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Evaluation of simple sequence length polymorphism for genetic discrimination and genotyping of aerobic rice genotypes

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Abstract
A study was conducted using a set of eight simple sequence repeat primer pairs and simple sequence length polymorphism was examined in five aerobic and one aromatic rice genotypes to investigate the nature and magnitude of differentiation and divergence among them at the molecular level. Additionally, the primer pairs were also tested and validated for their utilization in genetic purity assessment of these genotypes using twelve inter-genotypic mixtures consisting of pair-wise genotypic combinations. Altogether 38 allelic variants were detected with an average of 4.75 alleles per primer. Considerably greater percentage of unique alleles was generated by the primer pairs RM 153, RM 206, RM 332, RM 3530 and RM 5373. Polymorphic information content of the primers varied from 0.515 to 0.792 with an average of 0.659 per primer. Using the number of alleles along with the level of polymorphism and discrimination ability of the primers as the basis, RM 332 appeared to be highly polymorphic and comparatively more informative for the purpose of molecular characterization. Analysis of differentiation and divergence pattern based on amplification profiles allowed trenchant distinction of aromatic entry from rest of the entries belonging to aerobic rice. An intermediate and complementary banding pattern was observed in inter-genotypic mixtures that served as distinct molecular tags for distinguishing the genotypes and their inter-genotypic mixtures. Easily recognizable differentiation of elite genotypes from inter-genotypic mixtures finally led to validation of primer pairs RM 206, RM 224, RM 263, RM 3530 and RM 5359 for genetic purity analysis. These five primer pairs may be further utilized in genetic purity test and confirmation of aerobic rice genotypes.

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

Introduction
Aerobic rice is a revolutionary way of rice production in well-drained, non-puddled, and non-saturated soils without pounded water. This system uses input-responsive specialized rice cultivars and complementary management practices to achieve high yield using only limited amount of the water (Amudha, 2013) [1]. Aerobic rice is grown on dry soils with surface irrigations provided when necessary with intensive agronomic practices and substantial water savings (Parthasarathi *et al*., 2012) [21]. Rice varieties that are suitable for aerobic production systems grow in soil with moisture content at or below field capacity (Predeepa, 2012) [22] and emit lesser methane gas into the atmosphere, thus, keeping the environment safe.

Conventional methodology based on a specific set of phenotypic and agronomic characters to characterize genotypes is time-consuming, restricted to a few characteristics, influenced by environmental condition and inefficient also. Analyses of genotypic differentiation and divergence using morphological and biochemical markers in many cases lack the resolution power to reveal the distinct polymorphisms and to differentiate between closely related genotypes. The information pertaining to extent of variation present and genetic relationships among the existing genotypes is an important consideration for designing the future breeding programs. Further, unambiguous identification of elite genotypes is essential for their protection and prevention of unauthorized commercial use. A set of qualitative and quantitative characters known as descriptors are used for genotype identification and description. However, the presence of smaller amount of genetic variability among them makes it more difficult to unambiguously identify and distinguish them on the basis of morphological characteristics because of the influences by environmental factors. Further, molecular tools can facilitate in identification of genomic regions for traits potentially related to performance under water deficit (Lafitte *et al*., 2002) [16].

Recently, genomic markers assisted identification with high power of genetic resolution has emerged as a robust technique for genotype fingerprinting, identity profiling, estimating and comparing genetic similarity and variety protection.

Fingerprinting with molecular markers allows precise, objective and rapid genotype identification, which has been proved to be an efficient tool for crop germplasm characterization, collection and management. Amongst the several classes of genomic markers currently available, genetically mapped microsatellite markers, also known as simple sequence repeat markers, are of tremendous value. Such markers are considered as most amenable for several applications including interrelationship and genetic diversity studies in rice due to their multi-allelic nature, high reproducibility, co-dominant inheritance, abundance, extensive genome coverage and simple reproducible assays. These can be easily and economically assessed by polymerase chain reaction and polymorphic amplified fragments can be produced due to difference in the number of the repeat units.

Technical efficiency of microsatellite markers because of their multiplex potential, chromosome specificity, greater level of allelic diversity, high power of resolution, operational ease and low cost, make these markers more suitable and preferable for fingerprinting, high throughput mapping, genetic analysis and marker assisted improvement in rice including aerobic rice (Mahajan *et al*., 2011; Sandhu *et al*., 2012; Kumari and Sharma, 2014; Singh and Sengar, 2015; Kumari *et al*., 2018) [19, 24, 14, 27, 15]. A carefully chosen set of simple sequence repeat markers has been documented to facilitate molecular profiling of genotypes and testing of the genetic purity in rice (Chuang *et al*., 2011; Ma *et al*., 2011; Youseef *et al*., 2011; Deshmukh *et al*., 2013; Kumar *et al*., 2012; Kumar *et al*., 2015; Bora *et al*., 2016; Kumari *et al*., 2018) [4, 18, 32, 5, 12, 13, 3, 15]. Taking into consideration that such molecular profiles would be especially valuable to unambiguously distinguish the genotypes and to precisely test the genetic purity of genotypes, the present investigation was undertaken to evaluate simple sequence repeats based polymorphism for genetic discrimination and genotyping of rice genotypes and to validate the informative markers for genetic purity test of aerobic rice genotypes.

Materials and Methods

The materials of the present investigation comprised six rice genotypes and twelve inter-genotypic seed mixtures containing pair-wise combinations of the six genotypes. Seeds of all together eighteen entries comprising five aerobic rice genotypes, namely, AER-02 (IR 81449-8-8-128-1), AER-03 (IR 79913-8-176-8-4), AER-04 (IR 80312-6-8-3-2-8), AER-05 (IR 55423-1), AER-06 (IR 78875-207-8-1-8) and one aromatic rice genotype PRR-78 along with their twelve inter-genotypic mixtures, such as, MIX-01 (AER-06+AER-05), MIX-02 (AER-06+AER-04), MIX-03 (AER-06+AER-03), MIX-04 (AER-06+AER-02), MIX-05 (AER-06+PRR-78), MIX-06 (AER-05+AER-04), MIX-07 (AER-05+AER-03), MIX-08 (AER-05+AER-02), MIX-09 (AER-05+PRR-78), MIX-10 (AER-04+AER-03), MIX-11 (AER-04+AER-02), MIX-12 (AER-04+PRR-78) were planted in plastic pots. Leaf samples were collected from the three weeks old seedlings of each entry and used for DNA isolation. Total genomic DNA was isolated by using CTAB method following the standard protocol (Ferdous *et al*., 2012) [7] with slight modifications. The purification of isolated DNA samples was completed by RNase treatment and DNA pellet was dissolved in TE buffer.

Using standard protocol of polymerase chain reaction adjusted to laboratory conditions, amplification of specified genomic regions was selectively performed with known eight pairs (Table 1) of forward and reverse microsatellite primer pairs in 15 µl reaction mixture prepared by a combination of 2.8 µl water (Protease and Nuclease free), 3.0 µl 5X PCR buffer containing 15 mM $MgCl_2$, 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 amplification condition was optimized in a thermal cycler (Eppendorf) using initial denaturation at 95^0 C for 3 min, 35 cycles of denaturation at 94^0 C for 40 sec, primer annealing at 48 - 60^0 C (varied with different primers) for 40 sec and extension at 72^0 C for 1 min followed by final extension at 72^0 C for 10 min and cooling at 4^0 C. After completion of reaction cycles, the amplified products were resolved by agarose (2%) gel electrophoresis at 120 V for one and half hour and then visualized and documented under gel documentation system (Alpha Innotech, USA). Molecular size of amplified fragment was determined in relation to the size of markers in the ladder (50 bp) with the help of gel reader (Alpha View Gel Reader).

Assuming the location of well as initial position (Rf=0) and the position of migrated dye as final position (Rf=1) as a frame of reference, the Rf value was determined for each band. The position of the bands on the gel corresponded to the location along Y-axis (ranging from 0 to 1030). The polymorphism was recorded on the basis of presence or absence of bands in different entries under investigation. All the entries were scored for the presence and absence of bands and the data generated by this exercise were entered into binary matrix as discrete variables and then this data matrix was subjected to further analysis.

Table 1: List of eight microsatellite primers utilized for amplification of targeted genomic regions in eighteen entries of rice

Sl. No.	Primer	Ch. No.	Primer sequence (5'-3')	Repeat motif	Annealing temp. (^{0}C)
1.	RM 153	5	(F)GCCTCGAGCATCATCATCAG (R)ATCAACCTGCACTTGCCTGG	$(GAA)_9$	56
2.	RM 206	11	(F)CCCATGCGTTTAACTATTCT (R)CGTTCCATCGATCCGTATGG	$(CT)_{21}$	56
3.	RM 224	11	(F)ATCGATCGATCTTCACGAGG (R)TGCTATAAAAGGCATTCGGG	$(AAG)_8(AG)_{13}$	48
4.	RM 263	2	(F)CCCAGGCTAGCTCATGAACC (R)GCTACGTTTGAGCTACCACG	$(CT)_{34}$	60
5.	RM 332	11	(F)GCGAAGGCGAAGGTGAAG (R)CATGAGTGATCTCACTCACCC	$(CTT)_8(CTT)_{14}$	48
6.	RM 3530	1	(F)GTAGATCCGGTCAGCTCCTC (R)CAAGGAGATTCCCTTCCATG	$(CT)_{39}$	60
7.	RM 5373	10	(F)GGAGATGCTATAGCAGCAGTG (R)ATTGCTCCTTACCACCTTGC	$(TC)_{13}$	56
8.	RM 5359	1	(F)CGTGATCTCGTGCATCCC (R)CCCTCAGGAGCTTCATGAAC	$(TC)_{13}$	54

Suitability of the marker based polymorphism for characterization and differentiation of the entries was evaluated by computing the polymorphism per cent (Kumari *et al*., 2018) [15]. Allelic diversity revealed by the primers was assessed by computing the polymorphism information content (PIC) of the primer pairs (Anderson *et al*., 1993) [2] as follows:

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

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

Similarity with respect to targeted genomic regions amongst entries was evaluated on the basis of presence and absence of common bands. The genetic associations were analyzed by calculating the similarity coefficient (Dice, 1945) [6] for pair-wise comparisons based on the proportions of shared bands produced by primers as follows.

$$\text{Similarity coefficient} = 2a/(2a+b+c)$$

Where, a, b and c represent number of shared bands between J^{th} and K^{th} genotypes, number of bands present in J^{th} genotype but absent in K^{th} genotype and number of bands absent in J^{th} genotype but present in K^{th} genotype, respectively.

Cluster analysis was performed using the data on similarity coefficients. The method used for tree building in the cluster analysis involved sequential agglomerative hierarchical non-overlapping (SAHN) clustering and the dendrogram based on similarity indices was obtained by un-weighted pair-group method using arithmetic mean (UPGMA). Principal coordinate analysis was used to obtain a two-dimensional ordination of the genetic profiles of the elite genotypes and inter-genotypic mixtures. Computational analysis was performed with the help of NTSYS-pc software (Rohlf, 1997) [23]. The differentiation and divergence pattern of the entries under evaluation was examined by identifying the clusters at appropriate phenon levels and comparing the clusters and neighbor joining tree.

Result and Discussion

Ample genetic polymorphism among the entries was revealed by the amplification of targeted genomic regions using the eight primers specific to the unique flanking sequences of the simple sequence repeats. Genetic polymorphism among the entries manifested in the form of presence or absence of bands, in addition to variation in respect of number and position of bands (Fig. 1). Remarkable differential ability of the primer pairs to reveal genetic variability among the genotypes under evaluation was clearly evident from an analysis of the allelic diversity (Table 2). Although all the primer pairs generated unique alleles, the number and proportion of unique alleles varied considerably with the primer pairs (Mahajan *et al*., 2011; Sandhu *et al*., 2012; Kumari and Sharma, 2014; Kumar *et al*., 2015; Kumari *et al*., 2018) [19, 24, 14, 13, 15]. It was observed that some of the primers yielded several allelic variants due to variation in the length of simple sequence repeats among the genotypes. Contrarily, some of the primers generated only few alleles due to amplification of primer specific regions.

Table 2: Analysis of primer pairs used for the amplification of targeted genomic regions in eighteen entries of rice.

Primer	Allele size difference (bp)	Allele size range (bp)	No. of alleles	No. of unique alleles	No. of shared alleles	PP	PIC
RM 153	11	202 - 213	4	3	1	75.00	0.675
RM 206	38	132 - 170	3	2	1	66.60	0.515
RM 224	25	138 - 163	4	1	3	25.00	0.651
RM 263	51	165 - 216	6	3	3	50.00	0.601
RM 332	14	179 - 193	5	4	1	80.00	0.792
RM 3530	46	156 - 202	8	6	2	75.00	0.634
RM 5373	09	118 - 127	4	3	1	75.00	0.675
RM 5359	28	189 - 217	4	2	2	50.00	0.734

PP: Polymorphism per cent; PIC: Polymorphism information content

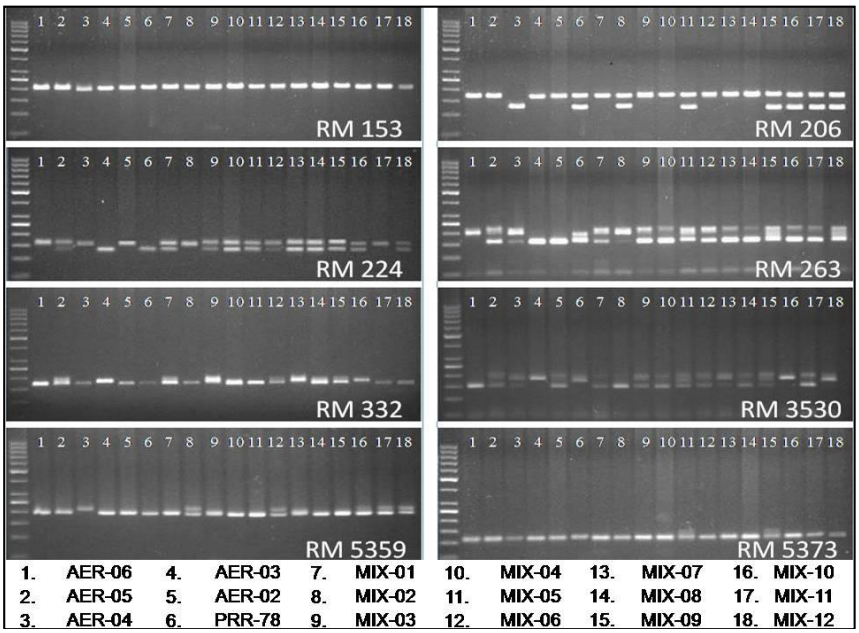

Fig 1: Amplification patterns of the targeted genomic regions in six rice genotypes and twelve inter-genotypic mixtures using eight microsatellite primers

Altogether 38 allelic variants were detected among the six genotypes with an average of 4.75 alleles per primer. The smallest (9 bp) and the largest (51 bp) differences in allele size range were recorded for RM 5373 and RM 263, respectively. Scoring of allelic variants further indicated that the number of alleles per primer pair ranged from three in the case of RM 206 to eight in the case of RM 3530. Using the panel of eight primer pairs, a total of 14 shared and 24 unique allelic variants were generated in the form of amplified products. The number of unique alleles per primer pairs ranged from one allele out of four alleles in the case of RM 224 to six alleles out of eight alleles in the case of RM 3530. Among the eight primer pairs used during molecular profiling, RM 153, RM 263, RM 332, RM 263, RM 3530 and RM 5373 generated considerably greater percentage of unique alleles. Only four primer pairs, namely, RM 153, RM 332, RM 5375 and RM 5359, amongst the eight primer pairs utilized in the study, generated single but polymorphic amplified product. Contrarily, the primer pairs RM 206, RM 224, RM 263 and RM 3530 yielded more than one amplified product in combinations with some of the genotypes under evaluation most probably because of the presence of residual heterozygosity in the genotypes.

Simple sequence length polymorphism exhibited by the eight primer pairs specific to the unique flanking sequences of the simple sequence repeats was further analyzed by a comparison of polymorphism information content (Anderson *et al*., 1993) [2] of each of the primer pairs. Allelic diversity and allelic frequency among the genotypes, as deduced from the numerical values, varied from 0.515 in the case of RM 206 to 0.792 in the case of RM 332 with an average of 0.659 per primer pair. Considerably greater magnitude reflecting higher polymorphic information content (Mahajan *et al*., 2011; Sandhu *et al*., 2012; Kumari and Sharma, 2014; Kumari *et al*., 2018) [19, 24, 14, 15] was obtained for the primer pairs RM 332, RM 5359, RM 5373 and RM 153 in descending order of magnitude. The primer pairs RM 3530, RM 263 and RM 332 generated considerably greater number of allelic variants due to variation in the length of simple sequence repeats based amplified products. Among the primer pairs which had higher polymorphic information content and generated greater number of allelic variants, RM 332 detected considerably greater percentage of unique alleles and appeared to be highly polymorphic and comparatively more informative for the purpose of molecular characterization of entries under evaluation.

The range of similarity coefficients of pair-wise combinations of the six genotypes (0.111 to 0.421) indicated a considerably greater extent of genetic variation and provided greater confidence for the evaluation of nature and magnitude of genetic differentiation and divergence amongst the genotypes. Numerically, the magnitude of similarity coefficient between AER-06 and AER-05 (0.421) was found to be the maximum amongst different pair-wise combinations followed by the similarity coefficient between AER-03 and AER-02 (0.352), AER-5 and AER-02 (0.300), AER-05 and AER-04 (0.285), AER-06 and AER-02 (0.235), AER-05 and AER-03 or AER-04 and AER-02 (0.210), AER-06 and AER-03 (0.125), PRR-78 and AER-03 or AER-06 and AER-04 or AER-04 and AER-03 (0.111) in descending order of magnitude. The magnitude of similarity coefficient between PRR-78 and AER-03 or AER-06 and AER-04 or AER-04 and AER-03 was found to be the same (0.111). Similarly, the similarity coefficient between AER-05 and AER-03 or AER-04 and AER-02 was observed to be equal in magnitude (0.210). Any similarity for targeted genomic regions between PRR-78 and the aerobic genotypes included in the study, with the exception of AER-03, was not displayed and the magnitude was equal to zero in all the cases.

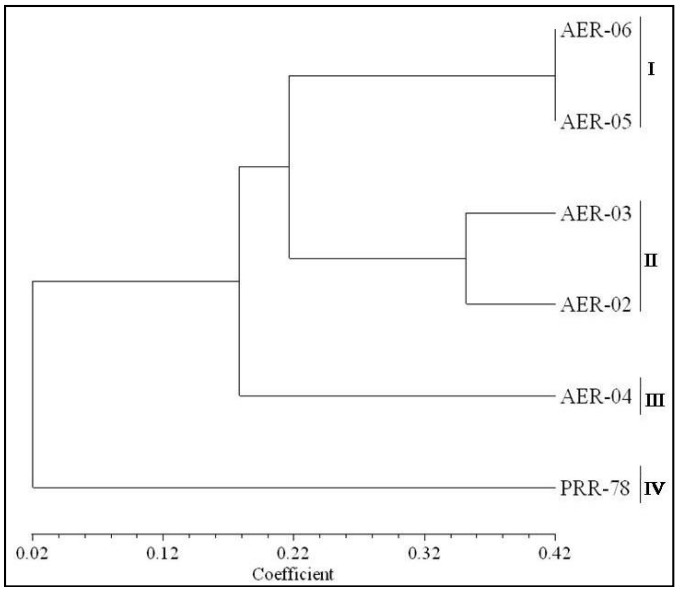

Fig 2: Dendrogram of six rice genotypes based on eight microsatellite primers dependent similarity indices

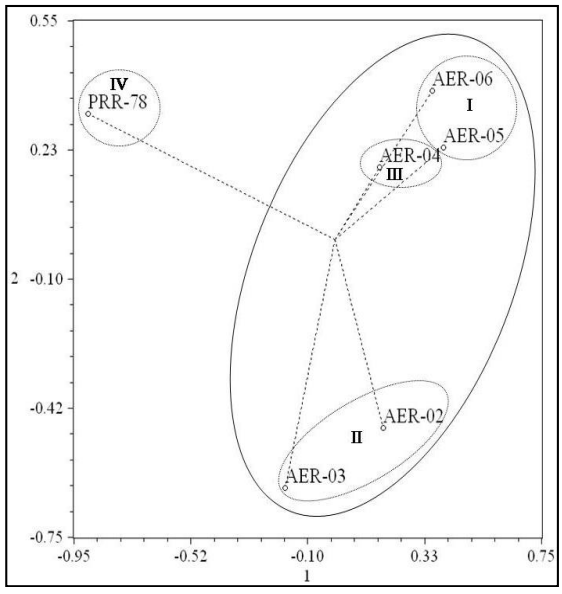

Fig 3: Spatial distribution pattern of eight microsatellite primers based genetic profiles of six rice genotypes

Taking into consideration broad classification pattern as inferred from a perusal of dendrogram (Fig. 2), the six genotypes seemed to be divided into two groups. The first group consisted of five aerobic rice genotypes and the second group accommodated the aromatic rice genotype PRR-78. While considering fifty or even seventy-five similarity units as cut off point, the multi genotypic group was further divided into three clusters; (a) di-genotypic cluster I accommodating AER-06 and AER-05; ((b) di-genotypic cluster II accommodating AER-03 and AER-02; and (c) a mono-genotypic cluster III that included AER-04. The di-genotypic cluster II was further sub-divided into two sub-clusters by drawing the phenon line at eighty-five similarity units as cut-off point. These two mono-genotypic sub-clusters included

the aerobic genotypes AER-03 and AER-04, respectively. Hierarchical classification pattern of rice genotypes was in complete agreement with the spatial distribution pattern of the microsatellite primers specific genetic profiles of the genotypes along the two principal axes (Fig. 3). Therefore, utilization of eight microsatellite markers in the molecular characterization of rice genotypes showed a high level of genetic polymorphism, which allowed unique genotyping and unambiguous classification of five aerobic and one aromatic rice genotypes included in the analysis.

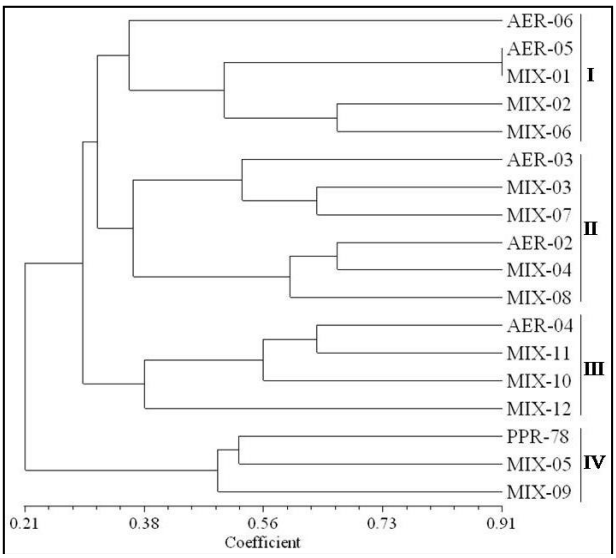

Fig 4: Dendrogram based on similarity indices of pair-wise combinations of six rice genotypes and twelve inter-genotypic mixtures

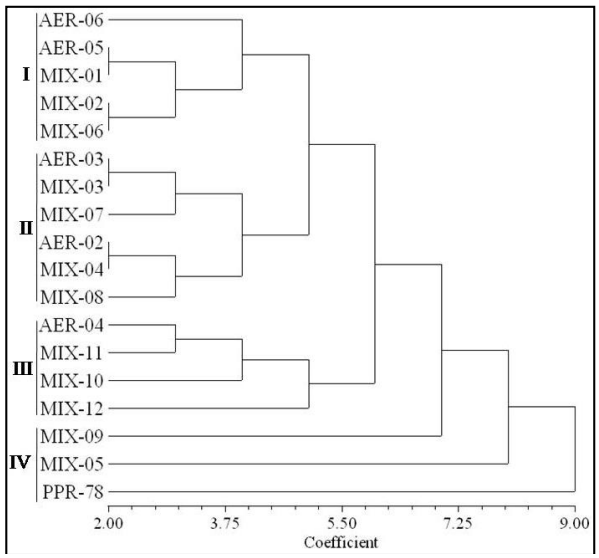

Fig 5: Neighbor joining tree based on similarity indices of pair-wise combinations of six rice genotypes and twelve inter-genotypic mixtures

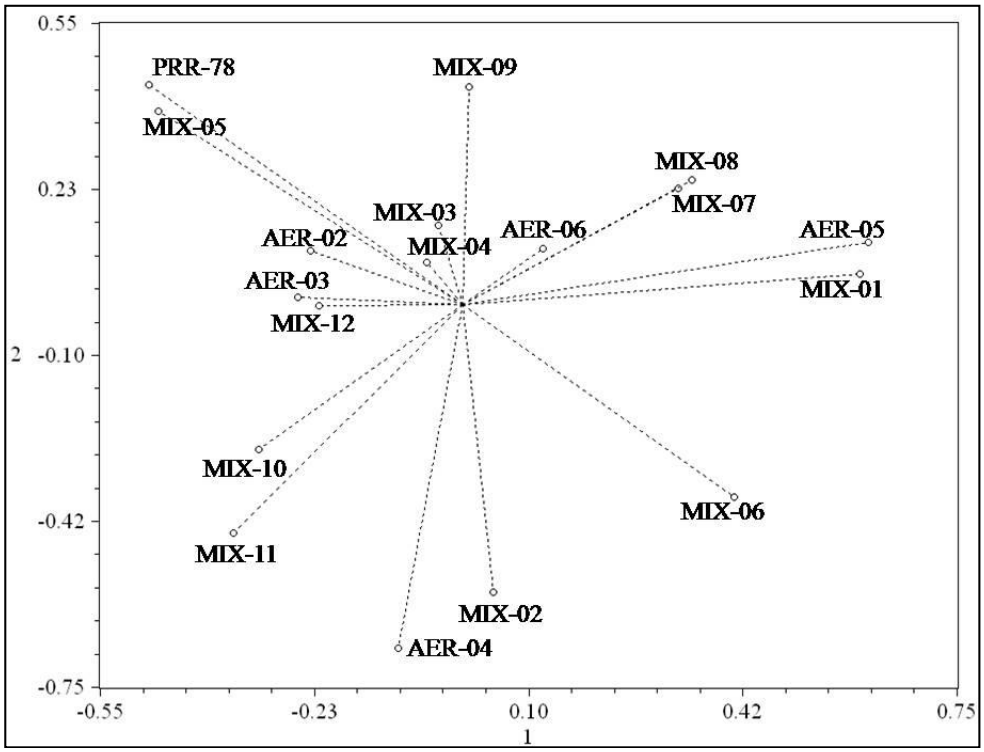

Fig 6: Principal coordinate analysis based two dimensional ordinations of eight microsatellite primers dependent genetic profiles of six rice genotypes and twelve inter-genotypic mixtures

Hierarchical classification based on analysis of marker generated polymorphism between the rice genotypes and their inter-genotypic mixtures established the usefulness of simple sequence length polymorphism for genetic purity test and confirmation of aerobic rice genotypes (Fig. 4). It is evident from a perusal of dendrogram that the genotypic mixture(s) containing pair-wise combinations of six genotypes were clearly discriminated but closely associated with the referral genotype(s) in question, suggesting that the panel of microsatellite markers utilized in the present study can be effectively and efficiently employed for individualization and confirmation of these aerobic rice entries. Neighbor joining tree (Fig. 5) and principal coordinate analysis exhibited more or less similar type of genetic associations amongst the aerobic rice genotypes and inter-genotypic mixtures under evaluation. Spatial distribution pattern of the genetic profiles of the genotypes and genotypic mixtures along the two principal axes showed that the inter-genotypic mixtures were

more or less invariably placed at nearly intermediate positions between the corresponding referral genotypes (Fig. 6), which were the components of genotypic mixture in question.

Molecular profiling with the primer pair RM 206 yielded two distinct bands in the Mixture-2, Mixture-5, Mixture-9, Mixture-10, Mixture-11 and Mixture-12, indicating the existence of complementary banding patterns in genotypic mixtures. Using primer pair RM 224 also, a complementary banding pattern in the form of two easily distinguishable bands with comparatively large allelic size difference was clearly observed in the Mixture-1, Mixture-3, Mixture-5, Mixture-6, Mixture-7, Mixture-8, Mixture-9, Mixture-10 and Mixture-12. Representing two different types of amplified products, presence of two distinct bands generated by primer pair 263 with comparatively large allelic size difference in the Mixture-1, Mixture-2, Mixture-3, Mixture-4, Mixture-5, Mixture-6, Mixture-7, Mixture-8, Mixture-10, Mixture-11 and Mixture-12, whereas three bands in the Mixture-9 showed a complementary banding pattern in genotypic mixtures. Similarly, appearance of two distinct bands generated by primer pair RM 3530 with comparatively large allelic size difference in the Mixture-1, Mixture-2, Mixture-3, Mixture-4, Mixture-6, Mixture-7, Mixture-8, Mixture-10 and Mixture-11, while three bands in the Mixture-5, Mixture-9 and Mixture-12 and two bands by the primer pair RM 5359 in the Mixture-2, Mixture-6, Mixture-10, Mixture-11 and Mixture-12 clearly demonstrated the complementary banding patterns generated by the primer pair directed amplification in genotypic mixtures.

Appraisal of the molecular fingerprinting data based on these five primer pairs apparently provided a basis for genetic purity test of referral aerobic rice genotypes at least with a single marker allele difference to differentiate the inter-genotypic mixtures from referral genotypes. The molecular profile of inter-genotypic mixtures based on these five primer pairs served as distinct molecular tags with complementary banding patterns, distinguishing the referral genotypes and inter-genotypic mixtures. Precise analysis of the amplification profiles, therefore, led to the validation of RM 206, RM 224, RM 263, RM 3530 and RM 5359 for genetic purity test of the six referral genotypes, since these primer pairs exhibited easily recognizable polymorphism between the genotypes and allowed unambiguous differentiation of the referral genotypes and genotypic mixtures. Usefulness of molecular markers in rice (Nandkumar *et al*., 2004; Xin *et al*., 2005; Hashemi *et al*., 2009; Tamilkumar *et al*., 2009; Youssef *et al*. 2011; Chuang *et al*., 2011; Kumar *et al*., 2012; Deshmukh *et al.*, 2013; Kumar *et al*., 2015; Bora *et al*., 2016) [20, 30, 9, 29, 32, 4, 12, 5, 13, 3] and other crop plants (Liu *et al*., 2007; Selvakumar *et al*., 2010; Gourishankar *et al*., 2013; Ye *et al*., 2013; Kumar *et al*., 2014; Jadhav and Verma, 2016) [17, 26, 8, 31, 11, 10] as more accurate and efficient approach in genotypic authentication and genetic purity analysis (Smith and Register, 1998; Scarano and Rao, 2014) [26, 25] has been established by several researchers.

The importance of two microsatellite markers, namely, RM 263 and RM 5359, for genetic differentiation and authentication of promising aerobic rice genotypes has been documented earlier (Kumari *et. al*., 2018) [15]. Critical 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 additional microsatellite primer pairs, which may be effectively and efficiently utilized for identification, authentication and genetic purity assessment of other promising genotypes of aerobic rice, provided these markers are found suitable and effective in revealing recognizable genetic polymorphism at molecular level among the genotypes.

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