Molecular characterization of Rice genotypes using molecular markers

Abstract

Rice, *Oryza sativa* L. (2n=24) belongs to the family Gramineae and subfamily Oryzoideae. *Oryza* has two cultivated species, *Oryza sativa* and *Oryza glaberrima*. *Oryza*, the common cultivated rice is grown worldwide. Breeding for good quality traits requires selection of parents with a wider genetic diversity. Molecular markers are used in molecular biology and biotechnology to identify a sequence of DNA. Molecular markers have played an increasing role in rice breeding for cultivar improvement, screening, selection and germplasm collections. The present investigation was undertaken to study the genetic diversity among thirty rice genotypes using ISSR marker. DNA was isolated using CTAB extraction method, with some modifications.The obtained DNA was of good quality and it was subjected to PCR amplification using 40 ISSR primers.

Out of 40 ISSR primers, 9 amplified and showed polymorphism viz., ISSR 807, ISSR 808, ISSR 809, ISSR 811, ISSR 816, ISSR 823, ISSR 826, ISSR 827, ISSR 829. Total of 76 loci were generated by amplification with 9 polymorphic primers, out of which 66 loci werepolymorphic with an average of 86.84 percent polymorphism. Among ISSR primers ISSR 807 produced maximum number of 11 loci. Dendrogram constructed by using NTSYSpc 2.02i software grouped all the 30 genotypes into two major clusters (clusters A and B). Cluster A Kalbhat alone is present in one sub cluster at 69.2 % similarity with Karjat 2, Jaya, Sairam, Saubhagya Dhan, RPBIO 226, Karjat 6, Ratnagiri 1, DRR Dhan 44, DRR Dhan 45, DRR Dhan 46, MTU 10010, Karjat 7, Swarna Shreya, Sugandha, and Madhumati. In Cluster B Karjat 3 alone forms one sub cluster and have 70 % similarity with MTU 1001, PKVHMT, JGL 1798, Indrayani, Phule Samruddhi, Phule Maval, Sonsal, Pavana, DRR Dhan 41, kundalika, Bhogawati, Ambemohar, and Phule Radha.

1. INRODUCTION

Rice, *Oryza sativa* L. (2n=24) belongs to the family Gramineae and subfamily Oryzoideae. *Oryza* has two cultivated species, *Oryza sativa* and *Oryza glaberrima*. *Oryza*, the common cultivated rice is grown worldwide. Rice (*Oryza sativa* L.) is a true diploid (2n=24) with twelve chromosome pairs and contains 5.8 x 10^5 kb/haploid genome (Bonnet and Smith, 1976). There is ample polymorphism in rice DNA and it is highly recombinogenic compared to other plants. Moreover, rice is also an ideal model plant for the study of grass genetics and genome organization due to its diploid genetics, relatively small genome size of 430 Mb (Causse *et al.,* 1994; Kurata *et al*., 1994). It is monocot plant; It is the staple food for more than half of the world's population and occupies almost one-fifth of the total land area covered under cereals It is an important food crop of the world both in terms of area (163.43 million ha) and production (498.95 million metric tons). In India, rice contributes to about 45 percent of cereal production and is the main food source for more than 60 percent of population in the country. We will have to produce 40 percent more rice by the year 2030 to satisfy the growing demand without affecting the resource base adversely.

The recent development of DNA markers has providednew opportunities for the genetic improvement of rice grain quality. Genetic diversity shows Genetic divergence among the genotypes that plays an important role in selection of parents having wider variability for different characters. Genetic divergence analysis quantifies thegenetic distance among the selected genotypes. For the assessment of genetic diversity molecular markers have been generally superior to morphological and biochemical data. Molecular markers provide reliable estimates of genetic diversity, may improve screening efficiency for many traits through their linkage having alleles with small (quantitative traits) and with large (qualitative traits) effects and will provide the first understanding of biology and architecture of quantitative traits at the DNA level. Molecular markers have played an increasing role in rice breeding for cultivar improvement, screening, selection and germplasm collections. Molecular marker based genetic diversity analysis also has potential for assessing changes in genetic

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Comment [U1]: In several parts of the manuscript, it is stated that 10 primers were used.

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diversity over time and space. Among various PCR based markers, SSR and ISSR markers are more popular in rice because they are highly informative, mostly monolocus, codominant, easily analyzed and cost effective. Inter simple sequence repeats (ISSR), which involves PCR amplification of DNA using a single primer composed of a micro satellite sequence anchored at the 3`" or 5`" end by 2-4 arbitrary nucleotides,is one of the DNA based molecular marker which could be used to assess genetic diversity (Qian *et al*., 2001). It has been successfully employed to assess genetic diversity within and between populations in several plant species (Liu and Wendell, 2001). The molecular characterization and fingerprinting of these released varieties using ISSR markers will provide sufficient knowledge on diversity among them at molecular level, which will help the breeders to develop strategies for the future, and the variety specific fingerprints will enable to identify and characterize each variety released.

Thus, the present investigation entitled **"Molecular characterization of rice genotypes using molecular markers"** was undertaken to analyze genetic diversity among different 30 rice genotypes using ISSR marker.

2. MATERIAL AND METHODS

Thise study ~~on **"Molecular characterization of rice genotypes using molecular markers"**~~ was carried out at the Division of Botany, Biotechnology laboratory, College of Agriculture, Pune. Country?

2.1 Plant Materials

Experimental material of thirty high yielding genotypes of rice seeds obtained from Agricultural Research Station, Maval, Pune. List of selected rice genotypes and their characteristic features given in Table 1.

Table 1: List of selected rice genotypes and their features

Sr. No.	Genotype Name	Characteristic Features
1	Karjat – 2	Dwarf stature, long slender grains, moderately resistant to blast and neck blast, recommended for rainfed region.
2	MTU-1010	Short slender, semi- dwarf (108 cm), resistant to blast & tolerant to Brown Plant Hopper.
3	Karjat – 3	Dwarf stature, short bold grain, resistant to blast suitable for rainfed uplands as well as irrigated areas for *kharif* and *rabi* season, Grains: short bold and scented.
4	Sairam	Short slender grain, early maturity.
5	Swarna Shreya	Long slender, suitable for rainfed low land and direct seeded aerobic condition with maturity period of 120-125 days, can withstand drought and also tolerance to many diseases and insects.
6	Sugandha	Tall (130-140 cm), grains: Medium Slender with aroma, white, moderately resistant to BLB and Pest complex.
7	Madhumati	Scented, long slender, late maturity.
8	Karjat – 7	Dwarf stature, long slender grain, early maturity, moderately resistant to brown plant hopper and bacterial leaf blast.
9	Karjat – 6	Short slender grain, resistant to bacterial leaf blast & brown plant hopper, suitable for midland under rainfed & irrigated conditions.
10	Ratnagiri-1	Long bold grain, Semi dwarf (100-105 cm), early maturity, moderately resistant to blast and neck blast, grains: long bold, moderately susceptible to BLB.
11	DRR Dhan-44	Short duration, medium slender, varieties with moderate tolerance to biotic (BPH and blast) and abiotic stress (drought).
12	DRR Dhan-45	Mid early duration culture (~130 days) with long slender grains, high zinc content (18.18 ppm) with high yield potential.

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Comment [JCWDA5]: There are only thirteen rice genotypes and not thirty!

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13	DRR Dhan-46	Long slender, mid late, high zinc with high yield potential and moderate tolerance to Brown Plant Hopper and blast and drought.

2.2 Growing of seedlings:

The seeds of thirty genotypes were grown in the tray at College of Agricultural, Pune. The leaves were plucked when the seedlings ~~were of~~were 20 days old for DNA extraction.

Plate 1: Raising of seedlings of different rice varieties

2.3. Extraction of genomic DNA (CTAB Method):

The genomic DNA was isolated from different rice genotypes using CTAB extraction method suggested by Doyle and Doyle (1990) with slight modifications. The buffer extraction was prepared according to Table 2. Two ~~2~~ gm leaves sample of rice varieties were crushed in a mortar and pestle using liquid nitrogen. After crushing, 1 ml of CTAB extraction buffer is added to the crushed sample and ~~mixed it~~mixed properly in centrifuge tube. Then tubes were incubated at 65ºC for 45 minutes in water bath with occasional mixing. Chloroform: Isoamyl alcohol (24:1) was added in equal volume and mixed by gentle inversion for 5-6 times. (This step was repeated ~~by~~ twice) These tubes were centrifuged at 12000 rpm for 10 minutes in a centrifuge machine. The contents got separated into two distinct phases. The aqueous phase containing DNA was pipette out and added equal volume of chilled iso- propanol, 100µl 3M sodium acetate to it and incubated at -20 ºC for overnight. On the next day the solution was centrifuged at 12000 rpm for 10 minutes. Pellet obtained was washed with 100 µl of 70 percent ethanol and centrifuged at 10000rpm for 10 minutes. Pellet was air dried and dissolved in 50 µl double distilled water. This DNA was stored at -20°C for further use.

Purification of DNA samples was done to remove RNA, proteins and Polysaccharides which were the major contaminants. RNA and proteins were removed with RNase A and Proteinase K treatment respectively. 1µl of RNase A and Proteinase K was added to the DNA sample and incubated at 37°C for 20 min. after each addition. The PVP used during extraction helpeds ~~to~~ the elimination of polysaccharides.

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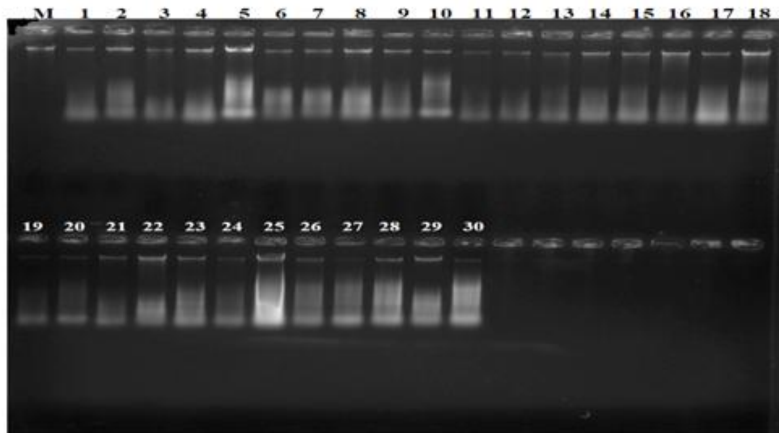

Plate 2: Isolated genomic DNA samples from 30 Rice genotypes

M-DNA Ladder, 1)Karjat – 2, 2)MTU-1010, 3)Karjat – 3, 4)Sairam, 5)Swarna Shreya, 6)Sugandha, 7)Madhumati, 8)Karjat – 7, 9)Karjat – 6, 10)Ratnagiri-1, 11)DRR Dhan-44, 12)DRR Dhan-45, 13)DRR Dhan-46, 14)Saubhagya Dhan, 15)Jaya, 16)Kalbhat, 17)MTU-1001, 18)RP-BIO-226, 19)PKV-HMT, 20)JGL-1798, 21)Indrayani, 22)Kundalika, 23)Phule Samruddhi, 24)Ambemohar, 25)Phule Radha, 26)Sonsal, 27)Phule Maval, 28)Pavana, 29)DRR Dhan-41, 30)Bhogawati.

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2.4. DNA quantification and quality checking:

~~Quality~~The quality of DNA in the sample was determined by agarose gel electrophoresis with isolated DNA on 0.8 percent agarose gel. ~~Agarose~~The agarose gel electrophoresis unit was cleaned with 70% ethanol properly before use. Agarose gel (0.8percent) was prepared by dissolving 0.8 g of agarose in 100 ml 1X TBE buffer and heated in microwave oven. 7 µl Ethidium bromide was added to it after cooling down to 45 to 50 ºC. The gel was poured in casting tray in which comb was inserted previously and kept for 30 min. After solidification of the gel, the comb was removed. 2 µl of DNA was mixed with 1µl of 6X gel loading dye and loaded on the gel. The electrophoresis was carried out at 3 to 5 volts per cm of run using 1X TBE buffer. The gel was then visualized under gel documentation unit to check the results.

2.5. Assessing the quality and quantity of DNA using spectrophotometers:

All the DNA samples were quantified spectrophotometrically. Before taking sample readings, the instrument was set to zero by taking 1µl autoclaved distilled water as blank. One micro~~-~~liter of nucleic acid samples was measured at a wavelength of 260 nm and 280 nm and OD_{260}/OD_{280} ratios were recorded to assess the purity of DNA. A ratio of 1.8 to 2.0 for OD_{260}/OD_{280} indicated good quality of DNA. The amount of DNA is calculated by using the following formula: ~~,~~

DNA (ng/µl) = A260 x 50 x dilution factor.

2.6. Molecular marker assay

Diversity analysis of rice was carried out using Inter Simple Sequence Repeats (ISSR) marker. ~~ISSR assay was performed to detect the polymorphism in amplification pattern in the region between two SSR's.~~ This was carried out by amplifying the DNA by using one ~~specific~~ primer~~s~~ designed ~~relating~~to be complementary ~~to~~ ~~the~~ SSR regions, the amplification could occur in the region between two SSR's, detecting polymorphisms in the amplification pattern~~flanking the ISSR~~. The good quality genomic DNA isolated from leaf samples of the selected genotypes was subjected to ISSR assay as per the procedure reported by Zietkiewicz *et al.* (1994). Total ten ISSR primers with good resolving power were selected after an initial forty primers screening listed in (Table ~~2~~3).

Comment [JCWDA8]: In the abstract, it is stated that nine primers were used

Comment [JCWDA9]: This is Table 3

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Table 2: Details components CTAB buffer

Sr. No.	Components	Stock concentration	Working concentration	Volume for 100ml
1	Tris HCl buffer	1M	100mM	12.11ml
2	NaCl	4M	1.4M	23.37ml
3	EDTA	0.5M	20Mm	14.61ml
4	CTAB (percent)	10	2	10ml
5	Bmercaptoethanol (percent)	4	0.2	4gm
6	PVP (percent)	10	1	1gm
7	Sterile double distilled water			
	Total			**100ml**

2.7. PCR reaction for ISSR analysis:

The PCR reaction was set for 20 µl reaction volume given in (table ~~3~~4). Master Mix was prepared for each primer with the above reagents and divided into different PCR tubes (each tube 20 µl). Two µl of different genomic DNA samples was added to master mix that led to final quantity of 20 µl. PCR tubes were placed in thermal cycler for amplification of the genomic DNA.

Table 3: Details of primer selected for ISSR assay

Sr.No	Primer	Nucleotide sequence (5'–3')	Sr. No	Primer	Nucleotide sequence (5'–3')
1	UBC801	ATATATATATATATATT	21	UBC821	GTGTGTGTGTGTGTGTT
2	UBC802	ATATATATATATATATG	22	UBC822	TCTCTCTCTCTCTCTCA
3	UBC803	ATATATATATATATATC	23	UBC823	TCTCTCTCTCTCTCTCC
4	UBC804	TATATATATATATATAA	24	UBC824	TCTCTCTCTCTCTCTCG
5	UBC805	TATATATATATATATAC	25	UBC825	ACACACACACACACACT
6	UBC806	TATATATATATATATAG	26	UBC826	ACACACACACACACACC
7	UBC807	AGAGAGAGAGAGAGAGT	27	UBC827	ACACACACACACACACG
8	UBC808	AGAGAGAGAGAGAGAGC	28	UBC828	TGTGTGTGTGTGTGTGA
9	UBC809	AGAGAGAGAGAGAGAGG	29	UBC829	TGTGTGTGTGTGTGTGC
10	UBC810	GAGAGAGAGAGAGAGAT	30	UBC830	TGTGTGTGTGTGTGTGG
11	UBC811	GAGAGAGAGAGAGAGAC	31	UBC831	ATATATATATATATATYA
12	UBC812	GAGAGAGAGAGAGAGAA	32	UBC832	ATATATATATATATATYC
13	UBC813	CTCTCTCTCTCTCTCTT	33	UBC833	ATATATATATATATATYG
14	UBC814	CTCTCTCTCTCTCTCTA	34	UBC834	AGAGAGAGAGAGAGAGYT
15	UBC815	CTCTCTCTCTCTCTCTG	35	UBC835	AGAGAGAGAGAGAGAGYC
16	UBC816	CACACACACACACACAT	36	UBC836	AGAGAGAGAGAGAGAGYA
17	UBC817	CACACACACACACACAA	37	UBC837	TATATATATATATATART
18	UBC818	CACACACACACACACAG	38	UBC838	TATATATATATATATARC
19	UBC819	GTGTGTGTGTGTGTGTA	39	UBC839	TATATATATATATATARG
20	UBC820	GTGTGTGTGTGTGTGTC	40	UBC840	GAGAGAGAGAGAGAGAYT

Note-Single letter abbreviations for mixed base positions R=(A,G);Y=(C,T).

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a. Agarose gel electrophoresis of amplified PCR products Requirements:

Agarose (2.0 g) was added to 100 ml of 1x TBE buffer and agarose was melted by heating the solution in microwave oven. Solution was cooled to about 55-60 °C and 7 to l of Ethidium bromide (0.7 to l/ ml) was added in it. The agar solution was poured into the gel casting unit after keeping the gel comb in the proper place. The gel was allowed to solidify at room temperature. Gel was placed in the electrophoresis apparatus in such a way that the end with wells is in line with the cathode. The apparatus was filled with 1x TBE buffer in order to submerge the gel in the buffer to prevent the entry of air bubbles while removing the gel combs

b. Resolutions of amplified product:

The amplified products were run on 2 percent agarose gel using 1X TAE buffer and stained with ethidium bromide along with DNA ladder. The profile was visualized under UV transilluminator and documented using gel documentation system. The documented ISSR profiles were carefully examined for polymorphism. ~~Number~~The number of bands produced by each primer were counted and tabulated.

c. Data Scoring and analysis:

Amplification profiles of all thirty rice genotypes with different primers were compared with each other and bands of DNA fragments scored manually as (1) or (0) depending on the presence or absence of a particular band respectively. The data was analyzed using Numerical Taxonomy System of Multivariate Statistical Program (NTSYS) software package. The dendrogram was constructed using Unweighted Pair Group Method of Arithmetic Averages (UPGMA).

3. RESULTS AND DISCUSSION

The present study on "Molecular characterization of rice genotypes using molecular markers" was conducted at the Division of Botany, Biotechnology laboratory, College of Agriculture, Pune. ISSR markers have high specificity, high reproducibility, multi-allelism, high polymorphism, more frequent and co-dominant nature, therefore have been used in many types of genetic analysis such as the construction of linkage maps, molecular characterization of germplasm and identification of molecular markers for marker- assisted selection. The results obtained based on analysis of 30 genotypes using UBC-ISSR primers are furnished in (Table-4).

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Table 4: Components of PCR reaction mixture

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Sr. No.	Components	Stock concentration	Working concentration	Volume for one tube
1	10X PCR buffer	10X	1X	2µl
2	$MgCl_2$	25mM	1.5mM	1.2µl
3	~~DNTPs~~dNTPs	10mM	200µl	0.4µl
4	Taq DNA polymerase	3U/µl	1U	1µl
5	Primer	100pmol/µl	10pmol/µl	1µl
6	Genomic DNA	30ng/µl	60ng	2.0µl
7	Sterile double distilled water			12.4µl
	Total			**20µl**

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The PCR amplification of template DNA produced a total of 172 bands among the 30 genotypes. The number of polymorphic markers obtained on analysis of 30 genotypes was 9. These were used to characterize and estimate genetic diversity among 30 genotypes of rice.

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3.1. Genomic DNA isolation

Plant genomic DNA of thirty cultivars of rice was isolated by using modified CTAB method (Doyle and Doyle, 1990). The protocol of CTAB DNA extraction method was used with some modification. ~~Pure~~A pure white pellet was ~~obtained~~obtained, and it was dissolved in double distilled water and was further analyzed for its

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quality and quantity.

3.2. Quantitative and qualitative analysis of DNA

The quantification of isolated DNA was done by measuring absorbance at 260nm wavelength and purity was checked by taking the ratio of absorbance (A260/A280). Qualitative analysis was also done by resolving DNA on 0.8% agarose gel. Detailed absorbance and purity ratio~~n~~s ~~were~~ are given in (T~~t~~able ~~no. 5~~6). The A260/A280 ratio was around 1.85 which indicated the purity of the genomic DNA obtained using modified CTAB method.

Table 5: PCR program for ISSR assay.

Step No.	**Temp. (°C)**	**Duration**	**Cycles**	**Function**
1	95	5min		Initial denaturation
2	95	1min	30 Cycles	Denaturation
3	50or52	1min		Annealing
4	72	1min		Extension
5	72	10min		Final extension
6	4	Pause		Final hold

3.3. PCR reaction for ISSR primers

The PCR amplification was done by using the 20µl PCR reaction. Annealing temperature for each of the primer pair was optimized by using different temperatures in gradient PCR. The annealing temperatures optimized for each of five primer set is given (Table ~~6~~5). Based on consistency of bands, the optimum concentration of PCR reaction mixture which gave reproducible results was used for ten ISSR primers analyzed in 30 rice varieties.

Table 6: DNA quality assay of taken genotypes

Sr. No.	Sample name	OD at 260 (nm)	OD at 280	Ratio of 260/280	Concentration
1	Karjat – 2	0.495	0.275	1.80	24.75
2	MTU-1010	0.393	0.256	1.54	19.65
3	Karjat – 3	0.386	0.196	1.97	19.3
4	Sairam	0.405	0.223	1.82	20.25
5	Swarna Shreya	0.401	0.221	1.81	20.05
6	Sugandha	0.393	0.215	1.83	19.65
7	Madhumati	0.395	0.210	1.88	19.75
8	Karjat – 7	0.414	0.233	1.78	20.7
9	Karjat – 6	0.405	0.216	1.88	20.25
10	Ratnagiri-1	0.413	0.226	1.83	20.65
11	DRR Dhan-44	0.415	0.238	1.74	20.75
12	DRR Dhan-45	0.414	0.239	1.73	20.7
13	DRR Dhan-46	0.413	0.219	1.89	20.65
14	Saubhagya Dhan	0.414	0.214	1.93	20.7
15	Jaya	0.416	0.224	1.86	20.8
16	Kalbhat	0.396	0.229	1.73	19.8
17	MTU-1001	0.413	0.219	1.89	20.65
18	RP-BIO-226	0.402	0.225	1.79	20.1
19	PKV-HMT	0.42	0.236	1.78	21
20	JGL-1798	0.417	0.249	1.67	20.85
21	Indrayani	0.398	0.219	1.82	19.9
22	Kundalika	0.406	0.217	1.87	20.3
23	Phule Samruddhi	0.432	0.236	1.83	21.6
24	Ambemohar	0.412	0.218	1.89	20.6
25	Phule Radha	0.401	0.209	1.92	20.05
26	Sonsal	0.435	0.239	1.82	21.75
27	Phule Maval	0.437	0.246	1.78	21.85
28	Pavana	0.419	0.220	1.90	20.95

Comment [JCWDA13]: This corresponds to Materials and methods section.

Comment [JCWDA14]: Ten or nine?

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29	DRR Dhan-41	0.417	0.234	1.78	20.85
30	Bhogawati	0.418	0.231	1.81	20.9

3.4. Molecular analysis using ISSR marker:

The molecular characterization of 30 genotypes of rice with 9 ISSR markers was done and presented in terms of PIC value for each primer & diversity analysis was done through similarity matrix and dendrogram obtained by using NTSYS-PC. All the selected ISSR primers were found to be polymorphic in the present study. These results are presented here under two ~~sub heads~~subheads. Among the ISSR primers, 807 produced maximum number of 11 bands followed by 811 and 827 (10 bands). However, ~~least~~the least number of bands was amplified by ISSR 823 primer (5 bands). The highest (100%) polymorphism was shown by ISSR 807, ISSR 808 primer while ISSR 827 primer showed minimum that is 60% polymorphism given in (Table 7).

Table 7: Annealing temperature optimized for ~~different~~ the chosen 9 ISSR primers

Sr. No.	Primer name	Annealing temp(^{0}C)	Sr. No.	Primer name	Annealing temp(^{0}C)
1	ISSR807	50	6	ISSR823	52
2	ISSR808	52	7	ISSR826	52
3	ISSR809	52	8	ISSR827	52
4	ISSR811	52	9	ISSR829	52
5	ISSR816	50			

The PIC values were calculated to find out the efficiency of primers in distinguishing individual genotypes. The polymorphism information Content (PIC) values of ISSR primers ranged from 0.40 to 0.80 in ISSR primer. Further it was observed that there was no correlation between per cent polymorphism and PIC values as ISSR primers ISSR 807, ISSR 808 showed maximum per cent polymorphism and ISSR primer 807 showed maximum PIC value followed by 808 and 823 Further it was revealed that minimum per cent polymorphism showed by ISSR 829 while ISSR 826 showed minimum PIC indicating ISSR 807 was more informative and ISSR primer 808 and 823 were moderately informative.

The ISSR primer, 823 amplified 5 ~~fragment~~fragments out of which 4 were polymorphic, 1 was monomorphic and 1 was unique (Table 7, plate 8). The unique fragment having size 400 bp was present in Kalbhat genotype. The ISSR 809 amplified 9 ~~fragment~~fragments out of which 8 were polymorphic, 1 was monomorphic and 1 was unique (Table 7, plate 5). The unique fragment having size 500 to 600 bp and was present in Kalbhat genotype only. The ISSR primer, 808 amplified 9 fragment and all 9 were polymorphic, and 1 ~~were~~was unique (Table 7, plate 4). The unique fragment having size 1 kb was present in Kalbhat and Ambemohar genotype only.

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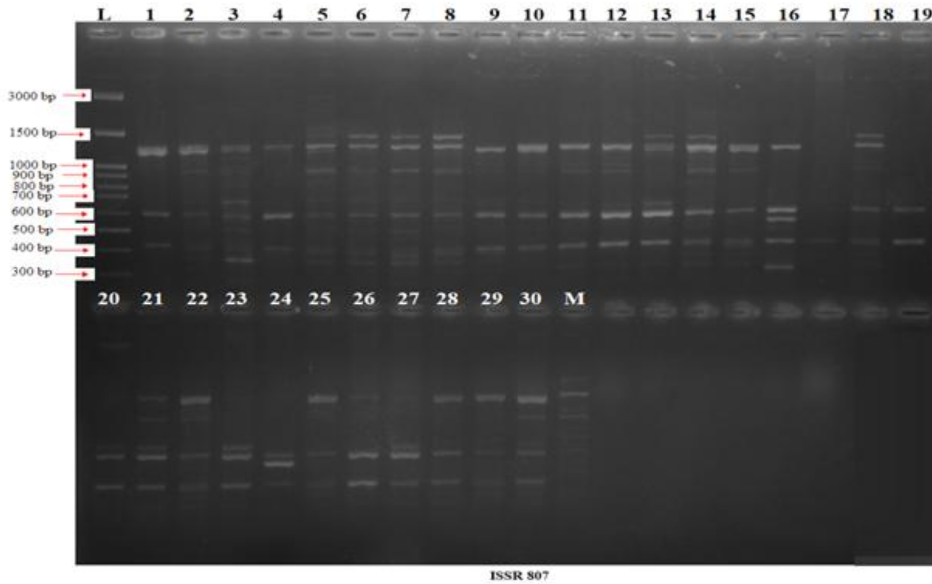

Plate 3: ISSR banding profile of thirty rice genotypes with ISSR 807 primer

L-DNA Ladder, 1)Karjat – 2, 2)MTU-1010, 3)Karjat – 3, 4)Sairam, 5)Swarna Shreya, 6)Sugandha, 7)Madhumati, 8)Karjat – 7, 9)Karjat – 6, 10)Ratnagiri-1, 11)DRR Dhan-44, 12)DRR Dhan-45, 13)DRR Dhan-46, 14)Saubhagya Dhan, 15)Jaya, 16)Kalbhat, 17)MTU-1001, 18)RP-BIO-226, 19)PKV-HMT, 20)JGL-1798, 21)Indrayani, 22)Kundalika, 23)Phule Samruddhi, 24)Ambemohar, 25)Phule Radha, 26)Sonsal, 27)Phule Maval, 28)Pavana, 29)DRR Dhan-41,30)Bhogawati.

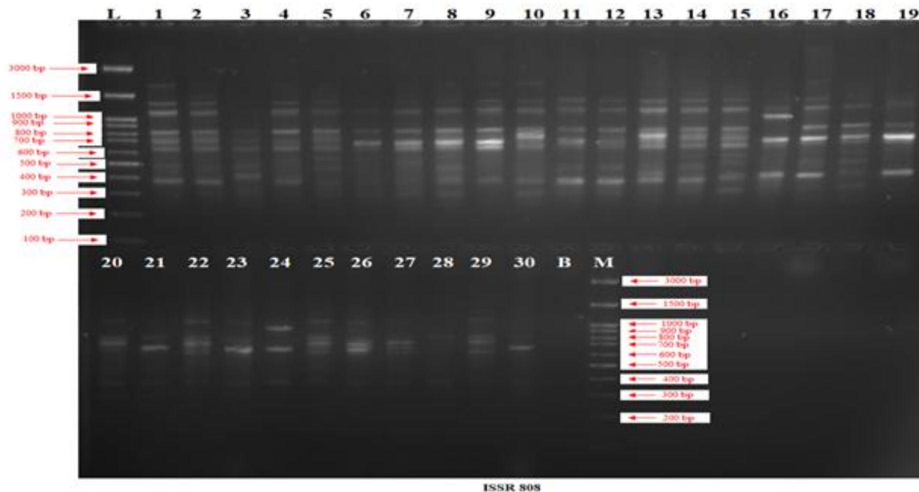

Plate 4: ISSR banding profile of thirty rice genotypes with ISSR 808 primer

L-DNA Ladder, 1)Karjat – 2, 2)MTU-1010, 3)Karjat – 3, 4)Sairam, 5)Swarna Shreya, 6)Sugandha, 7)Madhumati, 8)Karjat – 7, 9)Karjat – 6, 10)Ratnagiri-1, 11)DRR Dhan-44, 12)DRR Dhan-45, 13)DRR Dhan-46, 14)Saubhagya Dhan, 15)Jaya, 16)Kalbhat, 17)MTU-1001, 18)RP-BIO-226, 19)PKV-HMT, 20)JGL-1798, 21)Indrayani, 22)Kundalika, 23)Phule Samruddhi, 24)Ambemohar, 25)Phule Radha, 26)Sonsal, 27)Phule Maval, 28)Pavana, 29)DRR Dhan-41, 30)Bhogawati.

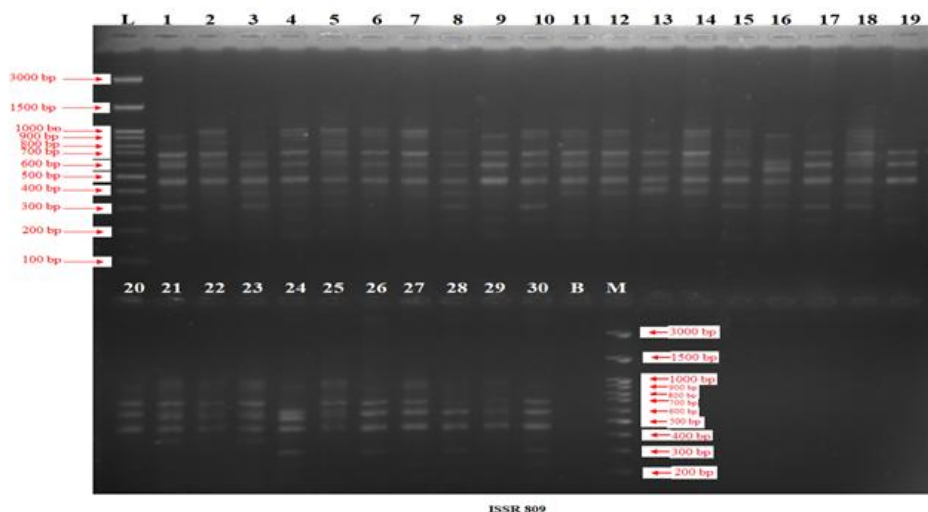

Plate 5: ISSR banding profile of thirty rice genotypes with ISSR 809 primer

L-DNA Ladder, 1)Karjat – 2, 2)MTU-1010, 3)Karjat – 3, 4)Sairam, 5)Swarna Shreya, 6)Sugandha, 7)Madhumati, 8)Karjat – 7, 9)Karjat – 6, 10)Ratnagiri-1, 11)DRR Dhan-44, 12)DRR Dhan-45, 13)DRR Dhan-46, 14)Saubhagya Dhan, 15)Jaya, 16)Kalbhat, 17)MTU-1001, 18)RP-BIO-226, 19)PKV-HMT, 20)JGL-1798, 21)Indrayani, 22)Kundalika, 23)Phule Samruddhi, 24)Ambemohar, 25)Phule Radha, 26)Sonsal, 27)Phule Maval, 28)Pavana, 29)DRR Dhan-41, 30)Bhogawati.S

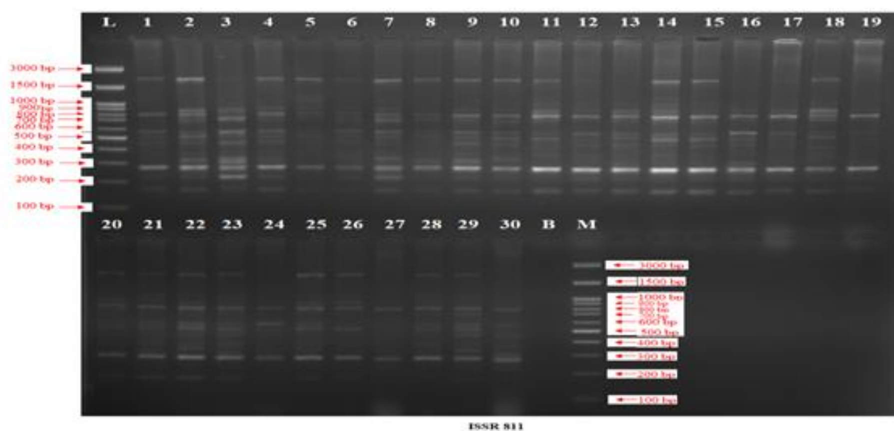

Plate 6: ISSR banding profile of thirty rice genotypes with ISSR 811 primer

L-DNA Ladder, 1)Karjat – 2, 2)MTU-1010, 3)Karjat – 3, 4)Sairam, 5)Swarna Shreya, 6)Sugandha, 7)Madhumati, 8)Karjat – 7, 9)Karjat – 6, 10)Ratnagiri-1, 11)DRR Dhan-44, 12)DRR Dhan-45, 13)DRR Dhan-46, 14)Saubhagya Dhan, 15)Jaya, 16)Kalbhat, 17)MTU-1001, 18)RP-BIO-226, 19)PKV-HMT, 20)JGL-1798, 21)Indrayani,

22)Kundalika, 23)Phule Samruddhi, 24)Ambemohar, 25)Phule Radha, 26)Sonsal, 27)Phule Maval, 28)Pavana, 29)DRR Dhan-41, 30)Bhogawati.

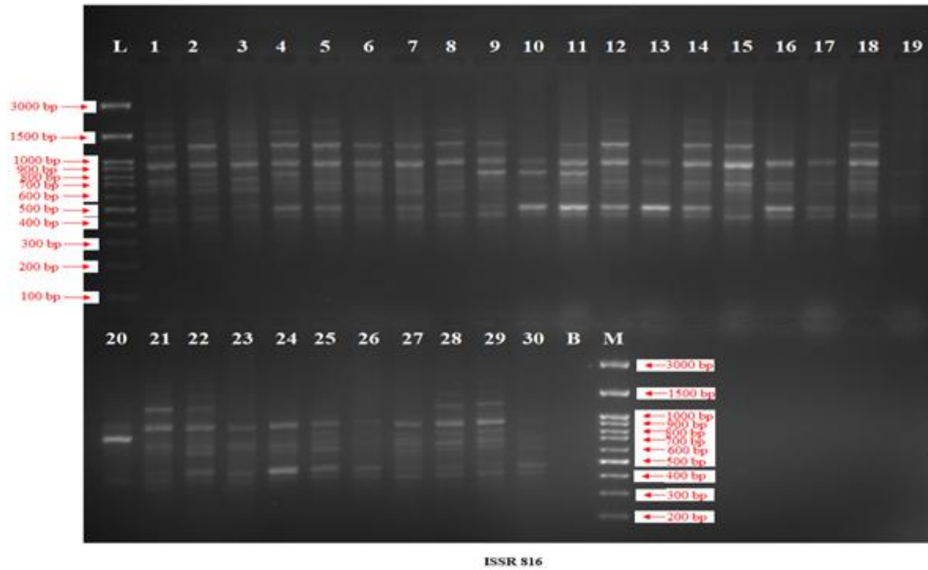

Plate 7: ISSR banding profile of thirty rice genotypes with ISSR 816 primer

L-DNA Ladder, 1)Karjat – 2, 2)MTU-1010, 3)Karjat – 3, 4)Sairam, 5)Swarna Shreya, 6)Sugandha, 7)Madhumati, 8)Karjat – 7, 9)Karjat – 6, 10)Ratnagiri-1, 11)DRR Dhan-44, 12)DRR Dhan-45, 13)DRR Dhan-46, 14)Saubhagya Dhan, 15)Jaya, 16)Kalbhat, 17)MTU-1001, 18)RP-BIO-226, 19)PKV-HMT, 20)JGL-1798, 21)Indrayani, 22)Kundalika, 23)Phule Samruddhi, 24)Ambemohar, 25)Phule Radha, 26)Sonsal, 27)Phule Maval, 28)Pavana, 29)DRR Dhan-41, 30)Bhogawati.

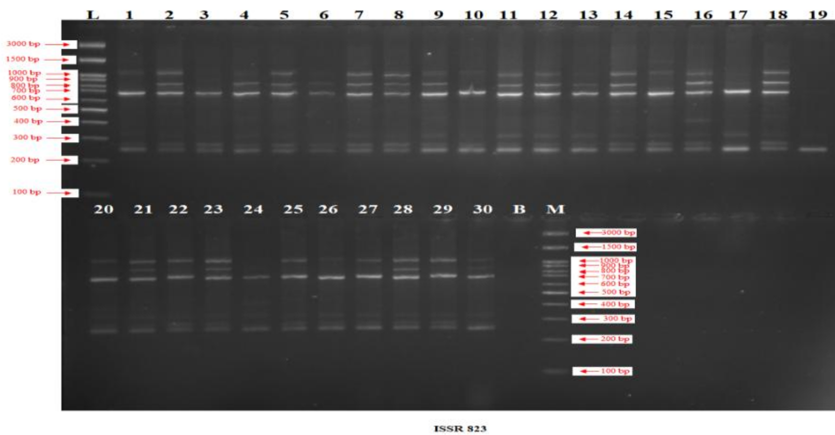

Plate 8: ISSR banding profile of thirty rice genotypes with ISSR 823 primer

L-DNA Ladder, 1)Karjat – 2, 2)MTU-1010, 3)Karjat – 3, 4)Sairam, 5)Swarna Shreya, 6)Sugandha, 7)Madhumati, 8)Karjat – 7, 9)Karjat – 6, 10)Ratnagiri-1, 11)DRR Dhan-44, 12)DRR Dhan-45, 13)DRR Dhan-46, 14)Saubhagya

Dhan, 15)Jaya, 16)Kalbhat, 17)MTU-1001, 18)RP-BIO-226, 19)PKV-HMT, 20)JGL-1798, 21)Indrayani, 22)Kundalika, 23)Phule Samruddhi, 24)Ambemohar, 25)Phule Radha, 26)Sonsal, 27)Phule Maval, 28)Pavana, 29)DRR Dhan-41, 30)Bhogawati.

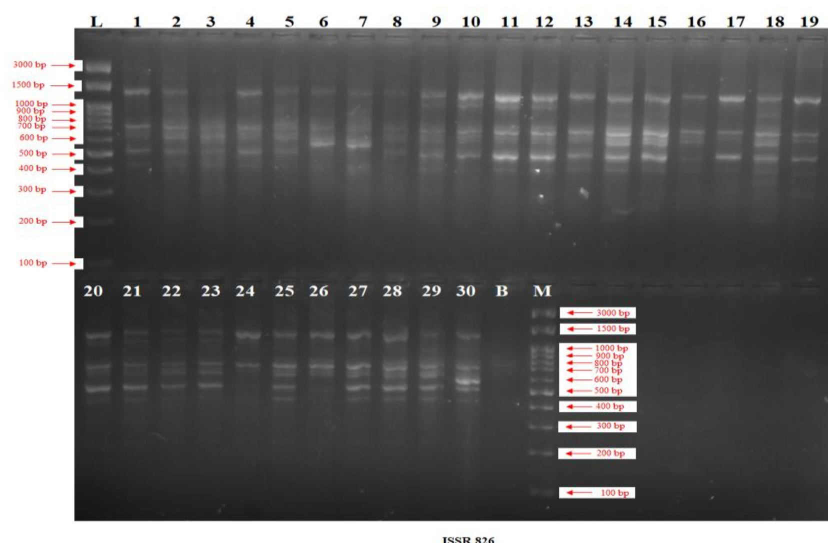

Plate 9: ISSR banding profile of thirty rice genotypes with ISSR 826 primer

L-DNA Ladder, 1)Karjat – 2, 2)MTU-1010, 3)Karjat – 3, 4)Sairam, 5)Swarna Shreya, 6)Sugandha, 7)Madhumati, 8)Karjat – 7, 9)Karjat – 6, 10)Ratnagiri-1, 11)DRR Dhan-44, 12)DRR Dhan-45, 13)DRR Dhan-46, 14)Saubhagya Dhan, 15)Jaya, 16)Kalbhat, 17)MTU-1001, 18)RP-BIO-226, 19)PKV-HMT, 20)JGL-1798, 21)Indrayani, 22)Kundalika, 23)Phule Samruddhi, 24)Ambemohar, 25)Phule Radha, 26)Sonsal, 27)Phule Maval, 28)Pavana, 29)DRR Dhan-41, 30)Bhogawati

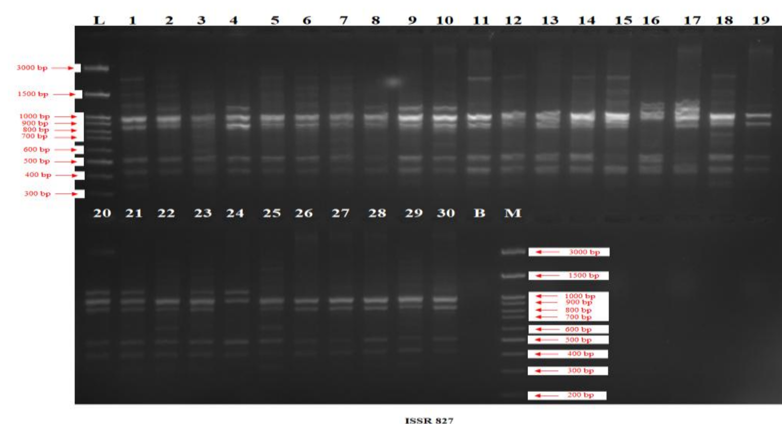

Plate 10: ISSR banding profile of thirty rice genotypes with ISSR 827 primer

L-DNA Ladder, 1)Karjat – 2, 2)MTU-1010, 3)Karjat – 3, 4)Sairam, 5)Swarna Shreya, 6)Sugandha, 7)Madhumati, 8)Karjat – 7, 9)Karjat – 6, 10)Ratnagiri-1, 11)DRR Dhan-44, 12)DRR Dhan-45, 13)DRR Dhan-46, 14)Saubhagya Dhan, 15)Jaya, 16)Kalbhat, 17)MTU-1001, 18)RP-BIO-226, 19)PKV-HMT, 20)JGL-1798, 21)Indrayani,

22)Kundalika, 23)Phule Samruddhi, 24)Ambemohar, 25)Phule Radha, 26)Sonsal, 27)Phule Maval, 28)Pavana, 29)DRR Dhan-41, 30)Bhogawati.

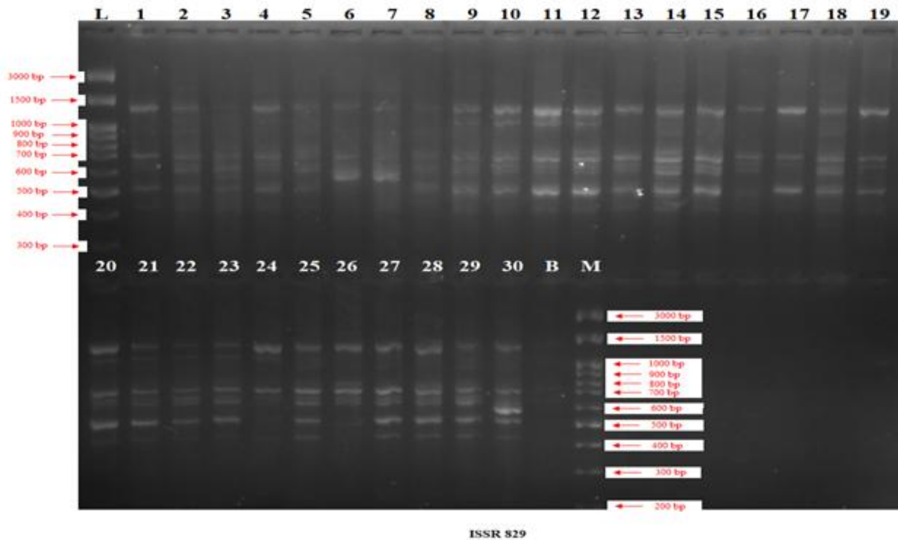

Plate 11: ISSR banding profile of thirty rice genotypes with ISSR 829 primer

L-DNA Ladder, 1)Karjat – 2, 2)MTU-1010, 3)Karjat – 3, 4)Sairam, 5)Swarna Shreya, 6)Sugandha, 7)Madhumati, 8)Karjat – 7, 9)Karjat – 6, 10)Ratnagiri-1, 11)DRR Dhan-44, 12)DRR Dhan-45, 13)DRR Dhan-46, 14)Saubhagya Dhan, 15)Jaya, 16)Kalbhat, 17)MTU-1001, 18)RP-BIO-226, 19)PKV-HMT, 20)JGL-1798, 21)Indrayani, 22)Kundalika, 23)Phule Samruddhi, 24)Ambemohar, 25)Phule Radha, 26)Sonsal, 27)Phule Maval, 28)Pavana, 29)DRR Dhan-41, 30)Bhogawati.

3.5. Genetic diversity analysis by ISSR primers:

Genetic similarity matrix was obtained by using NTSYS-PC for ISSR data (Table 8). The pair wise similarity values ranged from 0.50 to 0.92. Maximum similarity value of 0.92 was noticed between DRR Dhan 44 and DRR Dhan 45. Minimum similarity value of 0.50 was observed between rice genotype Ambemohar and Swarna Shreya. From these studies it is revealed that rice landraces are less divergent indicating that large part of the genome may be similar among themselves.

Table 8: PIC value, allele information and product size of ISSR primer

ISSR Primer	No. of allele	Polymorphic bands	Monomorphic bands	Percent Polymorphism	PIC Value	Product size (bp)
ISSR807	11	11	0	100	0.80	300to1500
ISSR808	9	9	0	100	0.61	400to3000
ISSR809	9	8	1	88	0.56	300to1000
ISSR811	10	9	1	90	0.51	300to3000
ISSR816	8	7	1	87.5	0.45	400to3000
ISSR823	5	4	1	80	0.53	400to1500
ISSR826	6	5	1	83.33	0.40	500to1500

UNDER PEER REVIEW

ISSR827	10	6	4	60	0.46	400to3000
ISSR829	8	7	1	87.5	0.42	400to1500

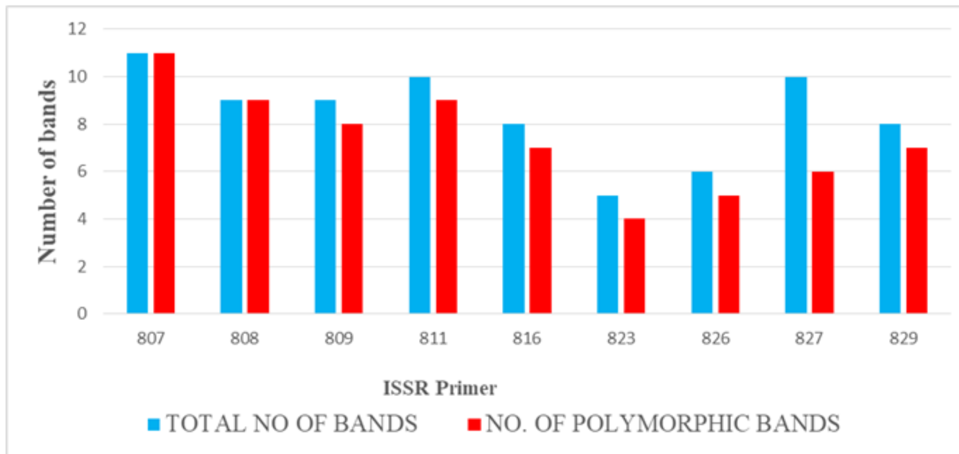

Fig.1 Total bands and polymorphic bands amplified by ISSR primer

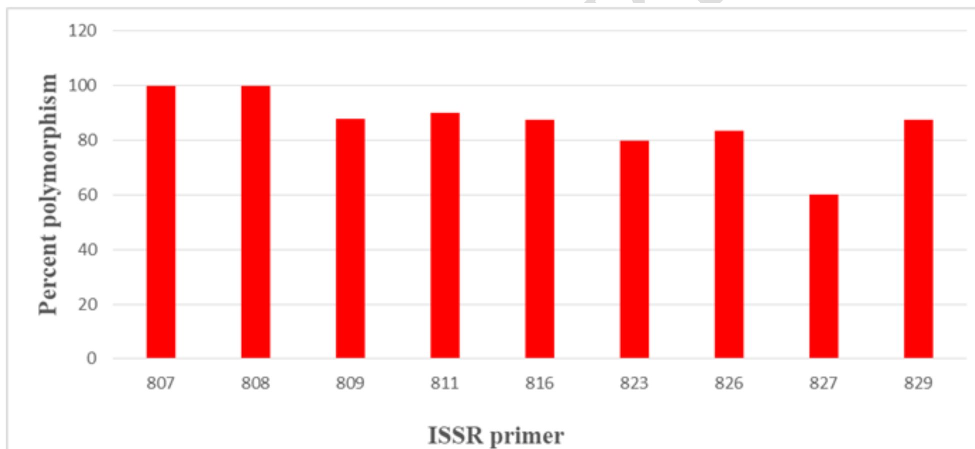

Fig.2 Total percent polymorphism detected by each ISSR primer used in this research~~s~~

The UPGMA based dendrogram of thirty rice genotypes generated with NTSYSpc 2.02i program~~me~~ was presented in (Fig.2). It was observed that two major clusters A and B were generated with dendrogram. Cluster A ~~consist~~consists of genotypes Karjat 2, Jaya, Sairam, Saubhagya Dhan, RPBIO 226, Karjat 6, Ratnagiri 1, DRR Dhan 44, DRR Dhan 45, DRR Dhan 46, MTU 10010, Karjat 7, Swarna Shreya, Sugandha, Madhumati, Kalbhat. Among cluster A, DRR Dhan 44 and DRR Dhan 45 has highest similarity that is 92 percent. Further cluster A is diversified in to two sub clusters. Kalbhat alone is present in one sub cluster with 69.2 % similarity with second sub cluster of A. The second sub cluster of A had varieties viz., Karjat 2, Jaya, Sairam, Saubhagya Dhan, RPBIO 226, Karjat 6, Ratnagiri 1, DRR Dhan 44, DRR Dhan 45, DRR Dhan 46, MTU 10010, Karjat 7, Swarna Shreya, Sugandha, Madhumati. The second sub cluster consists of two groups. The first of the two groups consist of genotypes Karjat 2, Jaya, Sairam, Saubhagya Dhan, RPBIO 226, Karjat 6, Ratnagiri 1, DRR Dhan 44, DRR Dhan 45, DRR Dhan 46. In the first group maximum similarity value of 0.92 was noted between DRR Dhan 44 and DRR Dhan 45. The second group ~~comprise~~is comprised of MTU 10010, Karjat 7, Swarna Shreya, Sugandha,

Madhumati. In the second group Sugandha and Madhumati have 85 % similarity.

Cluster B consists of Karjat 3, MTU 1001, PKVHMT, JGL 1798, Indrayani, Phule Samruddhi,Phule Maval, Sonsal, Pavana, DRR Dhan 41, kundalika, Bhogawati, Ambemohar, Phule Radha. Karjat 3 alone forms one sub cluster and has 70 % similarity with second sub cluster of B. The second sub cluster of B have varieties MTU 1001, PKVHMT, JGL 1798, Indrayani, Phule Samruddhi,Phule Maval, Sonsal, Pavana, DRR Dhan 41, kundalika, Bhogawati, Ambemohar, Phule Radha. This second cluster comprised of two groups at 71 % similarity. ~~First~~The first group had MTU 1001, PKVHMT with 75 % similarity.

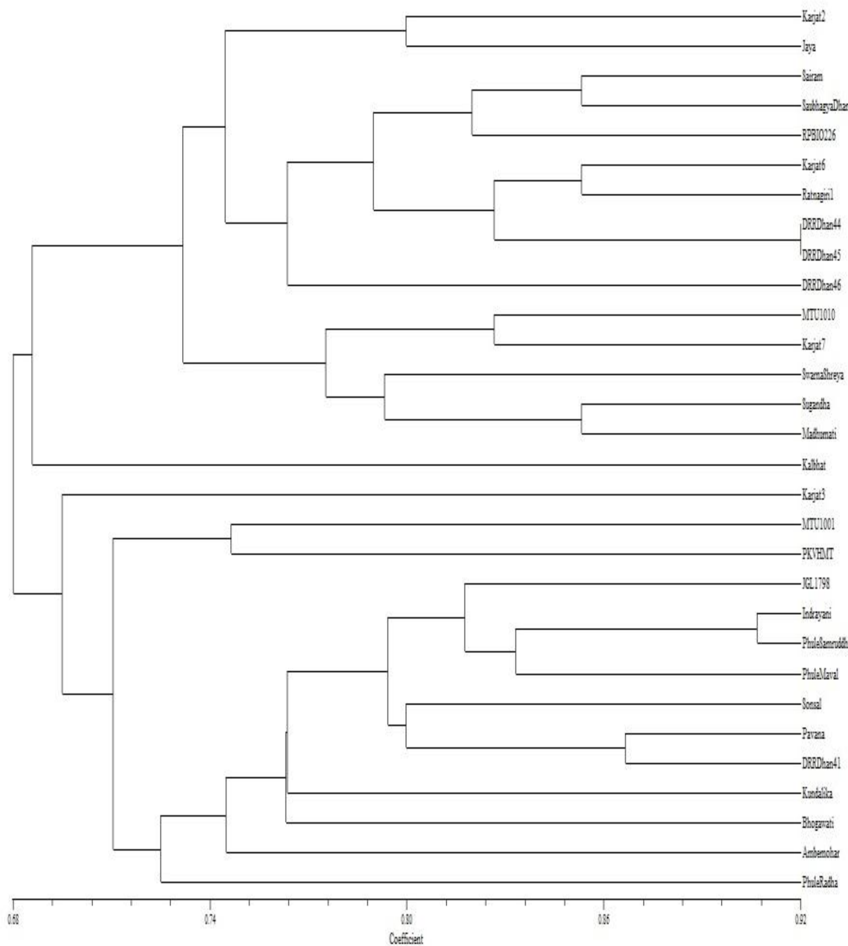

Fig. 3 Dendrogram showing phylogenetic relationship among thirty rice genotypes generated from ten ISSR primer

The second group has JGL 1798, Indrayani, Phule Samruddhi,Phule Maval, Sonsal, Pavana, DRR Dhan 41, kundalika, Bhogawati, Ambemohar, Phule Radha. In this group Indrayani and Phule Samruddhi has 91% similarity. From the dendrogram constructed on the basis of results obtained by ISSR 807, ISSR 808, ISSR 809, ISSR 811, ISSR 816, ISSR 823, ISSR 826, ISSR 827 and ISSR 829 primer, It is concluded that crosses should be made between cluster A and Cluster B, also crosses should be made between Kalbhat and other genotypes in cluster A and between Karjat 3 and other genotypes in cluster B. In this study, Swarna Shreya and Ambemohar were found to be most ~~diversed~~diverse than other genotypes and Kalbhat, Ambemohor and karjat 3 had shown some unique bands so they could be utilized for developing superior hybrids through various breeding strategies.

All screened primers are found to be polymorphic with high polymorphic information content which efficiently ~~discriminating~~discriminates the varieties at molecular level. By looking at the PIC values and polymorphic percentage, it can be concluded that the ISSR marker can be suitably used for assessing the genetic diversity among different rice genotypes.

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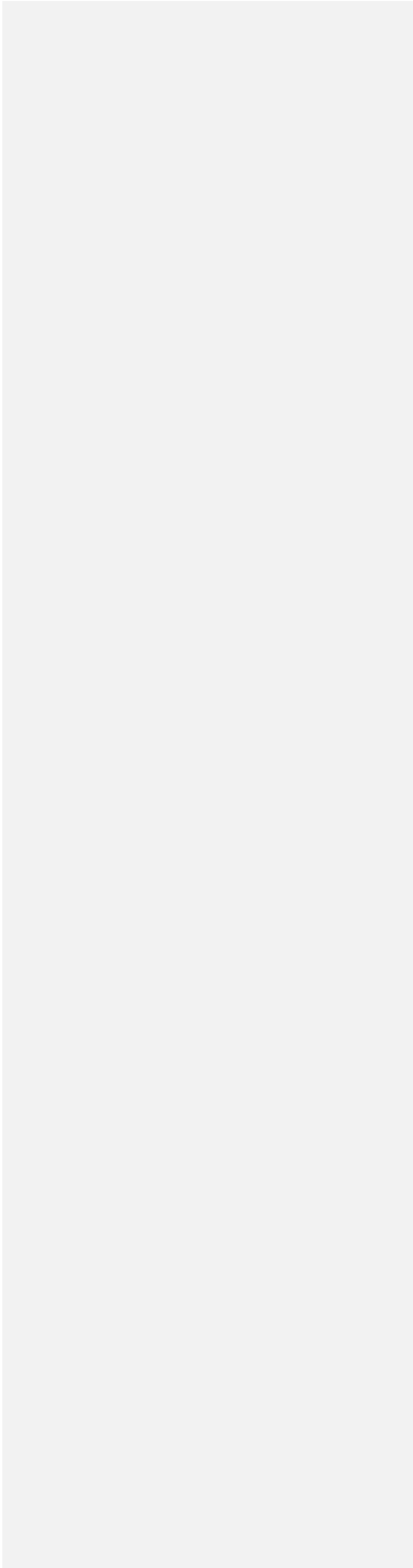

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