Rice Research: Open Access

Chemutai et al., J Rice Res 2016, 4:3
DOI: 10.4172/2375-4338.1000172

Research Article Open Access

Genetic Diversity Studies on Selected Rice (*Oryza sativa* L.) Genotypes based on Gel Consistency and Alkali Digestion

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Received date: May 26, 2016; **Accepted date:** August 01, 2016; **Published date:** August 09, 2016

Abstract

Knowledge of rice genetic diversity is necessary to ascertain the germplasm conservation and the development of improved rice genotypes with good quality traits through various breeding programs. The aim of this study was to determine the genetic diversity based on gel consistency and alkali digestion among selected Kenyan and Tanzanian genotypes using SSR markers. PowerMarker version 3.25, GenAlEx version 6.41 and DARwin 6.0.12 statistical software were used to carry out data analysis. The number of alleles per locus ranged from 2 to 4 with an average of 2.75 across the 8 markers used. Polymorphic information content (PIC) ranged from 0.5224 (RM577) to 0.1411 (RM85) with an average of 0.3673 observed across all the markers. Gene diversity ranged from 0.5764 (RM577) to 0.1528 (RM85) with an average of 0.4181 with one rare allele was detected at RM577 loci. Pairwise genetic dissimilarity matrix ranged from ranged from 0.9333 to 0.1818 with the least genetic distance being observed between *IR 54* and *BS 370* while the highest, 0.9333 being between *Saro 5* and *IR 2793*. The unweighted neighbour joining tree clustered the rice genotypes into three major clusters and subsequent sub clusters hence effectively differentiating the Kenyan and Tanzanian genotypes based on gel consistency and alkali digestion. This clustering was complemented with the findings in the principal coordinate analysis. These results show that determination of genetic diversity using SSR markers cam be successfully achieved. In this study RM577 was the best and most informative SSR marker given it showed the highest PIC, gene diversity and allele per locus. In addition, it's the only marker that showed a rare allele at 400 bp.

Keywords: Rice; Genetic diversity; Gel consistency; Alkali digestion

Introduction

Rice (*Oryza sativa* L.) is a monocotyledonous plant belonging to the grass family (Gramineae) and the genus *Oryza*. Rice is the major staple food for the 57% of the world's population and it provides approximately 23% of daily caloric intake. It was introduced into Kenya in 1907 from Asia and is an important cereal crop after maize and wheat in the country [1,2]. The crop is grown mainly by small scale farmers as food and cash crop with 80% of the crop grown in irrigation schemes and 20% produced under rain-fed conditions. In Kenya, there are 4 irrigation schemes that majorly grow rice and are under the management of the National Irrigation Board (NIB) (3). These paddy schemes are located in Mwea, West Kano, Ahero and Bunyala and the common genotypes that are grown includes *ITA 310, BS 370, IR 2793* and *BW 196*.

In Kenya and Tanzania, landraces and improved rice genotypes are grown with a resultant low yield. The low yield could be attributed to many production constraints including weed infestation such as *Striga* and the production of false finger millet, which lowers the quality and quantity of the produce. Pests such as the African gall midge and stem borer, rice diseases like the rice yellow mottle virus, bacterial leaf blight and the blast fungus affects rice in the irrigation schemes and in the rain fed plantation systems [3,4].

The level of phenotypic and genetic diversity of rice genotypes stored in various rice germplasm banks around the world is high [5]. The quality preferences of rice consumers have resulted in a wide diversity of the rice genotypes specific to different localities. In addition, the socio-cultural traditions have also increased the diversity of rice in terms of morphological and quality traits. These quality traits include grain size, shape and colour, aroma and endosperm properties [6].

Gel consistency (GC) is used in distinguishing cooked rice texture of high amylose genotypes. Genotypes are grouped based on the gel lengths into; hard (<40 mm), medium (41-60 mm), and soft (>61 mm) [7]. The associations of starch polymers determines whether the gels are weak or rigid. The gene coding for gel consistency is located within the *Wx* locus [8,9]. The *Wx* locus is located in chromosome six in the rice genome and encodes *granule-bound starch synthase-I (GBSSI)*. The *Wx* locus, has two variations namely the Wx^a and Wx^b. the Wx^a allele is predominant in *indica* rice while the Wx^b is found in the *japonica* rice. Rice with hard gel consistency hardens faster than genotypes with soft gel consistency. Therefore, consumers tend to prefer genotypes with soft gel consistency. Therefore, rice breeders tend to breed varieties with soft gel consistency [10]. Alkali digestion and gelatinization temperature have a significant correlation. Both traits are controlled by *alk* locus located on chromosome six, which encodes *soluble starch synthase (SSII)*. Alkali digestion is dependent on the nature of the amylopectin molecules [11,12]. There are many SSR markers that are tightly linked to QTLs controlling these traits and are available at a data-based search (http://www.gramene.org/).

The use of DNA markers is widespread among plant geneticists because of the substantial amount of useful information that can be

gathered from these markers. They are popularly known in the determination of the genetic diversity, generating gene map for tagging traits of interest, germplasm conservation and crop improvement [13]. Microsatellites also known as simple sequence repeats (SSR) markers have been applied in various studies to study rice genetic diversity [14-16], marker assisted selection [17,18] and in mapping of the QTLs [19,20]. In Kenyan and Tanzanian rice genotypes SSRs have been used to study diversity studies gelatinization temperature and amylose content [15]. This study was carried out using 12 rice genotypes selected on the basis of high farmer preference both in Kenya and Tanzania with inclusion of Philippines varieties as check varieties based on gel consistency and alkali digestion.

Materials and Methods

Plant material

A total of 500 g of the rice grains of twelve selected Kenyan and Tanzanian rice genotypes were collected from two repositories; Mwea Irrigation and Agricultural Development (MIAD) in Mwea, Kirinyaga county, Kenya on latitude -0.7°S, and longitude 37.37°E [21] and Kilimanjaro Agricultural Training Centre (KATC) in Moshi, Tanzania on latitude 3°27'7"S and longitude 37°23'49"E [22]. The rice grains were brought to Kenyatta University Plant Transformation Laboratory for molecular analysis. Fifteen rice grains were sowed in pots containing soil and watered daily until when the seedlings were 21 days where leaves were harvested for genetic diversity studies. *IR 64* was selected as a model genotype due to desirable combination of attributes such as the intermediate alkali digestion values, amylose content, and soft gel consistency [10]. The attributes and source of the genotypes are outlined in Table 1.

Genotype	Source	Attribute
IR 2793	Kenya	Improved genotype
BS 217	Kenya	Improved genotype
BS 370	Kenya	Improved genotype
BW 196	Kenya	Improved genotype
ITA 310	Kenya	Improved genotype
Red Afaa	Tanzania	Landrace genotype
IR 54	Philippines	Improved genotype
Kilombero	Tanzania	Landrace genotype
IR 64	Philippines	Improved genotype
Kahogo	Tanzania	Landrace genotype
Saro 5	Tanzania	Improved genotype
Wahiwahi	Tanzania	Landrace genotype

Table 1: Rice genotypes, origin and attributes of rice genotypes used in the study.

Marker	Chr*	Product Size(bp)	T* (ºC)	Repeat motif	Forward primer	Reverse primer	Locus
RM50	6	201	55	(CTAT)4(CT)15	ACTGTACCGGTCGAAGACG	AAATTCCACGTCAGCCTCC	*Wx*
RM501	7	179	55	(TC)10(TA)21	GCCCAATTAATGTACAGGCG	ATATCGTTTAGCCGTGCTGC	*Wx*
RM539	6	272	55	(TAT)21	GAGCGTCCTTGTTAAAACCG	AGTAGGGTATCACGCATCCG	*Alk*
RM577	1	193	55	(TA)9 (CA)8	GCTTTCCCTCTAACCCCTCT	GGATGTACCGCTGACATGAA	*Wx*
RM85	3	107	55	(TGG)5(TCT)12	CCAAAGATGAAACCTGGATTG	GCACAAGGTGAGCAGTCC	*Alk*
RM333	10	191	55	(TAT)19(CTT)19	GTACGACTACGAGTGTCACCAA	GTCTTCGCGATCACTCGC	*Alk*
RM314	6	118	55	(GT)8(CG)3(GT)5	CTAGCAGGAACTCCTTTCAGG	AACATTCCACACACACACGC	*Wx*
RM347	3	207	55	(GGC)5(AT)7	CACCTCAAACTTTTAACCGCAC	TCCGGCAAGGGATACGGCGG	*Alk*

Table 2: Marker name, chromosomal location, product size, annealing temperature, repeat motif and the sequence of the SSR markers used in the study.

Genomic DNA extraction and microsatellite analysis

Genomic DNA was extracted from leaf samples using modified cetyltrimethyl ammonium bromide (CTAB) extraction protocol method [23]. Per variety DNA was extracted from 4 seedlings and DNA pooled after the quality was determined to be of good quality. DNA quality was determined by loading 5 µl of each DNA sample in addition to 3 µl of loading solution and 2 microliter (µl) of SYBR Green I in 1% agarose. The gel was run at a constant voltage of 100 volts and allowed to run until the bromophenol blue had migrated and almost at the end of the gel. Details of the markers used in this study are listed in Table 2.

The PCR reactions were was carried out in 200 µl thin-walled PCR tubes in a thermal cycler (Applied Biosystem® 2720). A total of 35 PCR cycles were done where each reaction mixture totalled up to 25 µl which contained 5 µl of genomic DNA, 0.5 µl of each of the two primers (at a concentration of 10 pmole/µl), 12.5 µl of each of the 1X master mix which composed of 22 mMKCl; 20 mMTris-HCl (pH 8.9 @ 25°C);1.8 mM $MgCl_2$; 22 mM NH_4Cl; 0.05% Tween® 20; 5% glycerol; 0.06% IGEPAL® CA 360; 0.2 mM of each dNTPs; and 25 units/ml of one Taq DNA Polymerase and 6.5 µl of molecular water. The PCR conditions comprised of initial denaturation at 95oC for 4 minutes, 35 cycles at 94oC for 30 seconds; annealing temperature of 55-60oC (depending on the primer used) for 1 minute, extension at 68°C for 60s; and final extension at 68°C for 7 minutes. To 5 µl of each PCR product, 2 µl of SYBR Green I was added and run on 2% agarose gel alongside 3 µl 100 bp DNA ladder (Thermo Scientific™) at 80 volts for 1 hour placed under a UV trans-illuminator and photographed.

Data analysis

PowerMarker version 3.25 statistical software [24] was used to determine four statistical parameters; allele number, gene diversity and the polymorphic information content (PIC). Clearly resolved bands of PCR products were scored manually using presence or absence-coding index where 0 represented absence while 1 represented the presence of a band. Resultant scoring matrix was used to generate the genetic dissimilarity matrix based on the Jaccard's coefficient with the inclusion of 1000 bootstrap values and unweighted neighbour joining tree drawn using DARwin 6.0.12 statistical software. GenAlEx version 6.0 [25] statistical package was used to visualize the relationships between the studied genotypes in a principal coordinate analysis (PCoA). Analysis of molecular variance (AMOVA) was done to determine within and among the population variations with significance of variance tested with 1000 permutations using GenAlEx version 6.0.

Results

Assessment of SSR profiles

A total of 22 alleles were detected at the loci of the 8 SSR markers across the 12 genotypes. The number of alleles per locus ranged from 2 in RM 341, RM 539 and RM 85 to 4 in RM 577, with an average of 2.750. The highest number of allele per locus was observed in marker RM 577 while the least number of polymorphic alleles was observed in RM 314, RM85 and RM 539. The highest gene diversity was observed in RM577 at 0.5764, while RM85 had the lowest gene diversity of 0.1528 with an average of 0.4181 observed among all the microsatellite markers used. Polymorphic information content (PIC) showed an average of 0.3673 with RM577 showing the highest PIC value (0.5224) and RM85 showing the lowest values of 0.1411 (Table 3). An allele that is observed in less than 5% of the genotypes under study is considered as a rare allele. This was observed in Marker RM 577 loci at 400 bp. A total of sixty six null alleles were detected where the loci harbouring the highest number of the null alleles were found in RM501 (null alleles at 18 loci) while the lowest was observed in RM 577 and RM 333 (null alleles at 3 loci) (Figure 1).

Genetic dissimilarity

A dissimilarity matrix based on the Jaccard's dissimilarity index was used to determine the levels of relatedness of the 12 rice genotypes based on gel consistency and alkali digestion. The genetic dissimilarity ranged from 0.9333 to 0.1818. *Saro 5* and *IR 2793* had the highest genetic distance of 0.9333 while *IR 54* and *BS 370* had the least genetic distance of 0.1818. Rice genotypes *Wahiwahi*, *Red Afaa* and *Kahogo,* which are all landraces from Tanzania, had same dissimilarity value of 0.5000 with *BW 196* a Kenyan improved genotype. Details on genetic dissimilarity are shown in Table 4.

Marker	Major allele frequency	Allele number	Gene diversity	PIC
RM333	0.6667	3	0.4861	0.4235
RM314	0.75	2	0.3194	0.2545
RM577	0.5417	4	0.5764	0.5224
RM501	0.6111	3	0.5093	0.4552
RM50	0.5833	3	0.537	0.4694
RM539	0.8333	2	0.2639	0.2229
RM347	0.6667	3	0.5	0.4491
Mean	0.6962	2.75	0.4181	0.3673

Table 3: Genetic diversity profiles of 8 SSR markers used in the study.

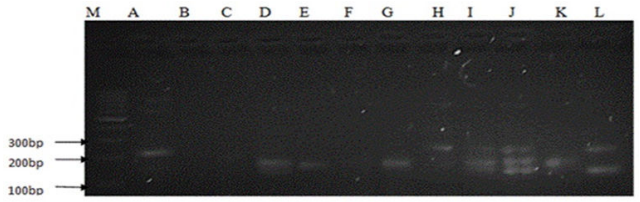

Figure 1: SSR marker RM 501 showing polymorphism pattern of the 12 rice genotypes in 2% agarose gel. The letters above represents; A - *IR 2793*, B - *BS 217*, C - *BS 370*, D - *BW 196*, E - *ITA 310*, F - *Saro 5*, G - *IR 64*, H - *Kilombero*, I - *Red Afaa*, J - *Kahogo*, K -*IR 54*, L – *Wahiwahi*. M- 100 bp molecular ladder.

Clustering of rice genotypes

Unweighted neighbor joining tree based on Jaccard's dissimilarity matrix with 1000 bootstraps was constructed using DARwin 6.0.12 statistical software. Cluster I consisted of all the landrace rice genotypes from Tanzania including *Kahogo*, *Red Afaa*, *Kilombero* and *Wahiwahi*. Major clusters II and III composed of improved genotypes from Kenya, Tanzania and genotypes from Philippines. Cluster II consisted of improved genotypes from Kenya and Philippines. Sub-cluster IIA consist of model rice genotype, *IR 64* and *Saro 5* and *ITA 310*. Sub cluster IIB contained *IR 54*, and two Kenyan basmati genotypes *BS 217* and *BS 370*. Cluster III consisted of only Kenyan improved genotypes *BW 196* and *IR 2793* with no further sub-clusters. Bootstrap values showed the confidence limits of the phylogenetic branches, where the sub cluster IIB and sub cluster IIA1 had the highest bootstrap value at 56%. The lowest bootstrap value of 19% was attained in major cluster II Details on the clustering of the 12 rice genotypes are outline in Figure 2.

Genotypes	IR 2793	BS 217	BS 370	BW 196	ITA 310	IR 64	IR 54	Saro 5	Kilombero	Red Afaa	Kahogo
BS 217	0.7273										
BS 370	0.6923	0.3333									
BW 196	0.5385	0.5833	0.4615								
ITA 310	0.6364	0.7	0.5455	0.6154							
IR 64	0.6364	0.8182	0.6667	0.875	0.6						
IR 54	0.7333	0.4545	0.1818	0.5333	0.6154	0.7143					
Saro 5	0.9333	0.8333	0.5833	0.8125	0.75	0.6364	0.6429				
Kilombero	0.5385	0.5833	0.5714	0.5333	0.5	0.6154	0.5333	0.8824			
Red Afaa	0.6	0.7333	0.625	0.5	0.5714	0.6667	0.5882	0.8333	0.2857		
Kahogo	0.6	0.7333	0.625	0.5	0.4615	0.6667	0.5	0.8333	0.2857	0.2667	
Wahiwahi	0.6154	0.6667	0.6429	0.5	0.8667	0.7857	0.5	0.875	0.5	0.4667	0.4667

Table 4: Jaccard's coefficient genetic dissimilarity matrix of the 12 genotypes.

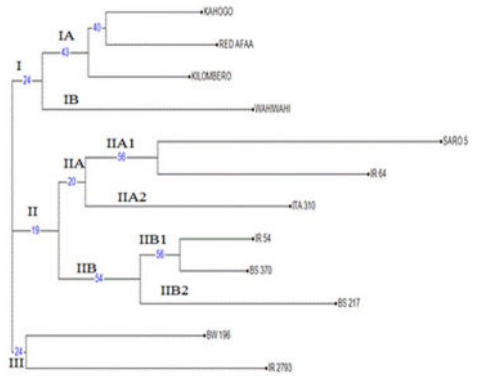

Figure 2: Unweighted neighbour joining dendrogram of the 12 rice genotypes based on gel consistency and alkali digestion using 8 SSR markers.

Analysis of molecular variance (AMOVA)

Analysis of molecular variance (AMOVA) determines distributions of variations within and among populations. From the total variation, 96% (P<0.001) of variation was distributed within population while 4% (P<0.001) was distributed among populations. Determination of variations between the Tanzanian and Kenyan rice genotypes revealed 89% and 11% of variation within and among populations respectively. Variation between Tanzania and Philippines and between Kenya and Philippines had a 100% (P<0.001) variation within population (Table 5).

Principal coordinate analysis (PCoA)

Principal coordinate analysis (PCoA) was done to visualize the genetic dissimilarities between genotypes. The PCoA supports the genetic relationships observed in the dendrogram. The two dimensional plot shows that the first principal coordinate accounts for 29.43% of the total variation while the second coordinate accounts for 19.84% of the total variation. The first quadrant comprised *Kahogo, Kilombero*, *Red Afaa* and *IR 2793*. The second quadrant consisted of *IR 64, ITA 310* and *Saro 5* while the third quadrant consisted of genotypes *BS 217*, *BS 370* and *IR 54* while the fourth quadrant consisted of *BW 196* and *Wahiwahi* (Figure 3).

Source of variation	Df	SS	MSD	Estimated Variation	% variation	P-Value
Among Populations	2	9.667	4.833	0.193	4%	
Within Populations	9	37	4.111	4.111	96%	
Total	11	46.667		4.304	100%	<0.001

Table 5: Summary of analysis of molecular variance (AMOVA).

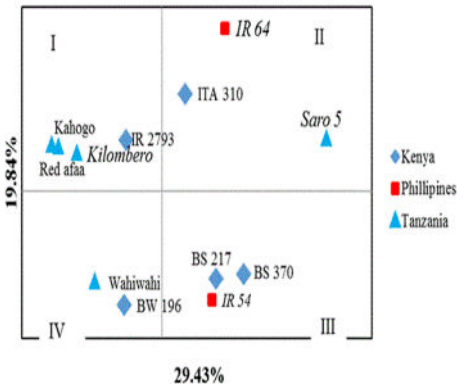

Figure 3: Principal coordinates analysis of the 12 rice genotypes.

Discussion

Knowledge of the genetic diversity in a plant's genotype is necessary to ascertain the germplasm conservation and management of genetic material achieved in a breeding program [26]. Assessment of the genetic diversity showed a significant level of genetic diversity. The number of alleles per locus detected varied per the marker used. This could be attributed to heterozygosity in the varieties studied. Similar findings have been reported in Pakistan basmati and non-basmati genotypes [27]. However, these results were less than what is reported by other studies using different genotypes and SSR markers [28,29]. This could be as a result of use of different rice germplasm accessions and the selection of SSR markers.

Gene diversity also referred to as the expected heterozygosity (He) attained had an average of 0.4181. This value was higher compared to 0.358 reported by Chen et al. [30] and lower than an average of 0.5513 reported in Kenyan and Tanzanian genotypes [16] and 0.68 in Asian rice accessions [31]. Evaluation of gene diversity based on the attributes of the genotypes, showed that improved genotypes had higher gene diversity value of 0.3729 compared 0.3167 of the landrace genotypes. Therefore, the low gene diversity is attributed to presence of a common gene pool amongst the landrace varieties unlike the improved genotypes where new genes have been introduced during crop improvement programs. The difference in gene diversity attained in this study with those in published literature could be attributed to the use different set of primers and rice genotypes under study.

The levels of polymorphism are determined using PIC values which reveals the allele diversity and it varies from one locus to another. The PIC values ranges from 0 to 1 where values that are greater than 0.5 are highly informative. The PIC values ranging from 0.25 to 0.5 shows a reasonably informative marker while those with values less than 0.25 are slightly informative [32]. Based on this classification, one marker (RM 577) was the most informative marker while 5 markers were reasonably informative markers. These informative markers can be utilised in marker assisted selection due to its ability to distinguish between genotypes. The mean PIC was less than 0.4680 observed by Nachimuthu VV, et al. [33] and 0.31 in Indian basmati and non-basmati rice genotypes [34].

The null alleles observed means that there exists a mutant at the loci which lacked normal gene function also it could be as a result of mutations in the binding regions of the markers hence inhibiting the primer annealing [35]. Similar null allele occurrence was reported by [36] in Indian rice genotypes. In general, the markers indicating a high number of alleles per locus also detected rare alleles. The many rare alleles observed in a germplasm indicates that there is a unique source of genetic diversity amongst the genotypes. Presence of rare alleles are important in fingerprinting rice genotypes [36].

The highest dissimilarity observed between *IR 2793* and *Saro 5* could imply that these genotypes have an uncommon origin. The wide variation could be due to genetic divergence that these rice genotypes have undergone during crop improvement strategies over time. Shortest genetic dissimilarity was observed between improved Kenyan genotype *BS 370* and *IR 54* a Philippine genotype. This could be attributed to presence of a common ancestry. Approximately 35 rice genotypes that are released by IRRI and grown in tropical Asia have a common genotype or wild species as an ancestor [37]. In addition, these two genotypes have been used as donor germplasm lines to generate more than 10 improved genotypes [38]. The dissimilarity matrix in this study was higher than 0.036 to 0.684 reported among basmati and non-basmati accessions [27].

The unweighted neighbour joining dendrogram was used in this study as it produces additive trees and does not assume identical evolutionary rates along its branches. Cluster I had all the landrace genotypes from Tanzania, which indicate that these rice genotypes have the same ancestral origin. They therefore portray similar characteristics in terms of gel consistency and alkali digestion. Aromatic genotypes *BS 370* and *BS 217* were categorized into sub cluster IIA while *IR 64*, a non-aromatic variety, was in sub cluster IIB. These finding is similar to findings that highlighted that the use of SSR markers can differentiate between the aromatic and the non-aromatic genotypes [27].

In sub cluster IIA, *Saro 5* had the longest branch length signifying that this genotype has undergone much evolutionary change compared to the other genotypes in this cluster. The *IR 64* genotype is a sister taxa to *Saro 5* an observation that is similar to the dissimilarity index obtained. This implies of a common evolutionary change and ancestry among these genotypes. Lower limits of at least 50% were considered statistically significant. Similar conclusions on bootstrap values have been made by Pervaiz et al. [14] on Pakistani rice landraces using microsatellite markers. The low bootstrap values attained in some branches could be attributed to the low redundancy in genetic dissimilarity dataset which is indicative of considerable variation within the genotypes [39]. Hence, in order to understand the level of genetic variation contributing to the low bootstrap values, AMOVA analysis using the Euclidean distance matrix [40] was conducted within and among populations.

Based on these percentage variations it is evident that diversity among the Kenyan, Tanzanian and Philippine genotypes is low, which could be due to factors such as common ancestry and interbreeding of rice genotypes during crop improvement. Within variation could be due to mutations that altered the repeat sequence of each SSR. Natural selection could be a mechanism contributing to the high within variation observed. The high within population variation in AMOVA table is confirmed by the large values that are shown in the dissimilarity matrix and also the low bootstrap values that were obtained for each of the cluster nodes [41]. The PCoA helps in visualizing the dissimilarity index among genotypes. Genotypes grouped together in a quadrant are interpreted as being closely related. Based on the obtained coordinates, coordinate 1 had the greatest variability while the second coordinate had the next greatest variability. Therefore, the genotypes that are located closer to one another have more similarity than those that are coordinated further away.

Conclusion

This study presents an overview of genetic diversity of selected Kenyan and Tanzanian rice genotypes based on alkali digestion and gel consistency. Molecular diversity shows that improved varieties had higher gene diversity compared to landrace genotypes. The PIC values obtained reveals that RM577 is the best marker for diversity studies on gel consistency and alkali digestion given it is highly informative compared to other markers. Kenyan rice genotypes have a higher similarity compared to Tanzanian genotypes that have a higher level of dissimilarity within its population.

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