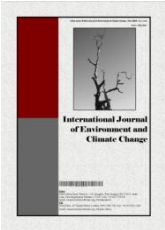

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Molecular Characterization of Land Races of Rice by Using SSR Markers (*Oryza sativa* L.)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The study was conducted to use thirty one rice land races for characterized the genetic diversity analysis at Seed Unit, UAHS, Shivamogga. The SSR markers (25 SSR markers) were used for the assessment of genetic diversity and relatedness among 31 rice landraces and all of them were found polymorphic. Twenty five SSR markers revealed 50 alleles in the 31 land races, the number of alleles per locus with an average of 2 per locus. Major allele frequency ranged from 0.58 (RM 25) to 0.94 (RM 229) with an average of 0.74 per marker. The PIC (polymorphism information content) value ranged from 0.12 (RM 229) to 0.66 (RM 481 and RM 25) with an average of 0.36 per marker. The PIC value of each marker, which was evaluated on the basis of its alleles, varied greatly all tested SSR loci-from 0.58 to 0.94 with an average of 0.74. The highest PIC value 0.49 was obtained for RM481 and RM25, followed by RM443 (0.47), RM224 (0.46) RM 159, RM 1054, RM226 and RM24 (0.44). Remaining markers were showed less PIC value. Lower PIC value result of closely related landraces and higher PIC values result of diverse landraces. The dendrogram based on UPGMA grouped the 31landraces into 10 clusters that were demarcated at a similarity coefficient of 0.68. Cluster III was the largest including 13 land races followed by cluster VI and II each of which had four and three land races respectively. The remaining clusters had one and two landraces each.

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1. INTRODUCTION

Land races played an important role in the local food security and sustainable development in agriculture, in addition to their significance as genetic resource for rice genetic improvement [1]. Genetic diversity is conventionally assessed by morphological traits. However, such traits are affected by environment, phenology or development stage of the plant and the type of plant material. Further, it requires testing in several replications and environments to establish the genetic contributions, which is highly influenced by several factors. Hence there is a need to go in for a highly reliable and precise method for assessment of genetic variability with no environmental effects. Molecular markers are considered an efficient, powerful tool for the assessment of genetic relationships [2]. Assessment of genetic diversity is important in plant breeding if there is to be improvement by selection. For the assessment of genetic diversity molecular markers have been generally superior to morphological, pedigree, heterosis and biochemical data. Genetic diversity is commonly measured by genetic distance or genetic similarity, both of which imply that there are either differences or similarities at the genetic level. Molecular Marker based Genetic Diversity Analysis also has potential for assessing changes in genetic diversity over time and space [3].

Several DNA markers are used for molecular diversity analysis of which Simple Sequence Repeats (SSR) or microsatellites are a class of repetitive DNA element. Microsatellites are PCR based markers that are technically efficient, cost effective, and common in rice [4]. Compared to RFLP, microsatellite markers detect a significantly higher degree of polymorphism in rice and are especially suitable for evaluating genetic diversity among closely related rice cultivars (or) accessions [2]. In the present study, thirty one landraces of rice were analyzed for genetic diversity using SSR markers. Specially, the objective of the study was DNA fingerprinting and genetic diversity analysis of landraces to measure the extent of genotypic differences, genetic relationship and to assist in broadening the germplasm base of future rice breeding programs.

2. MATERIALS AND METHODS

The materials used in the study comprise of thirty one rice land races collected from Organic Farming Research Centre. UAHS, Navile, Shivamogga. The lists of the genotypes are given in Table 1. A field trial was conducted using twenty nine rice land races (Plate 1). These genotypes were sown in raised nursery bed during *Kharif* 2021 and 25 days old seedlings were transplanted to the main field in a Randomized Completely Block Design replicated thrice. Each genotype was transplanted in five rows of 3m length adopting a spacing of 30cm between rows and 10cm between plants. All the recommended agronomic package of practices was adopted during the entire crop growth period.

Plate 1. General view of experimental plot

3. GENETIC DIVERSITY STUDIES BASED ON SIMPLE SEQUENCE REPEAT (SSR) MARKERS

3.1 Isolation of Genomic DNA

All the thirty one landraces were sown in laboratory condition for the extraction of DNA from leaf samples. DNA was isolated from 20 days old seedlings from each of the landraces for molecular diversity analysis. DNA was extracted by adopting the protocol developed by Van der beek et al. [5].

3.1.1 Reagents used Cetyl Trimethyl Ammonium Bromide (CTAB) extraction Buffer (100mL)

CTAB	2% (w/v)
Tris HCl (pH8.0)	200 mM
Sodium Chloride	1.4 M
EDTA (pH 8.0)	20 Mm

(Tris, sodium chloride and EDTA were autoclaved and 2% CTAB was added after autoclaving and pre heated before using the buffer)

Liquid Nitrogen
β Mercapta ethanol
Ice-cold Isopropanol
Chloroform: Isoamylalcohol (24:1) (v/v)
Ethanol (70 and 100%)

3.2 Extraction of Genomic DNA

One gram of fresh and clean leaves were cut into bits with the help of sterile scissors and transferred to pre-chilled mortar. The leaf tissues were frozen using liquid nitrogen and ground to fine powder. The fine powder was allowed to thaw in the presence of 800µl of preheated extraction buffer in the polypropylene centrifuge tubes, to this 100µl of β-mercapta -ethanol was added and incubated for 30-45 minutes at 65ºC in water bath with occasional mixing. The tubes were removed from the water bath, equal volume of 800µl of chloroform: Isoamyl alcohol mixture (24:1 v/v) was added and mixed by inversion for 1 hour. It was centrifuged at 10,000 rpm for 20 minutes at room temperature. The clear aqueous phase was transferred to a new sterile tube. Equal volume of Ice-cold isopropanol was added, mixed gently by inversion and then kept in the freezer until DNA was precipitated. The mixture was centrifuged at 13,000 rpm at room temperature for 10 minutes and the supernatant was decanted retaining the pellet. The pellet was washed with 100µl of 70 per cent ethanol and centrifuged at 6000rpm for 5 minutes. The alcohol was discarded and DNA was completely air dried. The dried pellet was resuspended in 100µl of sterile water.

Table 1. Lists of 31 landraces

Sl No.	Landraces	Sl No.	Landraces
1	Jasmine	17	Mysurumallige
2	Ratnachudi	18	Ramudi
3	Najarbad	19	Gowrisanna
4	Rajabhoga	20	Barmablack
5	Gandhasale	21	Doddabairunellu
6	Bangarasanna	22	Kempusale
7	Champakali	23	Navara
8	Dappavalya	24	Kempujiddu
9	Raichur sanna	25	Anekombinabhatta
10	Madras sanna	26	Kiruvani
11	Karigajavale	27	Ratnasagara
12	Karijiddu	28	Misebhatta
13	Neregulibhatta	29	Ambemohari
14	Puttabhatta	30	Jyothi
15	Jeerigesanna	31	MTU1001
16	Gilisale		

3.3 Quantification of DNA

The genomic DNA extracted from the young leaf samples was tested for its intactness by electrophoresis. The DNA was quantified with visually on agarose (0.8%) gel by staining with ethidium bromide. 10µl of each genotype DNA was loaded with tracking dye along with 1 Kb DNA. After the tracking reached one third of the gel, documentation was done using UV trans-illuminator. The quantity of isolated DNA was determined by its corresponding ladder DNA. Based on this, the dilution of DNA was done for SSR amplification.

3.4 PCR Amplification

Sequence of micro satellite primer pairs were downloaded from Genome data bases, Rice genes micro satellite markers (http://www.gramene.org/markers/microsat/ssr.html). The details of the primer sequences were given in Table 2. These primer sequences synthesized by Sigma Aldrich Inc., Bangalore were utilized for amplification.

The total reaction volume was 15µl, the cocktail for the amplification was prepared as follows in 0.2ml PCR tubes.

1. DNA 2.00µL
2. dNTPs (2.5mm) (Bangalore Genei Ltd., India) 1.50µL
3. Primer (Sigma Aldrich Inc., USA) 2.00µL
4. PCR buffer 1.50µL
5. Taq Polymerase (3 units/µL) (Bangalore Genei Ltd., India) 0.2µL
6. Magnesium chloride 0.3µL
7. Sterile distilled H_2O 7.50µL

Table 2. Details of SSR primers used for PCR amplification

Sl. No.	Marker	Nucleotide sequence	
1	RM 206	F 5 '-3'	CCCATGCGTTTAACTATTCT
		R 5 '-3'	CGTTCCATCGATCCGTATGG
2	RM 224	F 5 '-3'	ATCGATCGATCTTCACGAGG
		R 5 '-3'	TGCTATAAAAGGCATTCGGG
3	RM 229	F 5 '-3'	CACTCACACGAACGACTGAC
		R 5 '-3'	CGCAGGTTCTTGTGAAATGT
4	RM 235	F 5 '-3'	AGAAGCTAGGGCTAACGAAC
		R 5 '-3'	TCACCTGGTCAGCCTCTTTC
5	RM 240	F 5 '-3'	CCTTAATGGGTAGTGTGCAC
		R 5 '-3'	TGTAACCATTCCTTCCATCC
6	RM 208	F 5 '-3'	TCTGCAAGCCTTGTCTGATG
		R 5 '-3'	TAAGTCGATCATTGTGTGGACC
7	RM 220	F 5 '-3'	GGAAGGTAACTGTTTCCAAC
		R 5 '-3'	GAAATGCTTCCCACATGTCT
8	RM 236	F 5 '-3'	GCGCTGGTGGAAAATGAG
		R 5 '-3'	GGCATCCCTCTTTGATTCCTC
9	RM 248	F 5 '-3'	TCCTTGTGAAATCTGGTCCC
		R 5 '-3'	GTAGCCTAGCATGGTGCATG
10	RM 148	F 5 '-3'	ATACAACATTAGGGATGAGGCTGG
		R 5 '-3'	TCCTTAAAGGTGGTGCAATGCGAG
11	RM 226	F 5 '-3'	AGCTAAGGTCTGGGAGAAACC
		R 5 '-3'	AAGTAGGATGGGGCACAAGCTC
12	RM 338	F 5 '-3'	CACAGGAGCAGGAGAAGAGC
		R 5 '-3'	GGCAAACCGATCACTCAGTC
13	RM 590	F 5 '-3'	CATCTCCGCTCTCCATGC
		R 5 '-3'	GGAGTTGGGGTCTTGTTCG
14	RM 481	F 5 '-3'	TAGCTAGCCGATTGAATGGC
		R 5 '-3'	CTCCACCTCCTATGTTGTTG
15	RM 443	F 5 '-3'	GATGGTTTTCATCGGCTACG
		R 5 '-3'	AGTCCCAGAATGTCGTTTCG
16	RM 274	F 5 '-3'	CCTCGCTTATGAGCTTCG
		R 5 '-3'	CTTCTCCATCACTCCCATGG
17	RM 234	F 5 '-3'	ACAGTATCCAAGGCCCTGG
		R 5 '-3'	CACGTGAGACAAAGACGGAG

Sl. No.	Marker	Nucleotide sequence	
18	RM 578	F 5 '-3'	GGCGTCGTGTTTTCTCTCTC
		R 5 '-3'	CAAAAAGGAGGAGCAGATCG
19	RM 15780	F 5 '-3'	ACCTTCGACGCTATCAGATTTGG
		R 5 '-3'	ATAGCAAAGGAGTCGCAAAGACC
20	RM 251	F 5 '-3'	GAATGGCAATGGCGCTAG
		R 5 '-3'	ATGCGGTTCAAGATTCGATC
21	RM 273	F 5 '-3'	GAAGCCGTCGTGAAGTTACC
		R 5 '-3'	GTTTCCTACCTGATCGCGAC
22	RM 159	F 5 '-3'	GGGGCACTGGCAAGGGTGAAGG
		R 5 '-3'	GCTTGTGCTTCTCTCTCTCTCTCTCTCTC
23	RM 1054	F 5 '-3'	TGCATATGTACCGCAACCTC
		R 5 '-3'	TTTCTGCATGATCCCCTCTG
24	RM 25	F 5 '-3'	GGAAAGAATGATCTTTTCATGG
		R 5 '-3'	CTACCATCAAAACCAATGTTC
25	RM 42	F 5 '-3'	ATCCTACCGCTGACCATGAG
		R 5 '-3'	TTTGGTCTACGTGGCGTACA

The reaction mixture was given a short spin for thorough mixing of the cocktail components. Then the 0.2mL PCR-tubes were loaded on to a thermal cycler (MJ Research Inc. USA). The thermal cycler was programmed as follows.

Profile 1:	95ºC for 1 minute	Initial denaturation
Profile 2:	94ºC for 45 seconds	Denaturing
Profile 3:	55ºC for 1 minute	Annealing 34 cycles
Profile 4:	72ºC for 1 minute 30 seconds	Extension
Profile 5:	72ºC for 10 minutes	Final Extension
Profile 6:		4ºC for infinity to hold the samples

3.5 Agarose Gel Electrophoresis

After PCR amplification, products were separated by electrophoresis on agarose gels visualized by ethidium bromide staining.

3.6 Materials

Loading dye
Bromophenol Blue 0.5% (w/v)
10 x TBE (Tris Borate EDTA) Buffer
Trisbase – 10.76 gm
Boric acid – 5.5 gm
EDTA – 4 ml
(Dissolved in 800mL and made upto 1000mL and stored at 4ºC)

3.7 Protocol

The open ends of the Pyrex gel casting plate were sealed with cello tape and placed on a perfectly horizontal platform. Agarose (2.5 %) was added to 1X TBE, boiled until the agarose dissolved completely and then cooled to lukewarm temperature. Ethidium bromide was added. It was then poured in to the gel mould and then comb was placed properly and allowed to solidify. After solidification of the agarose, the comb and the cello tape were removed carefully. The casted gel was placed in the electrophoresis unit with wells towards the cathode and submerged with 1X TBE to a depth of about 1 cm.

3.8 Loading the DNA Samples

10µl of DNA samples were pipetted on to a parafilm and mixed well with 2µl of loading dye by pipetting up and down for several times. The gel was run at 90 volt until the tracking dye was one third of the gel and bands were visualized and documented in gel documentation system (Model Alpha Imager 1200, Alpha Innotech Corp., USA). The viewed picture was photographed and saved for further scrutiny.

3.9 Scoring

Clearly resolved, unambiguous bands were scored visually for their presence or absence with each primer. The scores were obtained in the form of matrix with ‘1’ and ‘0’ which indicate the presence and absence of bands in each variety respectively.

3.10 Data Analysis

Polymorphic Information Content (PIC) values were calculated for each of the SSR loci using the formula developed by Nei et al. [6].
$PIC = 1 - \Sigma x^2_k$

Where, x^2_k represents the frequency at the k^{th} allele.

The bands on the gel were scored for each of the SSR primer pairs in each genotype based on the presence or absence of bands generating a matrix of 1 and 0. Informative bands were used to calculate the genetic distance based on Jaccard's similarity coefficient using SIMQUAL procedure. The DNA data of SSR markers for 29 rice land races were clustered using an unweighted pair group method (UPGMA) with the module of with V5 software.

4. RESULTS AND DISCUSSION

4.1 Genetic Divergence Based on SSR Marker

4.1.1 Polymorphism of SSR markers

A total of 25 microsatellite markers were used to assess the extent of genetic diversity in the 31 rice land races and all of them were found polymorphic (Table 3).

In the present investigation, 25 SSR markers revealed 50 alleles in the 31 land races, the number of alleles per locus with an average of 2 per locus. This kind of less number of alleles per locus was already reported by Sundaram et al. [7] where an average of 4.86 alleles were detected, indicating less magnitude of diversity among the landraces.

Major allele frequency ranged from 0.58 (RM 25) to 0.94 (RM 229) with an average of 0.74 per marker. The PIC (polymorphism information content) value ranged from 0.12 (RM 229) to 0.66 (RM 481 and RM 25) with an average of 0.36 per marker (Plate 2).

The PIC value of each marker, which was evaluated on the basis of its alleles, varied greatly all tested SSR loci-from 0.58 to 0.94 with an average of 0.74 (Jain et al. 2004), 0.19 to 0.90 with an average of 0.75 [8], which is markedly higher than the result in our study. The highest PIC value 0.49 was obtained for RM481 and RM25, which indicates the efficiency of this primer in detecting the most heterogeneous landraces. This is in corroboration with the findings of Giarrocco et al. [9] followed by RM443 (0.47), RM224 (0.46) RM 159, RM 1054, RM226 and RM24 (0.44). Remaining markers were showed less Polymorphic Information Content (Table 4). Low PIC values for some other primers were earlier reported by Prabhakaran et al. [10]. Lower PIC value result of closely related landraces and higher PIC values result of diverse landraces. Upadhyay et al. [11] also reported the average PIC value of 0.78. These result revealed that the PIC value indicates that all these primers were highly informative and capable of distinguishing between landraces [12].

4.1.2 Cluster analysis of dendrogram constructed using SSR markers

The dendrogram based on UPGMA grouped the 31landraces into 10 clusters that were demarcated at a similarity coefficient of 0.68. Cluster III was the largest including 13 l and races followed by cluster VI and II each of which had four and three land races respectively. The remaining clusters had one and two landraces each. This dendrogram revealed that the land races that are originated from different places are not grouped together which is indicative of non parallelism exist between geographical origin and genetic diversity (Fig. 1).

Cluster I consisted of two landraces (Rajamudi and Gowrisanna), Cluster II contained (Rathnachudi, Raichurasanna and Kempujiddu) three landraces, largest Cluster III (Kempusale, Navara, Rathnasagara, Puttabhatta, Karijiddu, Dappavalya, Anekombinabhatta, Neregulibhatta, Champakali, Doddabirunellu, Madrassanna, Karigajavale and Rajabhoga), Cluster IV had two landraces (Bangarasanna and Kiruvani), Cluster V contained (Barmabl -ack and Mysurumallige) two landraces, Cluster VI were four (Nazarbad, MTU1001, Gilisale and Jyothi) landraces, Cluster VII only one landrace (Misebhatta) long awns in wild rice an indication of resistance to insect pest attack and efficient seed dispersal reported by Belinda et al. (2011). Cluster VIII were two (Gandasale and Ambemohari) landraces, Culster IX had only one (Jeerigesanna) and Cluster X consisted one (Jasmine) respectively. It's indicating these landraces were highly divergent and yield superior genotypes when crossed with landraces of the other clusters (Table.4).

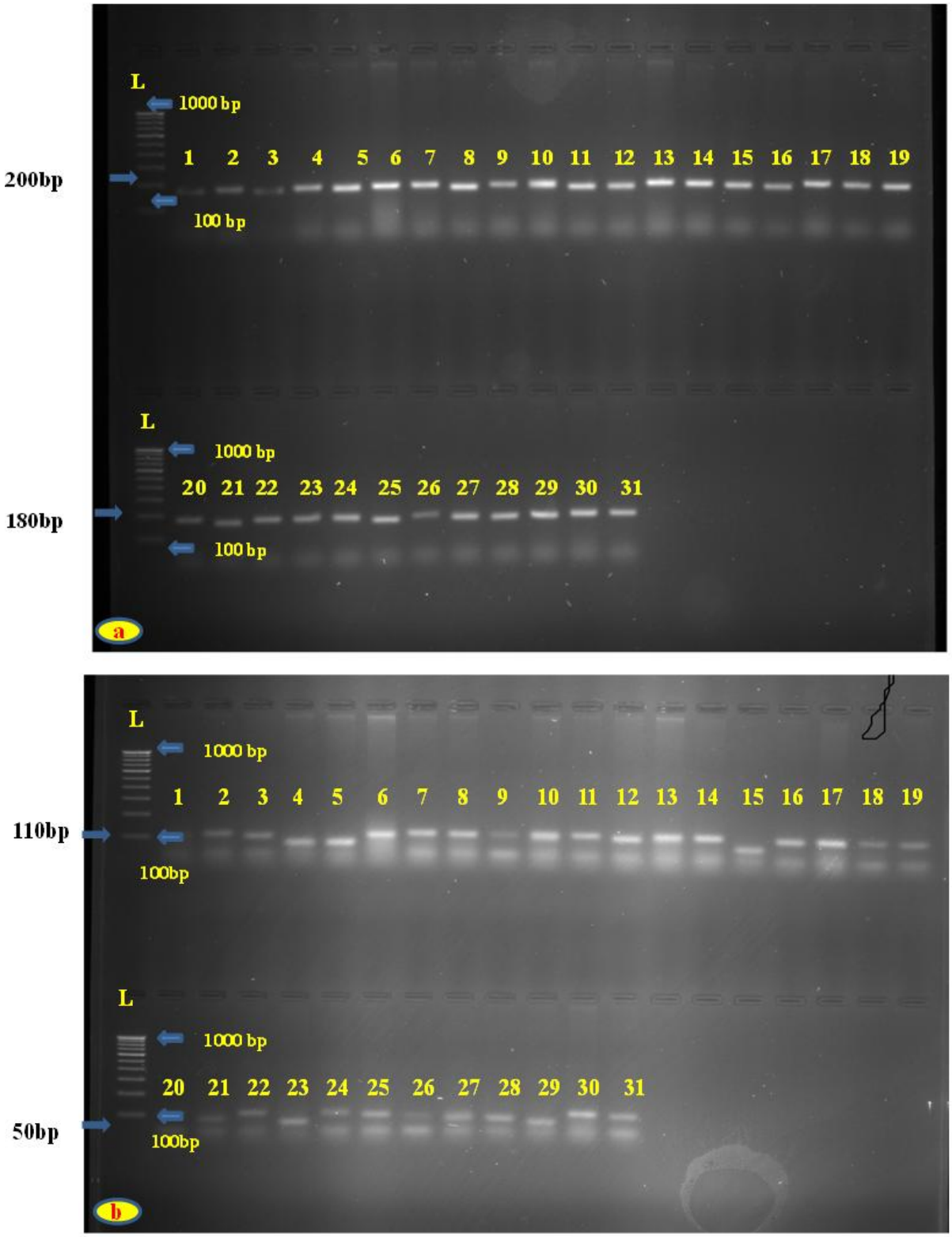

Plate 2. PCR amplification of rice landraces with RM 338 (a) and RM 248 (b) primers (L -100 bp ladder)

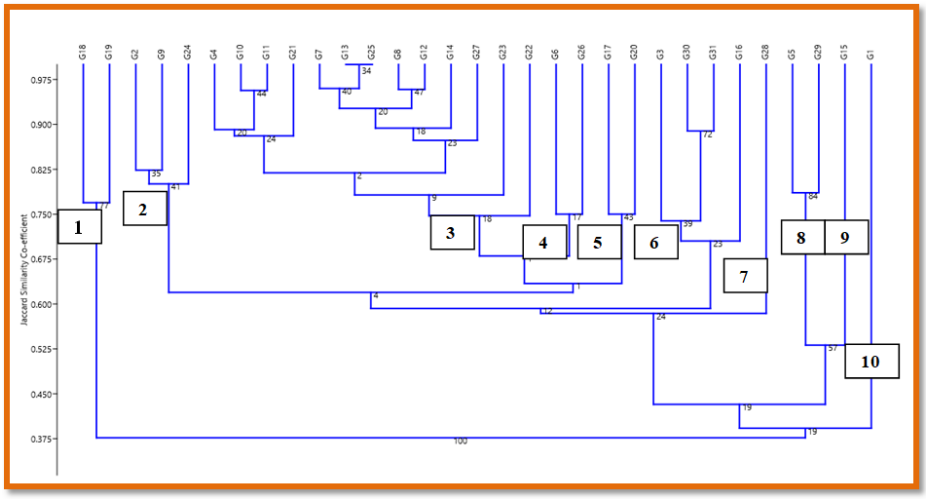

Fig. 1. Dendrogram representing distribution of 31rice landraces in ten different clusters

G1- Jasmine, G2-Ratnachudi, G3- Najarbad, G4- Rajabhoga, G5- Gandhasale, G6- Bangarasanna, G7-Champakali, G8- Dappavalya, G9- Raichurasanna, G10- Madrassanna, G11- Karigajavale, G12- Karijiddu, G13-Neregulibhatta, G14- Puttabhatta, G15- Jeerigesanna, G16- Gilisale, G17- Mysurumallige, G18- Rajamudi, G19-Gowrisanna, G20- Barmablack, G21- Doddabairunellu, G22- Kempusale, G23-Navara, G24- Kempujiddu, G25-Anekombinabhatta, G26- Kiruvani, G27- Rathnasagara, G28- Misebhatta, G29- Ambemohari, G30-Jyothi, G31-MTU1001

Table 3. SSR markers used to assess molecular diversity

Sl. No	SSR marker	Expected product size(bp)	Observed product size (bp)	No. Allele produced	Major allele frequency	PIC. value
1	RM 206	116-171	110-200	2	0.70	0.41
2	RM 224	123-155	110-180	2	0.65	0.46
3	RM 229	105-129	120-130	2	0.94	0.12
4	RM 235	109-136	100-180	2	0.77	0.35
5	RM 240	120-200	140-200	2	0.68	0.44
6	RM 208	150-200	180-200	2	0.74	0.38
7	RM 220	240-300	250-300	2	0.81	0.31
8	RM 236	170-200	180-200	2	0.87	0.22
9	RM 248	120-150	50-110	2	0.84	0.25
10	RM 148	100-130	100-120	2	0.87	0.22
11	RM 226	200-230	200-220	2	0.68	0.44
12	RM 338	160-200	180-200	2	0.71	0.41
13	RM 590	135-142	110-150	2	0.90	0.17
14	RM 481	169	120-200	2	0.58	0.49
15	RM 443	124	100-120	2	0.61	0.47
16	RM 274	160	180-200	2	0.88	0.22
17	RM 234	156	100-190	2	0.77	0.35
18	RM 578	220	200-210	2	0.77	0.35
19	RM 15780	130	100-120	2	0.71	0.41
20	RM 251	150	100-130	2	0.74	0.38
21	RM 273	170	110-150	2	0.77	0.35
22	RM 159	110-160	120-150	2	0.68	0.44
23	RM 1054	130-200	140-200	2	0.68	0.44
24	RM 25	146	110-150	2	0.58	0.49
25	RM 42	110-170	130-150	2	0.68	0.42
MINIMUM				2	0.58	0.12
MAXIMUM				2	0.94	0.49
MEAN				2	0.74	0.36

Table 4. Distribution of landraces to different clusters based on UPGMA method in genotypic dendrogram

Cluster No.	Number of landraces	Name of landraces
Cluster I	G18 and G19	Rajamudi and Gowrisanna
Cluster II	G2, G9 and G24	Ratnachudi, Raichursanna and Kempujiddu
Cluster III	G22, G23,G27, G14, G12, G8, G25 , G13, G7, G21, G11, G10 and G4	Kempusale, Navara, Ratnasagara, Puttabhatta, Karijiddu, Dappavalya, Anekombinabhatta, Neregulibhatta, Champakali, Doddabirunellu, Karigajavale, Madrassanna and Rajabhoga
Cluster IV	G6 and G26	Bangarasanna and Kiruvani
Cluster V	G17 and G20	Mysurumallige and Barmablack
Cluster VI	G16, G31, G30 and G3	Gilisale, MTU1001, Jyothi and Nazarbad
Cluster VII	G28	Misebhatta
Cluster VIII	G5 and G29	Gandasale and Ambemohari
Cluster IX	G15	Jeerigesanna
Cluster X	G1	Jasmine

5. CONCLUSION

The present study was concluded that the DNA fingerprinting and genetic diversity of 31 landraces of rice using SSR markers is effective. All of them 25 markers showed polymorphism. Major allele frequency ranged from 0.58 (RM 25) to 0.94 (RM 229) with an average of 0.74 per marker. The highest PIC value 0.49 was obtained for RM481 and RM25, followed by RM443 (0.47), RM224 (0.46) RM 159, RM 1054, RM226 and RM24 (0.44). Remaining markers were showed less PIC value. The dendrogram based on UPGMA grouped the 31landraces into 10 clusters that were demarcated at a similarity coefficient of 0.68. Cluster III was the largest including 13 land races followed by cluster VI and II each of which had four and three land races respectively. The remaining clusters had one and two landraces each.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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