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Choice of microsatellite markers for isolation of fertility restorers of wild abortive (WA) type cytoplasmic male sterility in rice

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Abstract

Simple sequence length polymorphism was examined in a set of eight effective restorers, four partial restorers, two weak maintainers and four complete maintainers of WA type CMS system in rice using a set of thirty-two microsatellite primer pairs in order to assess the nature and extent of genetic variation among them at molecular level. Altogether 248 allelic variants were detected with an average of 7.75 alleles per primer pairs. Polymorphic information content ranged from 0.508 for RM 6737 to 0.882 for RM 1 with an average value of 0.740 per primer pair. Amongst the primer pairs used, RM 1, RM 206, RM 591 RM 3873 and RM 8146 had remarkably higher number of allelic variants with greater gene diversity and genotype discrimination ability. Using similarity coefficient in numerical taxonomic approach of classification, the entries were differentiated and classified into different groups. Analysis based on molecular profiles allowed discrimination of effective fertility restorers from rest of the entries including partial fertility restorers, weak sterility maintainers and complete sterility maintainers. Principal coordinate analysis based two dimensional plotting of genetic profiles completely supported the results obtained from hierarchical classification of entries and effective fertility restorers were distinctly discriminated from the remaining entries. Four microsatellite primer pairs namely, RM 171, RM 315, RM 3873 and RM 6100, which allowed unambiguous discrimination of the eight effective fertility restorers from rest of the entries. These microsatellite primer pairs, may be further utilized in identification of effective fertility restorers of WA based CMS system in rice.

Key words: Rice, WA, CMS, SSR, PCA, gene diversity, fertility restoration

Introduction

Commercial exploitation of hybrid rice technology essentially demands that the hybrid should show appreciable extent of heterotic and economic advantage and there should be ease in large scale hybrid seed production because fresh seed has to be used for each planting. Nearly, five decades of experience has proved that strong and stable wild abortive (WA) type cytoplasmic male sterility (CMS) system based three-line method of hybrid seed production is an effective way to develop hybrid varieties of rice (Chen and Liu 2014; Horn et al. 2014; Huang et al. 2014) and will continue to be important in coming years. Identification of effective fertility restorers to ensure complete spikelet fertility is an important pre-requisite in hybrid rice breeding.

Traditional approach for identification of fertility restorer lines is based on an analysis of pollen fertility and spikelet fertility in test crosses involving cyto-sterile lines for the classification of pollen parents as maintainers or restorers. The pollen parents are considered as fertility restorers, if a high level of pollen fertility and spikelet fertility restoration is observed in test crosses (Priyanka et al. 2016). Recently, molecular markers have also been used by several researchers for tagging of fertility restorer genes and classification of pollen parents into the categories of sterility maintainers and fertility restorers (Huang et al. 2014; Chen et al. 2017). Amongst the several classes of molecular markers, microsatellite markers are regarded as the most amenable markers for various applications in rice due to their extensive genome coverage, multi-allelic nature, high reproducibility, co-dominant inheritance, abundance and simple reproducible assays. These can be easily and economically assessed by polymerase chain reaction using primers specific to the unique flanking sequences

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of the microsatellites and polymorphic amplified fragments can be produced due to difference in the number of the repeat units. Using microsatellite markers, the identification of fertility restorers and determination of genomic location of the fertility restorer loci have been attempted by earlier researchers (Jing et al. 2001; Ahmadikhan and Karlov 2006; Ahmadikhan et al. 2007; Bazrkar et al. 2008; Sattari et al. 2008; Sheeba et al. 2009; Alavi et al. 2009; Shah et al. 2012; Ghara et al. 2012; Revathi et al. 2013; Kumar et al. 2015). An attempt has been made in the present investigation to characterize the sterility maintainers and fertility restorers of WA type CMS system in rice using microsatellite markers and to generate information regarding suitability of different primer pairs for unambiguous discrimination and identification of fertility restorers.

Materials and methods

Experimental material of the present study comprised eighty five test crosses generated using four WA type CMS lines of rice in combinations with forty-five pollen parents having diverse genetic backgrounds. The test crosses were evaluated at the research farm of Dr. Rajendra Prasad Central Agricultural University, Pusa to identify commercially usable fertility restorers and sterility maintainers. Based on pollen and spikelet fertility analysis of test crosses, a set of eighteen pollen parents including eight effective restorers (PSRM 16-48, Ranvir Basmati, Sanwal Basmati, RAU 1-16-48, Rajendra Mahsuri, RAU 670, RAU 722 and RAU 1428-3), four partial restorers (RAU 1415-12, RAU 1415-32, RAU 1460-4 and RAU 1472-4), two weak maintainers (MTU 1120 and RAU 1515-32) and four complete maintainers (Rajendra Bhagwati, Rajendra Suwasni, Dhanlakshmi and Pusa 1121) were identified and subjected to microsatellite profiling. Seedlings of these entries were raised by placing the seeds over two layers of wet filter paper kept in the petridishes at room temperature. Total genomic DNA was extracted from young leaves of two weeks old seedlings using CTAB method (Saghai-Maroof, 1984). The quality and quantity of extracted DNA were determined spectrophotometrically by taking absorbance at 260 nm and 280 nm (Varian Cary 50 Spectrophotometer). The extracted DNA samples were diluted using T10E1 buffer to get the working concentration of 50ng/μl and utilized for microsatellite profiling.

Using a panel of 32 microsatellite primer pairs covering ten of the twelve chromosomes (Table 1), simple sequence length polymorphism amongst the eighteen entries was analyzed (Kumar and Sharma 2013). A 50 bp DNA ladder (Gene ruler, Fermentas) was used as size marker to compare the molecular weights of amplified products. The gels were documented using a gel documentation system (Alpha Innotech) and size of amplified product was determined in relation to the size of markers in the ladder using alpha view gel reader. The position of the amplified products corresponded to the location of the bands along y-axis (ranging from 0 to 1030). The Rf value for each band was determined assuming the location of well as initial position (Rf=0) and the position of migrated dye as final position (Rf=1) in a frame of reference. The polymorphism was recognized on the basis of presence or absence of bands, in addition to variation in respect of number and position of bands among the entries. All the entries used during molecular characterization were scored for the presence and absence of the bands. The efficacy of individual primers in differentiation of the genotypes was ascertained by computing the discrimination coefficient as *dp*/*np,* where, *dp* and *np* represent number of pairs of genotypes discriminated and total number of pairs of genotypes, respectively.

Principal coordinate analysis was conducted to obtain a two-dimensional ordination of microsatellite primers dependent genetic profiles of the entries under evaluation. The binary data matrix was also subjected to further analysis and genetic similarities among entries were calculated on the basis of presence and absence of common bands. Using the software NTSYS-pc (Rohlf 2000), genetic association among entries was analyzed by calculating the similarity coefficient (Dice 1945) for pair-wise comparisons based on the proportions of shared bands produced by the primers as $2a/(2a+b+c)$, where, a, b and c represent number of bands shared 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 clustering based on similarity coefficients. The dendrogram based on similarity indices was obtained by un-weighted pair-group method using arithmetic mean (UPGMA). An assessment of the nature of differentiation and divergence at the molecular level between the sterility maintainers and fertility restorers under evaluation in

Table 1. Analysis of primer pairs used for the amplification of targeted genomic regions in the eighteen entries

Primer	Ch. no.	Allele size range (bp)	Allele size difference (bp)	No. of alleles	No. of unique alleles	Gene diversity	Major allele frequency	DC	PIC	Heterozygosity
RM 1	1	100-167	067	12	6	0.892	0.166	0.948	0.882	0.111
RM 10	7	179-213	034	07	4	0.777	0.333	0.823	0.745	0.000
RM 17	12	178-233	055	10	6	0.844	0.305	0.876	0.829	0.055
RM 171	10	346-386	040	06	3	0.703	0.444	0.745	0.661	0.000
RM 201	9	142-340	198	08	5	0.808	0.277	0.856	0.782	0.000
RM 206	11	142-204	062	11	6	0.881	0.222	0.902	0.870	0.111
RM 216	10	142-169	027	06	2	0.765	0.333	0.810	0.729	0.000
RM 228	10	134-205	071	09	6	0.814	0.333	0.863	0.794	0.000
RM 234	7	144-184	040	09	5	0.851	0.222	0.902	0.834	0.000
RM 247	12	150-216	066	07	3	0.777	0.333	0.824	0.747	0.000
RM 250	2	160-176	016	06	2	0.784	0.277	0.830	0.750	0.000
RM 258	10	146-195	049	07	4	0.759	0.388	0.804	0.727	0.000
RM 263	2	175-223	048	10	6	0.851	0.277	0.902	0.837	0.000
RM 315	1	136-154	018	04	1	0.631	0.472	0.660	0.562	0.055
RM 407	8	183-200	017	04	1	0.635	0.444	0.673	0.565	0.000
RM 510	6	123-138	015	04	1	0.685	0.388	0.725	0.623	0.000
RM 524	9	206-550	344	07	2	0.672	0.527	0.791	0.644	0.222
RM 538	5	272-321	049	07	5	0.709	0.444	0.752	0.670	0.000
RM 558	12	250-317	067	08	5	0.796	0.333	0.843	0.770	0.000
RM 591	10	186-337	151	13	8	0.861	0.277	0.908	0.848	0.222
RM1108	10	131-150	019	08	2	0.851	0.222	0.732	0.834	0.000
RM 3530	1	161-205	044	05	2	0.683	0.444	0.660	0.630	0.055
RM3873	1	146-400	254	13	6	0.882	0.194	0.863	0.871	0.222
RM 5359	1	200-225	025	05	2	0.623	0.555	0.856	0.579	0.000
RM 5373	10	126-146	020	07	3	0.814	0.222	0.863	0.788	0.000
RM 6100	10	156-200	044	07	2	0.816	0.277	0.562	0.791	0.055
RM 6344	7	119-153	034	09	6	0.808	0.333	0.758	0.787	0.000
RM 6737	10	155-250	095	06	4	0.530	0.666	0.837	0.508	0.000
RM 7003	12	100-117	017	06	1	0.674	0.500	0.889	0.633	0.055
RM 7241	1	136-163	027	06	2	0.790	0.277	0.876	0.757	0.000
RM 8146	1	108-260	152	13	9	0.836	0.305	0.902	0.820	0.277
RM 10318	1	200-228	028	08	3	0.833	0.277	0.941	0.813	0.000

Ch. No.: Chromosome number; PIC: Polymorphism information content; DC: Discrimination coefficient

the present investigation was performed by identifying the clusters at appropriate phenon level.

Computational analysis for determining the major allele frequency, gene diversity, polymorphism information content and heterozygosity was performed using the software Power Marker (Liu and Muse 2005). Heterozygosity was expressed as the proportion of heterozygous genotypes in the population at a single marker locus in question. Gene diversity was estimated as the probability that the two randomly chosen alleles from the population are different at the

marker locus in question. Allelic diversity at each marker locus was assessed by comparison of the polymorphism information content (PIC) of the primer pairs (Botstein et al. 1980).

Results and discussion

Altogether 248 allelic variants including 125 shared and 123 unique alleles were detected using 32 microsatellite primer pairs with an average of 7.75 alleles per primer (Table 1). The size of the amplified products varied considerably with the entry and the primer. Polymorphism amongst entries was recognized in the form of presence or absence of bands, in addition to number and position of bands (Fig. 1). The difference in the size of the smallest and the largest amplified products generated by these primer pairs varied from 16 bp for RM 250 to 198 bp for RM 201. Allele size range and allele size difference clearly reflected a remarkably greater extent of variation in the length of simple sequence repeats flanked by the primer pairs utilized in the present study. The primer pairs RM 538, RM 8146, RM 17, RM 228, RM 6344, RM 201, RM 558, RM 591, RM 263, RM 10, RM 258, RM 234, RM 206, RM 1 and RM 171 generated considerably greater percentage of unique alleles. Amongst the primer pairs used in the present study, RM 1, RM 206, RM 591 RM 3873and RM 8146 had remarkably higher number of allelic variants with greater gene diversity and genotype discrimination ability.

Only one but polymorphic amplified product was generated by 22 primer pairs, namely, RM 10, RM 171, RM 201, RM 216, RM 228, RM 234, RM 247, RM 250, RM 258, RM 263, RM 315, RM 407, RM 510, RM 538, RM 558, RM 1108, RM 5359, RM 5373, RM 6344, RM 6737, RM 7241 and RM 10318 among the primer pairs used in combination with each of the 18 entries under evaluation. These primer pairs are well documented to amplify the simple sequence repeats located on chromosome number 7, 10, 9, 10, 10, 7, 12, 2, 10, 2, 1, 8, 6, 5, 12, 10, 1, 10, 7, 10, 1 and 1, respectively. Appearance of more than one band in the same entry was also noticed revealing most probably the existence of heterozygosity or possibly due to duplicated region in the genome. The primer pairs RM 1, RM 17, RM 206, RM 524, RM 591, RM 3530, RM 3873, RM 6100, RM 7003 and RM 8146 generated more than one amplified product in the same entry due to amplification of a specific microsatellite locus. However, further investigation under more stringent condition is required to confirm the existence of duplicated region because the intensity of bands was comparatively lower in some of the cases. These ten primers are well known to amplify simple sequence repeats located on chromosome number 1, 12, 11, 9, 10, 1, 1, 10, 12 and 1, respectively.

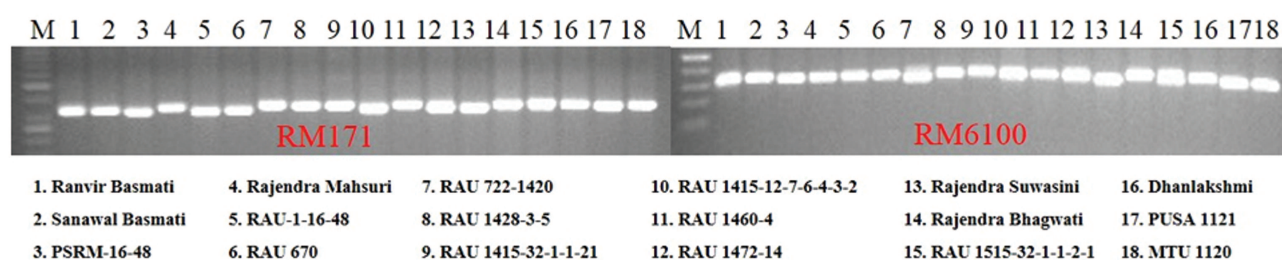

Fig. 1. Microsatellite primers dependent amplification patterns of targeted genomic regions in 18 entries of rice under evaluation in the present study

In general, the microsatellite loci with di-nucleotide and tri-nucleotide repeat motif tended to detect greater number of allelic variants than loci with tetra-nuclotide repeat motifs. The markers with GA, AT, AC and CT repeat motif revealed greater variability than marker with a TC, AG and GT repeat motif among the microsatellite loci having di-nucleotide repeat motif. Present study did not support some of the earlier reports in which a direct relationship between the repeat number involved in the microsatellite locus and the number of identified alleles was indicated and the discrepancy may be attributed to different primer pairs and entries used in the present study. Presence of stutter bands in the case of di-nucleotide repeat sequences detected by primer pairs RM 33, RM 524, RM 591, RM 3530, RM 3873 and RM 8146 indicated the presence of amplified minor products that had lower intensity than the main allele and normally lacked or had extra repeat units. Generally, the simple sequence repeat loci with di-nucleotide repeat motifs, in general, tend to detect higher number of alleles than the repeat

loci with tri-nucleotide repeat motifs or tetra-nucleotide repeat motifs or complex repeat motifs as reported earlier (Lapitan et al. 2007; Pervaiz et al. 2009) and consequently the primers amplifying di-nucleotide repeat motifs are found to be more polymorphic than those with tri-nucleotide, tetra-nucleotide and complex repeat motifs (Cho et al. 2000; Temnykh et al. 2001). Additionally, the total repeat counts of the di-nucleotide simple sequence repeat loci are generally recorded to be associated with the number of alleles detected per locus (Temnykh et al. 2001; Sajib et al. 2012). Apparently therefore, the repeat number involved in the simple sequence repeats directly relates to the number of identified alleles, reflecting that larger the repeat number involved in the simple sequence repeat locus, greater will be the number of detected alleles. Since, a change in the number of repeats results in the generation of allelic variants because of variation in the size of alleles, the total repeat count of di-nucleotide simple sequence repeat loci is mostly found to be associated with large number of alleles.

Frequency of markers among the entries, in addition to allelic diversity, is reflected by polymorphism information content (PIC) values. From a perusal of the pertinent data (Table 1), it is apparent that distinguishable extent of variability existed in respect of simple sequence length polymorphism based allelic diversity and frequency among the entries. Numerically, the value was found to vary from 0.508 in the case of RM 6737 to 0.882 in the case of RM 1 with an average value of 0.740 per primer, indicating that the primer pairs used for molecular characterization of entries under evaluation were, in general, highly informative. Noticeably, greater values were obtained in the cases of primer pairs RM 1, RM 10, RM 17, RM 201, RM 206, RM 228, RM 234, RM 247, RM 250, RM 263, RM 558, RM 591, RM 1108, RM 3873, RM 5373, RM 6100, RM 6344, RM 7241, RM 8146 and RM 10318. The appraisals, in general, reflected that the mean value was higher than that reported by some of the earlier investigators (Rahman et al. 2012; Sandhu et al. 2012; Mahajan et al. 2011; Yadav et al. 2011 and Tang et al. 2010). Contrarily, the mean value obtained in the present study was lower than the value of 0.810 as reported by earlier investigator (Bansal et al. 2013). The inconsistency among reports might be due to the inclusion of different sets of genotypes and utilization of different sets of primers during the investigation.

A similarity matrix obtained on the basis of comparison in respect of the presence and absence of amplified products generated by using thirty-two primer pairs specific to the unique flanking sequences of the microsatellites clearly revealed ample diversity at the molecular level amongst the eighteen entries under evaluation. The range of similarity coefficients (0.0 to 0.5) for pair-wise combinations of the entries indicated a considerably greater extent of variation among the entries and provided greater confidence for the classification of entries and assessment of genetic relationships. Similar inference has been derived in the studies conducted on the molecular markers based differentiation and divergence analysis in rice by earlier researchers (Neeraja et al. 2005; Lapitan et al. 2007; Herrera et al. 2008; Borba et al. 2009; Sivaranjani et al. 2010; Rani et al. 2010; Pervaiz et al. 2010; Singh et al. 2011; Rathi and Sarma 2012).

Using similarity coefficient as a measure of similarity, sequential agglomerative hierarchical nested (SAHN) clustering based on similarity matrix as the method for tree building and un-weighted pair group method using arithmetic mean (UPGMA) as the approach for generating dendrogram in numerical taxonomic approach of classification, an assessment of the nature and extent of differentiation and divergence was made and the clusters were identified at appropriate phenon level. While considering broad classification of entries, as indicated by dendrogram, basically the entries were divided into two groups. The first multi-genotypic group consisted of eight entries, whereas the second multi-genotypic group consisted of remaining ten entries. Therefore, eight effective fertility restorers were clearly discriminated from other entries comprising partial fertility restorers, weak sterility maintainers and complete sterility maintainers. By drawing the phenon line at greater similarity units as the cut off point and allowing the entries with comparatively more similar pattern for markers to be clustered together, the multi-genotypic groups were further divided into clusters, sub-clusters and sub-sub clusters. The inferences derived from the results of the similarity coefficients based numerical taxonomic approach of hierarchical classification were completely supported by the principal coordinate analysis (Fig. 2) based two dimensional plotting of microsatellite primer pairs dependent genetic profiles. Spatial distribution pattern of the genotypes along the two principal axes indicated that six amongst eight fertility restorer lines were placed far away from the centroid of the clusters and rest of the entries were placed more or less around the centroid. Using both the approaches, effective fertility restorers were distinctly discriminated from the

remaining entries into different genotypic groups without intermixing.

Clustering pattern further indicated that microsatellite primers based analysis revealed the genetic polymorphism at the molecular level in the form of variation in the length of simple sequence repeats as an efficient tool for unique genotyping and unambiguous differentiation of entries and diversity analysis. The markers utilized in the present study appeared to be sufficient for discrimination of eighteen entries of rice comprising effective fertility restorers, partial fertility restorers, complete sterility maintainers and weak sterility maintainers. Since the markers were chosen from ten amongst the twelve chromosomes of rice, the levels of diversity exhibited by them seemed to be unbiased and not due to chance (Bajracharya et al. 2006; Jaymani et al. 2007; Herrera et al. 2008; Borba et al. 2009; Vanaja et al. 2010; Rabbani et al. 2010; Singh et al. 2011; Rathi and Sarma 2012). So, these markers can be effectively and efficiently utilized for discrimination and unambiguous identification of entries and assessment of genetic diversity in rice.

When cluster analysis was performed by using the allelic diversity data generated from amplification pattern of the ten fertility restoration related primer pairs, namely, RM 1, RM 171, RM 206, RM 228, RM 258, RM 315, RM 6100, RM 10318, RM 6344 and RM 1108, the broad classification pattern of the entries was considerably similar. The dendrogram revealed that out of eight effective fertility restorers included in the analysis, six were clustered together. These primer pairs have also been used for classification of restorers from maintainers in the studies conducted earlier by several researchers (Jing et al. 2001; Ahmadikhan et al. 2007; Bazarkar et al. 2008; Sheeba et al. 2009; Shah et al. 2012). Principal coordinate analysis (Fig. 3) based two dimensional plot of these ten microsatellite primer pairs dependent genetic profiles of the entries was more or less in complete agreement with the results obtained from numerical taxonomic approach based classification of entries. Further analysis of the allelic diversity data obtained from amplification pattern of the six relatively more fertility restoration specific primer pairs, namely, RM 1, RM 171, RM 206, RM 315, RM 3873 and RM 6100, yielded almost similar results and well supported by the principal coordinate analysis (Fig. 4) based two dimensional plot of these six microsatellite primer pairs dependent genetic profiles of the entries, differentiating seven out of the eight effective restorers from rest of the entries included in the analysis. These six primer pairs have been reported to be closely related with

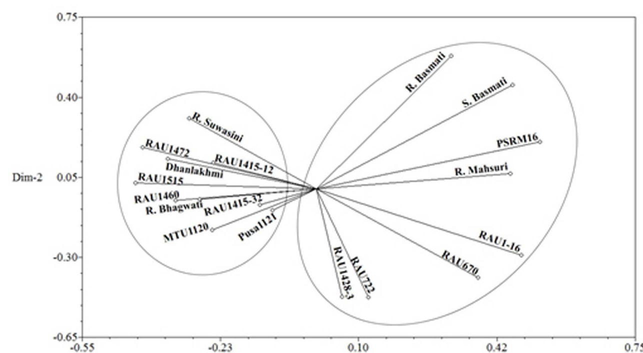

Fig.2. Principal coordinate analysis based two-dimensional ordination of 32 microsatellite primers pairs dependent genetic profiles from 18 rice genotypes

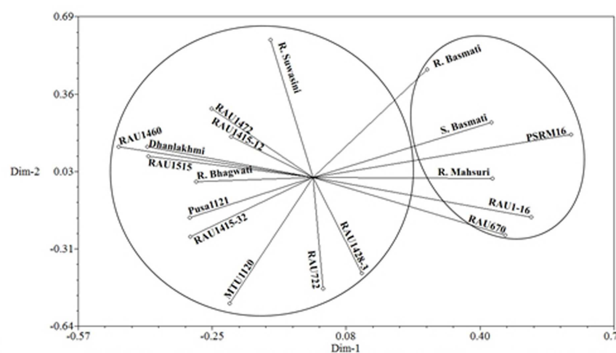

Fig.3. Principal coordinate analysis based two-dimensional ordination of 10 microsatellite primers pairs dependent genetic profiles from 18 rice genotypes. (Analysis using primer pairs RM1, RM171, RM206, RM228, RM258, RM315, RM6100, RM10318, RM3873 and RM1108)

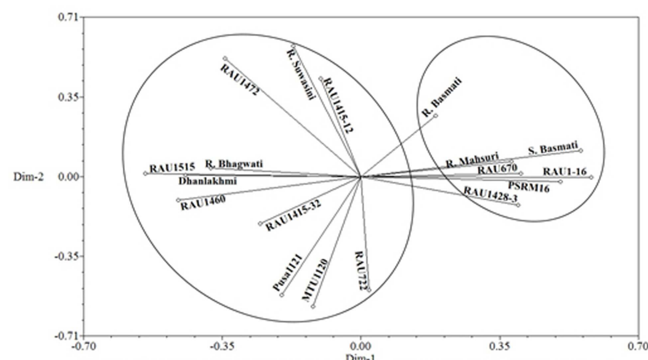

Fig.4. Principal coordinate analysis based two-dimensional ordination of 6 microsatellite primers pairs dependent genetic profiles from 18 rice genotypes. (Analysis using primer pairs RM1, RM171, RM206, RM315, RM6100 and RM3873)

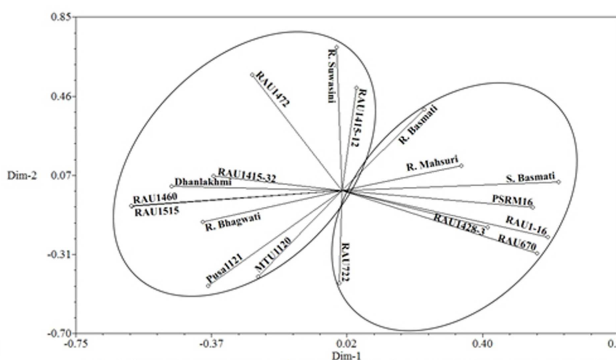

Fig.5. Principal coordinate analysis based two-dimensional ordination of 4 microsatellite primers pairs dependent genetic profiles from 18 rice genotypes. (Analysis using primer pairs RM171, RM315, RM3873 and RM 6100)

fertility restoration and therefore used for classification of maintainers and restorers in the studies conducted earlier by many research workers (Ahmadikhan and Karlov 2006; Ahmadikhan et al. 2007; Sattari et al. 2008; Revathi et al. 2013).

Interestingly, another analysis conducted by using allelic diversity data obtained from amplification pattern of the four relatively more effective fertility restoration specific primer pairs, namely, RM 171, RM 315, RM 3873 and RM 6100, provided more accurate and precise results, differentiating eight out of the eight effective restorers from rest of the entries included in the analysis. Spatial distribution pattern of the genotypes along the two principal axes as displayed by the principal coordinate analysis (Fig. 5) based two dimensional plotting of these four microsatellite primer pairs dependent genetic profiles of the entries also discriminated fertility restorers from rest of the entries. These four primer pairs are specific to the chromosomes known to harbor fertility restorer genes. Among the two fertility restorer genes known to be involved infertility restoration of WA type CMS system in rice, the effect of *Rf*4 located on chromosome 10 is stronger than the effect of *Rf3* located on chromosome 1 and additive effect of these two fertility restorer genes leads to effective fertility restoration (Cai et al. 2013; Huang et al. 2014; Chen et al. 2017). Some of these primers in combinations with other primer pairs have also been used in earlier studies for classification of fertility restorers from non-restorers (Alavi et al. 2009; Ghara et al. 2012). Using only a set of four microsatellite primers, as mentioned above, it was possible to detect sufficient variation at the molecular level among the entries (Fig. 5) which allowed unique genotyping of eighteen entries and discrimination of fertility restorers from rest of the entries included in the analysis. Therefore, the present study revealed that molecular characterization using such markers can be utilized as efficient tools for genotyping the entries with reasonable accuracy and for discrimination and unambiguous identification of fertility restorers from non-restorers of WA type CMS system in rice.

Authors' contribution

Conceptualization of research (VKS); Designing of the experiments (VKS, PK); Contribution of experimental materials (VKS, PK); Execution of field/lab experiments and data collection (VKS, PK, RK); Analysis of data and interpretation (VKS, PK, RK); Preparation of manuscript (VKS, PK, RK).

Declaration

The authors declare no conflict of interest.

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