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Original Article

Genetic diversity and identification of duplicates in selected Tanzanian farmer-preferred cassava landraces using simple sequence repeat (SSR) markers

Gladness Elibariki[1, 3*], Moses Njahira[2], Bramwel Wanjala[2], Kenneth Hosea[1] and Joseph Ndunguru[3]

[1]Department of Molecular Biology and Biotechnology, University of Dar es Salaam, P.O. Box 35179, Dar es Salaam, Tanzania;

[2]Biosciences eastern and central Africa-International Livestock Research Institute Hub (BecA-ILRI Hub), P.O. Box 30709, Nairobi, Kenya;

[3]Mikocheni Agricultural Research Institute, Biotechnology Section; P.O. Box 6226, Dar es Salaam, Tanzania

***Corresponding author**: E-mail: gladye2000@yahoo.com; Tel: +255-754841816;

Fax: +255 22 2410480.

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Abstract

Genetic variation at twenty simple sequence repeat (SSR) markers loci was used to evaluate 21 Tanzanian farmer-preferred cassava landraces collected and maintained at Mikocheni Agricultural Research Institute laboratory in Tanzania. Two West African cassava genotypes and one Kenyan cassava were included in the clustering analysis. Genotyping with twenty high polymorphic SSR markers grouped the genotypes into three clusters derived from Neighbour joining analysis. Clustering analysis was well supported by principle component analysis (PCA). The first three axes of PCA with positive Eigen values accounted for 22.76, 15.93 and 8.48 % of the total variations respectively. No genotype duplicates were detected among the cassava landraces. Average gene diversity among the Tanzanian cassava was high (0.71) with an average heterozygozity of 0.46. Total number of alleles across all loci was 127 with mean number of alleles per locus being 6.35 and SSR markers had a mean polymorphic information content of 0.67. The results obtained in this study facilitated on selection of genotypes to include in the ongoing cassava genetic improvement programs in Tanzania and germplasm conservation.

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Key Words: Cassava landrace, Farmer-preferred, Genetic diversity, germplasm collection, Tanzania

1. Introduction

Cassava (*Manihot esculenta* Crantz) is a well known root crop cultivated widely in many parts of sub-Saharan Africa as a source of food for almost 700 million people in the tropics [1] . According to FAO [2] Tanzania is the sixth producer of cassava in Africa with production of 4,646,520 tonnes and production area of 739,794 ha. In Tanzania, cassava serves as a staple and cash crop for low income generating families who sell cassava fresh tubers in the local markets.

Although several reports on genetic diversity of cassava landraces from East and West African countries exists [3,4,5] there is relatively much less information from Tanzania. Fregene and co-workers [6] included a few Tanzanian accessions collected from the Southern part in a genetic diversity study and differentiation of some African accessions. Besides, [7] assessed the genetic diversity of 24 arbitrary selected local Tanzanian cassava varieties using Randomly Amplified Polymorphic DNA. Otherwise inadequate information is available on the genetic diversity and relationships among Tanzanian farmer-preferred cassava landraces. Latin America is known to have the greatest genetic diversity of cassava since it is the centre of origin of this crop but a significant diversification has also taken place within Africa [6]. Genetic diversity can arise from the out crossing nature of cassava which produces volunteer seedlings which may adjust the diversity when farmer's select these seedlings for subsequent planting [8,9]. Elias and co-workers [8] reported that traditional farming of growing more than one cassava variety in the same field can endorse gene flow through hybridization and thus can alter the genetic diversity. This practice is also very common in Tanzania where farmers may grow even more than two cassava varieties in the same filed. Furthermore, conservative farmers never throw away any variety instead they keep even low yielding ones, a practice that maintains diversification. All these practices are considered as sources of cassava diversification in Africa.

Currently, SSR genetic markers are reported as good tools for genetic diversity studies among and between cassava

populations [10,11,5]. These markers are preferred for their multi-allelic nature, high polymorphism, co-dominance and reproducibility [12]. Moreover, SSRs are widely distributed across the cassava genome and thus can provide rich genetic information [10,11].

Tanzanian farmers have their own preferred cassava landraces that are well adapted to their local environmental conditions and have developed naturally through traditional breeding. Some characteristics of these cassava landraces that are preferred to farmers include early maturity, high yield, good taste, resistance to diseases and good ground storability. Farmers recognize those cassava landraces using morphological characters and each community has its own local name (s) for each. The genetic diversity and relationships of these Tanzanian farmer-preferred cassava landraces are not well documented. Furthermore, while germplasm collection and maintenance is very expensive, a small cassava germplasm collection at MARI has never been evaluated for genotype duplicates. This study intended to determine genetic diversity of selected farmer-preferred cassava landraces for proper management of cassava genetic resources while also identifying any duplication of genotypes in the germplasm collection.

2. Materials and Methods

2.1 Plant materials

Twenty one cassava landraces used in this study were collected from farmers in different ecological areas representing the cassava major growing areas in Tanzania (Table 1 and Fig. 1). Farmers provided the names, origin and agronomic traits (if known) of each landrace during sampling. Cuttings of about 25-30 cm were obtained from mature plants and brought to a screen house at Mikocheni Agricultural Research Institute (MARI), Tanzania. The cuttings were planted in seedling pots and watered for 2 to 3 weeks for sprouting. The apical meristems were used for micro propagation on MS media [13] and leaves of plantlets obtained were used to obtain the DNA used this study. *In vitro* cultures of one West African cassava variety (TME7) and a model cultivar (TMS60444) obtained from MARI laboratory were included in this genotyping.

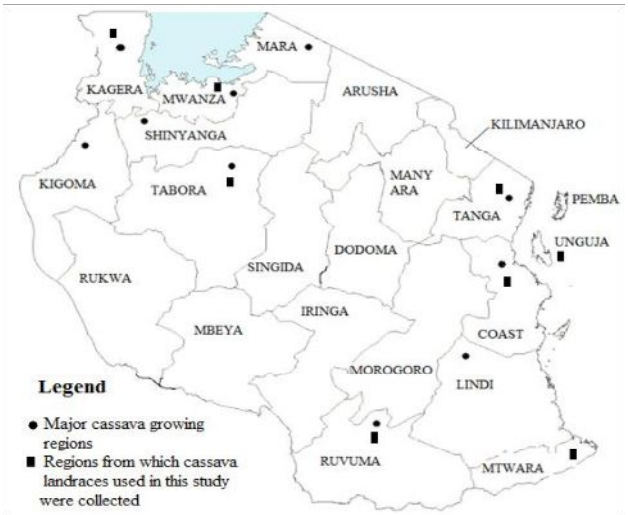


Figure 1 Map of Tanzania showing major cassava growing regions from which cassava landraces used in this study were collected

Table 1 Names and place of collection of all cassava samples used in this genetic diversity study

Name of landrace	Abbreviation	Region of collection	Part of a country
Gago	Gago	Tanga	Coast
Kaniki	Kani	Ruvuma	South
Karatasi	Kara	Tanga	Coast
Katakya	Kat	Kagera	Lake Victoria Zone
Kibandameno	Kibmeno	Tanga	Coast
Kibangili	Kib	Tanga	Coast
Kiroba	Kir	Mtwara	South
Lyongonyeupe	Lyong	Mwanza	Lake Victoria Zone
Machui	Mach	Zanzibar	Island
Mahonda	Mah	Zanzibar	Island
Milondikachini	Milo	Mwanza	Lake Victoria Zone
Mkongamwayo	Mkon	Kibaha	Coast
Mnazi	Mna	Bagamoyo	Coast
Mwafaka	Mwa	Zanzibar	Island
Nachinyaya	Nach	Kibaha	Coast
Paja la mzee	Paja	Tanga	Coast
Rangimbili	Rang	Mwanza	Lake Victoria Zone
Rushura	Rus	Kagera	Lake Victoria Zone
Sagalato	Sag	Tanga	Coast
Sepide	Sep	Unguja	Island
Shija	Shija	Tabora	West
TME7	TME7	West Africa	NA
TMS60444	TMS	West Africa	NA
Kiroba- Kenya	KirkE	ILRI-Lab	NA

2.2 DNA extraction and quantification

DNA extraction followed a slightly modified Dellaporta protocol [14] whereby 3-5 g of freshly cassava leaves harvested three weeks after planting were ground in liquid nitrogen using a motor and pestle. The fine powder obtained was transferred into 1.5 ml eppendorf tubes and mixed with 500 µl of a pre-warmed (65 °C) plant extraction buffer (50mM Tris, pH 8; 10mM EDTA, pH 8;100mM

sodium chloride;1% SDS; 1 % (w/v) PVP; 10mM beta-mercaptoethanol) and incubated in a water bath at 65 °C for 30 minutes with intermittent inversion. Thereafter, 200 µl of 3M sodium acetate (pH 5.2) was added to each tube, mixed thoroughly by brief vortexing and incubated on ice for 20 minutes, followed by centrifugation (Centrifuge Eppendorf, 5424) at 14,000 rpm for 10 minutes at room temperature. To digest contaminating RNAs, 450 µl of the supernatant was transferred into a new eppendorf and 10 µl of a pre-boiled 10 mg/ml RNAse was added, mixed gently by inverting the tubes 5 times and incubated in a water bath at 37 °C for 30 minutes. After a brief spinning of a mixture, 450 µl of chloroform:isoamyl (24:1) was added, mixed gently by inverting the tubes and centrifuged at 14,000 rpm for 10 minutes at room temperature. About 400 µl of the upper aqueous layer were transferred into a new 1.5 eppendorf tube and DNA precipitated using cold isopropanol and incubated at -20 °C for 30 minutes followed by centrifugation at 14,000 rpm for 10 minutes. The supernatant was discarded leaving a DNA pellet that was purified by washing twice with 500 µl of 70 % ethanol, centrifuged at 14,000 rpm for 5 minutes and then air dried for 30 minutes. The DNA pellet was finally re-suspended in about 40 µl of sterile distilled water and transported to Biosciences eastern and central Africa, International Livestock Research Institute (BecA-ILRI) Hub-Nairobi Kenya for genotyping. In addition, one DNA sample of cassava landrace (claimed to be of a Tanzanian origin) (KirKE) that had been kept at BecA-ILRI Hub was included in the SSR analysis making a total of 24 DNA samples. The integrity of all 24 DNA samples was checked on 0.8 % agarose gel stained with GelRed and ran in a 5 x Tris Borate EDTA buffer at 80 V for 30 minutes. The concentration and purity of all DNA samples were determined by using XNanoDrop® ND1000 (Thermo Scientific) at A260 nm and A280 nm. Thereafter all DNA samples were normalized to 50 ng/µl working concentrations

2.3 Polymerase chain reaction (PCR) with SSR markers

Twenty highly polymorphic SSR markers which have been reported as widely distributed in cassava genome were used in genotyping all the DNA samples. PCR was carried out in a GeneAmp®PCR system 9700 thermal Cycler (Applied Biosystems, USA). Each 10 µl of a PCR reaction mix contained 50 ng/µl of each gDNA sample, 1 X buffer (10 mM Tris-HCL pH 8.0, 1 mM EDTA pH 8.0), 0.25 mM dNTPs, 2.5 mM MgCl2; 0.1 µM of each of forward and reverse primers and 0.5 U Taq polymerase (Fermentas). PCR conditions were: Initial denaturation at 95 °C for 3 minutes followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 57 °C for 1 minute, extension at 72 °C for 1 minute and final extension at 72 °C for 30 minutes. To check for PCR amplification, 2 µl was loaded on 2 % agarose gel electrophoresis stained with GelRed and submerged in 0.5 X TBE buffer, run for 45 minutes at 80 V. ATTO gel documentation system (Japan) was used to visualize the gel and photographing using SCION Image (Scion Corporation) software.

2.4 Capillary electrophoresis

One microlliter of each PCR sample was mixed with a mixture of HiDi formamide and a size standard Liz (GeneScan™-500LIZ, Applied Biosciences) in a 96-well plate. Samples were denatured at 95 °C for 3 minutes and immediately chilled on ice ready for capillary electrophoresis. ABI 3500 DNA genetic analyser was used for fragment separation and allele calls made using GENEMAPPER software V.4.0 (Applied Biosystems). To avoid ambiguity during data scoring, co loading sets were pre-determined by taking into consideration a type of dye used in each primer and the expected PCR product size. Furthermore one PCR sample was duplicated in each reaction and at all loci purposely for tracing any duplicate genotypes.

2.5 Data analysis

Data generated were analysed using PowerMarker V3.25 and GenAlEx [15] softwares for genetic diversity parameters. The parameters included number of alleles per locus, allele frequency, percentage polymorphic loci, genetic differentiation and gene diversity (expected heterozygosity).Cluster analysis of the cassava landraces was performed on the dissimilarity matrix using the Neighbour-Joining algorithm [16] and the results displayed as dendrogram using a Data Asset Resource web interface **(**DARwin**)** V 5.0 [17]. Principal coordinate analysis (PCA) which gives spatial representations of genetic distances was done using DARwin 5.0 software. The 5 first axes (with positive Eigen value) were recorded based on dissimilarity index using simple matching with no missing data option and 1000 bootstraps. A two dimension scatter plot was plotted for all genotypes using scores of the first two principal components.

3. Results

3.1 DNA quality and quantity

DNA was successfully isolated from all cassava genotypes whereby its concentration ranged from 318.5 to 1843.6 ng/ µl. The quality of all DNA samples was in a generally accepted ratio of 1.8 at A260/A280 with a typical spectral pattern for nucleic acids except for two samples which had A260/A280 ratios below 1.8. However all DNA samples were of a good quality enough to be used for SSR genotyping.

3.2 PCR with SSR markers and capillary electrophoresis

All 20 SSR primers were able to amplify the SSR markers present at a particular locus and produced the expected PCR product sizes ranging from 122-315 bps. Some SSR primers revealed heterozygozity at some loci by producing two bands that were visible on 2 % agarose gel electrophoresis. Capillary electrophoresis was achieved with many SSR markers producing clear and sharp peaks. SSR primers namely SSRY04, SSRY69, SSRY100 and SSRY164 were among the best primers which produced clear and sharp bands which were very easy to score. On the other hand SSRY 38, SSRY106, SSRY103 and SSRY177 primers produced some false peaks and background noises of which some were difficult to score hence required some optimizations.

3.3 Genetic diversity among cassava landraces

All markers were highly informative with polymorphic information content (PIC) ranging from 0.45 to 0.84 (mean PIC=0.67). PIC value ranges from 0 to 1.0 hence the closer

Table 2 SSR markers used to assess genetic diversity in 21 Tanzanian farmer-preferred cassava landraces

SSR locus	Repeat motif	Forward primer (5'-3')	Reverse primer (5'-3')	Dye	Annealing T(C°)	Expected size(bp)
SSRY 4	(GA)16(TAGA)3	ATAGAGCAGAAGTGCAGGCG	CTAACGCACACGACTACGGA	6FAM	57	278-320
SSRY 9	(GT)15	ACAATTCATCATGAGTCATCAAC-	CCGTTATTGTTCCTGGTCCT	VIC	57	267-293
SSRY 12	(CA)19	AACTGTCAAACCATTCTACTTGC	GCCAGCAAGGTTTGCTACAT	NED	57	264-284
SSRY 19	CT(8)CA(18)	TGTAAGGCATTCCAAGAATTATCA	TCTCCTGTGAAAAGTGCATGA	6FAM	57	203-237
SSRY 20	(GT)14	CATTGGACTTCCTACAAATATGAAT	TGATGGAAAGTGGTTATGTCCTT	PET	57	146-188
SSRY38	(CA)17	GGCTGTTCGTGATCCTTATTAAC	GTAGTTGAGAAAACTTTGCATGAG	6FAM	57	122
SSRY 52	(GT)19	GCCAGCAAGGTTTGCTACAT	AACTGTCAAACCATTCTACTTGC	PET	57	266
SSRY 59	(CA)20	GCAATGCAGTGAACCATCTTT	CGTTTGTCCTTTCTGATGTTC	PET	57	146-202
SSRY 63	(GA)16	TCAGAATCATCTACCTTGGCA	AAGACAATCATTTTGTGCTCCA	PET	57	303-315
SSRY 64	(CT)13(CGCT)6	CGACAAGTCGTATATGTAGTATTCACG	GCAGAGGTGGCTAACGAGAC	VIC	57	192-220
SSRY 69	(CT)18(ATTTAT)2(CTTTCTTCTTT)2CCTTCT	CGATCTCAGTCGATACCCAAG	CACTCCGTTGCAGGCATTA	VIC	57	213-267
SSRY 100	(CT)17TT(CT)7	ATCCTTGCCTGACATTTTGC	TTCGCAGAGTCCAATTGTTG	PET	57	209-273
SSRY 102	(GT)11	TTGGCTGCTTTCACTAATGC	TTGAACACGTTGAACAACCA	6FAM	57	198-200
SSRY 103	(GA)22	TGAGAAGGAAACTGCTTGCAC	CAGCAAGACCATCACCAGTTT	PET	57	274-308
SSRY 105	(GT)6GC(GT)2(GA)16	CAAACATCTGCACTTTTGGC	TCGAGTGGCTTCTGGTCTTC	NED	57	225
SSRY106	(CT)24	GGAAACTGCTTGCACAAAGA	CAGCAAGACCATCACCAGTTT	PET	57	251-278
SSRY 161	(CT)11(TTCT)21(CA)19	AAGGAACACCTCTCCTAGAATCA	CCAGCTGTATGTTGAGTGAGC	NED	57	188-256
SSRY 164	(GA)29	TCAAACAAGAATTAGCAGAACTGG	TGAGATTTCGTAATATTCATTTCACTT	6FAM	45	156-204
SSRY 177	(CCT)6(CTN)65(CT)4(ATCT)18	ACCACAAACATAGGCACGAG	CACCCAATTCACCAATTACCA	PET	45	244-286
SSRY 181	(GA)22(G)3(CGA)3(GGAAGA)4	GGTAGATCTGGATCGAGGAGG	CAATCGAAACCGACGATACA	6FAM	57	192-216

the value to 1.0 the better the PIC value and the more the informative is the SRR marker. Therefore SSRY100 was the most informative marker (PIC= 84%) while SSRY38 was the least informative marker (PIC=45 %). Furthermore the chosen loci revealed a total of 127 alleles whereby SSRY12 recorded the highest number of alleles (12) while SSRY38 recorded only 3 alleles. Mean number of alleles per locus was 6.35. Similarly, an average heterozygozity (Ho) was 0.46, with SSRY38 revealing the least (0.16) and SSRY4 the highest (0.96). Gene diversity (He) among the landraces ranged from 0.53 to 0.85 and the average was 0.71. Summary of the SSR data showing the number of alleles per locus, gene diversity, heterozygozity and amount of polymorphic information content (PIC) obtained from each locus is presented in Table 3.

3.4 Cluster analysis and duplicates detection

Clustering analysis revealed three major clusters (I, II and III). Cluster I had three subdivisions A, B and C and cluster II subdivided into D, E and F. Cluster III had no subdivisions (Fig. 2). Cluster I was the largest and consisted of 11 landraces and three other non Tanzanian varieties namely TME7, TMS and KirKE. The second cluster (cluster II) comprised of 9 cassava landraces with one cassava landrace included as a duplicate (Kat and Kat2) to affirm the ability of the SSR markers to discriminate between closely related genotypes. Cluster III comprised of only two cassava landraces *Kibangili* (Kib) and *Paja la Mzee* (Paja). The PCA was calculated for dissimilarity coefficient whereby the first three axes with positive Eigen values accounted for 22.76, 15.93 and 8.48

Table 3 Genetic diversity parameters evaluated at 20 loci among all cassava landraces

Marker	Major Allele Freq	No of alleles	No of Genotypes	Gene diversity (He)	Heterozygosity (Ho)	PIC
SSRY9	0.62	6	10.00	0.58	0.32	0.56
SSRY12	0.4	12	10.00	0.79	0.36	0.77
SSRY19	0.3	6	10.00	0.78	0.56	0.75
SSRY38	0.6	3	3.00	0.53	0.16	0.45
SSRY59	0.48	4	5.00	0.65	0.08	0.59
SSRY63	0.46	5	6.00	0.7	0.32	0.65
SSRY69	0.32	10	11.00	0.79	0.4	0.76
SSRY100	0.26	11	15.00	0.85	0.6	0.84
SSRY105	0.4	9	8.00	0.75	0.08	0.71
SSRY181	0.54	4	6.00	0.61	0.56	0.55
SSRY64	0.32	5	7.00	0.73	0.4	0.68
SSRY102	0.32	5	8.00	0.73	0.36	0.68
SSRY4	0.46	6	6.00	0.7	0.96	0.66
SSRY20	0.34	7	11.00	0.79	0.76	0.76
SSRY52	0.56	6	7.00	0.63	0.24	0.6
SSRY103	0.44	7	11.00	0.74	0.68	0.72
SSRY106	0.4	7	10.00	0.77	0.68	0.75
SSRY161	0.5	4	7.00	0.63	0.48	0.57
SSRY164	0.42	4	6.00	0.66	0.64	0.59
SSRY177	0.38	6	10.00	0.76	0.56	0.73
Mean	0.43	6.35	8.35	0.71	0.46	0.67

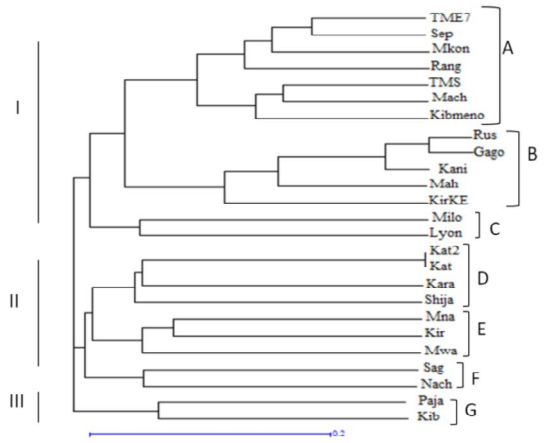


Figure 2 A dendrogram showing the genetic relationships among Tanzanian cassava landraces. I, II, and III represents three major clusters while A to G represents subgroups to which each cassava genotype belongs.

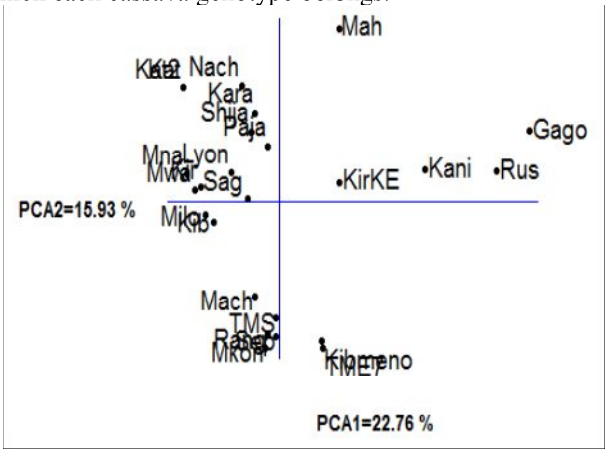


Figure 3 Principle coordinates analysis (PCA) from dissimilarity coefficients of all cassava genotypes.

% of the total variations respectively (Fig. 3). A two dimension PCA using the first 2 axes analysis and the phylogenetic tree results agreed to each other to a greater extent. For instance subdivision group A and B showed a strong relationship in both PCA and Neighbour Joining analyses.

4. Discussion

SSR genotyping of the selected Tanzanian cassava landraces was achieved using 20 SSR markers. The sample size used for this diversity study can provide a reliable results since the results depends more on the number of loci than number of individuals sampled [18,6]. Furthermore, twenty SSR primers used in this study (which represent 20 loci) were enough to give a clear picture of genetic diversity among the selected farmer-preferred cassava landraces. [19] reported that 16 polymorphic loci were enough to provide a reliable estimate of genetic diversity of a cassava population. Similarly, [20,21] obtained genetic diversity results comparable to the one reported here by using a few (10 and 16 respectively) polymorphic SSR markers. All markers used were highly informative with mean PIC value within a range reported elsewhere. Turyagenda et al [5] obtained mean PIC value of 61.1 % from 26 SSR markers used to study a genetic diversity of Ugandan farmer-preferred cassava landraces. Similarly, [4] reported a mean PIC value of 53 % from twenty SSR markers that were used to determine the genetic diversity of cassava germplasm in Ghana. Variations in these PIC values can be attributed by types of the chosen SSR markers and the cassava genotypes assessed but information obtained reflected the population structure studied.

Some SSR markers gave higher numbers of alleles across the assessed loci. In this study SSRY12, SSRY100 and SSRY69 gave high number of alleles a scenario that was

also reported by other researchers [3,5]. Furthermore, [6] reported a similar average number of alleles of 6.0 in the genetic diversity study of cassava landraces from Colombia while Montero-Rojas et al [9] observed a relatively higher number of alleles per locus in a diversity study of cassava in Puerto Rico.

High average gene diversity (He=0.71) revealed in this study is a good indication of a rich population that can be used in breeding strategies. Other researchers also reported similar results of high gene diversity within and among cassava accessions using SSR markers [3, 5, 6, 12,22].

Clustering analysis of the cassava landraces used in this study revealed three major groups with several sub divisions. Clustering revealed the relationship between cassava landraces without reflecting regions from which they were collected except for the cassava landraces *Paja la Mzee* and *Kibangili* (Paja and Kib) which are both from Tanga while *Milondikachini* and *Lyongonyeupe* (Milo and Lyong) are from Mwanza region. However these associations were not supported well by PCA. Possibly, the small sample size used from different regions might have affected the results. Some genotypes like *Sepide, Machui* and *Kibandameno* though classified as landraces clustered closely with TME7 and TMS (va.60444) suggesting that they might be hybrids. TME7 is a Nigerian cassava landrace and TMS60444 is a wild-type cassava mostly used as a model in various cassava experiments. However, since most of these TM series are improved varieties it might suggest that they share similar route with landraces. A cassava DNA sample obtained from BecA-ILRI Hub (KirKE) clustered with the Tanzanian ones suggesting that it might be of a Tanzanian origin although its name and origin could not be expounded. Nevertheless, the dendrogram showed a strong relationship between two samples (Kat and Kat2) which were actually duplicates that were intentionally included as a control. These duplicates showed a very strong association (100%), and thus eliminated a chance that the rest of the genotypes could contain any duplicates. Although there are some reports on the duplicates of gemplasms and misnaming of similar varieties with different names and vice versa it was not a case in this study. Duplicate cassava genotypes have been observed in other diversity studies [23,24]. Moreover, [5] observed two Ugandan cassava landraces (*Nyarare* and *Masindi4*) with 100 % association suggesting that they might be duplicates. Since none of the cassava landrace assessed in this study seem to be duplicates, it might be suggested that there is no misnomer and thus each landrace is genetically different from each other. Furthermore, duplicate samples that were intentionally included in the analysis supported the ability of SSR markers to distinguish even closely related species [6].

The PCA provided further information on the genetic distance between the Tanzania cassava landraces and non Tanzanian cassava genotypes. Generally the PCA plot demonstrated trends similar to the clustering revealed in a dendrogram. They both agreed to each other to a greater extent especially for a subcluster B which clustered in a similar manner. A 100 % association of a cassava landrace Kat and Kat2 was also consistent. However, a cassava landrace *Kibandameno* showed stronger association with TME7 in a PCA than it was illustrated in a dendrogram. Generally, cassava landraces evaluated in this study based on the twenty SSR loci suggested a relatively high genetic diversity among them. This is not surprising since they were collected from eight geographically distinct regions including two from Zanzibar Island. In addition, the genotyping data revealed high genetic diversity among the landraces probably due to the existing custom of planting more than one cassava variety in the same field as were found in the farmer's fields during sampling.

5. Conclusion

Although only a few farmer-preferred cassava landraces were evaluated in this study, this report is useful for cassava genetic improvement of good agronomic traits, breeding strategies and germplasm conservation. Besides, it is unnecessary and expensive to keep duplicates in any germplasm collections hence a follow up genotyping study of all Tanzanian farmer-preferred cassava landrace should be conducted to avoid duplicate genotypes into the cassava germplasm collection at MARI and within the region. Lack of duplicates among the cassava landraces evaluated in this study denotes farmers' ability to differentiate and maintain the same variety over long periods.

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