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

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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 273. 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 can 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, Athi 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* and *Wx*. The *Wx* allele is predominant in *indica* rice while the *Wx* 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.

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

| 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 |

Genetic diversity studies

IR 54 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.

Table 2: Marker name, chromosomal location, product size, annealing temperature, repeat motif and the sequence of the SSR markers 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 | ACTGTACCGGTGAAAGACG | AAATTCCAGTCAAGCC TCC | Wx |
| RM501  | 7    | 179              | 55      | (TC)10(TA)21 | GCCCAATTAATGTACAG GCG | ATATCGTTTAGCCGTG CTGC | Wx |
| RM539  | 6    | 272              | 55      | (TAT)21      | GAGGTTCTTGTATTTAAA CCG | AGTAGGGATACAGCC TCCG | Alk |
| RM577  | 1    | 193              | 55      | (TA)9(CA)8   | GCTTTCCCTCTAACCCTC TCT | GGATGTACCGCTGACA TGAAC | Wx |
| RM85   | 3    | 107              | 55      | (TG)5(TCT)12 | CCAAAAGTAAACCTGG ATTG | GCACAAGGTAGACG CATCG | Alk |
| RM333  | 10   | 191              | 55      | (TAT)19(CTT)19 | GTACGACTACGAGTGC ACCAA | GTCTTGGCAGATCAC TGC | Alk |
| RM314  | 6    | 118              | 55      | (GT)8(CG)3(GT)5 | CTACGAGAATCTCTTT CAGG | AAGCATCCACACAC AC CGG | Wx |
| RM347  | 3    | 207              | 55      | (GCC)5(AT)7 | CACCTGAAACTTATTC ACGC | TCCGGCAAGGATAC GCCG | Alk |

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 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 consisted of 22 mM MgCl₂, 20 mMTris-HCl (pH 8.9 @ 25°C); 1.8 mM MgCl₂; 22 mM NH₄Cl; 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 95°C for 4 minutes, 35 cycles at 94°C for 30 seconds; annealing temperature of 55-60°C (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, RM 85 and RM 539. The highest gene diversity was observed in RM577 at 0.5764, while RM85 had the lowest gene diversity of 0.2229. Saro 5 and IR 2793 had the highest gene diversity of 0.5224, which is observed in less than 5% of the genotypes under study is considered a rare allele.

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

| 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 |

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 IIIB contained IR 54, B - BW 196, E - ITA 310. Sub cluster IIIB 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 IIIB and sub cluster IIIB consisted of improved genotypes with 1000 permutations with 56% 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 | 0.6154  | 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.

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).

Table 5: Summary of analysis of molecular variance (AMOVA).
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 Pakistani 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 variation could be due to genetic divergence that these rice genotypes have undergone 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 taxon 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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