Morpho-molecular Diversity Analysis in Rice (*Oryza sativa* L.) Genotypes using Microsatellite Markers

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ABSTRACT

Background: The insight concerning genetic diversity and relationship among the rice genotypes is a basic contribution to the crop improvement programs. The present field experiment was conducted to study the level of diversity present in 47 rice genotypes using both morphological and molecular analysis.

Methods: The experiment was carried out at Agricultural Research Farm, Banaras Hindu University, Varanasi (U.P.), during *Kharif*-2019 with 47 rice genotypes. Mahalanobis' D^2 analysis was carried out to evaluate the morphological diversity present among the genotypes and 24 polymorphic SSR markers were used for molecular analysis using the NTSYSpc software.

Result: Mahalanobis' D^2 grouped 47 rice genotypes into 6 clusters based on the *inter-se* genetic distance. The highest inter-cluster distance (1134.14) was observed between clusters II and IV indicating the genotypes present in these clusters to be highly divergent. Molecular diversity analysis grouped the 47 rice genotypes into 3 main clusters *i.e.*, cluster I, cluster II and cluster III, which were further divided into sub-clusters. Polymorphic Information Content varied from 0.12 to 0.86, with an average of 0.465. The highest PIC value was observed for locus RM 507 (0.86). All the 24 primers showed polymorphism and the number of alleles was common for all *i.e.,* 2. Together, the morphological and molecular diversity analysis revealed that Desi Dhan and IR 91143-AC 293-1, Desi Dhan and BD 105, IR 85850 and Lal Sundiya, Chauli and Swarna were the most diverse genotypes among the 47 rice genotypes included in the study.

Key words: D^2 analysis, Dissimilarity coefficient, Microsatellite markers, Polymorphic information content, Rice.

INTRODUCTION

Rice (*Oryza sativa* L.) is a source of instant energy and has a high content of carbohydrates and is known to be the staple food for Indian consumption at a large scale. A large fraction of the world's population consumes rice, making it a major cereal food crop. According to United Nation, it has been expected that the world population might reach up to 8.5 billion by 2030 and thus, will create high pressure on rice breeding programs worldwide (Van Bavel, 2013). Therefore, to meet the increasing demand of the population, rice production must be increased by 50%.

Improvement in the genetic potential of the crop cultivars is one of the best approaches among very few options available to increase the yield potential of rice. Rice is rich in natural genetic diversity and there is humungous scope to exploit diversity for improvement of genetic base. Genetic diversity contributes to the production of superior recombinants and so it becomes an important element in crop improvement programs. Successful crop improvement program, mainly hybrid breeding, depends on genetic diversity present among the parents (Rana *et al.,* 2004); a lack of genetic variability would have the potential to significantly limit breeding progress and/or yield and quality crop improvements (Cornelious *et al.*, 2002). A quantitative assessment carried out on genetic diversity guides the breeder for the advancement in breeding programs. It aids in the selection of agronomically suitable diverse parents for hybridization and is also important to obtain desired recombinants in the segregating generations (Goulet *et al.,* 2017). The development of novel biotechnological techniques contributes towards the evaluation of genetic variation at both phenotypic and genotypic levels. The results derived from analyses of genetic diversity at the DNA level could be effective for designing better breeding programs with a promising aim to broaden the genetic basis of commercially grown varieties (Gaballah *et al.*, 2021).

Molecular markers are known to be a powerful tool for assessing genetic variation in rice genotypes. In contrast to morphological traits, molecular markers can provide insights about the variations among genotypes at the molecular level, providing more direct, reliable and efficient results for

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germplasm characterization, conservation and management. Additionally, the results are unaffected by environmental influence (Singh *et al.,* 2015). Among the various kinds of markers available, microsatellite markers or simple sequence repeat (SSR) markers can detect a high level of allelic diversity and they have been extensively used to identify genetic variation among rice subspecies (Chukwu *et al.*, 2020).

Morphological, genetic and physiological characters have been used efficiently to improve hybrid breeding in rice along with genetic estimates. D^2 analysis is done to evaluate the conventional heritable diversity (Manomani and Khan, 2003). This technique was developed by Mahalanobis (1936), is a multivariate analysis technique that is useful to the breeders for the estimation of genetic diversity in base population or germplasm collections concerning the characters considered together (Tejaswini *et al.*, 2018).

However, with the increasing world population, environmental condition is deteriorating every day, so in such conditions, food security has become a major challenge around the world, especially in Asia and Africa. Exploring the genetic aspects of germplasm using genotypic and phenotypic analysis is much dependable for the development of rice varieties that could pave the way towards the attainment of food security (Mohammad *et al.,* 2020). Thus, in the current study, a morpho-molecular approach was used to explore the diversity present among different rice varieties.

Materials and Methods

The current investigation was carried out during *Kharif* - 2019 at Agricultural Research Farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, U.P., India. The molecular analysis was performed at the Central Laboratory (Niche Area Lab) of Banaras Hindu University. The experimental material for this investigation comprised of forty-seven rice genotypes and are listed in Table 1. Field observations were recorded on ten quantitative characters *viz.,* days to 50% flowering, days to maturity, plant height (cm), total number of effective tillers per plant, panicle length (cm), plot yield (kg), biomass (kg/plot), 1000 grain weight (gm), harvest index and grain yield in kilogram per hectare (GYKGH) (kg). The calculated mean value for the recorded data was subjected to analysis of variance.

DNA isolation and polymerase chain reaction

Genomic DNA was extracted from young leaves collected from 15-20 days old rice seedlings and stored immediately at -20°C till further processing. DNA was extracted following the CTAB extraction method suggested by Doyle and Doyle, 1987 including few modifications made in the composition of DNA extraction buffer.

The polymerase chain reaction (PCR) was performed in a total volume of 15 µl per reaction containing 1.0 µl of template DNA (40 ng/µl), 1.0 µl of forward and reverse primers (10 µM stock concentration), 0.20 µl dNTPs (1 mM), 0.20 µl Taq polymerase (5 U/µl), 0.20 µl of $MgCl_2$ (50 mM), 1.50 µl 10 × PCR buffer and remaining volume was made up by HPLC Water. The PCR amplification was carried out on a thermal cycler at an initial temperature of 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s (primer annealing occurred with most of the primers while some were adjusted), 72°C for 1 min and at least 72°C for 5 min and then stored at 4°C. A total of twenty-four simple sequence repeat (SSR) markers were used for studying molecular diversity as denoted in Table 2.

Visualization and scoring of the PCR amplified fragments

The PCR products generated through SSR primers were separated by electrophoresis in 2.5% concentration agarose gel prepared in 1x TAE buffer (electrophoresis buffer). The amplified product mixed with 2 µl of 6x gel loading dye (Bromophenol blue 0.25%; Xylene. cyanol 0.25% and glycerol 30%) were loaded in each well using a micropipette.

Table 1: List of 47 rice genotypes used in the study.

1. Lal Sundiya	17. Pahadi Basmati	33. M-48
2. BRR dhan 64	18. BPT 5204	34. IR 64
3. Parwati	19. KU-45	35. Swarna
4. MTU1010	20. Dana Basmati	36. BD105
5. Chauli	21. Controli	37. BG102
6. Panfor	22. Patol	38. DRR Dhan 45
7. Askotioyan	23. KU-46	39. M399
8. Desi Dhan	24. Jlaagam	40. IR9143-AC-239-1
9. Goji	25. Sambhamasuri	41. IR91143-AC-293-1
10. DRR Dhan 48	26. Dudhkandar	42. IR85850-AC-157-1
11. Amkotiya	27. Karhani	43. IR2475-110-2-2-1-2
12. Khaldi	28. URG-1	44. Pusa 6B
13. HUR 105 (Check)	29. URG-22	45. IR-79156B
14. HUR 3022 (Check)	30. URG-19	46. IR-58025
15. Patol	31. URG-24	47. IR-68897
16. Jhimiya	32. Nagina-22	

Table 2: List of 24 SSR markers used in the study.

Marker	Forward and reverse sequence	Annealing temperature (°C)	Chr. no	No. of alleles	PIC value
RM 312	F- GTATGCATATTGATAAGAG	55	1	2	0.71
	R- AAGTCACCGAGTTTACCTTC				
RM 472	F-CCATGGCCTGAGAGAGAGAG	52	1	2	0.36
	R- AGCTAAATGGCCATACGGTG				
RM 5791	F- GAAGCAGAATACGCTTTCGC	53	1	2	0.73
	R- ACGTCACAAAGGGTTCTTGC				
RM 154	F- ACCCTCTCCGCCTCGCCTCCTC	56	2	2	0.57
	R- CTCCTCCTCCTGCGACCGCTCC				
RM 545	F- CAATGGCAGAGACCCAAAAG	52	3	2	0.84
	R- CTGGCATGTAACGACAGTGG				
RM 293	F- TCGTTGGGAGGTATGGTACC	55	3	2	0.25
	R- CTTTATCTGATCCTTGGGAAGG				
RM 280	F- ACACGATCCACTTTGCGC	55	4	2	0.36
	R- TGTGTCTTGAGCAGCCAGG				
RM 507	F- CTTAAGCTCCAGCCGAAATG	52	5	2	0.86
	R- CTCACCCTCATCATCGCC				
RM 178	F- TCGCGTGAAAGATAAGCGGCGC	55	5	2	0.45
	R- GATCACCGTTCCCTCCGCCTGC				
RM 162	F- GCCAGCAAAACCAGGGATCCGG	56	6	2	0.22
	R- CAAGGTCTTGTGCGGCTTGCGG				
RM 454	F- CTCAAGCTTAGCTGCTGCTG	55	6	2	0.32
	R- GTGATCAGTGCACCATAGCG				
RM 125	F-ATCAGCAGCCATGGCAGCGACC	55	7	2	0.65
	R-AGGGGATCATGTGCCGAAGGCC				
RM 11	F- TCTCCTCTTCCCCCGATC	55	7	2	0.54
	R- ATAGCGGGCGAGGCTTAG				
RM 152	F- GAAACCACCACACCTCACCG	55	8	2	0.59
	R- CCGTAGACCTTCTTGAAGTAG				
RM 284	F- ATCTCTGATACTCCATCCATCC	55	8	2	0.28
	R- CCTGTACGTTGATCCGAAGC				
RM 5493	F- GACAAAACACAAAGCAGGAC	52	8	2	0.15
	R- TAACAAACCAACCAACCAAG				
RM 410	F- GCTCAACGTTTCGTTCCTG	53	9	2	0.12
	R- GAAGATGCGTAAAGTGAACGG				
RM 105	F- GTCGTCGACCCATCGGAGCCAC	52	9	2	0.47
	R- CAAGGCTTGCAAGGGAAG				
RM6100	F- TCCTCTACCAGTACCGCACC	52	10	2	0.36
	R- GCTGGATCACAGATCATTGC				
RM 171	F- AACGCGAGGACACGTACTTAC	57	10	2	0.34
	R- ACGAGATACGTACGCCTTTG				
RM 552	F- CGCAGTTGTGGATTTCAGTG	54	11	2	0.67
	R- TGCTCAACGTTTGACTGTCC				
RM 536	F- TCTCTCCTCTTGTTTGGCTC	55	11	2	0.57
	R- ACACACCAACACGACCACAC				
RM 277	F- CGGTCAAATCATCACCTGAC	55	12	2	0.47
	R- CAAGGCTTGCAAGGGAAG				
RM 19	F- CAAAAACAGAGCAGATGAC	55	12	2	0.29
	R- CTCAAGATGGACGCCAAGA				

F: Forward primer, R: Reverse primer, PIC: Polymorphic information content.

After the run, the amplified products were visualized and photographed under UV light source in a gel documentation system (Gel Doc TM XR+, BIORAD, USA). The genotypes were manually scored using the binary coding system, '1' for the presence of band and '0' for absence of a particular band.

Data analysis

The quantitative trait mean values were calculated based on data of five randomly tagged plants in each genotype and were used for statistical analysis. Analysis of variance was performed to partition the total variation among the entries following randomized complete block design using WINDOSTAT version 9.0. Adjusted trait means of each of the genotype accession was estimated (Federer, 1956) and the same was used for all subsequent statistical analysis. In the present study, Mahalanobis' generalized distance as described by Rao (1952) was used to estimate the genetic divergence.

Standardization was done by dividing the deviation of the mean for a line from the mean for forty-seven genotypes with the standard deviation for the given trait; the STAND module of NTSYS software was used to furnish the same. The binary data matrices generated by polymorphic SSR markers were subjected to further analysis using NTSYSpc version 2.11W. The Jaccard's coefficient was calculated using SIMQUAL program. Unweighted pair group average (UPGMA) based clustering was done using SAHN module of NTSYSpc for dendrogram construction.

Polymorphic information content (PIC)

PIC value is used to estimate the discriminatory power of the SSR marker. The formula for PIC calculation for SSR markers:

$$PIC = 1 - \Sigma pi^2$$

Where,

pi stands for the frequency of i^{th} allele and summation extends over n patterns.

Results and Discussion

Analysis of variance (ANOVA) based on the mean values of ten quantitative traits in forty-seven rice genotypes recorded significant differences among the genotypes with respect to all the characters considered for the study (Table 3). This suggests that there is an inherent genetic difference among the genotypes for the traits studied.

Diversity analysis using morphological data.

D^2 analysis revealed the presence of significant diversity in the set of forty-seven genotypes assessed. The germplasm was distributed into six different clusters (Table 4). The genotypes within each cluster were comparatively closer to each other than the genotypes present in different clusters. Maximum number of genotypes (20) were included in cluster II followed by 18 genotypes in cluster I, cluster III and V are with 4 and 3 genotypes, respectively. Clusters IV and VI are solitary clusters. Similar results were observed by Islam *et al.* (2020), as they used 113 rice genotypes for D^2-analysis and observed that these genotypes were grouped into 4 clusters. Diversity analysis by Tejawini *et al.* (2018) and Salem and Sallam (2016) have also shown similar findings.

Cluster I showed the maximum intra-cluster distance (64.47) with 18 genotypes followed by cluster III (64.05) with 4 genotypes. The intra-cluster distance for clusters IV and VI was observed to be zero as these two clusters were monogenotypic. The maximum inter-cluster distance was observed between clusters II and VI (1134.14), followed by clusters II and III (802.93) and clusters V and VI (788.86). This suggests that the hybrids developed from the selected genotypes of these clusters would have a high chance to obtain heterotic combinations and produce a highly variable population in the segregating generations. One should not forget the fact that, along with the genetic distance, *per se* yield and yield contributing characters should also be considered while selecting the genotypes for breeding programs. In this respect, genotypes like Desi Dhan, IR 82475-110-2-2-1-2, DRR Dhan 48, *etc.* have the highest inter-cluster distance and high *per se* yield so they can be considered as high yielding parents in hybridization programs.

The lowest inter-cluster distance was observed between clusters IV and VI (112.81), followed by clusters I and IV (151.44) and clusters III and IV (159.57). The genotypes in these clusters are genetically very close and hence

Table 3: Analysis of variance for grain yield and yield component characters in rice.

Source of variation	Replication	Treatment	Error
df	2	46	92
Days to 50% flowering	5.37	24451.4**	111.95
Days to maturity	1.31	64360.31**	147.34
Plant height (cm)	4.56	86797.18**	1288.01
Number of tillers per plant	0.38	999.6**	96.48
Panicle length (cm)	10.94	1236.09**	236.67
Plot yield (kg)	0.002264	1.2**	0.12
Biomass (kg)	2.34	1759.39**	53.43
Harvest index	0.007	8.069**	1.42
1000 grain weight (g)	12.77	3536.56**	594.68
GYKGYH (kg)	12384.04	7438348.93**	786428.5

Table 4: Grouping of forty-seven genotypes of rice into six clusters by Tocher method.

Clusters	Number of genotypes	Name of genotypes	
Clusters I	18	15. Controli	12. Khaldi
		16. Askotiyan	22. BRR dhan 64
		14. Jhimiya	13. Jlaagam
		5. Amkotiya	8. Patol
		7. Goji	20. MTU1010
		9. KU-45	4. Dana basmati
		11. Parwati	10. KU-46
Clusters II	20	32. IR 91143-AC 290-1	47. IR 68897
		39. IR 91143-AC 293-1	46. URG 22
		25. Karhani	41. IR85850-AC 157-1
		42. IR 91143-AC 239-1	44. IR64
		45. BG-102	36. M-48
		43. Nagina-22	30. URG-1
		27. URG-30	29. Sathi
		26. BD105	40. URG-24
		28. Dudhkandar	33. URG-19
		37. IR 82475-110-2-2-1-2	38. M-399
Clusters III	4	21. DRR Dhan 48	23. Check1(HUR 105)
		19. BPT5204	6. Panfor
Clusters IV	1	3. Desi Dhan	
Cluster V	3	31. Sambhamahsuri	
		34. Swarna	
		35. URG-22	
Cluster VI	1	2. Pahadi Basmati	

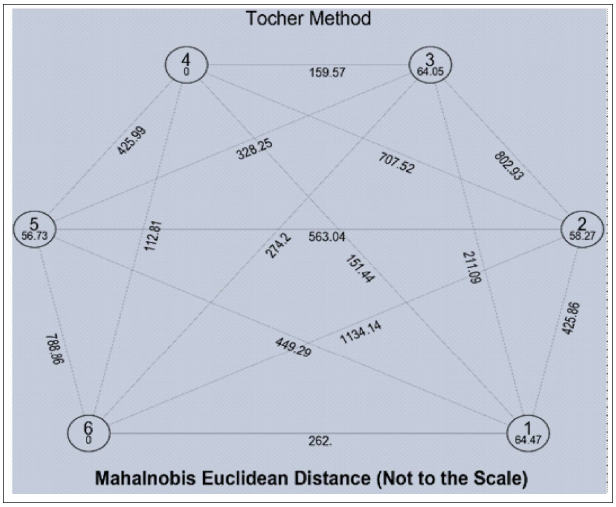


Fig 1: Relative disposition of clusters showing average genetic distance (D^2 values) between and within by Tocher method.

hybridization among these lines may not be very fruitful. These results are in accordance with the earlier reports of Thippeswamy *et al.* (2016), Devi *et al.* (2019) and Dey *et al.* (2020). The intra and inter-cluster distances (D^2 values) were given in table 5 and the cluster diagram was shown in Fig 1. The inter-cluster distances between all the clusters were higher than the intracluster distances suggesting wider genetic diversity among the genotypes of different cluster groups. This is in parallel with the findings of Mohan *et al.* (2015) and Singh *et al.* (2020). Fig 2 represents the dendrogram of forty-seven genotypes of rice by Tocher method.

Molecular diversity using microsatellite (SSR) markers

Molecular diversity study has been assessed by using chemically designed molecular markers. These sequences are the complementary sequences of DNA that lie close to a particular gene or QTL and through primer annealing, they amplify the target gene. In the present study diversity analysis among forty-seven rice genotypes was done using 24 rice SSR markers. Out of the 24 SSRs used, all the markers showed polymorphism.

Scoring of SSR bands and PIC value

The polymorphic information content (PIC) was calculated for each locus to assess the information of each marker and its discriminatory ability and it is an evidence of allele diversity and frequency among varieties (Table 2). The highest PIC value was observed for the locus RM 507 (0.86) followed by RM 293 (0.84), RM 5791 (0.74), RM 545 (0.73) and RM 312 (0.71) and lowest by RM 552(0.12) followed by RM 410 (0.15), RM 162 (0.22), RM 284 (0.28) and RM 19 (0.29). So, PIC value ranged from 0.86 to 0.12 with a mean value of 0.465. All the twenty-four primers showed polymorphism and the number of alleles were 2 in every case. A total of 50 alleles were amplified from 47 genotypes

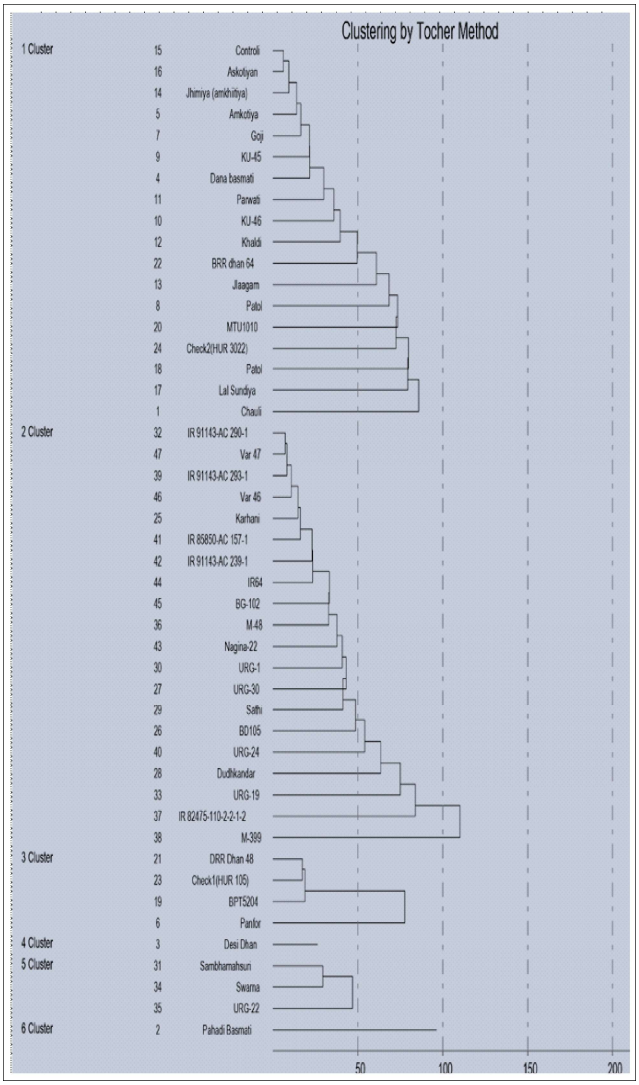


Fig 2: The dendrogram of forty-seven genotypes of rice by Tocher method.

and this shows significant variability among the genotypes. PIC value revealed that RM 507 was considered as best marker. Rafii *et al.* (2018) reported the genetic relationship and diversity analysis among 8 aromatic rice cultivars using 32 SSR primers, detecting a total of 131 alleles. The average number of alleles per locus was 4.09. Similar results were reported by Pathaichindachote *et al.* (2019) evaluated a set of 167 rice genotypes using 13 polymorphic SSRs to assess the genetic diversity and genetic relationship. A total of 110 alleles were amplified and the Polymorphic Information Content (PIC) values for SSR primers ranged from 0.27(RM542) to 0.87(RM21) with a mean of 0.59. So, PIC value is related to the polymorphism of markers and a higher PIC value indicates high polymorphism.

Dendrogram analysis

Dissimilarity coefficient was used to determine the level of relatedness among the genotypes. The dissimilarity coefficient varies from zero to one, a value closer to one reflects a higher dissimilarity, whereas, closer to zero reflects a higher similarity. The average dissimilarity ranged from 0.5516 to 0.7862. A dendrogram (Fig 3) based on Jackard's dissimilarity coefficient was constructed using UPGMA (Unweighted Pair Group Method with Arithmetic Averages) method and forty-seven rice genotypes were grouped into three main clusters *i.e.*, cluster I, cluster II and cluster III. Cluster I was further sub-divided into two minor sub-groups IA and IB and IA was differentiated into IA-1 and 1A-2. Cluster IIB was further sub-divided into two subgroups *i.e.,* IIB-1 and IIB-2. Cluster III was divided into three subgroups IIIA, IIIB and IIIC. The genotypes included in these clusters are presented in Table 6.

The total average of dissimilarity coefficient of all the 47 genotypes is 0.701. The dissimilarity coefficient varied with a highest value of 0.78 among the cultivar IR 85850 and Lal Sundiya followed by Chauli and Swarna (0.76) and DRR Dhan 45 and URG-1 (0.73). These values reflect high dissimilarity between them showing that they are highly

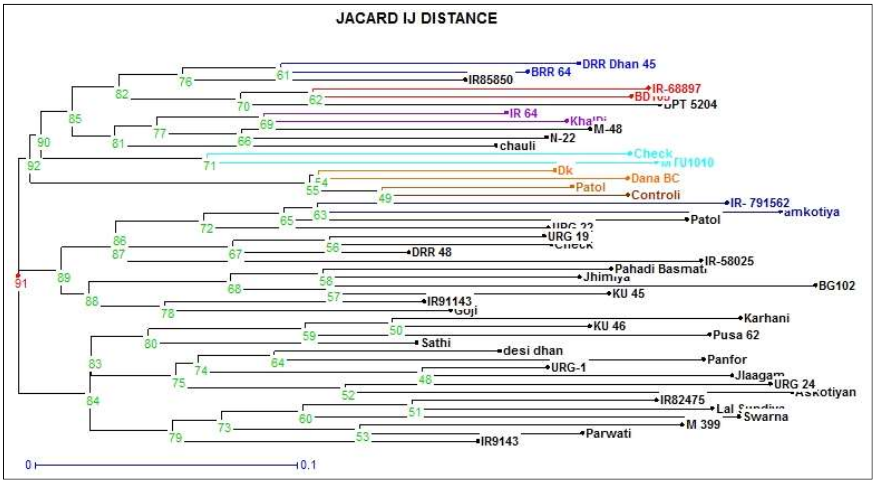


Fig 3: Dendrogram based on Jackard's coefficients.

Table 5: Average Intra and intercluster distance (D^2 values) among six clusters by Tocher method.

	Cluster I	Cluster II	Cluster III	Cluster IV	Cluster V	Cluster VI
Cluster I	64.47	425.86	211.09	151.44	449.29	262
Cluster II		58.27	802.93	707.52	563.04	1134.14
Cluster III			64.05	159.57	328.25	274.2
Cluster IV				0	425.99	112.81
Cluster V					56.73	788.86
Cluster VI						0

(Bold digits along the diagonal indicate intracluster D^2 values).

Table 6: Grouping of Forty-seven genotypes of rice based on dendrogram analysis.

	Cluster	No. of genotypes	Name of the genotypes
Cluster I			
Cluster-I A-1	11	DRR Dhan 45	N-22
		IR 64	BD 105
		BRR Dhan 64	Chauli
		Khaldi	BPT 5204
		IR 85850-AC 157-1	IR 68897
		M-48	
Cluster-I A-2	2	HUR 3022	MTU1010
Cluster-I B	4	Dudhkanda	Controli
		Dana basmati	Patol
Cluster II			
Cluster-II B-1	8	IR-791562	Patol
		URG 19	DRR Dhan 48
		Amkotiya	URG 22
		HUR 104	IR-58025
Cluster II B-2	6	Pahadi basmati	IR 91143-AC 239-1
		Jhimiya	Goji
		BG-102	KU-45
Cluster III			
Cluster III A	4	Karhani	Pusa-6B
		KU-46	Sathi
Cluster III B	6	Desi Dhan	URG-24
		Panfor	Askotiyan
		URG-1	Jlaagam
Cluster III C	7	IR 82475-110-2-2-1-2	M-399
		IR-42375	Parwati
		Lal sundiya	IR 91143-AC 290-1
		Swarna	

dissimilar from each other. The lowest value (0.48) was found between Jlgaam and URG-24 followed by 0.50 between Lal Sundiya and Swarna. This suggests there is a considerable diversity present among the genotypes in the study. Similar clustering of genotypes into various clusters and sub-clusters were reported by Das *et al.* (2013); Shahriar *et al.* (2014); Vengadessan *et al.* (2016); Dey *et al.* (2020) and Pathak *et al.* (2020).

Finally, according to the dendrogram and Jackard dissimilarity coefficient values, the most diverse cultivars among the 47 rice genotypes studied are IR 85850 and Lal Sundiya followed by Chauli and Swarna, DRR Dhan 45 and URG-1, there is a high chance that upon utilization of these genotypes in hybridization programs for crop improvement, will give useful segregants as they showed greater genetic distances. Additionally, these genotypes have lower relatedness making them genetically diverse which is of prime importance for any breeding program.

CONCLUSION

The present study shows the existence of a considerable level of morphological and molecular diversity among the 47 rice genotypes assessed. Analysis of variance discovered highly significant differences among all the

characters and reveals the presence of a high degree of variability in the studied material for effective selection. Based on morphological and molecular diversity analysis the most diverse genotypes observed were Desi Dhan and IR 91143-AC 293-1, Desi Dhan and BD 105, IR 85850 and Lal Sundiya, Chauli and Swarna. Breeders may attempt hybridization among the above genotypes that showed maximum diversity, for creating more variability in rice and further to obtain desirable segregants.

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Conflict of interest

All authors declare that they have no conflict of interest.

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