International Journal of Multidisciplinary Education and Research
ISSN: 2455-4588; Impact Factor: RJIF 5.12
www.multieducationjournal.com
Volume 1; Issue 4; June 2016; Page No. 59-65

Morpho-molecular diversity analysis of traditional and improved cultivars in rice

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Abstract
The study was carried out to access the genetic relatedness of 33 traditional and 12 improved rice cultivars based on morphological and molecular diversity analysis. Data observed for twelve morphological traits in traditional and improved cultivars rice were used for calculating dissimilarity index. Cluster analysis based on hierarchical clustering method grouped 45 cultivars into two major clusters. All the improved cultivars were grouped into one cluster expect ADT 43 found to be unique than other improved cultivars for the morphological characters. A total of 32 SSR markers were used for screening 45 traditional and improved rice cultivars. Clustering analysis for 33 traditional cultivars was grouped into nine major clusters, while improved cultivars revealed four cluster based on SSR marker data. Clustering pattern for pooled dissimilarity matrix of 45 rice cultivars reveals eleven clusters for SSR markers data. The tree obtained from the SSR marker revealed that the marker was more discriminatory, highly polymorphic and thus, more informative than the one obtained from morphological data. The improved rice cultivars collected for this study had a relative narrowing genetic base as compared to traditional cultivars. It is essential to rationalize conservation and use of traditional cultivars to ensure the maintenance of genetic variability, essential in plant breeding.

Keywords: Rice cultivars, traditional, improved, diversity, morphological and molecular data

1. Introduction
Genetic uniformity among modern rice cultivars is an alarming situation confronting the rice industry (Morishima and Oka, 1995) [10] as it has increase the vulnerability of the rice crop to insect infestation and disease epidemics. Selection for early maturing and high yielding rice cultivars is identified as key factors that had resulted in the loss of genetic variability in farmers' fields. Salazar (1993) [14] reported that with the introduction of these modern, high yielding cultivars, farmers are now planting fewer traditional landraces in their fields.

Traditional rice cultivars still extant in rice ecosystems may provide the diversity needed to diversify the desperate gene pool of improved rice cultivars. Owing to their adaptation to a wide range of agro-ecological conditions, traditional landraces represent tremendous genetic variability not found in modern cultivars. These cultivars can therefore be exploited to significantly enhance rice productivity in marginal areas. It is well known that diversity and wild relatives are fundamental sources of genetic traits needed for coping with environmental stresses, plant diseases and pests. Although these traditional cultivars and landraces appear to be inferior to modern cultivars in terms of yield potential, they possess many vital genes for pest resistance, drought-tolerance, high protein content, flavour, etc. Due to agricultural intensification, however, losses of traditional crop cultivars are increasing, as most farmers prefer high yielding modern cultivars (Maikhuri *et al.*, 2001) [8]. Therefore, to maintain crop diversity, collection, characterization, and conservation of traditional landraces are vital. With aforesaid points, present study was carried out to access the genetic relatedness of traditional and improved cultivars of rice based on morphological and molecular diversity analysis.

2. Materials and methods
2.1. Plant material
The plant material for diversity analysis consists of traditional and improved rice cultivars. The collection consists of 45 cultivars of which 33 are traditional cultivars and 12 improved cultivars (Table 1). About 29 traditional cultivars were collected from an NGO at Adhirangam and three cultivars were from Kerala. All the improved cultivars were collected from the central farmyard of Pandit Jawaharlal Nehru College of Agriculture and Research Institute (PAJANCOA & RI), Karaikal. The seed of traditional and improved cultivars were repacked and labelled.

2.2. Seed treatment and germination
Fifty seeds from each cultivar were taken and packed in individual covers followed by labelling. Covers were press-sealed and placed in hot air oven at 50^0C for five days for dormancy breaking. After incubation period, the seeds were soaked in wet germination paper to induce germination. Cabendazim (0.1%) was also sprayed to inhibit fungal growth during germination period. The seeds were maintained in germination paper for about 10 days. The germinated traditional and improved cultivars were subjected for planting

2.3. Sowing and transplantation
The germinated seeds were transplanted to hydroponic solution, and maintained the cultivar for about 3 weeks. After three weeks of time, cultivars were transplanted to the field. Each and every cultivar was planted in a single row at 30cm x 15cm spacing with the augmented experimental design with ADT 38 as check.

Table 1: List of traditional cultivars and improved rice cultivars

Sl. No.	Name	Collected Place	Sl. No.	Name	Collected Place
Traditional rice cultivars			**Traditional rice cultivars**		
1	Ananthanoor Sannam	Tamil Nadu	24	Varittarpana Sooli	Tamil Nadu
2	Kattu Ponni	Tamil Nadu	25	Vadan Samba	Tamil Nadu
3	Kanda Saali	Tamil Nadu	26	Vasanai Seeraga Samba	Tamil Nadu
4	Karupu Kavuni	Tamil Nadu	27	Vellai Kar	Tamil Nadu
5	Kanda Vari	Tamil Nadu	28	Halara Samba	Tamil Nadu
6	Kandavavi Samba	Tamil Nadu	29	Haluvupavu Patti	Tamil Nadu
7	Kari Satti	Tamil Nadu	30	Mundagan	Kerala
8	Kichili Samba	Tamil Nadu	31	Palliyaral	Kerala
9	Kuzhi Vedichan	Tamil Nadu	32	Thavalakannan	Kerala
10	Kuruvi Kalanjiam	Tamil Nadu	33	Vellaipunaran	Kerala
11	Kaivari Samba	Tamil Nadu	**Improved rice cultivars**		
12	Sannaki Patti	Tamil Nadu	34	ADT 39	PAJANCOA & RI
13	Singini	Tamil Nadu	35	ADT 43	PAJANCOA & RI
14	Chitti Sandai	Tamil Nadu	36	ADT 45	PAJANCOA & RI
15	Chinna Ponni	Tamil Nadu	37	ADT 46	PAJANCOA & RI
16	Chittika Patti	Tamil Nadu	38	ADT 48	PAJANCOA & RI
17	Thulasi Vasam	Tamil Nadu	39	ADT 49	PAJANCOA & RI
18	Thooyamalli	Tamil Nadu	40	ASD 18	PAJANCOA & RI
19	Thengaipoo Samba	Tamil Nadu	41	CR 1009	PAJANCOA & RI
20	Mappilai Samba	Tamil Nadu	42	KKL(R) – 1	PAJANCOA & RI
21	Muthirai Sannam	Tamil Nadu	43	Samba Masuri	PAJANCOA & RI
22	Mysore Malli	Tamil Nadu	44	TRY – 1	PAJANCOA & RI
23	Rathna Chutti	Tamil Nadu	45	White Ponni	PAJANCOA & RI

2.4. Morphological observation

The 33 traditional and 12 improved cultivars were subjected to observation of the following morphological traits based on the IRRI's Standard Evaluation System.

1. Leaf length
2. Leaf width
3. Blade pubescence
4. Blade colour
5. Basal leaf sheath colour
6. Leaf angle.
7. Ligule length
8. Ligule colour
9. Ligule shape
10. Collar colour
11. Auricle colour
12. Days to flower.

2.5. Molecular analysis

2.5.1. DNA extraction and quantification

The leaf samples were collected from young seedlings that were planted in hydroponic solution under green house. The leaf samples were cut by using sterilized scissor with 70% ethanol. The collected samples were stored in non absorbent covers and stored in deep freezer for further use. DNA was extracted from the collected sample using CATB method. From the stock DNA 5µl of samples were taken in cuvette and diluted with 95µl of water for quantification using spectrophotometer. Working samples were prepared with dilution of 10^{-1}

2.5.2. PCR amplification

A total of 60 SSR markers were evaluated for polymorphism in traditional and improved rice cultivars, of which 32 SSR markers were found to be polymorphic. The sequences for forward primer and reverse primer of 32 SSR markers are given in Table 2. The PCR reaction was carried in a total reaction volume of 15µl, containing the components are listed in Table 3. The reaction mixture was given a short spin for thorough mixing of cocktail components. Then the amplification was carried out in a thermal cycler. The temperature profile used in the PCR amplification is presented in Table 4.

2.5.3. Gel electrophoresis and scoring

After the completion of PCR steps, the products were separated in 3% agrose gel electrophoresis and visualized by ethidium bromide staining. Clearly resolved, unambiguous bands were scored visually for their presence and absence of allele types as 1 and 0, respectively. Homomorphic type I allele were scored as 1, 0; homomorphic type II allele as 0, 1; while the heteromorphic bands were scored as 1, 1 which indicates the presence of both alleles.

2.6. Diversity analysis

The data of morphological characters and SSR markers data were analyzed by using diversity analysis software DARwin5 (Perrier *et al.*, 2003 and Perrier, 2006) [13, 12]. The morphological data was converted to text tab limited format and imported in DARwin. The dissimilarity index was calculate for single data using as follows:-

Notations:

$$d_{ij} = \frac{u}{2m + u}$$

d_{ij} : dissimilarity between units i and j
u : number of unmatching variables
m : number of matching variables

Similarly the SSR allelic data was converted to text tab limited format and imported to DARwin software. The dissimilarity index was calculate for allelic data using the simple matching coefficient index as follows:-

Table 2: List of SSR primers used for screening of traditional cultivars and improved rice cultivars

Sl.No.	Name	Forward Primer	Reverse Primer
1	RM8094	AAAGTTTGTACACATCGTATACA	CGCGACCAGTACTACTACTA
2	RM318	GGCTACGCTCCAGTTAGTGAAGG	TGGTAGGAAGAAGGGAGAAGTTTAGG
3	RM20069	GCGAGCGAGAGGAGAGATAGACG	CGAATTCGGCACGAGTAATAGGG
4	RM3480	TAGACAATGGTCTGCAACTCTGC	CAAGGAGATTGGATTGCTGTACC
5	RM18	TTCCCTCTCATGAGCTCCAT	GAGTGCCTGGCGCTGTAC
6	RM248	AGAGAGCAAGTTTGAAGCGAAGC	ACCAAGAGGGTAGCCTAGCATGG
7	RM8300	GCTAGTGCAGGGTTGACACA	CTCTGGCCGTTTCATGGTAT
8	RM493	TAGCTCCAACAGGATCGACC	GTACGTAAACGCGGAAGGTG
9	RM23935	CGAGGCTGAGTGCTGAGTGACG	TAACCATGGACGGAGGGAGAGC
10	AP3206	TTCTCATCGCACCATCTCG	GGAGGAGGAGAGGAAGAAG
11	RM1287	CCATTTGCAGTATGAACCATGC	ATCATGCAATAGCCGGTAGAGG
12	RM12363	TGCCAGGATGGACAATTAGATGC	CTCTGAGATTGAGGCTGCTCTGG
13	RM6233	GCGGAGACCGGATGATCTGC	CCCATGATGGCCTCGTCATCC
14	RM12923	AAATGCACAGGCATTCGTAGACC	GAAGAAGTGGATGGAGGACATGG
15	RM13069	GGAGGGTTATTCTCGCGTAAAGG	GACCCTTCACATTTCGGTCAGC
16	RM6509	GTAAGACGGACAGCCATGGAAGC	GACAGCACCTGCTCGAACTGC
17	RM1024	AACTGCCATCTCTGAAACTCTGC	CATCTCACTTCAGAAGGATCATAGCC
18	RM12570	CTTCCATCAGGACCTTCAATTCG	GCACGTAAACCTACAACAACCTTTGG
19	RM16493	TCGGCAGCAACAACTCTTAAACC	TCAACAAGATGACTCCCTGTAGCC
20	RM14641	GCCTAGACGATGTAGCTGGATGC	TGTTACTGTTGCGAAGTGACTGG
21	RM 6	GTCCCCTCCACCCAATTC	TCGTCTACTGTTGGCTGCAC
22	RM318	GTACGGAAAACATGGTAGGAAG	TCGAGGGAAGGATCTGGTC
23	RM23865	TCATCCCATTCTCTTCCTCACC	CATACGGCCATACAAATGAACC
24	RM23867	GCCTAAACGAGAGCCTACTCATGG	GGGAGGTGGAGATGAGATGAGG
25	RM21976	CTTCCTCCTACCTTCCTCCATCC	GCACCATCACCTCCATCTCTAGC
26	RM420	CCTCTCACTCTGCCTGCTCTACC	TCTCTAACTCTTGAGTGACAGCAACC
27	RM6711	GGACAGTTTACAAGCATGGGAGTTGG	TGTAGGGCCCAAATTTAGTGATAGGG
28	RM12492	CCATGTACTGTGTGCTCTTCTTTGC	TGCTACCACCACGTTACACATGC
29	RM16493	TCGGCAGCAACAACTCTTAAACC	TCAACAAGATGACTCCCTGTAGCC
30	RM18276	ATGTTAGCACGTGTAGCAGTCC	AACGGAGTAGGAGGATAAGATACG
31	RM409	AGATGATCAATCCGTCTCTTGC	TGCTTTCTCTGTGATTCTCTGC
32	RM28011	CGCTATAATAGGCCGCTATTGG	GCACTATTGCGAATGCTAAGTTCC

Table 3: Components of PCR reaction

Sl. No.	Components	Volume
1	DNA	2.0 µl
2	Taq buffer	1.5 µl
3	dNTP'S	1.5 µl
4	Forward primer	2.0 µl
5	Reverse primer	2.0 µl
6	Taq polymerase	0.1 µl
7	Sterile water	4.9 µl

Table 4: Temperature profile used for PCR amplification

Profile	Steps	Temperature
Profile 1:	Initiation denaturation	95°C for 5 minutes
Profile 2:	Denaturation	94°C for 1 minute
Profile 3:	Annealing	(50 to 65)°C for 1 minute
Profile 4:	Extension	72°C for 2 minutes
Profile 5:	Final extension	72°C for 5 minutes
Profile 6:	For infinity to hold the samples.	4°C
	Programmed to run for 40 cycles.	Profile 2,3 and 4

$$d_{ij} = 1 - \frac{1}{L}\sum_{l=1}^{L}\frac{m_l}{\pi}$$

Notations:
d_{ij} : dissimilarity between units i and j
L : number of loci
P : ploidy
m_l : number of matching alleles for locus l

The tree were constructed by using hierarchical clustering for both morphological and maker data. The cluster analysis and dendrogram was constructed by Unweighted Pair Group Method (UPGMA) (Sneath and Sokal, 1973) [15].

3. Results

3.1. Diversity analysis for morphological traits

Data observed for twelve morphological traits in 45 traditional and improved cultivars rice were used for calculating dissimilarity index. Genetic dissimilarities were used to create a cluster diagram. Genetic dissimilarity among 45 traditional and improved cultivars as revealed by UPGMA cluster analysis based on morphological traits data is represented as radial tree structure in Figure 1. Cluster analysis based on hierarchical clustering method grouped 45 cultivars into two major clusters (Table 5). Cluster II is sub-grouped into seven clusters.

Table 5: Clustering of 45 traditional and improved rice cultivars for morphological traits

Cluster	Genotype No.
I	8,9,10,11,12,14,21,22,23,24,26,31,33,35,36,37,38,39,40,41,42,43,44,45
II A	2,16,17,18,27,30,32
II B	1,6
II C	3,5
II D	7,28
II E	13,25
II F	15,29,34
II G	19.20

3.2. Diversity analysis for molecular data

Genetic dissimilarities obtained from SSR marker data of 33 traditional and 12 improved cultivars were used to create a cluster diagram. Cluster analysis based on hierarchical clustering corresponds to the definition of neighborhood according to the minimal dissimilarity using UPGMA method. Clustering analysis for 33 traditional cultivars was grouped into nine major clusters (Table 6 and Figure 2). Improved cultivars revealed four clusters by UPGMA cluster analysis based on dissimilarity matrix of SSR markers data (Table 7 and Figure 3). Clustering analysis is also performed by combining the SSR markers data of 33 traditional and 12 improved cultivars, which represents eleven clusters (Table 8 and Figure 4).

Table 6: Clustering of 33 traditional rice cultivars using SSR markers data

Cluster	Genotype No.
I A	33
I B	31,32
II	2,3,4,8,9,14,19,20,22,25,36,30
III	15,17
IV	6,13,21
V	10,12,27
VI	1,7
VII	16,18
VIII	23,24
IX A	29
IX B	5,11,28

Table 7: Clustering of 12 improved rice cultivars using SSR markers data

Cluster	Genotype No.
I	1,10
IIA	2,4,5,6,7,12
IIB	8,11
IIC	3,9

Table 8: Clustering of 45 traditional and improved rice cultivars using SSR markers data

Cluster	Genotype No.
I	2,3,4,8,9,14,19,20,22,25,26,30,33,35,37,38,39,40,45
II	6,13,21
III	10,12,27
IV	1,7
V	16,18
VI	36,42
VII	23,24
VIII	34,43
IX	31,32
XA	29
XB	5,11,28
XI	15, 17, 41, 44

4. Discussion

Rice is the most important cereal of the world providing 21% of global human per capita energy and 15% of per capita protein (Maclean *et al*., 2002) [7]. India is the prominent rice growing country accounting for about 20% of all world rice production. Apart from the traditional cultivars, India is home to wide cultivars of rice cultivars, landraces and many lesser known cultivars that have been under cultivation since ages by farmers. Characterization of germplasm by means of DNA fingerprinting techniques provides a tool for precise germplasm identification and a quantitative estimate of genetic diversity. This estimate is important because a decrease in genetic variability might result in a reduction of the plasticity of the crops to respond to changes in climate, pathogen populations, or agricultural practices. Molecular markers are useful tools for evaluating genetic diversity and determining cultivar identity. The purpose of this study was to evaluate the genetic diversity within a diverse collection of rice accessions, and to determine differences in the patterns of diversity within the rice traditional and improved cultivars. The result of this study is discussed.

4.1 Diversity of traditional and improved rice cultivars for morphological traits

Morphological characterization is fundamental in order to provide germplasm information for plant breeding programs (Lin, 1991) [6]. Several researchers reported the use of morphological markers in the characterization and study of rice germplasm diversity. Yawen *et al.* (2003) [16] studied the genetic diversity on 5285 accessions of indigenous rice in China and found considerable morphological variation among accessions. Patra and Dhua (2003) [11] reported the morphological diversity of upland rice in India, while Fukuoka *et al.* (2006) [4] assessed the variability in agronomic characters among landraces of aromatic rice populations in Vietnam. The genetic relationships among 147 high altitude rice landraces from Jumla, Nepal was examined by Bajracharya *et al.* (2006) [1].

In present study dissimilarity index for the morphological characters were obtained among 45 rice cultivars. Clustering pattern is derived using dissimilarity index based on hierarchical clustering corresponds to the definition of neighborhood according to the minimal dissimilarity using UPGMA. The cluster analysis for the morphological characters using the dissimilarity coefficient classified the cultivars into two groups (Figure 1). All the improved

cultivars were grouped into cluster I expect genotype no. 34 (ADT 43) found to be unique than other improved cultivars for the morphological characters. Among the traditional cultivars no. 2 (Kattu Ponni) and no. 4 (Karupu Kavuni), found to have the unique morphological characters.

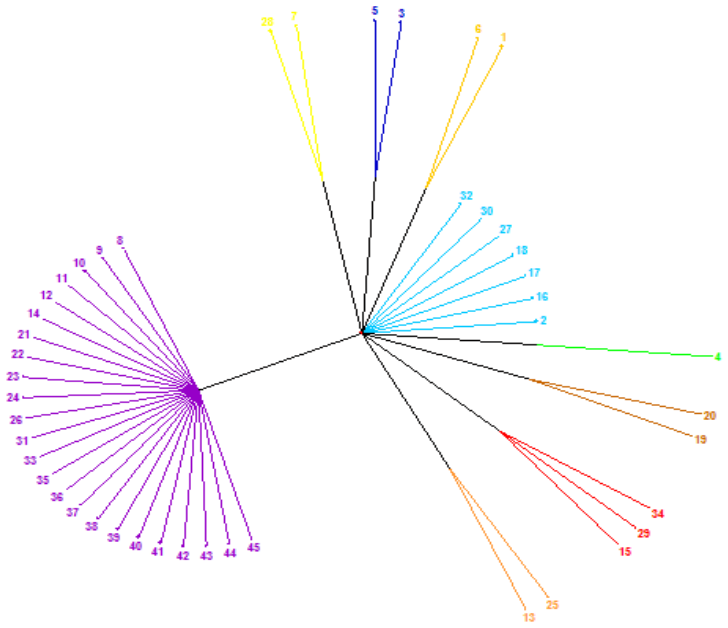

Fig 1: Radial tree structure of 45 traditional and improved rice cultivars for morphological traits.

4.2 Diversity of traditional and improved rice cultivars for SSR markers

Molecular marker based genetic diversity analysis has the potential for assessing the changes in genetic diversity over time and space (Duwick, 1984) [3]. DNA markers are predominantly used in molecular diversity studies due to their abundance and repeatability (McCouch *et al*., 1997) [9]. Among different based PCR markers, the simple sequence repeats (SSRs) markers are preferred over others due to their ease of application, rapid analysis, high reproducibility, easy scoring patterns, greater allelic diversity and low cost (Chen *et al*., 1997) [2]. Although rice germplasm diversity analysis has been done by several workers, variability studies of the traditional and improved cultivars grown in Tamil Nadu region is limited. Considering the wide cultivation and use of such cultivars in Tamil Nadu, the present study was undertaken with 45 rice cultivars which include 33 traditional and 12 improved cultivars of rice to assess their molecular diversity.

A total of 60 SSR markers were evaluated for their efficiency of polymorphism across 33 traditional and 12 improved cultivars of rice. About 32 SSR markers were found to be polymorphic and generated reproducible and informative allelic profiles. Data generated with 32 SSR markers in 33 traditional and 12 improved cultivars of rice is used diversity analysis. A dissimilarity matrix based on the proportion of shared SSR fragments was used to establish the level of relatedness between the cultivars. Cluster analyses was done to group the 33 traditional and 12 improved cultivars by constructing tree based on SSR marker dissimilarity matrix. The UPGMA-tree clustered the 33 traditional into nine major clusters (Figure 2). Cluster I has three traditional cultivars, i.e., no. 31, 32 and 33 namely viz., Palliyaral, Thavalakannan and Vellaipunaran, all these were cultivars collected from Kerala. Most of traditional cultivars of Tamil Nadu are grouped in Cluster II, which comprises of 12 cultivars. All other traditional cultivars are distributed in different clusters, among that genotype no. 29 (Haluvupavu Patti) have found to be distantly related one.

Molecular characterization is an important prerequisite to evaluate diversity within improved cultivars. It creates the basis to ensure effective utilization of the crop germplasm by both farmers and breeders otherwise unevaluated germplasm remain mere curiosities to the breeding programs. By examining the donors utilized in the development of the high-yielding cultivars, an indication is obtained of the genetic diversity. IRRI's elite germplasm has also been used extensively as a donor in Indian breeding programmes. Present study aimed to access the genetic diversity of 12 improved cultivars, widely planted in Cauvery delta region of Tamil Nadu using SSR marker data.

The tree generated from dissimilarity matrix provided an overall pattern of variation as well as the degree of relatedness among the 12 improved cultivars (Figure 3). Clustering analysis revelled only two main clusters, with very low dissimilarity values indicated narrow genetic variation among the improved rice cultivars. SSR marker data sets of 33 traditional and 12 improved rice cultivars were combined; a dissimilarity matrix was obtained between 45 rice cultivars for cluster analysis. Clustering pattern for pooled dissimilarity matrix of 45 rice cultivars reveals eleven clusters (Figure 4).

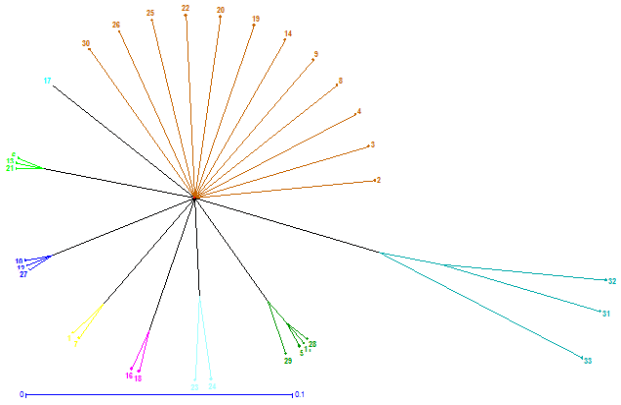

Fig 2: Radial tree structure of 33 traditional rice cultivars using SSR marker data.

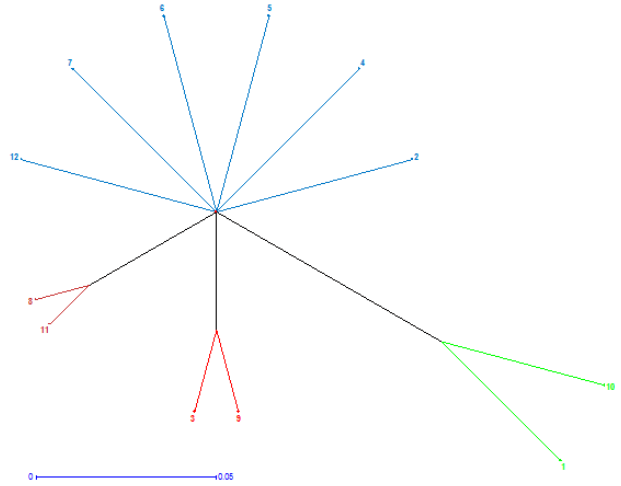

Fig 3: Radial tree structure of 12 improved rice cultivars using SSR marker data.

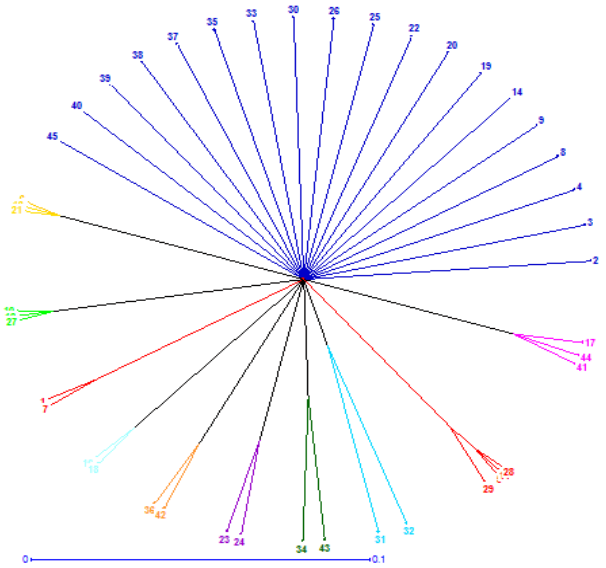

Fig 4: Radial tree structure of 45 traditional and improved rice cultivars using SSR marker data.

4.3 Comparison of morphological and molecular diversity pattern

Diversity analysis based morphological character among the 45 rice cultivars that comprises of 33 traditional and 12 improved cultivars reveals two main cluster using UPGMA method, while tree structure for 32 SSR molecular data with same set of 45 rice cultivars generated eleven clusters. The eleven major clusters together with their internal groups have demonstrated the polymorphic nature of the 45 rice cultivar used in the current study. The tree obtained from the SSR marker revealed that the marker was more discriminatory, highly polymorphic and thus, more informative than the one obtained from morphological characterization. Although, the morphological tree generated from dissimilarity or genetic distance matrices has provided an overall pattern of variation as well as the degree of relatedness among accessions, variations in environmental conditions such as soil types, and soil fertility levels, light, temperature and moisture regime allow for different results to be obtained using morphological grouping, particularly when experiments are repeated in time and space. Also, both the genetic make-up of seed, environment and field management practices has been reported to influence the morphology of a crop. The use of morphological characters in the classification of germplasm, particularly in rice, has been met with difficulties because the technique is inefficient. Observations above tend to emphasize the superiority of molecular dissimilarity grouping over and above the morphological grouping.

Both morphological and molecular variations exist between the 45 rice accessions that were evaluated. The tree obtained from the molecular was more discriminatory than the one obtained from morphological because the marker was able to make use of 32 SSR markers to sort out the 45 rice cultivars into eleven cluster groups than the morphological which was twelve traits. However, Li *et al.* (2000) [5] obtained consistent results analyzing the correlation between genetic and morphological differentiation in 111 accessions of rice from the Japonica and Indica groups.

5. Conclusion

Based on morpho-molecular diversity analysis, it was concluded that the improved rice cultivars collected for this study had a relative narrowing genetic base as compared to traditional cultivars. Genetic erosion and habitat destruction by modern agriculture has increased the significance of collection and preservation of plant germplasm. In many rice growing countries, the landraces carrying a vast amount of genetic diversity were distributed in remote villages. The number of landraces began to decline in 1970's when high-yielding cultivars were introduced. Most of the old landraces are now available in certain gene banks only, not in the hands of farmers. It is essential to rationalize conservation and use of genetic resources to guide in the establishment of strategies that ensure the maintenance of genetic variability, essential in plant breeding.

6. Acknowledgments

The research work reported in this article was carried out by the co-authors as project work for partial fulfilment of B.Sc (Agri) degree programme under the guidance and supervision of the first author. The authors are thankful to the Dean and Head (Department of Plant Breeding and Genetics), Pandit Jawaharlal Nehru College of Agriculture and Research Institute, for providing facilities to conduct this experiment.

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