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Sahu Shail
Department of Genetics and Plant Breeding, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India

NK Rastogi
Department of Genetics and Plant Breeding, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India

PK Chandrakar
Department of Genetics and Plant Breeding, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India

Ritu R Saxena
Department of Genetics and Plant Breeding, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India

Corresponding Author:
Sahu Shail
Department of Genetics and Plant Breeding, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India

Genotyping of rice accessions using SSR markers

Sahu Shail, NK Rastogi, PK Chandrakar and Ritu R Saxena

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Abstract
Molecular characterization of germplasm accessions of rice revealed genetic polymorphism and ensured unambiguous identification. A total of 24 SSR markers were used covering all the chromosomes of rice for their molecular characterization and discrimination. After analysis of the data generated, a total of 69 alleles were detected in 24 accessions of rice. The number of alleles per locus generated by each marker ranged from 1 to 6 alleles with an average number of 2.87 alleles per locus. The highest number of alleles (6) was detected in the locus RM 22565 while, lowest number of alleles (1) detected on each of locus OSR 13, RM 431, RM 454 and Xa5s. The PIC value ranged from 0.00 (OSR 13, RM 431, RM 454 and Xa5s) to 0.76 (RM 12146). Microsatellite markers (SSR) are also used to detect the genetic similarity of germplasm accessions of rice. The genetic similarity coefficient ranged from 0.24 to 1.00 as revealed by UPGMA cluster analysis using the 24 SSR markers. A total of five distinct groups resulted at a cut-off similarity coefficient of 0.46 among the 24 rice accessions, below which the similarity values narrowed conspicuously. Coefficient of similarity revealed that the rice accessions of cluster I were genetically distant from cluster IV. Thus, Peeleeluchai (135131) and Mahuwadeta Lal of cluster I; whereas, Kadamphool and Ram Karoni of cluster IV seems to be promising and should be utilized in hybridization programme. Molecular markers like RM 1, RM 12146, RM 215, RM 22710 RM 154 and RM 25 could potentially be used for molecular characterization of rice germplasm accessions from various sources on the basis of polymorphic reactions and high PIC values.

Keywords: Accessions, DNA fingerprinting, polymorphic, rice, SSR

1. Introduction
Genetic diversity probably serves as are insurance against crop failure (Subba Rao *et al.* 2001) [7]. Collection and characterization of the germplasm is not only important for utilizing the appropriate attitude based on donors in breeding programme but it is essential in the present era for protecting the unique rices. The amount of genetic variation in germplasm accessions and genetic relationships between genotypes are important consideration for designing breeding programme. Advances in plant genetics and molecular biology have led to the development of many types of molecular markers which can be used to characterize germplasm. Different types of DNA markers are available now-a-days, each method differing in principle, application, types and amount of polymorphism detected, cost and requirement. Simple Sequence Repeats (SSR) is codominant, abundant and highly reproducible and exhibits a high degree of allelic variations. SSR are excellent molecular marker system for many types of genetic analysis including linkage mapping, germplasm surveys and polygenetic studies. Microsatellites (also known as SSR) are simple, tandem repeated, di- to tetra nucleotide sequence and valuable as genetic markers because they detect high level of allelic diversity, coordinate easily and economically assayed by PCR and are easily automated. Characters may be qualitative or quantitative in nature. They may be governed by one or more genes. The quantitative traits are influenced by environment, which indicates that such characters are not stable and hence, cannot be used as marker traits whereas, qualitative traits may be used as morphological markers with low reliability because they are less influenced by environment.
On the other hand, molecular markers are DNA based markers and it represents the genetic constitution of any individual. DNA of any individual does not influenced by environment, hence the DNA based markers are supposed to be stable markers to diagnose any trait. The molecular markers are powerful tools in the assessment of genetic variation and having potential to detect genetic diversity in the management of plant genetic resources. In the present study, 24 rice germplasm accessions were used for molecular characterization and genetic diversity study.

2. Materials and Methods

2.1 Plant Material

A Field experiment was conducted during Kharif 2016 at Research cum instructional Farm, IGKV, Raipur. The experimental material consisted of 47 rice germplasm accessions including checks namely, Danteshwari, Indira Aerobic-1, IGKV R-1, IGKV R-2, and Safri 17 (Table 1). Each entry was transplanted in a single row at spacing of 20 cm between rows and 15 cm between plants. The standard agronomical practices were adopted for normal crop growth. All the 47 accessions were used for molecular characterization using 24 SSR and 31 ISSR primers to determine the genetic diversity.

Table 1: List of germplasm accessions of rice

S. No.	CGR Number	IC Number	Name of Accessions/ Genotypes	District
1.	CGR:1735	IC 132668	Lali Ajan	Bastar
2.	CGR:1737	IC 132671	Ajaniya	Bastar
3.	CGR:1760	IC 132694	Badshah Bhog (B)	Durg
4.	CGR:1761	IC132695	Badshah Bhog	Raigarh
5.	CGR:1768	IC 132702	Badshah Bhog (II)	Shahdol
6.	CGR:1774	IC 132708	Jogi Bhog	Mahasamund
7.	CGR:1795	IC 132732	Vishnu Bhog	Sarguja
8.	CGR:1803	IC 132740	Angur Guchcha	Durg
9.	CGR:1809	IC 132746	Anjan (II)	Shahdol
10.	CGR:1810	IC 132747	Anjan (I)	Mandla
11.	CGR:1811	IC 132748	Anjani	Mandla
12.	CGR:1813	IC 132750	Anjan	Mungeli
13.	CGR:1972	IC 132920	Dhabli Banko (I)	Raipur
14.	CGR:1978	IC 132935	Lal Banko	Raipur
15.	CGR:1990	IC 132398	Mudi Banko	Sarguja
16.	CGR:1991	IC 132939	Raj Banko	Bastar
17.	CGR:1994	IC 132942	Sugujihi Banko	Bilaspur
18.	CGR:2009	IC 132957	Banskupi	Shahdol
19.	CGR:3653	IC 135043	Luchai	Mandla
20.	CGR:3738	IC 135128	Peeleeluchai	Balaghat
21.	CGR:3741	IC 135131	Peeleeluchai	Balaghat
22.	CGR:3808	NA	Mahuwadeta Lal	Mandla
23.	CGR:3819	IC 135209	Mauhakuchi	Raipur
24.	CGR:3831	IC 135221	Makado	Bastar
25.	CGR:3863	IC 135253	Malpa (I)	Raipur
26.	CGR:3866	IC 135256	Mancha	Bastar
27.	CGR:4336	NA	Patel 27 Type 4	Durg
28.	CGR:4378	IC 135769	Kadamphool	Bastar
29.	CGR:4464	IC 135855	Rajniti (A)	Shahdol
30.	CGR:4521	IC 214037	Ram Karoni	Balaghat
31.	CGR:4909	IC 214410	Surmatia	Bastar
32.	CGR:4971	NA	X:5 Bhairamgarh	Bastar
33.	CGR:5123	IC 124706	Anjan (A)	Raipur
34.	CGR:5737	IC 113998	Barangi	Bastar
35.	CGR:5740	IC 113999	Barangi	Raipur
36.	CGR:5817	IC 114049	Moti Basmati	Seoni
37.	CGR:5818	IC 114050	Baspan	Raipur
38.	CGR:5854	IC 114073	Bauwara	Raipur
39.	CGR:5897	IC 114093	Bhaya	Raigarh
40.	CGR:5988	NA	Bhujani	Durg
41.	CGR:6046	NA	Bhurkund	Raigarh
42.	CGR:6261	IC 125488	Chitarboti	Bastar
43.			Danteshwari (Check)	IGKV Raipur
44.			Indira Aerobic 1 (Check)	IGKV Raipur
45.			Rajeshwari (Check)	IGKV Raipur
46.			Durgeshwari (Check)	IGKV Raipur
47.			Safri 17 (Check)	IGKV Raipur

2.2 Genomic DNA isolation

Total genomic DNA was extracted using CTAB method (Zheng *et al.* 1995) [16].

2.3 Procedure

1. Young plant leaves were collected at seeding stage, about one gram of leaves bits were cut by scissors and put in 2 ml of Eppendrof tube.
2. Add 700 μl of CTAB extraction buffer.
3. Grind the leaves with the help of tissue lyzer. After grinding add 300 μl of CTAB extraction buffer.
4. Keep it in water bath at 65 °C for 20 minutes.
5. Add 700 μl of Chloroform: Isoamyl alcohol (24:1).
6. Vertex the sample.
7. Centrifuge it for 10 min at 14000 rpm in centrifuge machine.
8. Transfer the supernatant in 1.5 ml of fresh Eppendrof tube and repeat the protocol twice form step 5-8).

9. Add 70 μl of sodium acetate and about 400 μl of pre-chilled isopropanol (equal volume of the supernatant transferred) in this and kept it for incubation at 4 °C for 2 hrs or -20 °C for overnight.
10. Centrifuge it for 3 min @ 1400 rpm.
11. Decant the solution and add 50 μl of 70% ethanol for washing and centrifuged at 14000 rpm for 5 minutes.
12. Decant the solution and dry the pellet for 2 hours or overnight until the smell of ethanol was evaporated.
13. Finally dissolved the pellets in 50 μl of TE buffer.
14. Stored at -20 °C until use.

2.4 PCR amplification and electrophoresis Data analysis

PCR Reaction: 2 μl of diluted template DNA of each genotype was dispensed at the bottom of PCR plate. Separately cocktail was prepared in an Eppendorf tube as described in Table 2. About 8 μl of cocktail were added to each sample and the PCR was set up as the profile depicted in Table 3.

Table 2: PCR mix for one reaction

Reagent	Stock Concentration	Volume (µl)
Sterile and nanopure H_2O	-	5.2
PCR Buffer A	10 X	1.0
dNTPs (Mix)	2.5 mM	0.5
Primer (Forward+reverse)	5 pmol	1.0
Taq polymerase	3 U/µl	0.3
DNA Tempelate	50 ŋg / µl	2.0
Total		10.0

Table 3: Temperature profile used for PCR amplification using micro-satellite markers

Steps	Temperature (˚C)	Duration (min.)	Cycles	Activity
1	94	5	1	Denaturation
2	94	0.5	↑	Denaturation
3	55	0.5	35	Annealing
4	72	1	↓	Extension
5	72	7	1	Final Extension
6	4	∞		Storage

Table 4: Detection of varietal polymorphism using simple sequence repeats (SSR) markers

S. No.	Name of Primers	Forward Sequence	Reverse Sequence
1	RM 25	GGAAAGAATGATCTTTTCATGG	CTACCATCAAAACCAATGTTC
2	RM 19	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA
3	RM 1	GCGAAAACAATGCAAAAA	GCGTTGGTTGGACCTGAC
4	RM 11	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG
5	RM 161	TCTCCTCTTCCCCCGATC	ATAGCGGGCGAGGCTTAG
6	RM 431	TCCTGCGAACTGAAGAGTTG	AGAGCAAAACCCTGGTTCAC
7	RM 152	GAAACCACCACACCTCACCG	CCGTAGACCTTCTTGAAGTAG
8	OSRqw 13	CATTTGTGCGTCACGGAGTA	AGCCACAGCGCCCATCTCTC
9	RM 408	CAACGAGCTAACTTCCGTCC	ACTGCTACTTGGGTAGCTGACC
10	RM 171	AACGCGAGGACACGTACTTAC	ACGAGATACGTACGCCTTTG
11	RM 154	ACCCTCTCCGCCTCGCCTCCTC	CTCCTCCTCCTGCGACCGCTCC
12	RM 527	GGCTCGATCTAGAAAATCCG	TTGCACAGGTTGCGATAGAG
13	RM 433	TGCGCTGAACTAAACACAGC	AGACAAACCTGGCCATTCAC
14	RM 22710	CGCGTGGGCGAGACTAATCG	CCTTGACTCCGAGGATTCATTGTCC
15	RM 22565	TCCACGCGTTGTCGTAGAAATTTAGC	AGCCCGAGCACCATGAAACACC
16	RM 287	TTCCCTGTTAAGAGAGAAATC	GTGTATTTGGTGAAAGCAAC
17	RM 316	CTAGTTGGGCATACGATGGC	ACGCTTATATGTTACGTCAAC
18	RM 447	CCCTTGTGCTGTCTCCTCTC	ACGGGCTTCTTCTCCTTCTC
19	RM 215	CAAAATGGAGCAGCAAGAGC	TGAGCACCTCCTTCTCTGTAG
20	RM 454	CTCAAGCTTAGCTGCTGCTG	GTGATCAGTGCSCCATAGCG
21	Xa 13	GGCCATGGCTCAGTGTTTAT	GAGCTCCAGCTCTCCAAATG
22	Xa 5s	GGCTCAATCTAGAAAGTCCG	TTACAGAGGTGGCGATAGAG
23	RM 12146	AGTATGCCCTGCCCTGCCCACTACACTAG	CAGCGAATGGCAAGAGCAAC
24	RM 242	GGCCAACGTCACGGAGTA	AGCCACAGCGCCCATCTCTC

Table 5: List of 24 microsatellite markers with their chromosome locations, number of alleles, allelic size and PIC value found among 24 rice accessions

SSR Primers	Chromosomal locations	Number of Alleles	Allele Size (bp)	PIC value
RM242	9	2	200, 250	0.079
RM11	7	2	130, 140	0.079
RM527	6	3	133, 120, 125	0.288
RM25	8	3	140, 150, 155	0.621
OSR13	3	1	100	0
RM1	1	4	100, 117, 125, 130	0.725
RM22710	8	4	145, 147, 150, 175	0.635
RM22565	8	6	220, 223, 275, 290, 293, 295	0.569
RM19	12	3	290, 300, 315	0.482

RM154	2	4	175, 183, 186, 190	0.635
RM431	1	1	310	0
RM316	9	4	200, 205, 212, 215	0.583
RM152	8	3	150, 155, 175	0.468
RM287	11	2	90, 100	0.375
RM433	8	3	295, 300, 305	0.569
RM408	8	3	110, 127, 132	0.402
RM171	10	3	395, 400, 405	0.531
RM447	8	3	105, 115, 125	0.496
RM161	5	2	170, 185	-0.001
RM215	9	3	110,130,133	0.692
RM12146	1	5	60, 72, 83, 86, 90	0.760
RM454	6	1	205	0
Xa13	8	3	225, 230, 237	0.402
Xa5s	5	1	275	0

3. Results and Discussion

A total of 24 SSR markers (Simple Sequence Repeats) were used for molecular characterization and discrimination of 24 rice germplasm accessions with the aim to access the genetic diversity (Table 4). After analysis of the data generated from 24 microsatellite markers (SSR), a total of 69 alleles were detected in 24 accessions of rice (Table 5). The number of alleles per locus generated by each marker ranged from 1 to 6 alleles with an average number of 2.87 alleles per locus. The value is lower than that of 3.02 alleles per locus (Anand *et al*., 2012) [1] and 5.66 alleles per locus (Haque *et al.*, 2014) [3]. In the present study, 2.87 alleles per locus were higher than 2.6 alleles (Joshi *et al.* 2017) [12] and 2.17 alleles (Gour *et al.* 2017) [2]. The highest number of alleles (6) was detected in the locus RM 22565 while, lowest number of alleles (1) detected on each of locus OSR 13, RM 431, RM 454 and Xa5s. The PIC value ranged from 0.00 (OSR 13, RM 431, RM 454 and Xa5s) to 0.76 (RM 12146). Markers with PIC value of 0.50 or higher are highly informative for genetic studies and are extremely useful in distinguishing the polymorphism rate of a marker at specific locus (De Woody *et al.* 1995) [8]. Markers having polymorphic reaction along with high PIC value *viz.*, RM 1 and RM 12146 should be potentially used for molecular characterization of rice accessions from various sources. On the other hand, four markers OSR 13, RM 431, RM 454 and Xa5s exhibited monomorphic reaction for all the accessions, which was also reflected by 'O' PIC value, whereas, rest of the markers also showed polymorphic reactions (Table 5).

Microsatellite markers (SSR) are also used to detect the genetic similarity of germplasm accessions of rice. The genetic similarity coefficient ranged from 0.24 - 1.00 as revealed by UPGMA cluster analysis using the 24 SSR markers. A total of five distinct groups resulted at a cut-off similarity coefficient of 0.46 among the 24 rice accessions, below which the similarity values narrowed conspicuously (Fig 1). The dendogram revealed a clear separation of the rice accessions into five groups. The accessions that are derivatives of genetically similar clustered in one group. Cluster I had four accessions and consisted of Luchai, Peeleeluchai (135128), Peeleeluchai (135131) and Mahuwadeta Lal. In this cluster Luchi and Peeleeluchai (135128) showed 100% similarity while, Peeleeluchai (135131) showed 84% similarity with luchai and Peeleeluchai (135128).

Moreover, Mauhakuchi, Makado and Rajniti (A) were formed Group II. While, Group III had eight accessions Barangi (113998), Bhijani, Chitarboti, Bhaya, Bhurkund, Bairamgarh, Barangi (113999) and Banwara. Group IV also included eight accessions namely, Malpa (1), Kadamphool, Mancha, Surmatia, Patel 27 type four, Ram Karoni, Anjan (A) and Moti basmati. On the other hand, Group V had only one accession Baspan.

Whereas, Mahuwadeta Lal exhibited 47% with rest of the accessions of Group I. In Group II Mauhakuchi and Makado showed 57% similarity. Whereas, Rajniti (A) had a low degree of similarity (53%) with Mauhakuchi and Makado.

In Group III Bhujani and Chitarboti were 91% similar, Barangi (113999) showed 64% similarity with Bhujani and Chitarboti. Whereas, Bhaya and Bhurkund were 63.5% similar and, Barangi (113999), Bhujani and Chitarboti were 55.5% similar to Bhaya and Bhurkund. On the other hand, X:5 Bairamgarh/ Bastar and Barangi (113998) showed a low degree of similarity (50%) with Bauwara.

In Group IV, Malpa (I) and Kadamphool showed 74% genetic similarity. Patel 27 Type Four and Ram Karoni were 71.55 genetically similar. Mancha and Surmatia also showed 64% similarity. Malpa (I) and Kadamphool showed a low degree of similarity (51%) with rest of the accessions in group IV.

Thus, SSR markers provide adequate power of resolution to discriminate between rice accessions of rice and it could serve as a potential tool in the identification and characterization of genetically distant cultivars from various sources.

The present study addresses the utilization of 24 microsatellite markers to reveal genetic polymorphism and ensures unambiguous identification of 24 accessions of rice. The mean allele (2.87) across 24 loci in the present study was comparable with the result reported by Anand *et al.* (2012) [1] for 3.02 alleles per locus in 25 rice hybrids; Singh *et al.* (2016) [15] for 3.11 alleles per locus in 729 rice varieties; Meti *et al.* (2013) [14] who found an average of 2.08 alleles per locus among 48 traditional indigenous aromatic rice germplasm using SSR Markers with the range of 1 to 5 alleles per locus. Gour *et al.* (2017) [2] found an average of 2.17 alleles in the range of 1 to 3 alleles per locus with a total of 26 alleles among 19 accessions. Sajib *et al.* (2012) [6] detected 3.3 alleles per locus who used a total of 24 SSR markers across 12 elite aromatic rice genotypes for their characterization and discrimination. The number of alleles per locus ranged from 2 to 6 alleles. In another study, Kumar *et al.* (2014) [4] found an average of 2.93 alleles per locus among 72 rice genotypes using 15 polymorphic SSR markers. The number of alleles ranged from 1 to 4 per locus. Whereas, Hossain *et al.* (2007) [10] reported 3 to 9 alleles per locus with an average of 4.53 alleles per locus for 30 microsatellite markers; Marathi and Maliha (2018) [13] reported 2 to 4 alleles per locus with an average of 2.39 alleles per locus for 23 SSR markers in 53 varieties. Similarly, Rahman *et al.* (2012) [5] found an average of 4.18 alleles per locus. In another study, Jain *et al.* (2004) [11] found 3-22 alleles per locus with the average of 7.8 for 30 SSR markers.

Larger range of similarity values for cultivars revealed by microsatellite markers provides a greater confidence for the assessment of genetic diversity and relationships, which can be used in future breeding programme. With the aid of microsatellite makers and clustering data, different distantly related rice genotypes may be combined by inter-crossing genotypes from different clusters to get hybrids with the highest heterosis.

The present study revealed a wide variation among the accessions. The results indicated that SSR markers are neutral and co-dominant and could be a powerful tool to assess the genetic variability of the accessions. Coefficient of similarity revealed that the rice accessions of cluster I were genetically distant from cluster IV. Thus, Peeleeluchai (135131) and Mahuwadeta Lal of cluster I; whereas, Kadamphool and Ram Karoni of cluster IV seems to be promising and should be utilized in hybridization programme on the basis of kernel L:B ratio and grain yield. Molecular markers like RM 1, RM 12146, RM 215, RM 22710 RM 154 and RM 25 could potentially be used for molecular characterization of rice germplasm accessions from various sources on the basis of polymorphic reactions and high PIC values. The information about the genetic diversity will be very useful for proper identification and selection of appropriate parents for breeding programme, including gene mapping and ultimately for emphasizing the importance of marker assisted selection in germplasm of rice improvement worldwide,

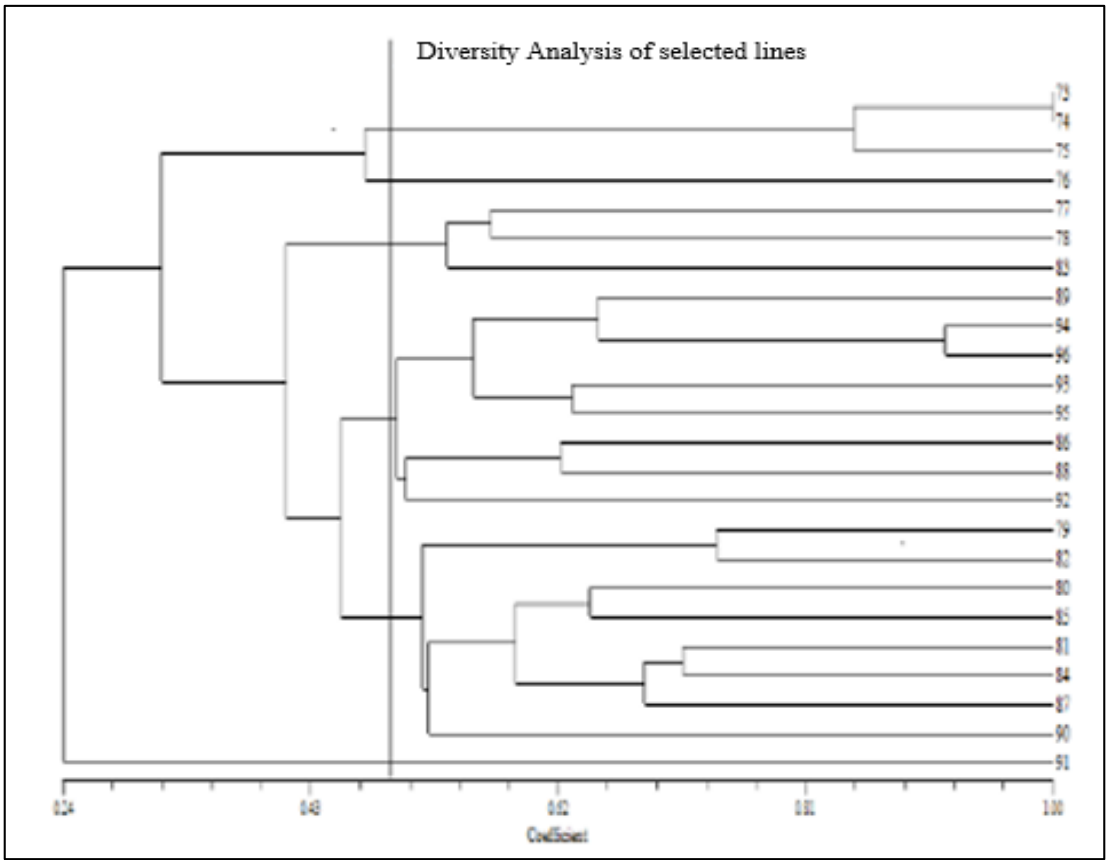

Fig 1: An UPGMA cluster dendogram showing the genetic relationships among 24 germplasm accessions of rice based on the alleles detected by 24 SSR markers

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