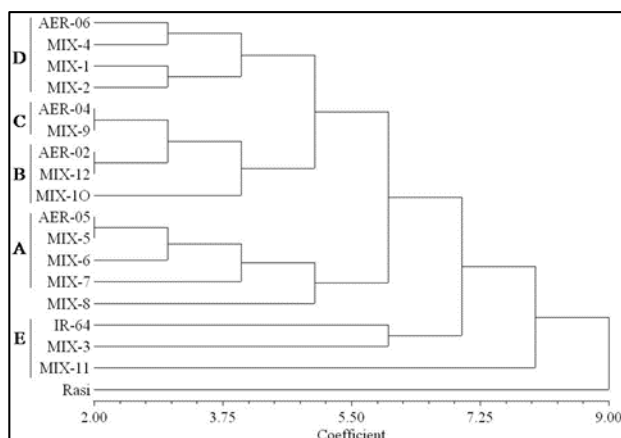

Fig 3: Similarity indices based neighbor joining tree of the six aerobic rice genotypes and twelve inter-genotypic mixtures
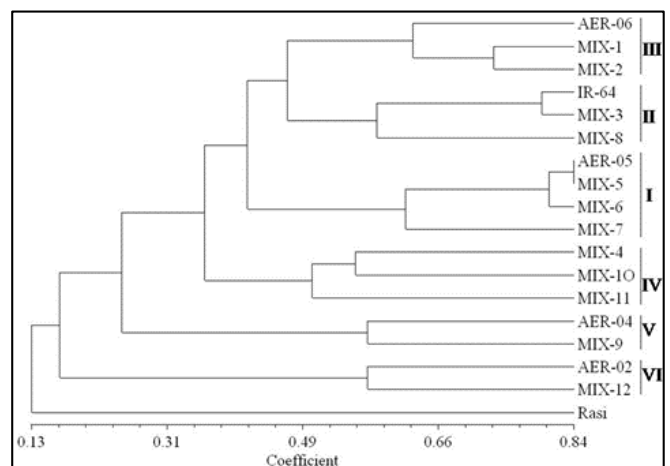

Fig 4: Similarity indices based dendrogram of the six aerobic rice genotypes and twelve inter-genotypic mixtures

Table 2: Analysis of primer pairs used for the amplification of targeted genomic regions in the six genotypes of aerobic rice

Primer	Allele size range (bp)	Allele size difference (bp)	No. of alleles	No. of unique alleles	PP	PIC	DC
RM 538	284 - 293	09	4	2	50.00	0.722	0.866
RM 3530	152 - 197	45	5	3	60.00	0.417	0.933
RM 263	165 - 217	55	6	5	83.33	0.750	1.00
RM 558	236 - 247	11	4	2	50.00	0.723	0.866
RM 5359	197 - 221	24	5	4	80.00	0.777	0.933
RM 337	160 - 210	50	6	4	66.66	0.528	0.933

PP: Polymorphism per cent; PIC: Polymorphism information content; DC: Discrimination coefficient

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