



Application of SSR Markers in Determination of Putative Resistance to SPVD and Genetic Diversity among Orange Fleshed Sweet Potato

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SMK and LSK designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors MAO and FNW performed the statistical analysis, managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Viruses are the second most important production constraint after the sweet potato weevil. Orange fleshed OFSPs) are characterized by medium to low resistance to sweet potato virus disease (SPVD). Therefore this studies were aimed at evaluating OFSPs for resistance to SPVD and their genetic diversity from six families.

Thirteen OFSPs clones from five families and other four genotypes were selected by their moderate SPVD responses after challenge to viruses. They were screened using ten Simple sequence Repeats (SSR) markers, six of them being polymorphic. Neighbor joining tree was generated with DARwin Version 6.0.010 using unweighted pair group method with arithmetic

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means (UPGMA). Principal component analysis (PCA) and Pearson Correlation matrix were obtained with XLSTAT 2015 version. The test clones clustered in two groups separately from virus susceptible genotypes. This implied that SSR markers are useful in discriminating virus susceptible and resistant genotypes. A total of 18 alleles were detected with an average of 3.0 alleles per locus. The highest major or abundant alleles (0.94) was observed in marker IBJ- 324 Major allele frequency mean of 0.69 for the six markers was obtained. Mean genetic diversity of the markers was 0.41. Average polymorphism information content was 0.36. Pearson's correlation coefficient was used to determine relationship between genotypes screened with SSR markers. Virus susceptible genotypes Ejumula and Carrot C had the highest similarity matrix of 0.83. Lowest matrix was between F1C9 and F3C1 at -0.03. Pearson's correlation coefficient revealed an average similarity of 0.54 among genotypes. This study therefore indicates that there is robust genetic diversity in SPVD resistant sweet potato genotypes. These genotypes can be used as parents in breeding programs aimed at improving the crop for the multiple traits.

Keywords: SPVD; resistance; simple sequence repeats; OFSPs.

1. INTRODUCTION

Sweet potato ranks fourth in importance in the developing world after rice, wheat, and maize [1]. The potential utility of sweet potato includes use as food, feed, processed products, and a source of income for millions of resource-poor farmers in developing countries [2]. Some of food utilization options include *chapattis* and *mandazi*, flour for porridge and making chips has been developed since sweet potato is cooked and eaten in a limited variety of forms [3]. The yellow and OFSPs varieties are a source of the vitamin A that has been reported as a deficiency in developing countries [4]. Sweet potato is referred to as a subsistence crop, food security or famine relief crop and its utilization has diversified significantly in the developing countries [5]. Its cultivation and production is primarily carried out within Lake Victoria basin where it is key in households' food security [6-7]. In this region, farmers grow different cultivars such as the white, yellow, cream and light to deep types. The most nutritious of cultivars grown in the Lake Basin region are orange-fleshed cultivars (OFSPs) that contain beta-carotene [8]. In East and Central Africa, antagonistic effects of pests and diseases on production of maize, cassava and banana has led to increased importance of [9-10]. Since the crop is a vegetatively propagated crop, accretion and dissemination of viruses can become a major constraint for production. Virus diseases often cause reduction in yield and quality of storage roots [11].

SPVD is caused by dual infection of the aphid transmitted Feathery Mottle Virus (SPFMV genus *Potyvirus*, family *potyviridae*) and whitefly transmitted Chlorotic Stunt Virus (SPCSV genus *Crinivirus*, family *Clesteoviridae*). SPVD is the

most destructive disease globally and particularly in the East Africa region [12]. Sweet Potato Mild Mottle Virus (SPMMV genus *Ipomovirus* family *Potyviridae*) also infects sweet potato. Viral synergism involving SPFMV and SPCSV cause severe SPVD in which potyvirus titers increase by 600 fold whereas no rise is observed in titers of SPCSV [13]. Improvement of germplasm for resistance to SPVD by use of conventional breeding is lengthy and slow. Use of Marker Assisted Selection (MAS) has been used to accelerate and complement breeding programs in many food crops. OFSPs resistance to multiple virus infection and determination of resistance traits and genetic diversity. Evaluation of the responses of various OFSPs clones for resistance to multiple virus infection and determination of resistance traits and genetic diversity of selected OFSPs clones using SSR has not been carried out. Assessment of reaction to SPVD challenge and ascertaining the presence of resistant genes in OFSPs genotypes is not only fast but also broadens the source of resistance to SPVD among sweet potato varieties.

Simple Sequence Repeats (SSRs) have been most commonly used in genomic characterization principally in maize, cassava, soybean, barley, wheat and sunflower among other crops. SSR markers have since been developed for diversity studies [14]. High levels of polymorphism have been reported in white and orange-fleshed sweet potato farmer varieties from East Africa [15]. DNA markers have been identified and associated with economically important traits such as resistance to SPVD [16], knot nematodes resistance [17], dry-matter and starch contents, and beta-carotene content [18]. SSR markers have been used to characterize

sweet potato genotypes for SPVD resistance and high dry matter content in the germplasm collection in Kenya and Tanzania [19-20]. This study was aimed at identifying OFSPs genotypes with resistance traits to SPVD with acceptable levels of dry matter and micronutrients content, especially beta-carotene. The study was also aimed at identifying SSR markers that can be used to characterize sweet potato for SPVD resistance in future.

2. MATERIALS AND METHODS

2.1 Establishment of the Experiment, Study Site and Data Collection

Germplasm from six families was obtained from International Potato Center (CIP) in Mozambique. Seeds from the 6 families (MUSG 0622-58 MUSG 0608-22, 108196G07-06, UW 11906-289, MUSG 0608-02 and MUSG 0704-16) were acid scarified using concentrated sulphuric acid. The seeds were later put in a petridish with pre-wetted filter paper to enable germination to occur. Pre-germinated seeds were planted on forest soil and sand which was sieved and mixed at the ratio of 3:1. Controlled watering was carried out as excessive watering which would cause rotting of seeds. Germination counts were taken three weeks after planting. Control of SPVD vectors was carried out by spraying insecticides Actara® and Dynamec® from Syngenta in two weeks intervals.

Detection of viruses was carried out using Nitrocellulose membrane ELISA (NCM-ELISA) [21]. The kit was obtained from the manufacturer (CIP, Peru) and used to detect the following viruses: Chlorotic stunt virus, Feathery mottle virus (SPFMV), Mild mottle virus (SPMMV), Chlorotic fleck virus (SPCFV), Latent virus (SPLV), Mild speckling virus (SPMSV) Chlorotic fleck virus (SPCFV), Caulimo-like virus (SPCaLV), C-6 virus and Cucumber mosaic virus (CMV). RT PCR protocol was used to validate results obtained by NCM-ELISA [22].

Only virus free clones were selected for planting in the field. The virus free clones were planted in a field with high SPVD pressure using Randomized Complete Block Design (RCBD) with three replications. The experiment was laid out at a field adjacent to breeding blocks as source of natural infection at Kenya Agricultural and Livestock Research Organization–KALRO in Njoro. Disease severity was determined based on a score of 1-9 where 1 = no visible symptoms

and 9 represents very severe symptoms including purpling /yellowing or mosaic on leaves, severe leaf distortion, reduced leaf size, severe stunting and chlorosis. Disease scores were taken after every two months.

2.2 Selection of Clones for Resistance Traits and Genetic Diversity

The selection of clones for resistance traits and genetic diversity analysis was primarily based on responses to virus infection data. Clones with scores of 6 (score of 1 refers to no symptoms while a score of 10 are very severe symptoms) and below were selected in addition to other criteria such as carotene content, low weevil damage and harvestable roots.

2.3 Dry Matter and Micronutrient Analysis

Dry matter analysis was carried out using oven drying method [23]. Iron and zinc were determined in the clones after maturity using a method described by [24]. β carotene content in different parts of the same root was determined by UV light absorbance spectrophotometry at a wavelength of 450 nm [25].

2.4 Simple Sequence Repeats (SSRs) Analysis for SPVD Resistance

Ten primers pairs for SSR loci linked to the known SPVD resistance genes sourced from [26-27], [28] and [29] (Table 1) were used for PCR amplification of the sweet potato genomic DNA test samples (test clones). Two checks (Kenspot 4 and Kenspot 5) known to have moderate virus resistance and two virus susceptible varieties (Ejumula and Carrot C [30-31] were included in SSR analysis.

Total nucleic acids extraction from leaves was carried out using CTAB method [32] with some modifications. Each 20 μ l PCR reaction contained 10 pmol/ μ l of each primer, 2.5 mM MgCl₂, 10 mM dNTPs, 0.1 Units of Taq DNA polymerase and 5X PCR buffer (Qiagen, Germany). Amplifications were carried out using the Gene-Amp PCR system 2720 (Applied Biosystems, USA) under the following thermocycling conditions: 1 cycle of 94°C for 2 minute (Initial Denaturation), followed by 15 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 2 minute, extension at 72°C for 1.5 minute, another 30 cycles were carried out using the following cycling conditions. 94°C for 1 minute, 50°C for 2 minutes and 72°C

Table 1. SSR markers used in the study

Lab no	Name of locus	Repeat motif of the SSR marker	Annealing temperature (°C)	Size range of the PCR product (bp)	Number of alleles per locus
1	IB-R03	(GCG)5	58	302-312	5
2	IB-S07	(TGTC)7	60	193-211	4
3	IB-R12	(CAG)5A	60	356-395	5
4	IB242	(CT)3 CA(CT)11	58	136-155	6
5	IB297	(CT)13	58	150-182	4
6	IB316	(CT)3C(CT)8	58	151-167	5
7	IBCIP-1	(ACC)7A	63	155-167	4
8	IB324	*	56	*	*
9	IBJ 522a	(CAC)6-7	57	*	*
10	JB 1809	(CCT)6 (CCG)6	60	225-298	9

*Required information in the fields not available

for 1.5 minutes and a final extension step of 10 minutes at 72°C in order to lessen the likelihood of false scoring of stutter bands as true alleles. The PCR products of each sample and a 100 base pair molecular weight marker that was loaded in one of the wells were resolved on a 2% agarose gel in 1X TBE buffer. Bands were be scored for presence as 1 and for absence as 0.

Table 2. Genotypes evaluated for resistance traits

Sample number	Family/clone	Test attribute(s)
1	F1/C2	Test clone
2	F1/C7	Test clone
3	F1/C9	Test clone
4	F2/C13	Test clone
5	F2/C9	Test clone
6	F3/C1	Test clone
7	F3/C13	Test clone
8	F3/C3	Test clone
9	F3/C5	Test clone
10	F3/C9	Test clone
11	F4/C15	Test clone
12	F4/C9	Test clone
13	F4/C11	Test clone
14	Kenspot 4	Medium resistance to virus diseases
15	Kenspot 5	Medium resistance to virus diseases
16	Ejumula	Virus susceptible variety
17	Carrot C	Virus susceptible variety

2.5 Data Analysis

The SSR data was analyzed using Dissimilarity Analysis Representation for Windows (DARwin

version 6.0.010) to generate neighbor joining tree for the 17 genotypes. Pearsons correlation coefficient was used in generation of the tree. The dendrogram was constructed using UPGMA (Unweighted Paired Group Method with Averaging with Arithmetic Averaging). Power Marker Version 3.0 was used to compute summary statistics.

3. RESULTS

3.1 Determination of SPVD Resistance Traits in OFSP Clones Using SSR Markers

Genotypes were scored for the presence (1) or absence (0) of each fragment. Only distinct fragments were scored. Fragments with the same mobility on the gel but with different intensities were not distinguished from each other when genotypes were being compared.

3.1.1 Major allele frequency, allele number, gene diversity and polymorphism content

Six (SSR) markers for 6 loci were used to characterize 17 sweet potato genotypes. Power Marker Version 3.0 was used to give summary statistics output. A total of 18 alleles were detected. The number of alleles per locus ranged from 6 for marker IB-316 and 2 for markers IBJ-324, IB-1809, IB R03 and IB-CIP with an average of 3.0 alleles per locus. The highest number of alleles was observed in marker IBJ-324(0.94) whereas marker IB-316 showed the lowest value of 0.29. Major allele frequency mean for the six markers was 0.69. Gene diversity among the six markers was highest in marker IB-316 at 0.78

while the least diversity was recorded in marker IBJ-324. Mean genetic diversity of the markers was 0.41. The polymorphic information content (PIC) ranged from the lowest, 0.10 for marker IB-316 to 0.74 for marker IB-316. Average polymorphic information content was 0.36 (Table 3).

3.1.2 Genetic diversity of OFSP clones with putative resistance traits based on simple sequence repeats

Genetic Similarity among the 17 genotypes ranged from - 0.033 to 1.0. An average of 0.54 among the genotypes was revealed. Similarity matrix showed that the most closely related genotypes were Carrot C and Ejumula, F1/C1 and F1/C7, F1/C2 and Ejumula, F1/C9 and F3/C5, F2/C3 and F1/C2, F2/C13 and F4/C11, F2/C9 and F4/C11, F2/C9 and F4/C9 and F2/C9 and Kenspot 4 among others. The most remotely related genotypes were F1/C9 and F3/C1, F3/C1 and Kenspot 4, F3/C1 and Ejumula, F3/C1 and Carrot C, F1/C7 and F3/C1 and F4/C15 and F2/C9. The similarity matrices showed that the genotypes such as Ejumula and Carrot C usually characterized by low virus resistance are not genetically similar to test clones such as F3/C1.

3.1.3 Phylogenetic analysis

SSRs used in this study revealed different levels of polymorphism across test genotypes. Different profiles were obtained when Family 1 (F1/C2, F1/C7 and F1/C9) were screened against markers IB-R03, IB-CIP, IB-316 and IBJ 522 (plate not shown). The screening for putative resistance traits in the 17 genotypes using different SSR markers revealed varying alleles among test samples. Reliability of SSRs in identifying resistance traits was revealed by absence of some alleles in susceptible genotypes which were available in the test clones using marker IB-316.

The resultant dendrogram constructed revealed three clusters. Cluster 1, 2 and 3 represented 53% 29.4% and 17.6% of the 17 genotypes analyzed. Test clones clustered independently from the checks except Kenspot 5 and virus susceptible varieties. Carrot C and Ejumula were clustered so closely revealing their SPVD susceptibility trait. However, Kenspot 4 was placed in the same cluster. Summarized data on tested attributes indicated that cluster 1 had high beta carotene contents with an average of 25.59 ppm compared to cluster 2 which had an average of 6.73 ppm. However, three out of nine had an average virus score. Cluster 2 had four out of five clones with virus score average below 5. Cluster 2 had higher dry matter content with an average of 27.26 gm compared to 25.59 gm in cluster 1. Similarly, cluster 2 had higher iron content averaging 4.80 ppm against 3.96 ppm in cluster 1. Difference in zinc content was not significant since cluster 1 had an average of 17.33 ppm while cluster 2 averaged at 17.33 ppm. Cluster 1 revealed a higher average virus score of 4.70 while cluster 2 had an average of 4.60. Generally, there was acceptable dry matter, iron content and medium severity score among clusters (Table 4).

3.1.4 Principle component analysis (PCA) based on genetic distances of the 17 sweet potato genotypes

Four principle components were obtained in the PCA plot. Generally, the dendrogram and the PCA agree since there was no unique pattern in the two outputs. The clustering pattern in the PCA plot was similar to the neighbor joining tree since almost all clones in cluster 1 of the tree fell closely among themselves in the PCA plot. Similarly clones in cluster 2 of the tree grouped closely among themselves and distantly from the virus susceptible varieties (Ejumula and Carrot C) which overlapped each other in the in the PCA plot (Fig. 2).

Table 3. Major allele frequency, allele number, genetic diversity and polymorphism information content in the 17 genotypes

Marker	Major allele frequency	Sample size	Number of observations	Allele numbers	Availability	Gene diversity	PIC
IB-316	0.29	17.0	17.0	6.0	1.0	0.78	0.74
IBJ-324	0.94	17.0	17.0	2.0	1.0	0.11	0.10
IBJ-522	0.64	17.0	17.0	3.0	1.0	0.49	0.41
IB 1809	0.70	17.0	17.0	3.0	1.0	0.45	0.41
IB-R03	0.70	17.0	17.0	2.0	1.0	0.41	0.32
IB-CIP	0.88	17.0	17.0	2.0	1.0	0.20	0.18
Mean	0.69	17.0	17.0	3.0	1.0	0.41	0.36

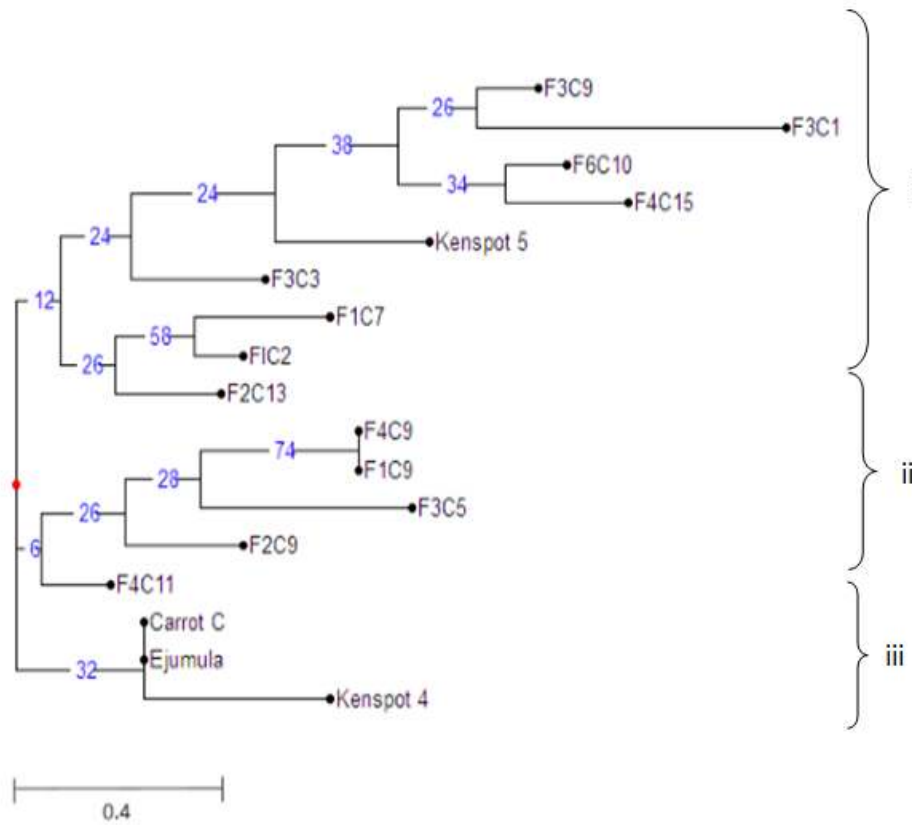


Fig. 1. Neighbor joining tree showing 17 sweet potato genotypes grouping in 3 distinct clusters

Table 4. Micronutrients content and virus score averages of the analyzed clones

Cluster	Family/clone	Carotene (ppm)	Dry matter (gm)	Iron (ppm)	Zinc (ppm)	Average virus score
Cluster 1	F3/C9	17.33	19.67	4.33	35.00	5.67
	F3/C1	4.00	26.33	4.67	18.33	5.00
	F6/C10	10.00	29.33	1.67	4.67	5.67
	F4/C15	47.33	24.67	4.67	3.33	4.00
	Kenspot 5	19.67	28.67	3.33	24.67	4.67
	F3/C3	2.33	30.00	4.00	36.33	4.00
	F1/C7	52.00	23.67	4.00	19.67	4.67
	F1/C2	7.00	30.00	5.67	6.00	4.00
Cluster 2	Average	21.51	25.59	3.96	17.33	4.67
	F2/C13	34.00	18.00	3.33	8.00	4.33
	F4/C9	17.00	28.67	5.00	44.33	5.33
	F1/C9	6.00	20.33	6.00	14.67	4.00
	F3/C5	0.33	34.33	3.67	2.33	4.33
	F2/C9	6.67	21.33	5.67	5.67	4.67
Cluster 3	F4/C11	3.67	32.67	3.67	21.67	4.67
	Average	6.73	27.47	4.80	17.73	4.6
	*Carrot C	-	-	-	-	-
	*Ejumula	-	-	-	-	-
	Kenspot 4	30.67	24.67	3.67	47.67	4.67

* Genotypes that were used as virus susceptible varieties in SSR analysis

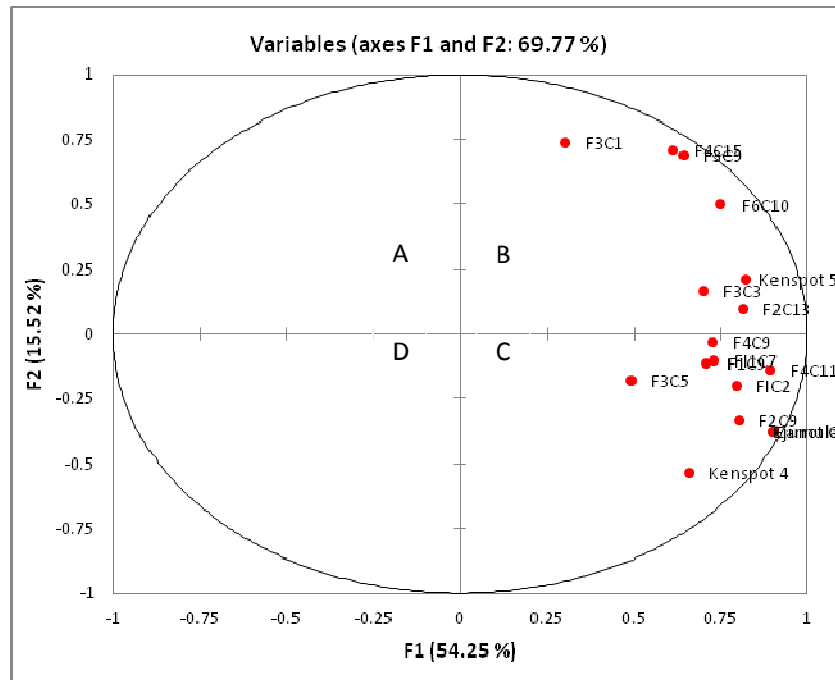


Fig. 2. Principal component analysis plot for 17 genotypes revealing four clusters

4. DISCUSSION

Availability of putative resistance traits for characterizing OFSPs using SSR markers is important in marker assisted breeding and selection of genotypes as sources of resistance to SPVD. In recent times, SSR markers have been used to characterize genotypes for SPVD resistance and high dry matter content in the germplasm collection in Kenya [19]. The present study used 10 SSR markers of which six of the markers showed considerable levels of polymorphism. Neighbor joining tree revealed clustering of Ejumula, Carrot C and Kenspot 4 in the same group, exhibiting the ability of SSR markers to discriminate virus tolerant sweet potato genotypes from the susceptible ones. Test clones did not cluster with the virus susceptible varieties used in the study. This implies that they possess the putative viruses' resistance genes. The findings corroborate earlier works that SSRs are useful in identification of resistant genotypes [19]. Analysis of 57 Tanzanian genotypes against SPVD infection revealed that a high proportion (43.8%) of genotypes has promising resistance against SPVD [21]. Thirteen test clones and Kenspot 4 clustered into two distinct clusters. Presence of genetic differences among the SPVD resistant genotypes revealed by their clustering into discrete groups suggests the

occurrence of different sources of resistance to SPVD clones. Neighbor joining tree revealed two groups (Cluster 1 and 2) of SPVD resistant genotypes. Ideally, the relationship between genotypes in the clusters groups cannot be solely attributed to their resistance to SPVD but also to other attributes such as dry matter or micronutrients among other traits. This agrees with findings by [19] where Kenyan genotypes with SPVD resistance clustered into three independent clusters. The clustering observed in this study is indicative of a trend implicating the ability of markers to be used for selection of carotene, iron and other attributes in OFSPs.

Results obtained in PCA in this study backed by the result of the neighbor joining tree clustering show that SSR markers are adept in detecting genetic relationships in sweet potato genotypes with response traits (resistant or susceptible) to SPVD and other attributes such as micronutrients. This is so because even with one principal component, virus susceptible varieties grouped closely as in the neighbor joining tree. Test clones did not group close to Ejumula and Carrot C as demonstrated in the tree. Absence of other principal components could be attributed to similarity in a trait such as micronutrient content or dry matter. Genetic relationships among 17 genotypes using Pearson's correlation coefficient

revealed an average of 0.54 among genotypes. This represented 54% variation among the 17 genotypes. Similarity matrix ranged from - 0.033 to 1.0. The Pearson's coefficients were slightly lower to Jaccard's coefficients obtained by [19], who similarly employed simple sequence repeats to analyze sweet potato cultivars. SSR based Jaccard's coefficient ranged from 0.5 to 1, with an average of 0.752, accounting for 50% variation among the 89 genotypes. In the present study, Carrot C and Ejumula were the most closely related genotypes as earlier confirmed in the neighbor joining tree, PCA and in Pearson's correlation. This could be attributed to their response to virus infection. The lowest similarity among the genotypes was between F1C9 and F3C1. This distant relationship can be attributed to fact that the clones arose from different families and they were a result of open pollination. The genetic distinctiveness among the two clones however is not on the basis of dry matter and micronutrient contents. Clone F3C1 was uniquely different from Ejumula, and Carrot C. This was revealed in the tree, PCA plot where it was distantly placed from the virus susceptible varieties and in the similarity matrix where clone and the two susceptible varieties had a low value of 0.03. Distinctiveness of this clone against the virus susceptible genotypes is largely attributed to virus tolerance/ resistance. However, the clone was characterized by low carotene content.

Six markers used in the computation of major alleles revealed that marker IB 324 and IB-CIP had the highest value of major allele frequency and the lowest gene diversity and PIC value. This implies that the markers are not ideal for trait and diversity analysis. Marker IB-316 had the lowest major allele frequency but with the highest allele number, gene diversity and PIC value indicating its usefulness in diversity information. The microsatellite markers used in this study were generally informative in unraveling the genetic relationships of 17 sweet potato genotypes in the study. The mean genetic similarity coefficient of 0.54 obtained in this study is moderately high, implying occurrence of large diversity among the studied genotypes. Comparably, [33] reported a higher similarity coefficient (0.58) among sweet potato accessions from South America which is also known to be a center and origin of diversity of sweet potato. Observed similarity coefficient in OFSPs were slightly lower. Higher mean similarity coefficient values of 0.64, 0.69, and 0.79 have been reported [34,27] and [35]

respectively, and they concluded occurrence of low diversity among studied germplasm.

5. CONCLUSIONS

Screening orange fleshed sweet potato clones for disease resistance traits and micronutrients is the key to identification of suitable candidates for sweet potato breeding and germplasm maintenance. SSRs have been useful in different crops for various traits analysis. The present study findings reveal the discriminatory ability of SSR markers. Grouping of susceptible varieties carrot C and Ejumula into a discrete clusters is a promising finding since future SSR analysis on SPVD tolerance can be successful through the use of these two varieties as controls and a set of markers used in this study. Evaluation of sweet potato SSR markers is proposed in order to identify suitable markers that can be used to evaluate germplasm for various traits such as dry matter, micronutrients, weevil resistance and other attributes that can contribute positively to improved sweet potato production such as drought resistance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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