Original Research Article

Molecular characterization of different varieties of rice (*Oryza sativa* L.) using SSR markers

Please review the english of the whole article.

Abstract

The present study was performed to analyze molecular diversity among different varieties of rice using SSR markers, which are effective and reliable tools for this type of analysis. A total of 34 different alleles were generated using 17 SSR primers. Out of these 33 alleles were found to be polymorphic and only one was monomorphic. On an average, 2 alleles per primer and 1.94 polymorphic alleles per primer was calculated. In the cluster analysis three varieties KATKIRICE, SONUMRICE and 1010 were found to be diverse from other varieties of rice. These varieties may be used as diverse parents for future breeding programme for rice improvement. According to Jaccard's similarity coefficient, the highest genetic diversity was observed between KATKIRICE and CHANDRAHASNI. Both varieties were grouped distantly. Out of 17 primers, the best 10 primers were selected based on polymorphic banding pattern for genetic diversity analysis. These selected 10 primers are sufficient to discriminate the group of rice varieties. These findings not only highlight the capacity of the SSR technique but also help in the selection of diverse rice varieties for conservation and crop improvement.

Key words: Rice (*Oryza sativa* L.), SSR markers, genetic diversity, molecular markers, variety, similarity, phylogenetic analysis **Key words must not be in the title**

Introduction

I suggest inserting biographical references from the last five years.

Rice (*Oryza sativa* L.) is one of the maximum vital food crops in the world. Around three billion inhabitants consume rice as an essential food which gives on the subject of 50 to 80% of their daily calories. India is considered as big diversity centres for rice and reports are available on diversity both at inter and intra specific levels (Roy et al. 2015). In any crop improvement programme, yield, valuable characters and presence of tolerant genes for biotic and abiotic stresses are main targets (Nachimuthu *et al*. 2015). These targets have participated in the increase of agricultural production in the country (Dudley, 1994). A common problem with the uninterrupted assortment among genetically related cultivars is reduction of the hereditary establishment of the crops. The foundation of current agriculture is the choice of genetically diverse genotypes as parents for crossing purposes (Plucknett et al. 1987).

Identification of diversity at genetic level is a pre-requisite for any crossing programme for crop improvement. It contributes in the establishment of genetic affiliation in the collection of germplasm. This identification also helps in selection of diverse parental combinations with higher genetic variation and possibilities of recombination to transfer advantageous genes (Islam et al. 2012; Thompson et al. 1998). At present plentiful molecular markers are

available to estimate the genetic variation and create the molecular fingerprint of rice genotypes. Among all markers, simple sequence repeat (SSR) markers have proved their efficiency in rice for estimation of genetic variation (Kuleung et al. 2004). Several researchers have used SSR markers in molecular diversity analysis of rice. SSR based fingerprinting allows rapid cultivar classification, which is a proven tool for genotypic categorization, assortment, and execution (Zhu et al. 2012; Tang et al. 2015). Additionally, SSR markers have their own benefits in comparison to single nucleotide polymorphism (SNP) markers for genetic diversity analysis at low cost (Hamblin et al. 2007).

The objectives of the present investigation were to analyze the molecular variability and genetic relatedness of 45 rice genotypes using SSR markers. This study will improve our understanding of genetic diversity among different genotypes of rice and make possible the exploitation of dissimilar genotypes in rice improvement.

Materials and Methods

Rice materials

A total of 45indica inbred rice varieties were chosen to represent a wide diversity (Table-1). These materials were collected from different locations of Madhya Pradesh and Chhattisgarh.

Table-1 Rice varieties used in the present study

Sl.	Varieties	Location	Place	State
1	SARNA	Local market	Chhuikhdan	Chhattisgarh
2	BASMATIPS3	JNKVV	Jabalpur	Madhya Pradesh
3	IGKVR2	IGKVV	Raipur	Chhattisgarh
4	1010	Local market	Chhuikhdan	Chhattisgarh
5	KARIKAMOD	Local market	Chhuikhdan	Chhattisgarh
6	IR64	JNKVV	Jabalpur	Madhya Pradesh
7	IGKVR1	IGKVV	Raipur	Chhattisgarh
8	CULTURERICE	Local market	Chhuikhdan	Chhattisgarh
9	AMPORICE	Local market	Chhuikhdan	Chhattisgarh
10	SAMBLESHWARI	IGKVV	Raipur	Chhattisgarh
11	DUBRAJ	Local market	Chhuikhdan	Chhattisgarh
12	BASMATIP1460	JNKVV	Jabalpur	Madhya Pradesh
13	MTU1010	JNKVV	Jabalpur	Madhya Pradesh
14	CHANDRAHASNI	IGKVV	Raipur	Chhattisgarh
15	DANTESHWARI	IGKVV	Raipur	Chhattisgarh
16	MTU1011	Local market	Chhuikhdan	Chhattisgarh
17	KAROKAMOL	Local market	Chhuikhdan	Chhattisgarh
18	SATARISFARI	KVK	Korea	Chhattisgarh
19	SONAMRICE	Local market	Birutola	Chhattisgarh
20	BAIJARRICE	KVK	Korea	Chhattisgarh
21	HATHWA	KVK	Korea	Chhattisgarh
22	JHIGAFULL	KVK	Korea	Chhattisgarh
23	BAMLESHWARI	KVK	Korea	Chhattisgarh
24	LALURICE	KVK	Korea	Chhattisgarh
25	M2	JNKVV	Jabalpur	Madhya Pradesh
26	SUKNANDAN	KVK	Korea	Chhattisgarh
27	WGL32100	JNKVV	Jabalpur	Madhya Pradesh
28	IR81	JNKVV	Jabalpur	Madhya Pradesh
29	KRANTI	JNKVV	Jabalpur	Madhya Pradesh
30	SAFADSARNA	Local market	Birutola	Chhattisgarh
31	SAHBAGHI	JNKVV	Jabalpur	Madhya Pradesh

32	JR201	JNKVV	Jabalpur	Madhya Pradesh
33	CHINDEMORI	KVK	Korea	Chhattisgarh
34	KATKIRICE	KVK	Korea	Chhattisgarh
35	SURJARICE	KVK	Korea	Chhattisgarh
36	KARHANI	KVK	Korea	Chhattisgarh
37	GHINMORI	KVK	Korea	Chhattisgarh
38	HMT	Local market	Birutola	Chhattisgarh
39	RANIKAJAL	KVK	Korea	Chhattisgarh
40	IGKVR1244	KVK	Korea	Chhattisgarh
41	KANASHRI	KVK	Korea	Chhattisgarh
42	GERAFULL	KVK	Korea	Chhattisgarh
43	SATHARHSAFRI	KVK	Korea	Chhattisgarh
44	MAHAMAYA	Local market	Birutola	Chhattisgarh
45	CHOTISAFRI	KVK	Korea	Chhattisgarh

I suggest entering the geographical coordinates of the areas where the samples were collected

Genomic DNA extraction and molecular analysis

Leaf samples were collected after transplanting. DNAwas extracted following the CTAB procedure as described by Saghai-Maroof et al (1980) with required modifications.Total 17 SSR primers (Table-2) were used to detect polymorphism of selected genotypes. The primers were procured fromIntegrated DNA Technology, USA. PCR was performed in a 10µL reactionmixture containing 25 ng template DNA, 0.5 µmol/L of each primer, 200 µmol/L of each dNTP, 1.5 mmol/L $MgCl_2$ and 1 U *Taq* polymerase and 1.0µL of 10 x PCR reaction buffer. The PCR procedure was done as 5 min initial denaturation at 94 °C; 35cycles of 30 sec denaturation at 94 °C, 30 sec anneal at 55 °C and 1 min extension at 72 °C; and 5 min final extension at 72 °C. The amplified products were electrophoresed on 2.5% agarose gel.

Table-2 SSR primers and their sequences used in the study

Sl.	Primer	Forward 5'-3'	GC %	Reverse 5'-3'	GC %
1	RM474	AAGATGTACGGGTGGCATTC	50	TATGAGCTGGTGAGCAATGG	50
2	RM413	GGCGATTCTTGGATGAAGAG	50	TCCCCACCAATCTTGTCTTC	50
3	RM484	TCTCCCTCCTCACCATTGTC	55	TGCTGCCCTCTCTCTCTCTC	60
4	RM19	CAAAAACAGAGCAGATGAC	42.1	CTCAAGATGGACGCCAAGA	52.6
5	RM212	CCACTTTCAGCTACTACCAG	50	CACCCATTTGTCTCTCATTATG	40.9
6	RM249	GGCGTAAAGGTTTTGCATGT	45	ATGATGCCATGAAGGTCAGC	50
7	RM250	GGTTCAAACCAAGCTGATCA	45	GATGAAGGCCTTCCACGCAG	60
8	RM280	ACACGATCCACTTTGCGC	55.5	TGTGTCTTGAGCAGCCAGG	57.8
9	RM541	TATAACCGACCTCAGTGCCC	55	CCTTACTCCCATGCCATGAG	55
10	RM11	TCTCCTCTTCCCCCGATC	61.1	ATAGCGGGCGAGGCTTAG	61.1
11	RM152	GAAACCACCACACCTCACCG	60	CCGTAGACCTTCTTGAAGTAG	47.6
12	RM153	GCCTCGAGCATCATCATCAG	55	ATCAACCTGCACTTGCCTGG	55
13	RM21	ACAGTATTCCGTAGGCACGG	55	GCTCCATGAGGGTGGTAGAG	60
14	RM475	CCTCACGATTTTCCTCCAAC	50	ACGGTGGGATTAGACTGTGC	55
15	RM247	TAGTGCCGATCGATGTAACG	50	CATATGGTTTTGACAAAGCG	40
16	RM256	GACAGGGAGTGATTGAAGGC	55	GTTGATTTCGCCAAGGGC	55.5
17	RM259	TGGAGTTTGAGAGGAGGG	55.5	CTTGTTGCATGGTGCCATGT	50

Data analysis

Electrophoresed SSR gels were scored for the presence (1) or absence (0) of bands of molecular weight size in the form of binary matrix for all the varieties studied. Data were analyzed to obtain Jaccard's coefficients among the isolates by using NTSYS-PC Version 2.02e software (Rohlf, 1998). The SIMQUAL program was used to calculate the Jaccard's coefficients. A dendrogram was constructed using UPGMA (unweighted pair-group method with arithmetic averages) with the SAHN (sequential, agglomerative, hierarchical, and nested clustering) routine.

Allele frequency, Genetic diversity and Polymorphism Information Content (PIC) values for each marker were analyzed using Power Marker software 3.25 (Liu and Muse, 2005).

Results and Discussion

Rice is the most important cereal crop that has been referred as global grain because of its use as prime staple food in about 100 countries of the world. Most of the world's rice is cultivated and consumed in Asia, which constitutes more than half of the global population. It is planted about 154 million hectares annually or on about 11 percent of the world's cultivated land. The rising demand, saturation of cultivable field and climate change cause a supply shortage of a crop in the future. By the near 2025, about 785 million tones of paddy which is 70 percent more than the current production will be needed to meet the growing demand. To achieve the target yield that is required to sustain the world population, rice varieties with a yield advantage of about 20 percent over currently grown varieties must be developed (Chakravarthi et al., 2006).

In the present investigation, initially 17 primers were screened for amplification of DNA samples of rice varieties. All the 17 primers were amplified successfully and selected for varietal identification on the basis of sharp and clear banding pattern. Further PCR reactions were carried out using 17 SSR primers. Selected 17 SSR primers amplified 34 different loci in all the samples. The band size of amplified product ranged from 100bp-300bp. Maximum numbers of allele (3) were scored by the primers RM21 while minimum numbers of allele (1) were scored by the primers RM484. Out of these amplified loci 33 were found to be polymorphic (94.11%). Average numbers of band per primer was 2.00 while, average numbers of polymorphic band per primer was 1.94. Sixteen primers were able to amplify polymorphic alleles and only one primer i.e. RM484 found to be monomorphic (Table-3). PCR amplification gave good and clear banding profile which has been obtained by the primers with 50 to 60% GC content. Brondani (2006) observed an increasing GC content of the primer with increased number of bands. The explanation for the correlation between GC content and the number of bands may be the stability of base complementation of A with T. The amplified DNA profiling was scored according to the presence and absence of bands. Fritsch et al. (1993) also reported that the importance of the GC content of primers on yield of PCR amplified products.

Table-3 Banding pattern in ricevarieties using 17 SSR markers

S.N.	Primer	TB	PB	PP	PIC	Allele No	Band size (in bp)	Allele Frequency	Genetic Diversity
1	RM474	2	2	100	0.71	2	140,160	0.4000	0.6775
2	RM413	2	2	100	0.71	2	160,180	0.3556	0.7230
3	RM484	1	0	0	0.00	1	200	1.0000	0.0000
4	RM19	2	2	100	0.72	2	250, 300	0.3333	0.7299
5	RM212	2	2	100	0.71	2	100, 150	0.5333	0.6044
6	RM249	2	2	100	0.73	2	140, 160	0.2889	0.7477
7	RM250	2	2	100	0.70	2	160, 180	0.4000	0.7457
8	RM280	2	2	100	0.79	2	180, 200	0.3556	0.7042
9	RM541	2	2	100	0.73	2	80, 100	0.4444	0.6064
10	RM11	2	2	100	0.70	2	180, 200	0.3778	0.6746
11	RM152	2	2	100	0.70	2	150, 200	0.5556	0.6153
12	RM153	2	2	100	0.72	2	200, 220	0.5556	0.5956
13	RM21	3	3	100	0.89	3	140, 180, 200	0.2667	0.8128
14	RM475	2	2	100	0.72	2	200, 230	0.3778	0.7605
15	RM247	2	2	100	0.73	2	160, 200	0.4889	0.7032
16	RM256	2	2	100	0.68	2	100, 150	0.6889	0.4770
17	RM259	2	2	100	0.75	2	160, 180	0.3778	0.6854

Total	34	33	-	-	34	-	-	-
Mean	2.00	1.94	94.11%	0.68	2	-	0.4588	0.6390

TB- Total Band, PB- Polymorphic Bands, PP- Percentage of Polymorphism, PIC-Polymorphism Information Content

According to Parida et al. (2008) out of 52 SSR markers selected 41were found to be polymorphic while 11 were found to be monomorphic among parental lines. A total of 77 alleles were detected and the number of alleles per locus ranged from 1 to 6. Similar results were observed in rice by Sundaram et al., (2008); Nandakumar et al., (2004); Seetharam et al., (2009). During the present investigation similar type of result was obtain for varietal identification; all SSR markers were polymorphic except one.

According to Jaccard's similarity coefficient the highest similarity 97% was found between MTU1011 and SONAMRICE and lowest (25.7%) between KATKIRICE and CHANDRAHASNI. The range of Jaccard's similarity coefficient 25.7-97% indicates higher level of diversity among rice varieties. To evaluate the Polymorphism information content (PIC) value of a marker the frequencies of each marker allele must be determined. Markers with greater numbers of alleles tend to have higher PIC values and thus are more informative (Meti et al., 2013). For genetic diversity analysis, PIC value evaluated based on the specific locus/marker. The overall PIC value ranged between 0.0 (RM341) to 0.582 (RM256) with a mean value of 0.324 reported by Gour et al. (2016). Similar results were observed for evaluation of PIC value by many researchers such as (Seetharam et al., 2009; Singh et al., 2011; Shrivastava et al., 2015). In the present investigation, the highest PIC value (0.89) was observed in RM21 with 3 alleles among the 45 varieties. Primers RM280, RM259, and RM249 also exhibited higher PIC scores and high number of alleles and lowest PIC value was recorded in RM484 (0.00). Whereas the pic value ranged 0.00 (RM484) to 0.89 (RM21) with a mean value of 0.68 in different sets of rice varieties which was closer to the result as previous studies.

In UPGMA cluster analysis, the varieties were grouped into two clusters (Fig.1). The major group contained 44 varieties and minor group containing only one variety KATKIRICE. Major group further classified into two sub-groups 'A' and 'B'. Sub-group 'A' contained 37 varieties whereas sub-group 'B' contained 7 varieties. Variety KATKIRICE was found to be diverse from others and placed at end of the cluster. Similar clustering patterns have also been reported in rice (Keshavulu et al., 2015; Seetharam et al., 2009 and Vhora et al., 2013).

Principle components analysis of 45 rice varieties was carried out according to the similarity coefficient. In this analysis three groups were divided into group 'A', group 'B' and group 'C'. Group 'A' holding 26 varieties namely KARIKAMOL, KARIKAMOD, BASMATIP1460, IGKVR2, HATHWA, BAIJARRICE, SAMBLESHWARI, DANTESHWARI, JHIGAFULL, AMPORICE, IR81, SAHBAGHI, KRANTI, DUBRAJ, JR201, KATKIRICE, SATARISAFARI, BAMLESHWARI, CULTURERICE, SUKNANDAN, SONAMRICE, MTU1011, MTU1010, IR64, IGKVR1 AND CHANDRAHASNI and these varieties were placed closely due to more similarity among them. Group 'B' varieties has 16 varieties namely KANASHRI, IGKVR1244, SATHARHSAFRI, CHOTISAFRI, MAHAMAYA, SARNA, GHINMORI, HMT, GERAFULL, SAFADARNA, M2, 1010, WGL32100 AND BASMATIPS3 were placed closely with more similarity and group 'C' possessed five varieties namely LALURICE, RANIKAJAL, KARHANI, SURJARICE and CHINDEMORI were more diverse from other varieties (Fig.2).

Three dimensional scaling of 45 rice varieties also revealed similarity conferring principle component analysis. These varieties were divided into three groups namely group 'A', group 'B' and group 'C', group 'A' contained 24 varieties namely KRANTI, DUBRAJ, SAHBAGHI, JR200, DANTESHWARI, SANBLESHWARI, IR81, JHIGAFULL, GERAFULL AMPORICE, MAHAMAYA, 1010, SARNA, WGL32100, MTU1010, SUKNANDAN, BAMLESHWARI, CULTURERICE, BASMATIPS3, IR64, IGKRV1, CHANDRAHASNI, SONAMRICE and MTU1011 exhibiting high similarity amongst them. Group 'B' contained 17 varieties namely CHINDEMORI, KARIKAMOL, KARIKAMOD, BASMATIP1460, SAFADSRNA, M2, BAIJERRICE, SATARISAFRI, HMT, HATHWA, IGKVR1244, IGKVR2, GHINMORI, SATHARHSAFRI, KATKIRICE, CHOTISAFRI and KANASHARI. Group 'C' contained four varieties such as LALURICE, RANIKAJAL, KARHANI and SURJARICE (Fig.3).

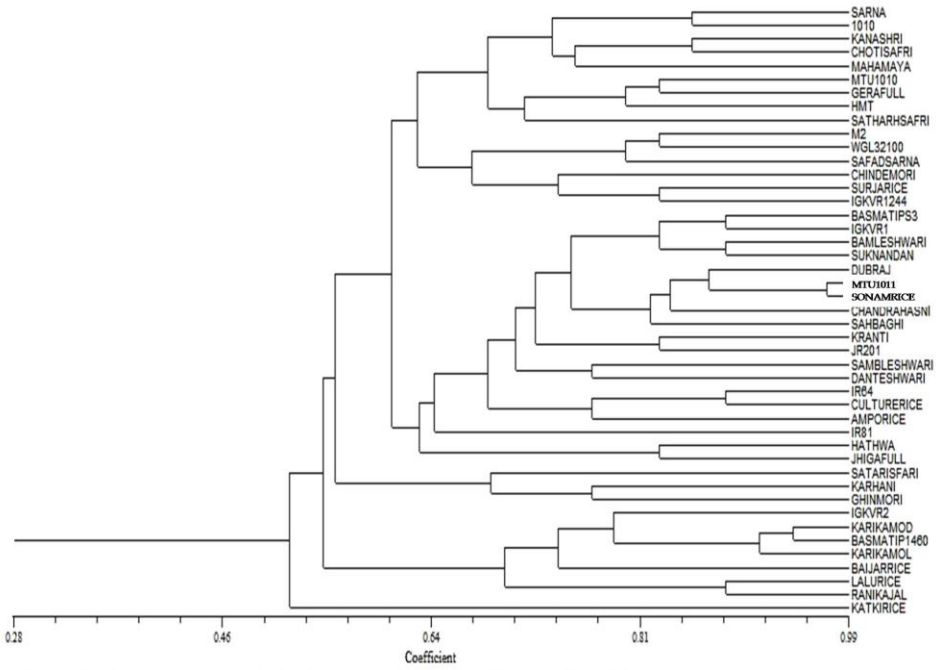

Fig.1. Phylogenic tree showing relationship among rice varieties based on SSR data

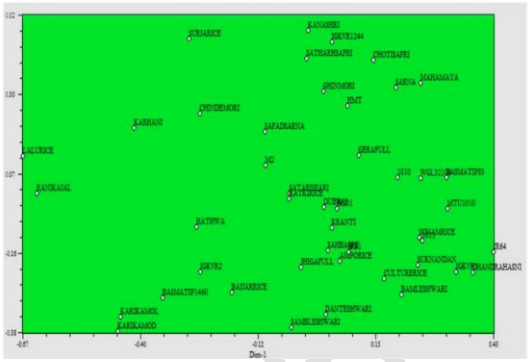

Fig.2. Two dimensional scaling of rice varieties based on Principal Component Analysis (PCA)

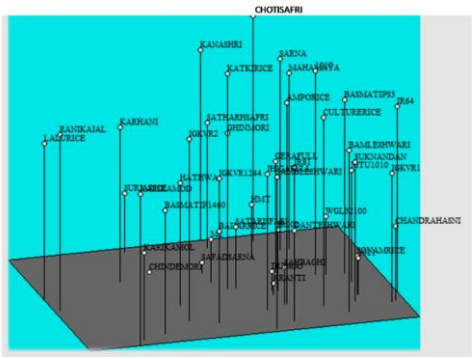

Fig.3. Three dimensional scaling of rice varieties based on SSR data

Authors should explore the results further.

Conclusion

The molecular marker technique used in this study has proved to be successful in elucidating relationships among the 45 rice varieties, in identifying species-specific markers and generating a fingerprinting key as the important resources for the breeding and management of rice germplasm/ varieties. In addition, some of these markers can be used for marker assisted selection (MAS) for genetic improvement of rice.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

References

Please review all bibliographic references and place in accordance with the journal's guidelines.

Brondani C, Borba TCO, Rangel PHN and Brondani RPV. 2006. Determination of genetic variability of traditional varieties of Brazilian rice using microsatellite markers. Genetics and Molecular Biology. 29(4): 676-684.

Chakravarthi BK and Naravaneni R. 2006. SSR marker based DNA fingerprinting and diversity study in rice (*Oryza sativa* L.). African Journal of Biotechnology. 5(9): 684-688

Dudley JW. 1994. Plant breeding- a vital part of improvement in crop yields, quality and production efficiency. In: Frey KJ, editor. Historical Perspectives in Plant Sciences. Ames: Iowa State University Press; p. 163–77.

Fritsch P, Hanson MA, Spore CD, Pack PE and Rieseberg LH. 1993. Constancy of RAPD primer amplification strength among distantly related taxa of flowering plants. Plant Molecular Biology Reporter. 11(1): 10-20.

Gour L, Koutu GK, Singh SK and Mishra DK. 2016. Genetic potency identification of indigenous rice (*Oryza sativa* L.) lines using quality assessment and SSR marker analysis. Int J Chem Stud 5(4): 97-101.

Hamblin MT, Warburton ML, Buckler ES. 2007. Empirical comparison of simple sequencerepeats and single nucleotide polymorphisms in assessment of maize diversity and relatedness. PLoS One 2(12): e1367

Islam MR, Gregorio GB, Salam MA, Collard BCY, Singh RK and Hassan L. 2012. Validation of *SalTol* linked markers and haplotype diversity on chromosome 1 of rice. Molecular Plant Breeding. 3(10): 103-114.

Kunusoth K, Vadivel K, Sundaram RM, Sultana R, Rajendrakumar P, Maganti S, Subbarao LV and Chin-Wo SR. 2015. Assessment of genetic diversity of elite indian rice varieties using agro-morphological traits and SSR Markers. Journal of Experimental Agriculture International. 6(6): 384-401.

Kuleung C, Baenziger PS and Dweikat I. 2004. Transferability of SSR markers among wheat, rye, and triticale. Theoretical and Applied Genetics. 108(6): 1147–1150.

Meti N, Samal KC, Bastia DN and Rout GR. 2013. Genetic diversity analysis in aromatic rice genotypes using microsatellite based simple sequence repeats (SSR) marker. African Journal of Biotechnology. 12(27): 4238-4250.

Nachimuthu VV, Muthurajan R, Duraialaguraja S, Sivakami R, Pandian BA, Ponniah G, Gunasekaran K, Swaminathan M, Suji KK, Sabariappan R. 2015. Analysis of population structure and genetic diversity in rice germplasm using SSR markers: an initiative towards association mapping of agronomic traits in *Oryza sativa*. Rice. 8(1):1-25.

Nandakumar N, Singh AK, Sharma RK, Mohapatra T, Prabhu KV and Zaman FU. 2004. Molecular fingerprinting of hybrids and assessment of genetic purity of hybrid seeds in rice using microsatellite markers. Euphytica. 136(3): 257-264.

Plucknett DL, Smith NJH, Williams JT and Anishetty NM. 1987. Gene Banks and the World's Food. Princeton, New Jersey: Princeton University Press; p. 247

Roy S, Banerjee A, Mawkhlieng B, Misra AK, Pattanayak A and Harish GD. 2015. Genetic diversity and population structure in aromatic and quality rice (*Oryza sativa* L.) landraces from North-Eastern India. PloS One 10(6): e0129607

Seetharam K, Thirumeni S and Paramasivam K. 2009. Estimation of genetic diversity in rice (*Oryza sativa* L.) genotypes using SSR markers and morphological characters. African journal of Biotechnology. 8(10): 2050-2059

Shrivastava A, Mishra DK, Koutu GK, Singh SK, Prakash V and Sohgaura NE. 2015. Assessment of genetic diversity of restorer and maintainer lines of rice using SSR markers. The Ecoscan. 9(1&2): 265-269.

Singh D, Kumar A, Sirohi A, Kumar P, Singh J and Kumar V. 2011. Improvement of Basmati rice (*Oryza sativa* L.) using traditional breeding technology supplemented with molecular markers. African journal of Biotechnology. 10(4): 499-506.

Sundaram RM, Kumar NB, Biradar SK, Balachandran SM, Mishra B, Ilyas AM, Viraktamath BC, Ramesha MS and Sarma NP. 2008. Identification of informative SSR markers capable of distinguishing hybrid rice parental lines and their utilization in seed purity assessment. Euphytica. 163(2): 215–224.

Tang H, Yu HY and Zhang XM. 2015. Analysis on the diversity of DNA fingerprinting of the example varieties used for the test of rice new varieties. Journal of Plant Genetic Resources. 16(1):100–6.

Thompson JA, Nelson RL and Vodkin LO. 1998. Identification of diverse soybean germplasm using RAPD markers. Crop Science. 38(5):1348-1355.

Vhora ZA, Trivedi RU, Chakraborty SU, Ravikiran RA and Sasidharan N. 2013. Molecular studies of aromatic and non-aromatic rice (*Oryza sativa* L.) genotypes for quality traits using microsatellite markers. Bioscan. 8(2):359-362.

Zhu YF, Qin GC, Hu J, Wang Y, Wang JC and Zhu SJ. 2012. Fingerprinting and variety identification of rice (Oryza sativa L.) based on simple sequence repeat markers. Plant Omics. 5(4):421-426.