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Molecular marker based genetic relatedness assessment for short duration Rice (*Oryza sativa* L.) varieties using microsatellite (SSRs) markers

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Abstract
Knowledge on genetic relatedness between genotypes that are used in hybridization programmes is very useful for plant breeders as it supports the decisions on the selection of cross combinations from large sets of parental lines. It also helps to widen the genetic base of varieties in a breeding programme. With this in view this investigation was carried out with the objective of estimating genetic relatedness present in a set of 29 short duration rice varieties recommended for cultivation in Tamil Nadu and Puducherry, using SSR makers. Genetic relatedness assessed with 18 polymorphic SSRs had generated 51 alleles and maximum of five alleles were observed in RM-7 and RM-515 loci. Maximum PIC value of 0.742 was observed in RM-515. Cluster analysis performed using Jaccard's similarity coefficient had generated a dendrogram producing two major clusters at 30 per cent similarity and resulted in the identification of diverse clusters. The genetic relatedness of the 29 short duration rice varieties was observed to be narrow and, therefore, it is necessary to broaden the rice gene pool through introduction of new genes from new and diverse sources.

Keywords: Genetic relatedness, short duration rice and SSR markers

Introduction
Rice (*Oryza sativa* L.) is the well-known holder of two important titles: the global grain of the world, as it is consumed by more than 50 per cent of the world population, and a model cereal species, owing to its small genome size and genetic relatedness to other cereals. The projected increase in global population to 9 billion by 2050 and predicted increase in water scarcity, decrease in arable land, the constant battle against new emerging pathogens and pests, and possible adverse effects from climate change pose great challenges for rice breeders and agricultural scientists. (Collard *et al*., 2008) [7]. Rice is cultivated on all the continents except Antarctica, over an area of 150 million ha, but most rice production takes place in Asia (Jena and Mackill, 2008) [16]. In India, rice is the most important crop and is grown widely in diverse ecological niches with a wide range of selected cultivars. Most rice varieties developed by rice research stations in India are regionally adapted. Self-sufficiency and reasonable stability in rice production in India was made possible by development and widespread cultivation of ideotype-based, high yielding varieties (Davierwala *et al*., 2000) [8].
In the past four decades, India has made a spectacular progress in rice production and productivity. The rice production, at the time of independence, was 21 million tonnes which was boosted to 93 million tonnes by 2001-02 (Sharma, 2006) [34]. This nearly five-fold increase was due to improved high yielding rice varieties (HYV) with cultural practices, developed during 1960s to 1970s, which heralded the so called "Green revolution". A product of an unprecedented international effort (IRRI, Philippines), the Green Revolution is certainly one of the most important accomplishments of the 20th century (Jauhar, 2006) [15]. Breakthrough in rice yields were achieved with the development of semi dwarf varieties characterized by lodging resistance and nitrogen responsiveness (Khush, 1999) [20]. Thus, the introduction of dwarfing genes by conventional breeding had revolutionized rice production in Asia, averting mass scale starvation (Jauhar, 2006) [15]. However, during past decade, production potential of modern cultivars has remained stagnant (Khush, 2005) [21], which may have resulted from the narrow genetic base of released varieties (Carmona, 1990) [5].
Green revolution led to the depletion of genetic base of rice varieties, as thousands of traditional varieties (land races) were replaced by a handful of modern rice varieties, resulting in genetic uniformity (Shivkumar *et al*., 1998) [35]. This reduction in biodiversity, coupled with luxuriant vegetative growth and continuous cropping, increased the vulnerability of the rice crops to increased pest and disease incidences (Mishra *et al*., 2003) [26]. Similar situations have been reported from Japan (Kaneda, 1985) [18], United States (Dilday, 1990) [10], Taiwan (Lin,

1991) [24] and Australia (Ko *et al*., 1994) [22], documenting a widespread reduction in genetic relatedness of modern rice cultivars due to intensive breeding efforts. Crosses between genetically diverse parents are, therefore, important in hybridization programs to increase heterosis, maximize heterozygosity and maintain high levels of genetic variability in the progeny (Messmer *et al*., 1993) [25], ideally leading to an increase in grain yield. Currently, the narrow genetic base of breeding programmes has resulted in a bottleneck effect in rice cultivar development (Tanksley and McCouch 1997) [39]. Unlocking the tremendous genetic potential from wild rice might break the genetic bottleneck and improve the modern cultivar with the aid of molecular selection (Zamir, 2001) [45] to achieve higher productivity and resistance to biotic and abiotic stresses. Therefore, knowledge of genetic relatedness among modern rice cultivars is of paramount importance to breeders for understanding of germplasm usage to avoid development of varieties with a narrow genetic base (Davierwala *et al*., 2000) [8].

Several approaches have been used to estimate genetic relatedness to select appropriate parental genotypes in crossing programmes. Genetic relatedness in plants has been assessed using morphological traits which may not be a reliable as gene expressions are highly influenced by environmental conditions (Yeo *et al*., 1990) [44] although it is essential in plant breeding as it reveals important traits to plant breeders. Characterization of genotypes based on polymorphisms at the DNA level with molecular markers is a powerful tool for determine the extent of genetic divergence (Hashimoto *et al*., 2004) [12]. Commonly used measures of genetic similarity are morphological and biochemical characteristics and the DNA markers, such as Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeats (SSR) and Amplified Fragment Length Polymorphism (AFLP). Among them, RFLP and microsatellites are Codominant markers and their map positions on the rice genome are well known, while RAPD and AFLP markers involve the use of random, largely dominant markers. Compared with RFLPs, microsatellite markers (SSRs) detect a significantly higher degree of polymorphism in rice (Wu and Tanksly, 1993) [43] and are especially suitable for evaluating genetic diversity among closely related rice cultivars (Akagi *et al.*, 1997) [1]. SSRs are highly reliable, co-dominant in inheritance and generally transferable between mapping populations. The genomic distribution of microsatellites in rice seems to be random, with no obvious bias for particular regions or clustering in particular regions (Chen *et al*., 1997) [6]. With this in background the present study was, therefore, undertaken to quantify the genetic relatedness short duration rice varieties (less than 115 days), recommended for cultivation in Tamil Nadu and Puducherry (U.T) during June-September was investigated by using SSR markers.

Materials and Methods

Plant Materials

The present investigation was carried out in the Department of Plant Breeding and Genetics, Pandit Jawaharlal Nehru College of Agriculture and Research Institute (PAJANCOA & RI), Karaikal. The materials chosen in the study comprised 29 short duration rice varieties maturing in less than 115 days. Of these 25 are recommended for cultivation in Tamil Nadu and Puducherry during *kuruvai* (June–September) and *Navarai* (December-April) seasons. Two varieties *viz*., Annada and Tulasi, as popular national varieties and another two varieties: IR 72 and IR 74 as popular International varieties (IRRI bred varieties cultivated in many countries) were included for comparison. Details of parentage, year of release and breeding station of rice varieties are given in Table 1.

Table 1: Details of parentage, year of release and breeding station of rice varieties

Sl. No.	Variety	Parentage	Year of release	Breeding station
1.	ADT 36	Triveni x IR 20	1980	TRRI, Aduthurai
2.	ADT 37	BG 280 x PTB 33	1987	TRRI, Aduthurai
3	ADT 41	Natural mutant from Basmathi 370	1992	TRRI, Aduthurai
4.	ADT 42	AD 9246 x ADT 29	1994	TRRI, Aduthurai
5.	ADT 43	IR 50 x Improved whiteponni	1998	TRRI, Aduthurai
6.	ADT 45	IR 50 x ADT 37	2001	TRRI, Aduthurai
7.	ADT 47	ADT 43 x Jeeragasamba	2005	TRRI, Aduthurai
8.	ADT 48	IET 11412 x IR 64	2005	TRRI, Aduthurai
9.	IR 36	IR 1561 x IR 24 x *Oryza nivara* x CR 94	1979	IRRI, Philippines
10.	IR 42	IR 2042 x CR 94	1983	IRRI, Philippines
11.	IR 50	IR 2153 x IR 28 x IR 36	1982	IRRI, Philippines
12.	IR 64	IR 5657 x IR 2061	1989	IRRI, Philippines
13.	IR 72	TN 1 x Chianung 242	1989*	IRRI, Philippines
14.	IR 74	IR 19661 x IR 15795	1991*	IRRI, Philippines
15.	TKM 9	TKM 7 x IR 8	1978	RRS, Tirur, Tamilnadu
16.	TKM 11	C 22 x BJ 11	1998	RRS, Tirur, Tamilnadu
17.	TKM 12	TKM 9 x TKM 11	2002	RRS, Tirur, Tamilnadu
18.	PY 2	Kannagi x Cul 2032	1980	PKKVK, Puducherry
19.	PY 3	IR 3403 x PTB 33 x IR 36	1984	PKKVK, Puducherry
20.	PY 5	Swarnadhan x NLR 9674	1994	PKKVK, Puducherry
21.	PMK 1	CO 25 x ADT 31	1985	ARS, Parmakudi
22.	PMK 2	IR 13564 x ASD 4	1994	ARS, Parmakudi
23.	PMK 3	UPLRI 7 x CO 43	2003	ARS, Parmakudi
24.	ASD 16	ADT 31 x CO 39	1986	RRS, Ambasamudram
25.	ASD 18	ADT 31 x IR 50	1991	RRS, Ambasamudram
26.	CO 47	IR 50 x CO 43	1999	PBS, Coimbatore
27.	MDU 5	*Oryza glaberrimma* x Pokkali	1996	AC and RI, Madurai

28.	ANNADA	MTU 15 x Waikoku	1987	CRRI, Cuttack
29.	TULASHI	CR 151 x CR 1014	1988	CRRI, Cuttack

*Released in Indonesia

Genomic DNA isolation and SSR analysis

Total genomic DNA was extracted by adopting the protocol developed by Dellaporta *et al*. (1983) [9]. A total of 24 SSR primer pairs synthesized by M/s SIGMA Aldrich Inc. Bangalore, were used for PCR amplification. These primers were selected based on their uniform distribution across the 12 rice SSR chromosomes and the details of SSR primer pairs used for PCR amplification are given in Table 2. PCR amplification reactions were done in 15 µl reaction mixtures, containing 2µl l of diluted template DNA, 1 x assay buffer, 200 mM of deoxyribonucleotides, 2 µM of $Mgcl_2$, 0.2 µM of each primer (both forward and reverse primers) and 1 unit of Tag DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore). An DNA thermal cycler (Model: MJ, Research Inc. USA) was used along with the following PCR profile: an initial denaturation step for 2 mins at 95°C, followed by 34 cycles of denaturation (94°C) for 45 seconds, annealing (55°C) and primer elongation (72°C) for 1 minute and 30 seconds each and then a final extension at 72°C for 10 mins and at 4^0C for cooling. Annealing temperature of 55ºC was adopted for all the primers used in the study as identified by their specific Tm requirement.

Table 2: Details of SSR primer pairs used for PCR amplification

Sl. No.	Loci	Ch. location	Sequence (5'-3')	Repeat motif	Product Size (bp)
1.	RM-428	1	AACAGATGGCATCGTCTTCC F CGCTGCATCCACTACTGTTG R	(AG)15	266
2.	RM-443	1	GATGGTTTTCATCGGCTACG F AGTCCCAGAATGTCGTTTCG R	(GT)10	124
3.	RM-110	2	AAA TTC GAA GCC ATC CAC CAA CG F GCC GAC GAG GTC GAG TAG AAG G R	(GA)15	156
4.	RM-53	2	ACG TCT CGA CGC ATC AAT GC F CAC AAG AAC TTC CTC GGT AC R	(GA)14	182
5.	RM-569	3	GACATTCTCGCTTGCTCCTC F TGTCCCCTCTAAAACCCTCC R	(CT)16	175
6.	RM-7	3	TTCGCCATGAAGTCTCTCG F CCTCCCATCATTTCGTTGTT R	(GA)19	180
7.	RM-273	4	GAAGCCGTCGTGAAGTTACC F GTTTCCTACCTGATCGCGAC R	(GA)11	207
8.	RM- 261	4	CTA CTT CTC CCC TTG TGT CG F TGT ACC ATC GCC AAA TCT CC R	C9(CT)8	125
9.	RM-153	5	GCC TCG AGC ATC ATC ATC AG F ATC AAC CTG CAC TTG CCT GG R	(GAA)9	201
10.	RM- 403	5	GCTGTGCATGCAAGTTCATG F ATGGTCCTCATGTTCATGGC R	(GA)8	241
11.	RM-510	6	AACCGGATTAGTTTCTCGCC F TGAGGACGACGAGCAGATTC R	(GA)15	122
12.	RM- 343	6	CCACGAACCCTTTGCATC F GTGATGATGCGTCGGTTG R	(CAT)5 (CAC) 5CAT (CAC)4	233
13.	RM-234	7	ACAGTATCCAAGGCCCTGG F CACGTGAGACAAAGACGGAG R	(CT)25	156
14.	RM- 429	7	TCCCTCCAGCAATGTCTTTC F CCTTCATCTTGCTTTCCACC R	(TG)10	159
15.	RM-152	8	GAAACCACCACACCTCACCG F CCGTAGACCTTCTTGAAGTAG R	(GGC)10	151
16.	RM-515	8	TAGGACGACCAAAGGGTGAG F TGGCCTGCTCTCTCTCTCTC R	(GA)11	211
17.	RM-464	9	AACGGGCACATTCTGTCTTC F TGGAAGACCTGATCGTTTCC R	(AT)21	262
18.	RM- 524	9	TGAAGAGCAGGAACCGTAGG F TCTGATATCGGTTCCTTCGG R	(AT)11	198
19.	RM- 590	10	CATCTCCGCTCTCCATGC F GGAGTTGGGGTCTTGTTCG R	(TCT)10	137
20.	RM- 596	10	ATCTACACGGACGAATTGCC F AGAAGCTTCAGCCTCTGCAG R	(GAC)10	188
21.	RM-206	11	ATC GAT CCG TAT GGG TTC TAG C F GTC CAT GTA GCC AAT CTT ATG TGG R	(CT)21	147
22.	RM-21	11	ACAGTATTCCGTAGGCACGG F GCTCCATGAGGGTGGTAGAG R	(GA)18	157
23.	RM-247	12	TAGTGCCGATCGATGTAACG F CATATGGTTTTGACAAAGCG R	(CT)16	131
24.	RM-313	12	TGCTACAAGTGTTCTTCAGGAC F GCTCACCTTTTGTGTTCCAC R	(GT) 6CAC (CG) 5-6-(GT)8	111

F – Forward primer
R – Reverse primer

Prior to electrophoresis, each PCR product was mixed with gel loading dye (bromophenol blue) and electrophoresis was carried out in a horizontal electrophoresis tank, run on 2.5% agarose gel prepared in 1X TBE buffer stained with Ethidium Bromide (0.5µg/ml). 10 µl of the sample were loaded in each well and run at constant voltage of 90 V for a period of 60 to 90 minutes. The gel was visualized in UV transilluminator and photographs were taken using gel documentation unit linked to a PC. (Model Alpha Imager 1200, Alpha Innotech Corp., USA). Qualitative multistate traits that depict an array of characters were converted into binary characters (Sneath and Sokal, 1973) [37] based on the variations present. Primers that showed polymorphic banding patterns were selected whereas primers that showed monomorphic banding patterns were excluded. Only the clear and unambiguous bands were scored. Markers were scored for the presence and absence of the corresponding band among the genotypes. The score 1 and 0 indicates the presence and absence of bands respectively.

The Polymorphism Information Content (PIC) value described by Botstein *et al.* (1980) [4] and modified by Anderson *et al.* (1993) [3] for self-pollinated species was calculated as follows:

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

Where P_{ij}, is the frequency of the j^{th} allele for the i^{th} marker, and summed over n alleles. The genetic variation was measured in terms of genetic diversity and was computed by averaging PIC estimates over all loci (Weir, 1996) [41].

The scoring data in the form of binary values was used for the construction of dendrogram. The genetic associations between accessions were evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on the proportions of shared bands produced by the primers (Jaccard, 1908) [14]. Similarity matrix was generated using the SIMQUAL programme of NTSYS-pc software, version 2.02 (Rohlf, 1998) [31]. The similarity coefficients were used for cluster analysis and dendrogram was constructed by the Unweighted Pair-Group Method (UPGMA) (Sneath and Sokal, 1973) [37].

Results and Discussion

Number of alleles, Allele variation and Polymorphic Information Content (PIC)

A total of 57 alleles were amplified using 24 SSR primer pairs. Out of the 24 SSR primer pairs, 18 SSR primer pairs were found to be polymorphic and generated 51 polymorphic alleles in the 29 rice varieties under investigation (Table 3). Six SSR primer pairs that exhibited monomorphism were RM-403, RM-343, RM-429, RM-524, RM-590 and RM-596.

Table 3: Allele variation and PIC value for SSRs markers identify in 29 rice varieties

Sl. No.	Loci	Chromosomal location	Total	PIC value
1.	RM-428	1	2	0.452
2.	RM-443	1	2	0.279
3.	RM-110	2	3	0.660
4.	RM-53	2	3	0.205
5.	RM-569	3	2	0.493
6.	RM-7	3	5	0.684
7.	RM-273	4	2	0.303
8.	RM-261	4	2	0.490
9.	RM-153	5	3	0.551
10.	RM-403	5	1	0.00
11.	RM-510	6	2	0.286
12.	RM-343	6	1	0.00
13.	RM-234	7	3	0.137
14.	RM-429	7	1	0.00
15.	RM-152	8	4	0.477
16.	RM-515	8	5	0.742
17.	RM-464	9	3	0.220
18.	RM-524	9	1	0.00
19.	RM-590	10	1	0.00
20.	RM-596	10	1	0.00
21.	RM-206	11	3	0.546
22.	RM-21	11	3	0.618
23.	RM-247	12	2	0.498
24.	RM-313	12	2	0.469

The number of alleles amplified by each primer pair ranged from 2 to 5 with an average of 2.83 per locus. This is significantly lower than the average number of alleles reported by Spada *et al*. (2004) [38] with 7.2 and Salgotra *et al*. (2015) [32] with 3.0. In contrast, the average alleles per locus identified by Wong *et al*. (2009) [42] with 2.6 and Umadevi *et al*. (2014) [40] with 2.7 which is lower than our report. The higher values may be due to large number of diverse accessions assayed at more SSR loci by these authors. The reason for the wide variation in the number of alleles detected was due to the different sets of genotypes, number and distribution of SSR loci and method of gel electrophoresis.

Maximum of five alleles were noticed in two SSR loci (RM-7 and RM-515), followed by four alleles in one loci (RM-152) and three alleles in seven loci. In earlier studies RM-7 in chromosome 3 was reported to produce five alleles in *indica* lines (Ishii *et al*., 2001) [13], eight alleles in cultivated varieties and wild *sp.* (Ren *et al*., 2003) [30], six alleles in breeding lines (Zeng *et al*., 2004) [46] and five alleles in salinity lines (Kanawapee *et al*., 2011) [17]. Similarly, RM-515 in chromosome 8 was reported to generate six alleles (Ren *et al*., 2003) [30].

The PIC values for these 18 SSR primer pairs varied from 0.137 (RM-234) to 0.742 (RM-515) with an average of 0.33.

The SSR marker profile obtained with primer pair of RM-510 is shown (Figure 1). A report with similar PIC was earlier observed by Pachauri *et al*. (2013) [27] in 41 rice genotypes (0.38). Although the average PIC value in this study was higher than that determined by Singh *et al*. (2013) [36] in 375 Indian rice varieties (0.25), it was lower than that determined by Panaud *et al*. (1995) [28] in 24 rice cultivars and wild *sp*. (0.69) and Kumbhar *et al.* (2015) [23] in 50 rice genotypes (0.79). In the present investigation higher proportion of closely related cultivars and lesser number of markers used may be the reason for lower PIC when compared to earlier published reports.

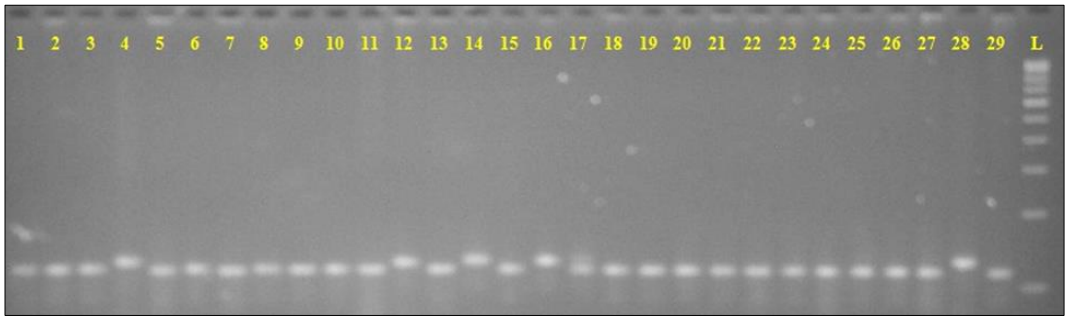

Fig 1: PCR products of SSR primer RM 510 for 29 rice varieties

In this study higher PIC of 0.742 was observed in RM-515 which is higher than that reported by Ren *et al*. (2003) [30] in rice germplasm (0.539) and Seetharam *et al*. (2009) [33] in salt tolerant rice germplasm (0.278). Another marker RM-7 was observed with PIC of 0.684 which is higher than that observed by Zeng *et al.* (2004) [46] (0.640) and Ramdan *et al*. (2015) [29] (0.570) but lower than that reported by Alvarez *et al*. (2007) [2] (0.750). The wide variation in PIC for these markers in earlier studies may be due to the choice of the genetic materials and number of samples analyzed.

Cluster analysis

UPGMA cluster analysis was performed using Jaccard's similarity coefficient matrices calculated from SSR markers on 51 alleles to generate a dendrogram of the 29 rice varieties (Figure 2). The similarity coefficient ranged from 0.30 (406 pairs) to 1.0 (ADT 43 and ADT 47) with an average of 0.47 indicating high diversity. This is in accordance with the findings of Garland *et al*. (1999) [11] in genetic diversity analysis of 43 rice cultivars with 0.50 mean genetic similarities. The UPGMA dendrogram showed the grouping pattern of the 29 rice varieties considered in the present investigation. All the 29 rice varieties were grouped into two major clusters with 33 per cent similarity among them. The first major has two sub-clusters, the first sub-cluster consisting of ten rice varieties (ADT 36, ADT 37, ADT 43, ADT 47, ADT 45, IR 42, IR 74, ADT 41, ADT 42 and ADT 48) at genetic similarity of 40 per cent. Genotypes ADT 43 and ADT 47, located within this sub-cluster, showed genetic similarly of 100 per cent. Similarly genotypes ADT 36 and ADT 37 also located within the same sub cluster, showed genetic similarity of 87 per cent. The second sub cluster comprising three rice varieties (IR 50, IR 72 and IR 64) was grouped at similarity of 48 per cent. The rice varieties IR 50 and IR 72, within this sub cluster, recorded 68 per cent similarity.

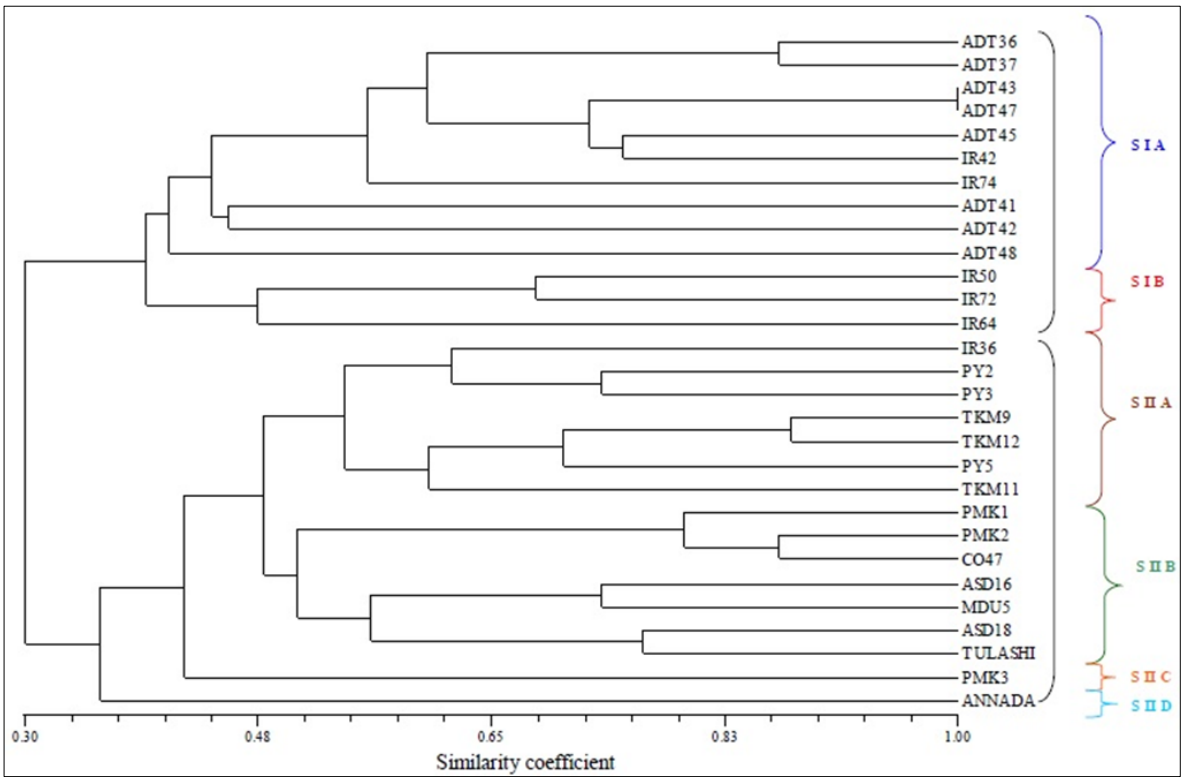

Fig 2: Dendrogram showing genetic relatedness among 29 rice varieties using SSR markers

The second major cluster also had, as first major cluster, two sub clusters at 48 per cent similarity. The first sub cluster comprised of seven rice varieties (IR 36, PY 2, PY 3, TKM 12, PY 5 and TKM 11) showed 54 per cent similarity. Two varieties PY 2 and PY 3, with 74 per cent similarity, recorded 62 per cent similarity with IR 36. Similarity TKM 9 and TKM 12, with 88 per cent similarity, revealed 71 per cent similarity with PY 5 and second sub cluster consisted of seven varieties (PMK 1, PMK 2, CO 47, ASD 16, MDU 5, ASD 18 and TULASHI) grouped at similarity of 52 per cent ASD 18 and TULASHI, with 76 per cent similarity, grouped with ASD 16 and MDU 5 at 56 per cent similarity and subsequently with PMK 1, PMK 2 and CO 47 at 51 per cent similarity. The varieties PMK 3 and ANNADA formed separate clusters branched from this second major cluster at a level of 42 per cent and 36 per cent similarities respectively from the varieties of second major cluster. Clustering together of rice varieties, bred at various research stations, based on SSRs may be due to the molecular similarities with respect to repeat motifs of SSRs.

Of the six clusters formed cluster SIA with ten varieties (ADT 36, ADT 37, ADT 47, ADT 45, IR 42, IR 74, ADT 41, ADT 42 and ADT 48) and clusters S II C and S II D with one variety each (PMK 3 and ANNADA) were found to be diverse genetic stocks involving these varieties may widen the variability.

Genetic relatedness of the 29 short duration rice varieties was observed to be narrow as revealed by SSR marker-based analysis. It is, therefore, necessary to broaden the genetic base of the present-day rice cultivars and also to reveal the gene-pool comprising land races and wild *spp*. to exploit valuable genes for agronomic value to generating new elite rice cultivar and also develop genetically diverse parents for the hybridization program.

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