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CHARACTERIZATION AND IDENTIFICATION OF RICE GENOTYPES BASED ON THE MICROSATELLITE SSR MARKER FOR VARIETAL PURITY ASSESSMENT

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ABSTRACT
Microsatellite markers prove highly valuable for both assessing genetic purity and diversity. Moreover, efforts have been made to identify an alternative to the Grow-Out-Test, a traditional but laborious and time-consuming method used for seed genetic purity testing in the history of quality seed multiplication. The primary objective of the present study was to examine genetic purity and diversity among ten distinct rice varieties recently released from TNAU i.e., ADT-53, ADT-54, ADT-55, ADT-57, MDU-6, TKM-15, CO-52, CO-55, TRY-5, VGD-1.The results of the experiments revealed that, out of the 50 SSR primers examined, 28 were identified as polymorphic and were subsequently employed in the analysis of these ten rice varieties. Among the primer pairs used for molecular profiling, namely RM 60, RM 3345, RM 5463, RM 205, and RM 512, RM 60, RM 3345, RM 5463, RM 205, and RM 512 emerged as particularly effective in revealing discernible and predictable patterns of polymorphism for genetic purity testing. Major allele frequencies varied from 0.18 (RM 3345) to 0.66 (RM 520), averaging at 0.42. A dendrogram, constructed through cluster analysis based on microsatellite polymorphism, showcased the relationships among the ten rice varieties. These findings have practical implications for the monitoring of purity, genotype identification and the protection of plant varieties.

KEYWORDS: Rice, characterization, cluster analysis, dendrogram, genotype, molecular marker

1.INTRODUCTION

Rice (*Oryza sativa* L.) holds a crucial position as one of the most significant crops in the worldwide, serving as a primary food source for over half of the global population. It plays a vital role in bolstering India's national food security and serves as a primary calorie source for both urban and rural communities. Given the evolving preferences of consumers and the demands of the export market, there has been notable attention directed towards rice breeding programs in India, specifically aimed at enhancing yield and grain quality. Despite the development of more than 900 rice varieties in India, only a selected number of elite rice strains have been officially released to accommodate various agro-climatic conditions. To foster the creation of improved rice varieties in the future, it is imperative to assess the variability among these elite rice strains. This assessment will facilitate the identification of genetically distinct varieties that can be instrumental in future breeding initiatives (Dwivedi *et.al.*2022).

Varietal determination is an important step in determining the seed quality. Additionally, it holds significant importance in the context of seed production, certification, cultivar propagation, and the implementation of India's Protection of Plant Varieties and Farmer's Rights Act. Traditionally, the assessment of distinctness and the creation of broad descriptions for rice varieties have relied on morphological traits. However, it's important to note that rice varieties cultivated in diverse agro-climatic regions exhibit substantial differences in agronomical characteristics, making it challenging to solely rely on the morphological criteria recommended by the International Union for the Protection of New Varieties of Plants (UPOV) descriptors (Roja *et.al.*2024). Furthermore, rice breeding often results in the development of elite varieties that may exhibit limited phenotypic distinctiveness due to the narrow genetic base of their populations. In such cases, morphological traits prove inadequate as they are influenced by environmental factors. Morphological variation doesn't consistently reflect genuine genetic diversity, primarily due to genotype-environment interactions and the complex genetic control of polygenic morphological and agronomic traits (Mishra *et.al.*2003).

Molecular marker-based approaches offer a valuable opportunity to delve into the genetic foundation underlying the observed variations in phenotypes among released rice varieties. Various types of molecular markers, including RAPDs, RFLPs, AFLPs and SSRs have been utilized to assess genetic diversity and create molecular

profiles for rice. Among these options, simple sequence repeats (SSRs) or microsatellites stand out as the preferred markers in rice research (Khalid *et al*.2023). SSR markers have gained prominence as the markers of choice for conducting molecular characterizations and studies on genetic diversity in rice. In India, the breeding of rice varieties has primarily focused on a selected set of key traits, including plant height, flowering, resistance to pests and diseases, maturity, and yield. Remarkably, no comprehensive study based on molecular markers, in particular, has been documented for the well-established rice varieties that are popular in the country (Creste *et al.*,2005). Given this context, we aim to undertake the characterization and estimation of genetic diversity in elite rice varieties cultivated in India by employing SSR markers is undertaken.

2.MATERIALS AND METHODS

2.1.Planting material

Genetically pure seed samples from ten different rice varieties of TNAU (ADT 53, ADT 54, ADT 55, ADT 57, TKM 15, MDU 6, CO 52, CO 55, VGD 1, TRY5), which were obtained from various rice research stations located in Tamil Nadu were utilized. The experiments were conducted at the Department of Seed Science & Technology at the Agricultural College and Research Institute in Madurai, TNAU, Tamil Nadu, India.

2.2. DNA extraction and PCR amplification

DNA was extracted from young leaves of forty-five-day-old plants using the CTAB method. For this study, fifty SSR markers that span various genomic regions of rice were employed. PCR reactions were performed in a Thermal cycler (Bio Rad) with a total reaction volume of 10 µl, comprising 8 µl of PCR master mix, 1 µl of genomic DNA, and 1 µl each of the forward and reverse primers. The PCR cycling conditions were as follows: an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension step at 72°C for 7 minutes. The amplified products were separated on a 3 percent agarose gel prepared in 1X TAE buffer. The gel was run at a constant voltage of 100 V for a duration of 1 to 2 hours and was subsequently stained with ethidium bromide. Subsequently, the gel was visualized under a UV trans-illuminator, and photographs were captured using a gel documentation instrument. The presence or absence of clearly resolved and unambiguous bands with each primer was visually scored, generating scores in the form of a matrix consisting of '1' and '0,' representing the presence and absence of bands in each rice variety, respectively (Joydip *et al*.,2012).

2.3. Simple Sequence Repeat (SSR)

Analysis of the polymorphism information content (PIC) for the primer pairs involved calculating their values using the following formula:

$$PICi=1-\sum_{j=1}^{k} P^2ij \qquad \text{(Eqn. 1)}$$

In this context, Pij represents the frequency of the j^{th} allele for the i^{th} marker, with the summation covering n alleles. The assessment of polymorphism was based on variations in their allelic sizes.

2.4. Cluster analysis

Genetic associations and relationships between entries were assessed by computing the similarity coefficient for pairwise comparisons, which relied on the proportions of shared bands generated by SSRs. The procedure employed for constructing the tree involved sequential agglomerative hierarchical non-overlapping (SAHN) clustering, which was built upon similarity coefficients. Dendrograms, formed using similarity indices, were derived using the unweighted pair-group method with arithmetic mean (UPGMA). This analysis was conducted using NTSYS-pc software version 2.1 (Rohlf and James. 1998).

3.RESULTS

Analysis of ten rice varieties involved the examination of 50 SSR markers encompassing all 12 rice chromosomes. Out of these, only 28 SSR markers displayed polymorphism, featuring two to three alleles across all the varieties. The degree of divergence varied among the different varieties for 24 microsatellite loci. The allelic diversity per locus ranged from 2 to 3 alleles, with an average of 2.5 alleles. Among the markers, RM 464 and RM 520 exhibited the maximum number of three alleles each. In contrast, 26 markers (RM 226, RM 3252, RM 573, RM 1106, RM 60, RM 168, RM 16030, RM 303, RM 142, RM 551, RM 3345, RM 30, RM 400, RM 527, RM 5463, RM 214, RM 234, RM 420, RM 547, RM 205, RM 474, RM 24933, RM 2110, RM 512, RM 5746, and RM 7619) amplified two alleles each, while the remaining 22 markers showed a monomorphic allelic pattern. Notably, RM 60, RM 16030, RM 3345, RM 5463, and RM 205 exhibited dimorphism in the varieties CO-52, MDU-6, TKM-15, CO-55, and VGD-1, respectively (Table 1, Figure 1 and Figure 2)

3.1. Polymorphism Information Content (PIC)

The SSR markers analysed proved to be both highly informative and polymorphic, as demonstrated by the calculated PIC values. Across all tested SSR loci, the PIC values, indicative of allele diversity, exhibited significant variation, ranging from 0.18 to 0.66, with an average of 0.42. The RM 520 primer pairs displayed the highest PIC value at 0.66, while the RM 3345 primer pairs showed the lowest value at 0.18. (Table 1).

3.2. Cluster analysis

A dendrogram was constructed using UPGMA cluster analysis, utilizing the similarity coefficient derived from SSR markers, to visualize the relationships among ten rice varieties. These varieties were grouped into two major clusters, referred to as CI and CII. Cluster CI comprised seven varieties and was further subdivided into two distinct groups. Group A1 included two subgroups, A1a (comprising ADT-57, MDU-6and TKM-15) and A1b (comprising CO-52, TRY-5and CO-55). Meanwhile, group A2 consisted of ADT-55. Notably, the SSR-based dendrogram revealed a remarkable 90% similarity between TRY-5 and CO-52, suggesting a close genetic relationship between these two varieties. Cluster CII, on the other hand, encompassed three rice varieties: ADT-53, ADT-54and VGD-1. The similarity coefficients within this cluster ranged from 79% to 92%, with an average similarity of 85%. The pairwise similarity matrix analysis unveiled that within the 55 comparisons, 23% displayed similarity scores in the range of 71% to 80%, 51% exhibited similarity scores falling between 81% to 90%and the remaining 25% indicated similarity scores ranging from 91% to 100% (Table 2 and Figure 3)

4.DISCUSSION

Evaluating the genetic purity of seed varieties stands out as a crucial aspect in ensuring the quality of seeds sold for agricultural cultivation, ultimately contributing to enhanced productivity. It's worth noting that the potential for seed plot contamination exists through various pathways, including physical mixtures that can occur during post-harvest handling procedures like threshing, drying, cleaning, grading, and bagging. Maintaining a high level of genetic purity in seeds is vital to harness heterosis, which is traditionally assessed through a Grow-Out Test (GOT) using a representative sample of quality seeds before they are introduced into the market. The GOT primarily relies on achieving morphological uniformity. This procedure is inherently empirical and somewhat subjective, relying on the expression of these traits. Both qualitative and quantitative characteristics can be influenced significantly by environmental factors. However, the introduction of molecular markers has effectively mitigated these limitations associated with morphological markers. Microsatellite/SSR markers exhibit consistent stability across diverse environmental conditions, retaining their reliability. Additionally, their plant stage specificity and attributes such as reproducibility, multi-allelic nature, codominant inheritance, relative abundance, and comprehensive genome coverage (Powell *et al.,*1996), position microsatellite markers as a highly convenient and preferred choice. These markers serve the purpose of assessing the distinctness of varieties by generating unique amplicon profiles, which are crucial for future protection (Law *et.al.*1998). This quality is especially valuable in the evaluation of genetic purity, particularly for high-quality rice varieties.

Various techniques have been employed to assess the genetic diversity of rice varieties. These methods encompass the examination of their morphological and physiological characteristics (Mondal *et.al.,*2014), isozymes (Glaszmann,1987), RFLP markers (Yi *et.al.,*2001 and Kojima *et.al.* 2005), RAPD (Qian *et.al.,* 2001), or microsatellite markers (Wang *et.al.*2009). However, these studies typically treated each rice landrace or cultivar as a single sample, without delving into the internal diversity within them. Conversely, rice landraces have been shown to exhibit heterogeneity within their populations (Fukuoka *et.al.*2006). Microsatellite markers have proven to be a valuable tool for elucidating the genetic diversity among rice landrace genotypes.

Therefore, an effort was made to identify the most recent rice varieties released by TNAU using microsatellite polymorphisms. These ten rice varieties, which are the focus of this study, have been officially approved for commercial cultivation in various regions throughout India. As a result, it is of paramount importance to conduct molecular fingerprinting on these varieties to safeguard Plant Breeders' Rights (PBR) and to counter the prevalent issue of biopiracy in India and neighbouring rice-growing nations. In this study, a set of fifty SSR primer pairs was selected, and they successfully amplified a total of 80 alleles, with each primer pair yielding between 1 and 3 alleles, averaging at 1.6 alleles per primer pair. This outcome is in line with the findings of Tamilkumar *et al.* 2009, who reported 2-4 alleles per primer pair with an average of 2.9 alleles using 11 primer pairs.

The SSR primer RM 520 exhibited the highest PIC value at 0.66. The PIC value serves as an indicator of allele diversity and their frequency among the different rice varieties. A higher PIC value at a locus corresponds to a greater number of detected alleles. This pattern aligns with the findings of Lapitan *et al.* (2007) and Wang *et al.* (2009). The average PIC value for the remaining SSR loci stood at 0.3785. The microsatellite markers detected varying numbers of alleles, ranging from 2 to 4, with an average of 2.6957 alleles per locus. These results were in accordance with those reported by Wang *et al.* (2009) and Jalaluddin *et al.* (2007), who utilized a different set of rice germplasm. However, it's worth noting that the average allele counts in this study were lower compared to those reported in previous studies (Siwach *et al.,*2004; Brondani *et al.,*2006; Jayamani *et al*.2007; Thomson *et al.,*2007, Pervaiz *et al.,*2009 and Ghneim *et al*.,2008). Numerous approaches, including morphological and anatomical characterization, organoleptic markers like odour, colour, and texture, and chemical testing, have been devised for the purpose of authentication and the detection of adulterants stated by Shaw *et al.*(1997). Among these methods, molecular analysis for varietal identification stands out as the most reliable and authenticated.

The analysis of the pairwise similarity matrix unveiled that out of the 55 comparisons made, 23% displayed similarity scores falling in the 71% to 80% range, while 51% exhibited similarity scores ranging from 81% to 90%, with the remaining 25% reflecting similarity scores between 91% and 100%. These findings align with

earlier studies on the application of SSR markers for genotype characterization, as documented by Singh *et al.* (2008) in pigeon pea, and Latif *et al.* (2013) in rice.

5.CONCLUSION

This study highlights the effectiveness of SSR markers, including RM 60, RM 3345, RM 5463, RM 520, and RM 512, in both purity and diversity analysis of rice varieties. These markers demonstrated high genetic polymorphism, enabling precise genotyping of ten rice varieties: ADT-53, ADT-54, ADT-55, ADT-57, MDU-6, TKM-15, CO-52, CO-55, TRY-5, and VGD-1. The findings offer valuable insights for breeders and researchers in varietal development.

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Table 1. Polymorphic SSR marker details.

S. No	Marker	Chr. number	Annealing Temperature (^{O}C)	Forward Primer	Reverse Primer	PIC Value
1	RM 226	1	55 ^{O}C	GAAGCTAAGGTCTGGGAGAAACC	AATGGCCTTAACCAAGTAGGATGG	0.47
2	RM 3252	1	55 ^{O}C	ATGCAAGCATCTGCTTATGG	GTTGGTAACTTTGTTCCCATGC	0.42
3	RM 573	2	55 ^{O}C	TCATGTTGACGCACACATACACG	CTCTTCTTCCCTGGACCACACC	0.42
4	RM 1106	2	55 ^{O}C	GATCAACTGCTCCGCCCTTCG	ACACCACCTCGCCCAATTAAACC	0.48
5	RM 60	3	55 ^{O}C	CAAGTTCACCCGCCTTCTCG	TTTCCATCATTAGCAGGCAGTAGC	0.50
6	RM 168	3	55 ^{O}C	TGTCGTCGAGGATTTGGAGATCG	GAATCAATCCACGGCACAGTCC	0.66
7	RM 520	3	55 ^{O}C	ACGATAACGCCGACATCACTGG	GCTAAGCATCCACGGTTTCTCTCC	0.66
8	RM 16030	3	55 ^{O}C	GCGAACTATGAGCATGCCAACC	GGATTACCTGGTGTGTGCAGTGTCC	0.18
9	RM 303	4	55 ^{O}C	ATCGATGTAGGTAGAGGGACACC	CAGATCTAGTCGACATGGTTGG	0.50
10	RM 142	4	67^{O}C	TCTTCCTCTCCACTTCCATTTCC	AAGAAGCTCGGGATCTTCACC	0.66
11	RM 551	4	55 ^{O}C	CTTACTCCATTGGGCTGGAACC	TGTAGGGTGGTAAGAGATCCACTCC	0.32
12	RM 3345	5	55 ^{O}C	CAGAGCTTCTTTGCTTCCTATGG	ATTCATGACTGAGGAGCTAGTGG	0.18
13	RM 30	6	55 ^{O}C	GTCACATTCCGTTTCCCATCATTCC	CCTCACCTCACCACACGACACG	0.42
14	RM 400	6	55 ^{O}C	TTACACCAGGCTACCCAAACTCG	TTGCTGAGTTCCCTCGTCTATCC	0.48
15	RM 527	6	55 ^{O}C	CGGTTTGTACGTAAGTAGCATCAGG	TCCAATGCCAACAGCTATACTCG	0.55
16	RM 5463	6	55 ^{O}C	AGTGCCTGTTTGTTTCCTCTTCG	CATGGTCAGAGCAAGTTAGTGTGG	0.18
17	RM 214	7	55 ^{O}C	GAACATGCTTTCAACCATCAGG	GATCCTCTCAGTTCAGTGCAAGC	0.50
18	RM 234	7	55 ^{O}C	TTCAGCCAAGAACAGAACAGTGG	CTTCTCTTCATCCTCCTCCTTGG	0.42
19	RM 420	7	55 ^{O}C	CCTCTCACTCTGCCTGCTCTACC	TCTCTAACTCTTGAGTGACAGCAACC	0.32
20	RM 547	8	55 ^{O}C	TTGTCAAGATCATCCTCGTAGC	GTCATTCTGCAACCTGAGATCC	0.32

21	RM 205	9	55 ^{O}C	CCTAAGAGGAGCCATCTAA CAACTGG	CTTGGATATACTGGCCCTT CACG	0.32
22	RM 464	9	55 ^{O}C	GAAGCAGGAAACAAGAAG AGAAGG	GTCTTCACCACAGTAAATG CTTGC	0.58
23	RM 474	10	55 ^{O}C	TACACGAGGGAGTACTCG AATGG	CATGGAGGTATAGAAGAG CATTGG	0.32
24	RM 24933	10	55 ^{O}C	AGTGGCATGTGACTGATAT GACG	TCTGGGTGAAGCTGTCGTA GC	0.50
25	RM 2110	11	55 ^{O}C	TGTGTCAACCTCTGTCCTC ACACC	GCTTCGTTGGTTTGCTTGT GG	0.48
26	RM 512	12	55 ^{O}C	TGCAGTGAATGGAGACCA CTAGC	CGGTGAGTCCCATATCTTC AACC	0.18
27	RM 5746	12	55 ^{O}C	CAGCTTCGGCAAAGCAAA GC	CTCGCTACGTCGACTGATT TGG	0.48
28	RM 7619	12	50 ^{O}C	TCTTGGTATGTATTGGCAG CGAAAGC	AGGATGTGAATGAAGGCG AATGG	0.32

Table 2. SSR marker similarity index for rice varieties.

Variety	ADT-53	ADT-54	ADT-55	ADT-57	MDU-6	TKM-15	CO-52	CO-55	TRY-5	VGD-1
ADT-53	1.0000									
ADT-54	0.8194	1.0000								
ADT-55	0.7638	0.7778	1.0000							
ADT-57	0.7638	0.8333	0.7500	1.0000						
MDU-6	0.8056	0.7917	0.8750	0.8472	1.0000					
TRY-5	0.7083	0.8056	0.8333	0.8889	0.9028	1.0000				
CO-52	0.7361	0.8333	0.8056	0.8611	0.8472	0.8611	1.0000			
CO-55	0.7500	0.8472	0.8194	0.8472	0.8333	0.8472	0.9028	1.0000		
TRY-5	0.7917	0.8333	0.8333	0.8333	0.8750	0.8611	0.9167	0.9028	1.0000	
VGD-1	0.8056	0.8472	0.7639	0.7639	0.7778	0.7639	0.8194	0.8056	0.8750	1.0000

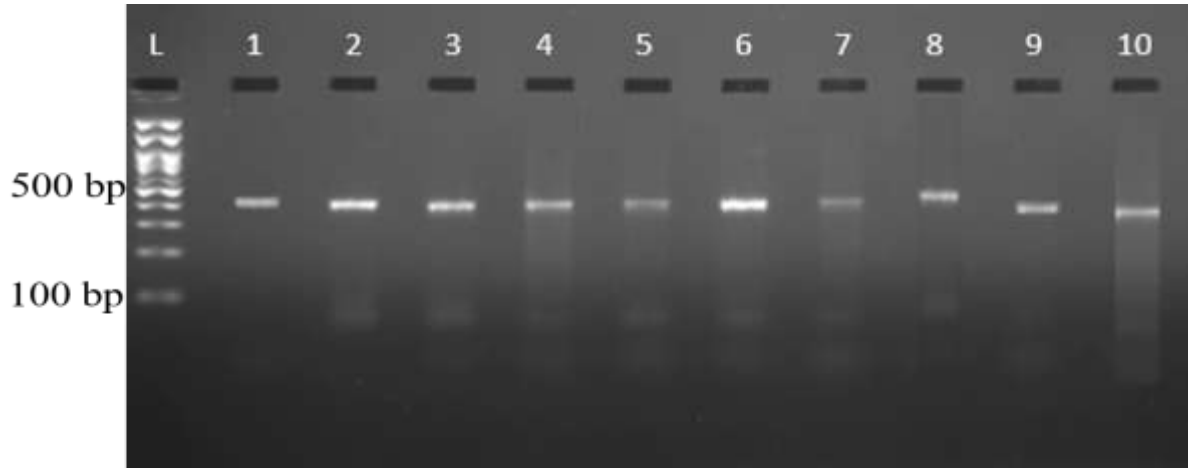

Fig. 1. Rice genotypes characterization using SSR markers- RM 5463

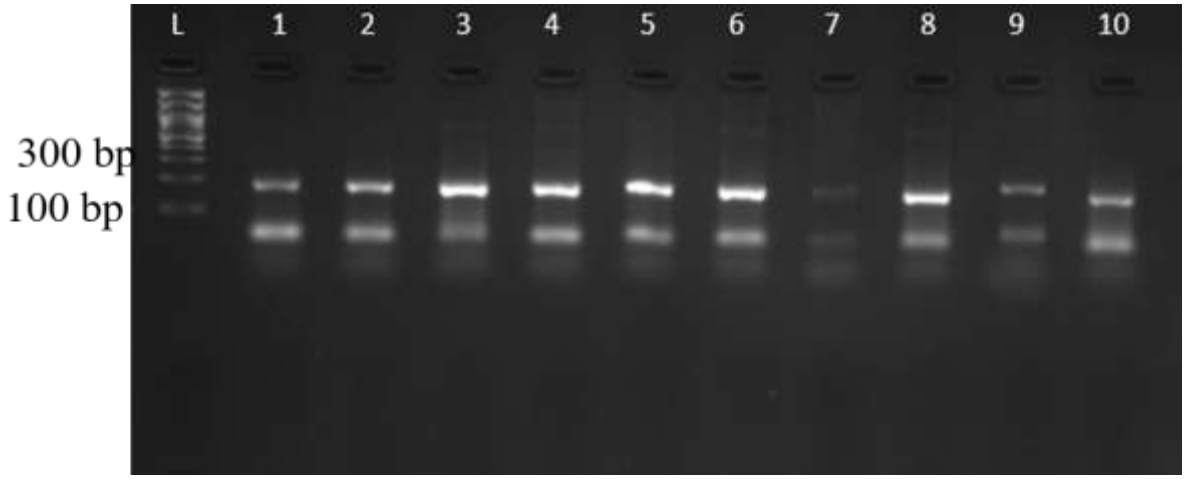

Fig. 2. Rice genotypes characterization using SSR markers-RM 512

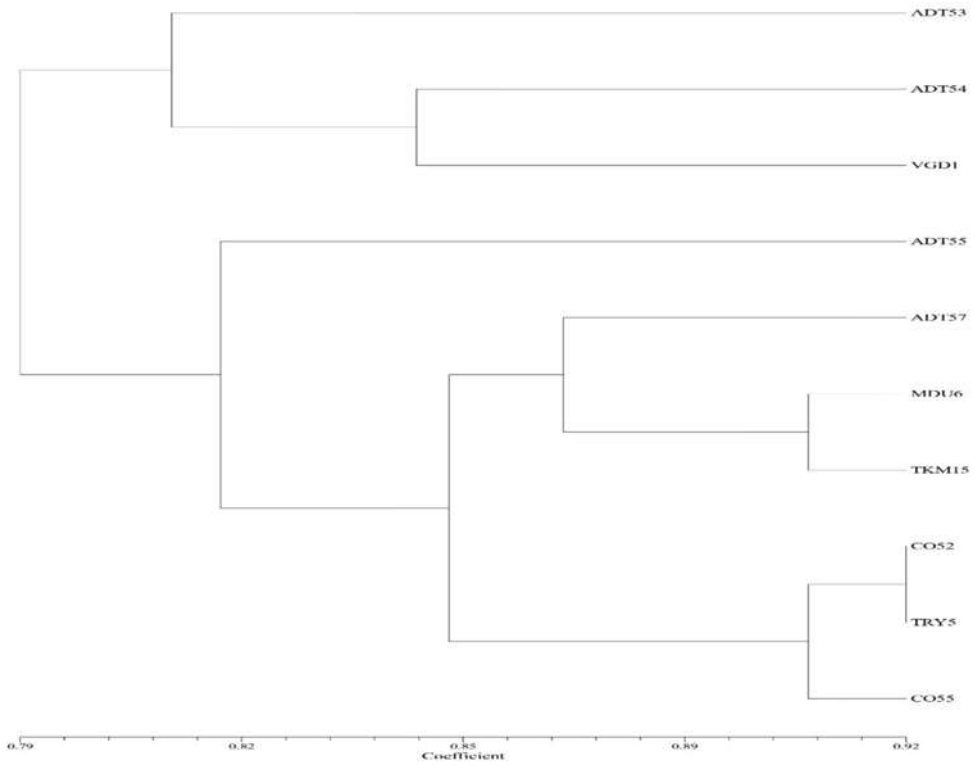

Fig. 3. Dendrogram depicting the classification of ten rice varieties formed through UPGMA method based on molecular marker