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Characterization of Selected Zambian Rice (*Oryza sativa*) Accessions using Simple Sequence Repeat (SSR) Markers

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ABSTRACT

Background: Rice (*Oryza sativa* L.) is grown in various parts of Zambia. Response of available varieties towards biotic (bacterial and fungal pathogens) and abiotic factors such as water stress remains unavailable. A systematic rice seed system or breeding program appears to be non-existent. This study was undertaken to assess the genetic diversity among some selected Zambian rice accessions using simple sequence repeat (SSR) molecular markers.

Methods: Thirty accessions were selected for genetic diversity analysis using ten SSR markers (RM5, RM168, RM7, RM13, RM225, RM452, RM211, RM205, RM413 and RM46) selected from various chromosomal loci of the rice genome. Rice genomic DNA was amplified by the polymerase chain reaction and products were analyzed by 1% agarose gel electrophoresis. Most products appeared as single alleles in form of single bands averaging between 79-200 base pairs (bp). Significant relatedness within and among accessions was observed while most of the primers showed distinct polymorphism. Cluster analysis *via* a dendrogram revealed that most of the accessions were closely related and clustered into two major groups designated A and B.

Result: Only one accession (ZM8321) appeared to be distinct and distantly related to all the other 29 accessions. Cluster B contained the rest of the 29 accessions which were subdivided into 8 sub-clusters. Of the 29, accessions ZM8295 from Samfya District and ZM8313 from Kaputa district showed 100% similarity meaning that they are the same accession.

Key words: Genetic diversity, *Oryza sativa,* SSR markers.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the staple crops, globally which accounts for providing 50 to 80% daily calorific need (Aljumaili *et al*., 2018) and is distributed across a variety of agro-climatic zones having developed a wide array of genotypes and phenotypes. As an agricultural commodity, it is the third-highest worldwide in terms of production after maize and sugarcane (FAOSTAT-2012). In Zambia, crops like maize have been extensively studied to create hybrids. However, limited efforts aimed at variety selection take place in crops such as rice, groundnuts, pigeon pea, cowpea and tree and plantation crops (Zambia National Rice Development Strategy Report 2011, Version 1).

The Agronomic importance of varieties of rice depends on factors like efficacious and qualitative yield, resistance to pathogens, pests and unfavourable environmental determinants. Studies based on genetic diversity of rice are of paramount importance in breeding, cultivars rating, identification, conservation, purity and improvement program to ensure germplasm utilization, efficacious breeding of related species (Saini *et al*. 2004) which in turn helps predict possible genetic potentials (Chakravarthi and Naravaneni, 2006). The measure of genetic diversity is based on differences in morphology with identifiable procedural gaps like time, cost and labour-intensive (Thomson *et al.* 2007).

DNA dependent evaluation of genetic diversity is the most reliable approach in terms of repeatability and stability. Molecular Markers (MM) are routinely used in diversity analysis, Marker-Assisted Selection and identification of candidate genes are based on nucleic acid amplification technologies like restriction fragment length polymorphism (RFLP) using restriction enzymes, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) and single nucleotide polymorphisms (SNP) utilize the polymerase chain reaction (PCR) (Jeung *et al.* 2005). SSR markers known as DNA microsatellites are non-coding regions of DNA with one to five repeat nucleotides of AT

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and GA in plants. They are preferred because of properties like abundance, distributed well in genome hyper-allelic, co-dominant, ease of assaying and reproducible (Viera *et al.* 2016). They have been meticulously utilized not only for the assessment of genetic diversity of both cultivated and wild species of rice but also genetic polymorphism and differentiation among genotypes (Dhama *et al.*, 2018). The use of SSR markers has been explored in *Cucumis melo* L (Palomares-Rius *et al.* 2011). Singh*et al.* 2010 used a set of validated SSR molecular markers in rice parental lines and demonstrated their suitability for Qualitative Tract Locus (QTL) mapping and fingerprinting studies in the crop. Therefore, genetic diversity analysis of the Zambian rice will help measure the extent of genotypic differences, genetic relationships and assist in broadening the germplasm base of future aromatic rice breeding programs.

MATERIALS AND METHODS

The study was divided into the following phases:

Phase I: Experimental location

The experiments were conducted at the Zambia Agricultural Research Institute (ZARI), Mount Makulu Research Station in Chilanga, Lusaka Province that lies on latitude 15.547745 and longitude 28.249392.

Phase II: Processing of plant materials

Thirty rice accessions were collected from the major rice-growing regions in Zambia and curated at the National Plant Genetic Resource Centre (National Gene Bank) at ZARI in Chilanga. Fig 1 elucidates the processing of rice seedlings. Table 1 elucidates details and passport information of the accessions.

Phase III: Genomic DNA extraction

All chemicals used for DNA extraction and nucleic acid amplification were of analytical grade and procured from HiMedia Laboratories, Mumbai-India. Genomic DNA was isolated from young leaves of 10-day old plants following the mini-modified DNA extraction method (Zheng *et al.* 1995), (Fig 2).

Phase IV: Polymerase Chain Reaction (PCR)

PCR amplification was carried out using 10 SSR markers selected from 7 of the 12 chromosomes listed in Table 1. Lyophilized primers were reconstituted in nuclease-free water to a stock solution of 100mM from which working solutions of 50ng/il were made. Template DNA extracted

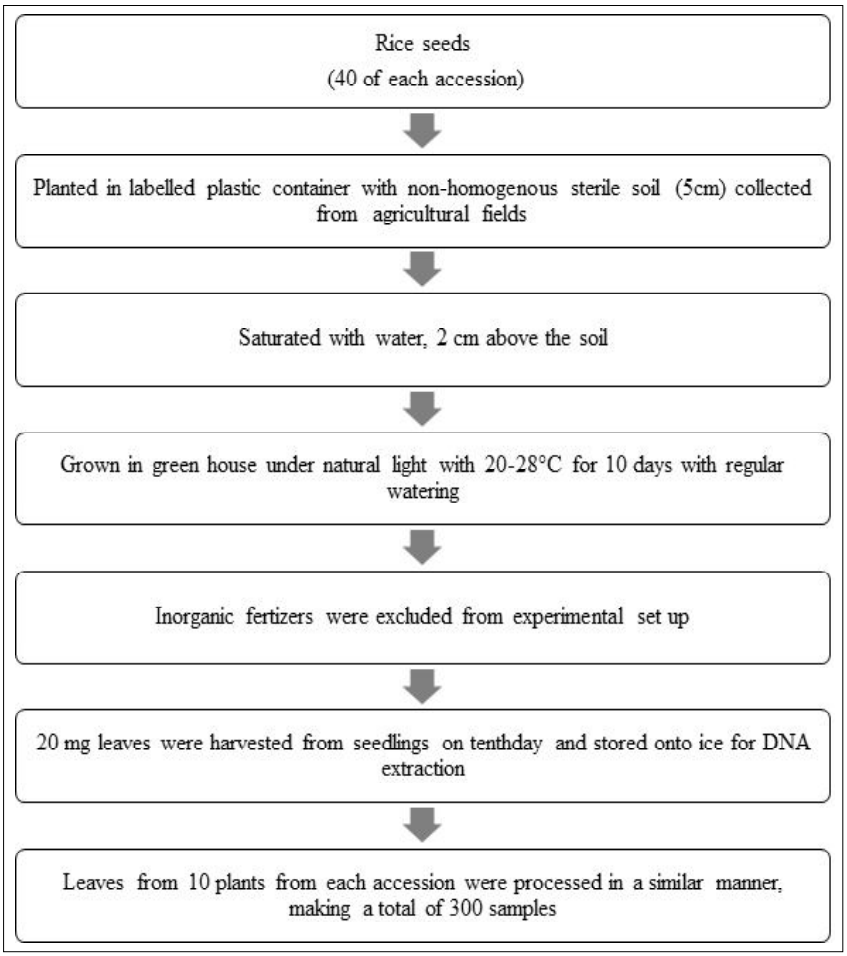

Fig 1: Processing of plant material.

from 10 independent plants from each of the 30 accessions was amplified using the selected SSR primers.

The PCR reaction mix was prepared by mixing 2 µl of template DNA, 2 µl 10x PCR buffer with $MgCl_2$ (25 mm), 1 µl dNTP mix (10 mM), 1µl each of forward and reverse primers (50ng), 0.2 µl of *Taq* DNA polymerase and molecular grade nuclease-free water was then added to make a final volume of 21 µl. The PCR reaction was preceded by an initial template denaturation at 94°C for 5 minutes followed by 35 cycles of 1 minute denaturation at 94°C, 1-minute annealing at 55°C, 1 minute extension at 72°C and a final extension at 72°C for 5 minutes.

RESULTS AND DISCUSSION

SSR markers reveal polymorphism in local rice varieties

The size of most intensely amplified fragments was determined by comparing the migration distance of amplified fragments relative to 100 bp molecular weight DNA marker. polymorphic information content (PIC) values were calculated for each SSR locus based on Anderson *et al.*, 1993. The amplified bands were scored for each SSR primer pair based on the presence or absence of bands, generating a binary data matrix of 1, 2 and 0 for each marker system. The matrices were then analyzed using the Genstat statistical package 18th edition. Dendrograms displaying relationships among the 30 rice accessions were constructed using the Genstat statistical package (Fig 4).

PCR fragment analysis reveals distinct product band patterns

The markers were found to be polymorphic among the rice accessions and generated reproducible and informative allelic profiles. Fragment profile as a measure of polymorphism was observed in DNA samples amplified using RM5 and RM 13 for instance. For RM5, a DNA fragment of 110 base pairs was observed in all accessions with examples of ZM84338, ZM8316, ZM8328 and ZM8321 shown in Fig 3a. In the case of ZM8338 DNA extracted from seven independent plants amplified using RM5 showed variation with reference to PCR products. Three of these plants (36, 37 and 40) had amplification products whereas four (34, 35, 38 and 39) showed no amplification products. In the case of ZM8316 DNA from 10 independent plant samples was amplified to produce the expected fragment of 110 base pairs. In the case of ZM8328, the RM5 marker

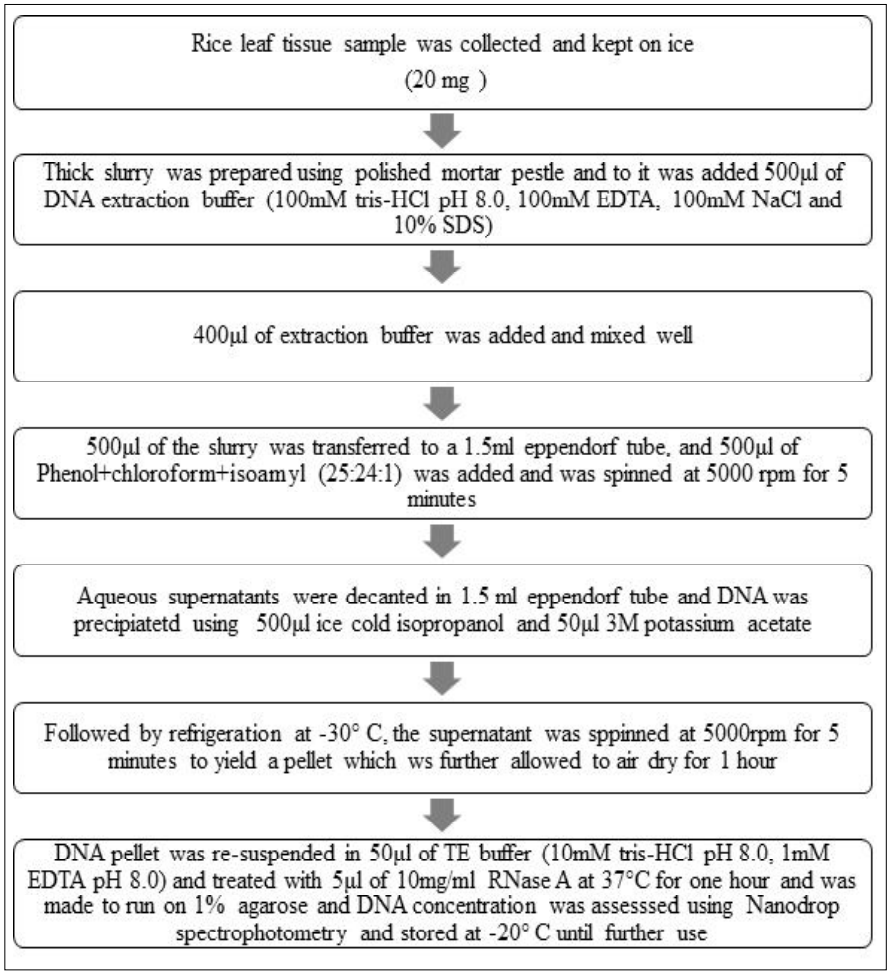

Fig 2: Genomic DNA Extraction.

was able to amplify all the ten samples except for two (51 and 57), Fig 3a.

DNA from 10 independent plants of accessions ZM8338, ZM8316, ZM8328 and ZM8321 were also amplified as the case was for the rest of the samples in the study. Fig 4 shows that there was an amplification of template DNA from five of the samples (33, 34, 37, 38 and 39) while no amplification products were observed in three samples (35, 36 and 40). Molecular marker RM225 showed diversity in terms of DNA amplification profiles in fragment numbers and sizes as shown in Fig 3c. For instance, nine DNA samples from ZM8338 amplified with RM225 showed amplification of three samples (43, 47 and 49) giving a fragment size of 140 base pairs while the rest (42, 44, 45, 46, 48 and 50) showed no amplification products. In the case of ZM8316, only one sample (54) showed an amplification product with two fragments of 500 bp and 140 base pairs respectively. Similar results were obtained in the use of RM225 on 10 DNA samples of ZM8328 except sample 69, Fig 3c.

Among the 10 markers, only RM 2 generated 2-3 alleles, an accession was assigned a null allele for a microsatellite locus whenever an amplification product could not be detected for a particular genotype-marker combination. The amplicon size of all 30 genotypes for each marker alleles ranged between 79-200bp.In all the reactions, a 110 bp band was obtained except in lanes 38 and 58 where no band was observed. DNA band intensity also differed with some reactions showing higher band intensity than others as the case was for lanes 39 and 46 as shown in Fig 3a. Fig 3a, 3b and 3c elucidate gel pictures of the amplified fragments using primers designed for the SSR marker RM 5, RM 13 and RM225.

Clustering of rice accessions

Cluster analysis was done to group the accessions by constructing a dendrogram illustrated in Fig 4 based on allelic information gathered from the genotype-marker interaction. The Genetic Similarity (GS) index ranged from a minimum of 80% to a maximum of 100%. The Genstat-dendrogram clustered the 30 genotypes into two major clusters (A and B). Cluster A comprised of only one accession, ZM8321 commonly called Ndelema from Chama district in Muchinga province. Cluster-B consisted of the 29 accessions which were subdivided into 10 sub-clusters that showed more genetic relatedness among the accessions with ZM8295 (Supa-Samfya district; Luapula Province) and ZM8313 (Kaputa District, Northern Province) demonstrating 100% relatedness.

The success of a crop improvement program depends on the magnitude of genetic variability and the extent to which the desirable characters are heritable (Ravi *et al.* 2003). The assessment of genetic diversity has been demonstrated to be essential in establishing relationships among different cultivars (Sivaranjani *et al.* 2010). Molecular characterization could reveal their phylogeny and this information would be quite useful in utilizing the rice germplasm in genetic improvement of the existing rice accessions. This research aimed at characterizing selected rice accessions in Zambia at the genetic level to create baseline information that could be useful in the genetic improvement of the crop. This suggested that the observed absence of amplification products were not polymerase chain reaction artifacts which could be interpreted as failed reactions. On the contrary, it showed that the seed accessions from which the DNA was isolated did not carry the targeted alleles. This also indicated that the observed presence or absence of PCR product bands revealed polymorphism between or even within what are currently considered to be pure accessions. The other possibility was that the PCR products were absent in some of the reactions

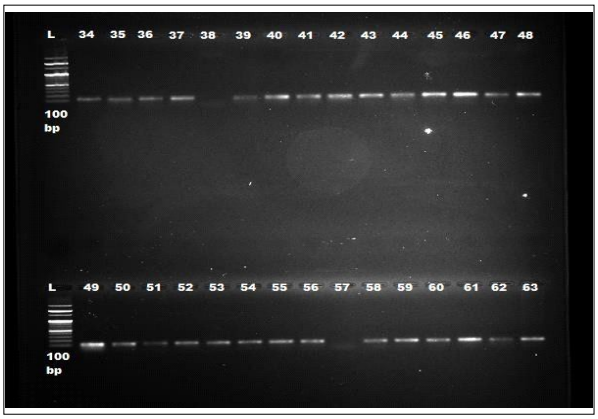

Fig 3a: DNA profile of four accessions with SSR marker RM 5.

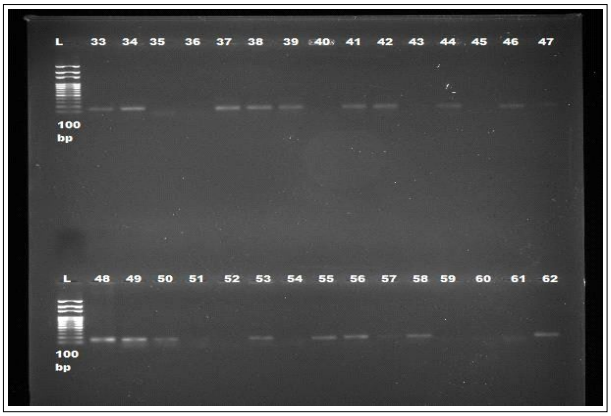

Fig 3b: DNA profile of four accessions with SSR marker RM 13.

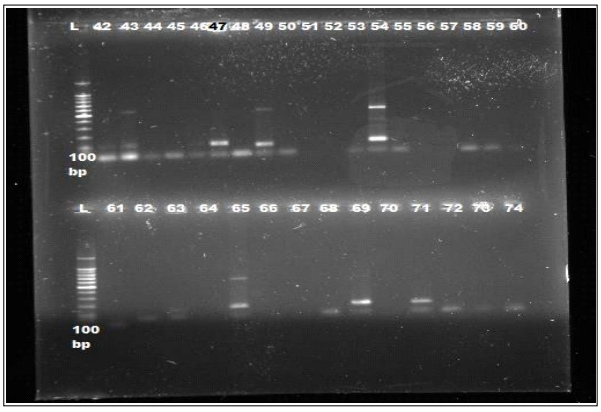

Fig 3c: DNA profile of four accessions with SSR marker RM 225.

due to the absence of the target DNA sequence in those rice accession, thereby contributing to the diversity of the accessions. The amplification reactions were repeated with similar results, ruling out reaction failure as the cause of the absence of band fragments. Matin *et al.* 2012 proposed PIC as an accurate indicator of allele diversity and frequency among accessions. The PIC values for the tested markers in this work ranged between 0.2 and 0.8. The SSR marker RM225 revealed the highest PIC value also backed by results from agarose gel electrophoresis analysis of PCR products obtained with this particular marker, Fig 1c in harmony with Martin *et al.* 2012 findings. The frequencies of null alleles were not included in the calculation of PIC values for each SSR locus. Cluster analysis *via* a dendrogram revealed that most of the accessions were closely related and clustered into two major groups designated A and B. Only one accession (ZM8321) appeared to be distinct and distantly related to all the other 29 accessions. Cluster B contained the rest of the 29 accessions which were subdivided into 8 sub-clusters. Of the 29, accessions ZM8295 from Samfya District and ZM8313 from Kaputa District showed 100% similarity (cluster K) meaning that they are the same accession despite the different names by which they are identified. Furthermore, despite the different names and accession numbers assigned to these two accessions, visual examinations revealed morphological similarities which confirm the genetic information obtained from the study. These were closely related to ZM8346 (Chandege-white and brown- Chama, Muchinga) than the rest of the accessions. MLK020 and MLK003 showed relatedness at almost 99% and are from the same agro-ecological zones but are known by different district's local names. ZM 8338 (Senga from Chama, Muchinga) and ZM 8316 (Malawi faya from Nyimba, Eastern) in cluster E showed 97.5% relatedness, which was a very high level of relatedness despite being known by different names in two closely located provinces as well as an ecological zone in Zambia. Nonetheless, clusters F, G, H, J and K were closely related but quite distant from clusters A, B, C, D and E.

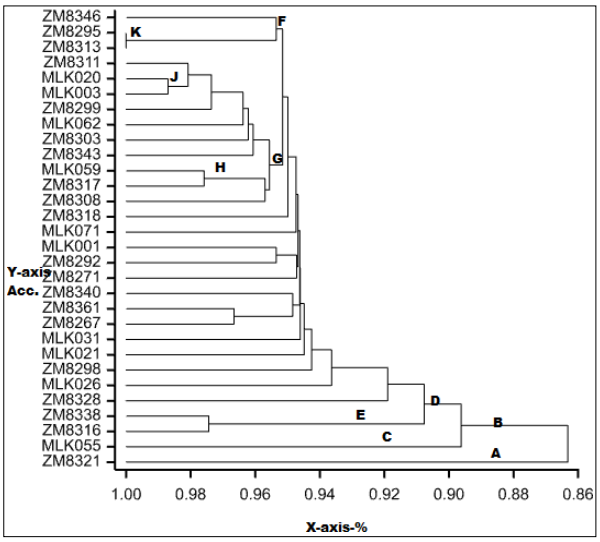

Fig 4: A genstat clustering dendrogram showing the genetic relationships among 30 accessions on the alleles detected by 9 microsatellite markers.

From the clustering, it was observed that a number of accessions were very similar to each other; for example, clusters E, H, J and K; this could mean these accessions paired together are the same despite being known by different local names and coming from distantly spaced growing regions. This information would help in decongesting the gene bank and also reduce the possibility of breeders dealing with the same accessions while thinking they are distantly related. For all the sub-clusters, few accessions where showed genetic differences with a different local name, agro-ecological zone from which it was collected and outstanding morphological distinctiveness. Some few accessions showed the exact opposite. They were observed to be quite distant in terms of relatedness with other accessions despite them being known by the same local name in different ecological zones *e.g.* Supa. This may mean that most of the accessions analyzed in this study were heterogeneous even within those collected from the same agro-ecological zones and believed to be homogeneous and treated as being the same accession. Therefore, it can be said that there is genetic distinctness among accessions selected in this study, but with a higher level of relatedness. It can also be seen that genotypes from far distinct cluster groups when crossed can give rise to a genetically wide range of off spring, giving a diversity of genotypes to select from.

Table 1: Rice SSR markers (primer pairs).

Locus name	Chr. No.	Forward primer	Reverse primer	Size(bp)
RM-5	1	TGC ACC TTC TAG CTG CTC GA	GGG TTC TAG CCT AGC	113
RM225	6	TGC CCA TAT GGT CTG GAT G	CGGA AGG ACT AGG TGA AAG	140
RM-7	3	TTC GCC ATG AGG TCT CTC G	TT GTT GCT TTA CTA CCC TCC	180
RM-168	3	TGC TGC TTG CCT GCT TCC TTT	CGGCA CCT AAC TAA GCA AAG	116
RM-13	5	TCC AAC ATG GCA AGA GAG AG	GAC CTT AGC TTA CGG TGG	141
RM-452	2	CTGATCGAGAGCGTTAAGGG	GTCTT TGC ACC AAA CTA GGG	105
RM-463	12	TTCCCCTCCTTTTATGGTGC	GCGTC ACT GAC TCC TCT TGT	192
RM-413	5	GGCGATTCTTGGATGAAGAG	CTTCT GTT CTA ACC ACC CCT	79
RM-211	2	CCGATCTCATCAACCAACTG	GG AAA CTC TAG GAG CAC TTC	161
RM-11	7	TCTCCTCTTCCCCCGATC	GAT TCG GAG CGG GCG ATA	140

The study also revealed that although two variants could be present in the same cluster and share only about 75% similarity at the genetic level. By convention, it is assumed that the two variants should have been the same and must be genetically similar, but our genetic diversity analysis contradicts this fact. Such a kind of cultivar identity crisis is often witnessed throughout the world (Matin *et al.* 2012). Such discrepancies emphasize the importance of molecular characterization of rice germplasm prior to their deployment in varietal improvement. The results revealed that all the primers showed distinct polymorphisms among the accessions studied, indicating the robust nature of microsatellites in revealing polymorphism. Therefore microsatellite marker-based molecular fingerprinting could serve as a potential basis in the identification of genetically distant genotypes as well as in sorting of duplication for morphologically close accession.

CONCLUSION

The present study revealed a range of genetic variations among the selected rice accessions. The results obtained showed that there was genetic distinctiveness among the selected rice accessions but with a higher degree of relatedness. It can also be concluded that the existing naming system of the accessions does not imply ultimate distinctiveness, neither does the growing region. The results indicated that the SSR molecular markers are neutral and co-dominant and could be a powerful tool to assess the genetic variability of the cultivars. The information about genetic diversity may be very useful for the proper identification and selection of appropriate parents for breeding programs. Morphological and molecular characterization of such cultivars followed by their use in rice varietal improvement could lead to substantial gain.

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Conflict of interest

Authors declare none competing/conflicting interests.

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