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FLUORESCENT SSR MARKERS AND CAPILLARY ELECTROPHORESIS REVEAL SIGNIFICANT GENETIC DIVERSITY IN NATURALIZED PUMPKIN ACCESSIONS IN KENYA

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ABSTRACT
This study was undertaken as a preliminary step in determining genetic diversity of naturalized pumpkins in Kenya. It entailed allelic patterns, frequency, inbreeding coefficient, molecular variance, cluster and inter-population genetic analyses. It utilized 96 pumpkin accessions and five fluorescent SSR markers in capillary electrophoresis. Data were captured using ABI 3730 software, and analyzed using GeneMapper V 4.1 software. Measures of genetic variability were determined using GenAlEx 6.5, genetic diversity within and among accessions using Power Marker V 3.25 and data contained in the electrophenograms by GeneMapper V 4.1 software. XLstat 2014 was used for cluster and GenAlEx 6.5 software for principal coordinates analyses. DNA quantity ranged from 62.7 to 2992 ng/µl and quality from 0.56 to 2.1 of 260/280 absorbance ratio. Fluorescent SSR markers detected 23 alleles with an average of 4.6 alleles per marker, with size ranging from 181 to 326 bp. A total of 934 distinct DNA fragments were identified. Mean PIC was 0.49, observed heterozygosity 0.5048, genotype number 6.8, gene diversity 0.5491, and polymorphism 98.5% across the markers. Mean allelic patterns showed great variation among the accessions. Cluster and principal coordinate analysis revealed distinct accession groups independent of their geographic origin. AMOVA indicated that genetic differentiation was significant (P=0.02). Total molecular diversity of 3% was attributed to regional differences, 9% to accession differences within regions, while 88% to differences within accessions. The F_{ST} of 0.026 indicated very little genetic differentiation due to continuous selection of pumpkin seeds by farmers. The present study proved that fluorescent SSR markers and capillary electrophoresis are effective in estimating genetic diversity and detecting polymorphisms present in pumpkin accessions in Kenya. The genetic diversity should be related with desirable quantitative and qualitative traits and used in improving pumpkin into commercial cultivars. The KK-3 and KK-56 accessions with unique, private and locally common alleles should be prioritised during conservation efforts.

KEYWORDS: Agrobiodiversity, Alleles, *Cucurbita moschata*, Molecular characterization, Vegetables.

INTRODUCTION

Pumpkins belong to the family cucurbitaceae, with several sub-families, 8 tribes, 118 genera and 825 species (Jeffrey, 1990). *Cucurbita moschata* has a wide range of variability (Naik *et al*., 2015) and genetic diversity in the array of landraces adapted to local conditions (Ntuli *et al.*, 2013). Driven by survival motives, farmers produce diversity as a positive externality (Virchow, 2003) and maintain local landraces (Thies, 2000). *Cucurbita moschata* is indistinguishable from *C. pepo* and *C. maxima* due to similar plant habit (Paris, 2000). Phenotypic diversity is high and includes variation in fruit shape, size, flesh quality, colour, thickness, and production precocity, as well as seed number and size (Whitaker and Robinson, 1986; Hernandez *et al*., 2005). Intercropping enhances gene flow among cucurbit species due to random bee pollination. Occasional introduction of seeds and informal seed exchange among farmers influence gene exchange. Determining the degree of variability of local pumpkin landraces is the preliminary step in studying their genetic diversity (Ferriol *et al*., 2001). Users of plant germplasm want to know the favourable as well as undesirable traits associated with particular accessions (Poulos, 1993).

Molecular genotyping takes advantage of variation in highly polymorphic genes (Gupta *et al*., 2010). Molecular markers support detailed characterization (El-Assal and Gaber, 2012). The SSR markers are useful for cultivar identification (Watcharawongpaiboon and Chunwongse, 2007) and characterization of germplasm diversity (Khanam *et al*., 2012). The fluorescent SSR markers are powerful due to their genetic co-dominance, abundance, dispersal throughout the genome, multi-allelic variation, high reproducibility and polymorphism. They are amenable to automated allele detection and sizing (Khanam *et al*., 2012). The SSR markers allow heterozygotes in diploid genomes to be distinguished (Serra *et al*., 2007). Capillary electrophoresis (CE) increases test sensitivity and discriminatory power. It provides automated and accurate estimates of allele sizes. In combination with fluorescent-labeled SSR markers, CE provides high detection sensitivity of amplified DNA fragments (Wang *et al*., 2009). Capillary electrophoresis

can measure the size of polymerase chain reaction (PCR) products with very high resolution (Gupta *et al*., 2010). Improvement of preferred pumpkin is constrained by limited characterization, selection of desirable traits (Adebooye *et al*., 2003), and information per species (Hamisy *et al*., 2002). In most institutions, research efforts with powerful biotechnological tools are concentrated on staple crops and no adequate extension to pumpkins has been done, leaving them unimproved to suit consumer demands (Maundu *et al*., 1999; Onyango, 2002). Nonetheless, Kenya is endowed with great genetic diversity suitable for pumpkin improvement into commercial cultivars. The present study characterized the pumpkins, using fluorescent SSR markers in capillary electrophoresis to assess relationships and genetic diversity that could be used to improve commercial utility and conservation.

MATERIALS AND METHODS

DNA Extraction

Accessions for molecular characterization were planted in Chuka University (CU) research farm on 10th January, 2013 in a completely randomized design (CRD) with three replications. The farm lies at 0° 19` S, 37° 38` E and 1535 m above sea level. Leaves of 139 accessions were picked for DNA extraction in the Jomo Kenyatta University of Agriculture and Technology (JKUAT) Institute Biotechnology Research (IBR) laboratories using the method described by Doyle and Doyle (1987). The extracted DNA was quantified using the NanoDrop-1000 spectrophotometer (Beauman, 2007). DNA extracts were subjected to polymerase chain reaction to generate products separated by 1.5% w/v agarose gel electrophoresis. DNA samples of 96 accessions showing clear and sharp bands were selected for genotyping in the International Livestock Research Institute (ILRI) laboratories.

Quantification, qualification and normalization of DNA samples

The concentration and quality of DNA was determined using optical density reading on NanoDrop (ND-8000) spectrophotometer (ND Technologies, Inc., Wilmington, DE). A control was established by loading 1 µL of blank after which 1 µL of each DNA sample was loaded on eight pedestals present. Laser light was then shot from fiber optics through the eight pedestals containing DNA, and the ratio of wavelength absorbance and DNA concentration recorded for each sample. The realized concentrations were used as a normalization guide for each sample to a concentration of 30 ng/µL. The ratio of 260/280 nm was used to give an indication of the purity of the DNA samples. A resultant ratio of 1.7-2.0 was considered indicative of good quality DNA.

Polymerase chain reaction and capillary electrophoresis

PCR using fluorescent SSR markers on 96 accessions DNA samples amplified the samples for screening on capillary electrophoresis on the ABI Prism 3730 (Applied Biosystems). Forward SSRs were labeled at the 5' end of the oligonucleotide with fluorescent dyes (Table 1). PCR was performed with a Gene-Amp 9700 (Applied Biosystems) in a 10 µL final volume containing PCR mixture for each reaction (Table 2). Thermocycling reactions were programmed for initial denaturing at 94°C for 3 minutes, 30 cycles of 30 seconds at 94°C, 1 minute for 55°C, and 2 minutes at 72°C, elongation at 72°C for 20 minutes and a final hold at 4°C.

TABLE 1: Fluorescent labeled SSR primer pair sequences and dye colours

Primer code	Primer sequence (5' to 3')	Fluorescence dye colours
PKCT-47	Forward: GGT CCC AAT AAT AGC AAC CAA, Reverse: GTG GGA CAC ATC TTG AGC A	6-FAM
PKCT-62	Forward: GAA GTT CGT GGT CTG TGC AAG TC Reverse: CCT GAG TAA CCT CCG TGC TTC C	VIC
PKCT-111	Forward: GTT GCA GCG ACC GTT CTT CTT C Reverse: GCA TCT GAA GAC GAT GCG TCG T	PET
PKCT-122	Forward: CTA AAC AGG ATG CCT CTG ACA C Reverse: CGG GAT TTC CGA AAC AAC GT	NED
PKCT-133	Forward: TCG GAA TCG TCT TCA GCA ATA GTC Reverse: TCC TCT TCC ATT CCA CTT TCT CCT	6-FAM

Purification of PCR products

PCR products for DNA samples of the 96 accessions were purified using Qiagen kit (QIAquick PCR purification kit) to remove any remaining dNTPs, primers, Taq, and Mg ion. These components are impurities that interfere with subsequent DNA genotyping. PCR products purification protocol described in QIAquick spin handbook of November, 2006 was used for the purification process.

TABLE 2: PCR components, stock solution concentration and reaction conditions in each labeled SSR pairs

Components	Stock concentration	One reaction
Buffer	10 X	1.0 µL
$MgCl_2$	10 mM	0.8 µL
dNTPs	2.5 mM	0.8 µL
Primer forward	5.0 pmoles/µL	1.0 µL
Primer reverse	5.0 pmoles/µL	1.0 µL
Taq polymerase	5 U/µL	0.1 µL
H_2O	Nil	4.3 µL
Genomic DNA	30 ng/µL	1.0 µL
Final Volume		10.0 µL

Capillary electrophoresis

The purified PCR products were screened on capillary electrophoresis using ABI 3730 (Applied Biosystems). The PCR products were co-loaded with an electrophoresis cocktail prepared by pipetting 1.0 ml of HIDI into a 1.5 ml Eppendorf tube and 12.0 ul of LIZ-500 size standard added and mixed by vortexing. A 9.0 µl of the mixture was added into the 96 well-plate followed by addition of 1.2 µl of the PCR products. The cocktail was denatured at 95°C for 3 minutes and quickly chilled on ice for 5 minutes then run on the ABI-3730 PCR machine. DNA fragments were then size-fractioned on capillary electrophoresis using ABI 3730. The GeneMapper v4.1 software was used to size peak patterns, using the internal GenescanTM-500LIZTM size standard and Genotyper 3730 for allele calling. Genotyping was carried out on capillary electrophoresis using the ABI prism 3730. A fluorescent based capillary detection system that uses polymer as the separation matrix facilitated accurate sizing of the microsatellite allele to within ± 0.3 base pairs (Buhariwalla and Crouch, 2004).

Fragment analysis

Size calling which included peak detection and fragment size matching were performed using GeneMapper v4.1 software. Bins which represent a fragment size or base pair range and dye colour that define an allele, were constructed from reference data. Algorithms were used to determine if peaks represented alleles. When a peak from a given data sample matched the location of a bin, the software made an allele call. Alleles were automatically assigned allele calls based on the bin definitions. The results were stored in the GeneMapper v4.1 database.

Data Collection and Analysis

Capillary data capture was done by ABI 3730 (Applied Biosystems), and the resulting fragments analyzed the allele scores using Genemapper V 4.1 software. Any value greater than 1.0 was designated "1" and values less 1.0 were designated "0". This binary coding compared the fluorescent intensity of each fragment and determined the discriminatory power of fluorescent SSR markers for each accession. Measures of genetic variability including total number of common and private alleles, Polymorphic Information Content (PIC), Inbreeding Coefficient (F_{IS}, F_{IT}, F_{ST}), Shannon information index (I) and analysis of molecular variance (AMOVA) were determined using GenAlEx 6.5 software (Peakall and Smouse, 2012). Heterozygosity and number of alleles for each marker, genetic diversity within and among accessions were generated by PowerMarker V 3.25 (Liu and Muse, 2005). Data contained in the electrophenograms were analyzed using GeneMapper v4.1 software. XLstat 2014 software was used in cluster analysis using genetic distance matrices generated by Euclidean distance method (Rousseeuw and Kaufman, 1990) and unweighted pair group method of arithmetic averages (UPGMA) to minimize within cluster variance (Hintze, 2001). Cluster analysis grouped accessions that showed dissimilarity in several traits (Goda *et al*., 2007). GenAlEx 6.5 generated principal coordinates. Cluster analysis and principal coordinate results were graphically presented as dendrograms and scatter bi-plots to infer relationships among accessions.

RESULTS

Quantity and quality of DNA was revealed in 88 accessions DNA samples, with eight DNA samples failing to produce any results. The concentration ranged from 62.7 - 2992 ng/µL with an absorbance ratio ranging from 0.56 - 2.17 of 260/280 nm wavelengths. The absorbance ratio ranged from 1.7 - 2.1 in 67 samples considered to be pure and of good quality. The ratio ranged from 1 - 1.7 in 19 samples, and 0.56 - 0.99 in two samples.

Accession Allelic Analysis Across Fluorescent SSR Markers

The allelic sizes ranged from 181 to 326 bp. The number of genotypes detected by each SSR locus ranged from 4 - 9, with a genotype frequency range of 90 – 96%, and polymorphism 93.8 - 100%. The number of alleles per SSR locus ranged from 3 – 5 alleles, PIC 0.29 - 0.64, observed heterozygosity 0.1684 - 0.9271, and gene diversity 0.3404 - 0.6884 across the five fluorescent SSR markers. A total of 23 alleles and 934 distinctive DNA fragments were detected using the five fluorescent SSR markers (Table 3).

TABLE 3: Characteristics of fluorescent SSR markers indicating major allele frequency, genotype and allele number, gene diversity, observed heterozygosity, polymorphic information content and inbreeding coefficient

SSR marker code	Allele size range (bp)	Major allele frequency	Genotype number	Allele number	Gene diversity	Observed heterozygosity (fixation index /inbreeding	A allele frequency	Polymorphic information content (PIC)	Genotype frequency (GF)	Polymorphism percentage (%)
PKCT-47	205-269	0.7135	8.0	5.0	0.451	0.3854	180	0.41	96	100
PKCT-62	300-326	0.7895	4.0	3.0	0.340	0.1684	190	0.29	95	98.95
PKCT-111	187-210	0.4611	9.0	5.0	0.688	0.5222	180	0.64	90	93.75
PKCT-122	181-225	0.4375	6.0	5.0	0.675	0.9271	192	0.62	96	100
PKCT-133	201-251	0.5729	7.0	5.0	0.590	0.5208	192	0.53	96	100
Mean		0.5949	6.8	4.6	0.5491	0.5048	186.8	0.499	94.6	98.5

Allelic Patterns and Frequency Across Sub-counties

The average number of different alleles was 2.2. Khwisero and Nyeri Central accessions had the highest average number of different alleles. Kakamega Central, Mathira East and Nyeri South accessions had high number of alleles with a frequency of ≥5%. Number of effective alleles was high in Nyeri Central. Shannon's information index was high in Nyeri South and low in Tetu. Private

alleles were found in Butere and Kakamega Central for SSR PKCT-133 and PKCT-111 in accessions KK-3 and KK-56, respectively. The number of locally common alleles ≤25% were found in Kakamega East and South, Khwisero, Mathira East, Nyeri Central and South. The number of locally common alleles ≤50% was high in Kakamega South and Nyeri Central, and low in Butere, Kakamega Central and Mathira West. Expected heterozygosity was high in Nyeri Central and low in Kakamega South (Table 4). Allelic patterns in accessions across subcounties are illustrated in Figure 1. Allele frequency displayed by the fluorescent SSR markers ranged from 0.029 to 0.969. The SSR PKCT-62 and PKCT-47 detected high and low allele frequency in accessions from Kakamega South, respectively (Fig. 2).

TABLE 4: Mean allelic patterns across Subcounties

Sub counties	Na	Na Freq. ≥5%	Ne	I	Private Alleles	L Comm Alleles (≤25%)	L Comm Alleles ≤50%)	He	UHe	F_{IS}
Kakamega Central	3.2	3.2	2.6	1.0	0.2	0	0.2	0.6	0.6	0.2
Kakamega East	3.0	2.2	1.9	0.7	0	0.2	0.4	0.4	0.5	-0.3
Kakamega South	2.8	2.4	1.9	0.7	0	0.2	0.6	0.4	0.5	-0.3
Butere	2.6	2.6	2.3	0.9	0.2	0	0.2	0.6	0.6	0.3
Khwisero	3.4	2.8	2.2	0.9	0	0.2	0.4	0.5	0.5	0.1
Mathira East	3.2	3.2	2.5	1.0	0	0.2	0.4	0.6	0.6	0.2
Mathira West	2.8	2.8	2.5	0.9	0	0	0.2	0.6	0.6	-0.2
Nyeri Central	3.4	3.0	2.7	1.0	0	0.2	0.6	0.6	0.6	0.3
Nyeri South	3.2	3.2	2.1	0.9	0	0.2	0.4	0.5	0.6	0.1
Tetu	2.2	2.2	1.8	0.7	0	0	0.4	0.4	0.5	-0.4

Na = Number of different alleles; **Na Freq. ≥5% =** Number of different alleles with a frequency of ≥5%; **Ne-** Number of effective alleles, **I =** Shannon Diversity index; **Private Alleles =** Number of alleles unique to a single population; **Number of L Comm Alleles (≤25%)**-Number of locally common alleles found in 25% or fewer populations; **Number of L Comm Alleles (≤50%) -** Number of locally common alleles found in 50% or fewer populations; **He =** Expected heterozygosity; **UHe =** Unbiased expected heterozygosity; **F** = fixation index (inbreeding coefficient)

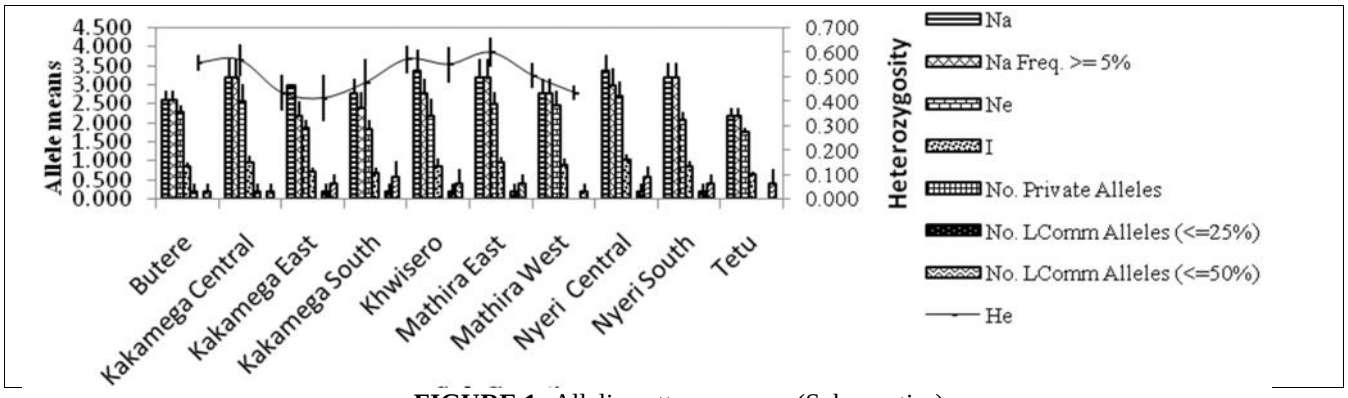

FIGURE 1: Allelic patterns across (Subcounties)

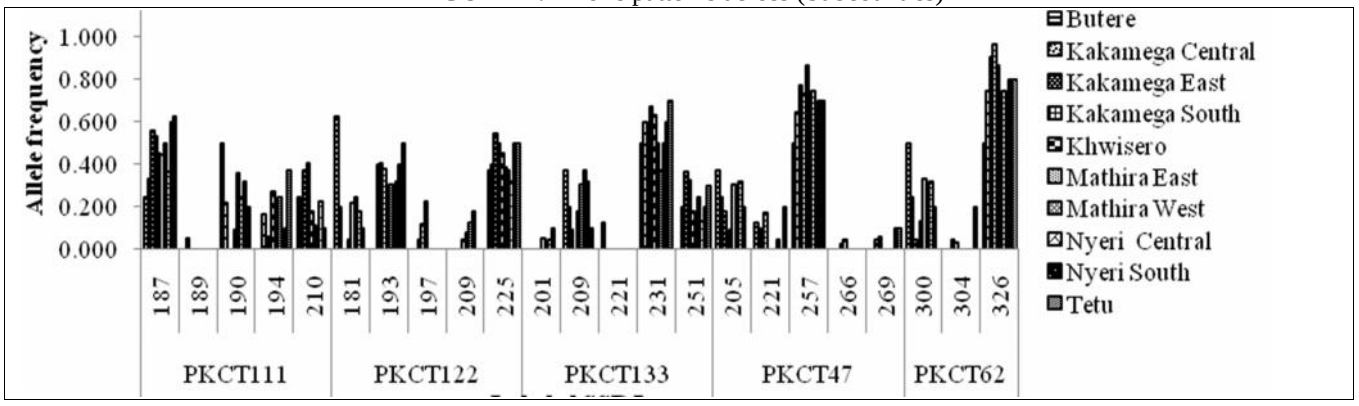

FIGURE 2: Allelic frequency across fluorescent labeled SSR loci

Inbreeding Coefficient Measures (F-statistics)

The F_{IS} values were negative, with a mean of -0.316 within accessions in four subcounties. The F_{IS} values were positive within accessions in six subcounties (Table 4). The mean F_{IS} value of individual accessions was 0.093, F_{IT} value of individual accessions relative to total accessions within subcounties was 0.116 and the mean F_{ST} among subcounty accessions was 0.026 (Table 5). The average $F_{ST,}$ F_{IS} and F_{IT} across the SSR loci was 0.089, -0.025 and 0.111, respectively. The SSR PKCT-122 recorded negative F_{IS} and F_{IT} values (Table 6).

TABLE 5: F-statistics fixation indices and *P*-values for individual and accessions within and among subcounties

F-Statistics	Value	*P*-value
F_{ST}	0.026	0.028
F_{IS}	0.093	0.026
F_{IT}	0.116	0.007

TABLE 6: Genetic variability as estimated by each labeled SSR primer pair

Primer codes	F_{IS}	F_{IT}	F_{ST}
PKCT-111	0.185	0.268	0.101
PKCT-122	-0.448	-0.312	0.094
PKCT-133	0.037	0.101	0.067
PKCT-47	0.018	0.084	0.067
PKCT-62	0.336	0.414	0.118
Mean	-0.025	0.111	0.089

F_{IS} = inbreeding coefficient of individuals within sub populations; F_{IT} = inbreeding coefficient of individuals in relation to total sub populations; F_{ST} = inbreeding coefficient in relation to total population

Analysis of Molecular Variance (AMOVA)

Table 7 shows data for individual accessions and subcounties of Kakamega and Nyeri across five fluorescent SSR loci. AMOVA revealed that molecular diversity was 88% within individual accessions as opposed to 9% among individuals within subcounties and 3% in accessions among subcounties. There were significant (P<0.026) differences in accessions in subcounties, but there were no significant differences among accessions within subcounties and individual accessions (P>0.093 and P>0.116), respectively (Table 7).

TABLE 7: Analysis of molecular variance for fluorescent SSR data across Sub counties

Source of variation	df	SS	MS	Estimated variation	Variation	*P*-value
Among subcounties	9	19.594	2.177	0.036	3%	0.028
Within subcounties	86	129.036	1.500	0.128	9%	0.026
Individual accessions	96	119.500	1.245	1.245	88%	0.007
Total	191	268.130		1.409	100%	

Co-dominance of SSR markers

The fluorescent SSR markers displayed their co-dominance for the accessions characterized. A single peak denoted homozygous genotypes while two peaks indicated heterozygous genotypes (Figure 3).

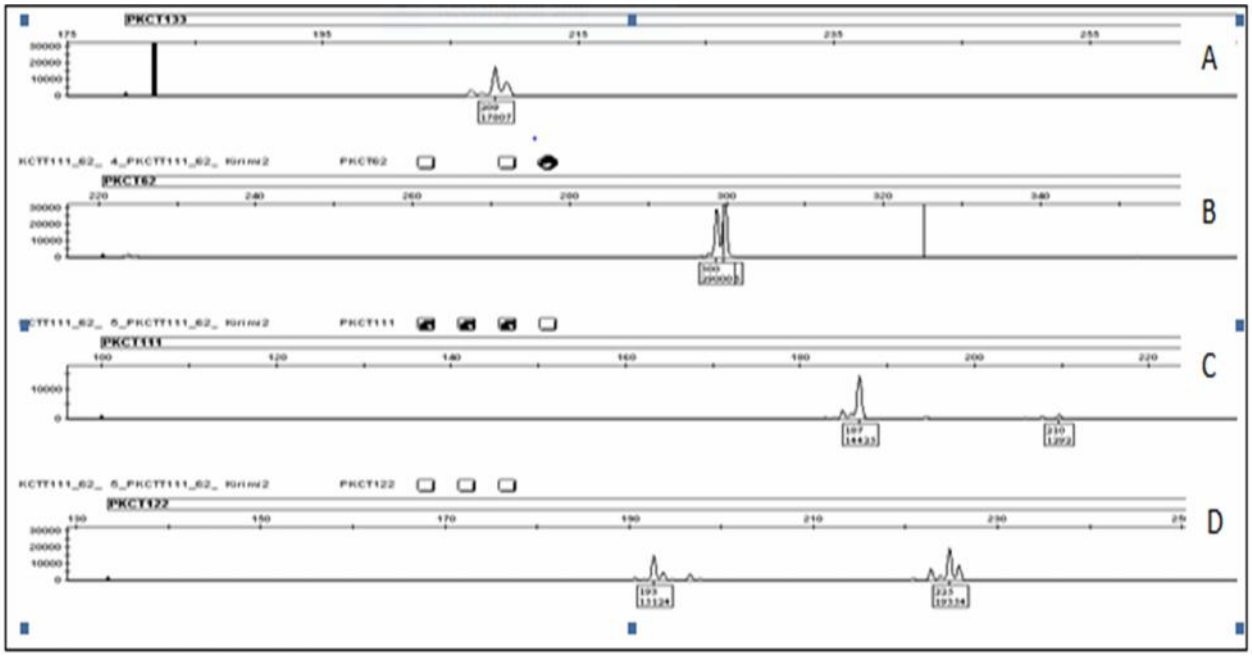

FIGURE 3: Electrophenogram displaying homozygous (A; B) and heterozygous (C; D) nature of SSR PKCT-133, 62, 111 and 122, respectively. The x and y-axis represent allele sizes and peak intensities, respectively.

Inter-population Genetic Variability

A principal coordinate analysis plot for the first two coordinates derived from fluorescent SSR on capillary electrophoresis data displayed multidimensional relationship among 96 accessions. Three components resulted in a cumulative of 71.9%. The first two principal coordinates accounted for 64.4% of the total variation among accessions within subcounties. The first coordinate accounted for 55% and the second 9.4% of the total variation (Figure 4). A third component contributed 7.6%

to the total variation. A significant overlap was noted for most of the subcounties with accessions from Mathira East being dispersed widely in the plot (Figure 4).

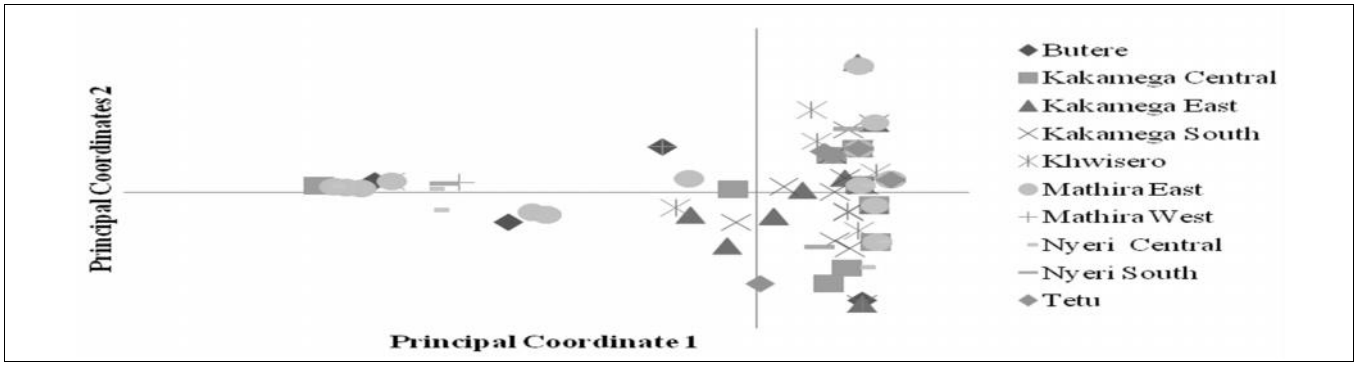

FIGURE 4: A graphical representation of the principal components analysis percentage of variation explained by the first 3 axes in the 10 sub counties

Genetic Diversity and Relationships among Accessions

Cluster analysis identified 17 clusters from 90 accessions. Six accessions with missing data were not included. The Euclidean dissimilarity coefficient ranged from 0 - 6.6 based on UPGMA (Figure 5). The largest cluster grouped 33 and the second largest 23 accessions. Seven clusters grouped accessions in simplicifolious, 2 in bifolious, 2 in trifolius, 3 in quadrufolious and 1 cluster in pentafolious. The cluster variance ranged from 0 - 441.5. The variance within clusters was 18.2%, and between clusters 81.8%. There was no variance for accessions clustered in simplicifolious and in quadrufolious. The Euclidean distance coefficient dissimilarity was high at 5.5 in accession KK-26, and low at 0.5 in KK-3, KK-32 and NY-129, respectively (Figure 5). The accessions were not clustered according to their geographical origin.

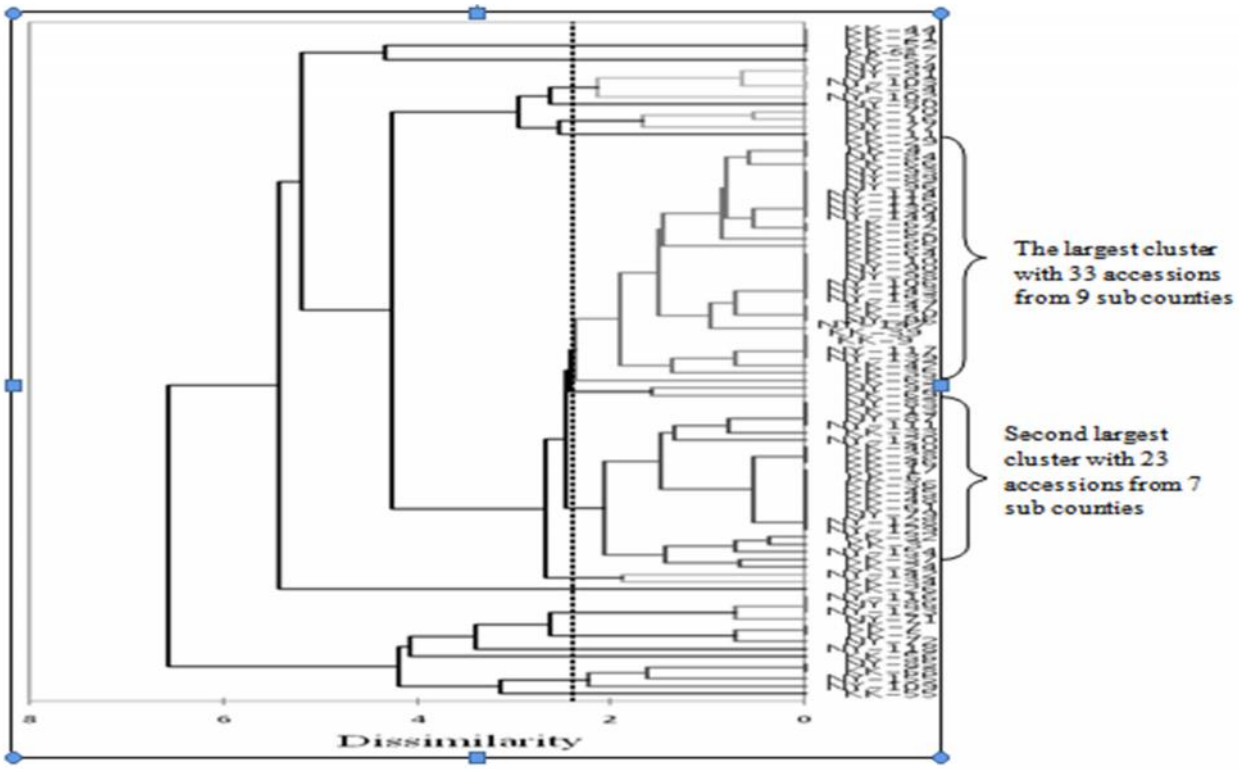

FIGURE 5: UPGMA cluster analysis showing the relationship and diversity among 90 pumpkin accessions produced by fluorescent labeled SSR markers on capillary electrophoresis data

DISCUSSION

Effect of Quantity and Quality of DNA on Genetic Diversity Analysis

In the present study, the quantity of extracted DNA varied among accessions. High molecular weight and too much DNA interfere with the enzymatic reactions, results in false priming and poor DNA synthesis due to obstructed diffusion of large *Taq* polymerase molecules. Extremely small DNA is often lost through clotting, adsorption, chemical or enzymatic degradation and contamination with impurities that come into contact with it (Sahu *et al*., 2012). In the present study, most of the accessions had high quality DNA with an absorbance ratio ranging from 1.7 to 2.0. Contaminants such as polysaccharides inhibit restriction, enzymatic and *Taq* polymerase activity by co-precipitating with DNA, giving a viscous glue-like

appearance (Healey *et al.,* 2014). The oxidized form of polyphenols covalently binds to DNA giving a brown colour and reduces maintenance time making DNA useless for molecular studies (Sahu *et al*., 2012). Phenolics such as terpenoids and tannins undergo rapid oxidation upon release from leaf tissue and irreversibly bind to the phosphate backbone of DNA, causing browning of leaf material. These contaminants prevent use of DNA for PCR procedure by inhibiting the action of polymerases (Healey *et al.,* 2014).

Fluorescent SSR Markers and Capillary Electrophoresis Allelic Pattern Analysis

Genetic diversity is measured in terms of allelic (average number of alleles per locus) and gene diversity (heterozygosity across loci), or nucleotide differences within a species (Gaston, 2010). The size of alleles in base pairs (bp) detected across the five fluorescent SSR markers showed differences among accessions. The differences in size of alleles suggested distant relationships among accessions. Accessions with different size of alleles were more distantly related than alleles of similar size (Hoshino *et al*., 2012). The differences in allelic size occur due to variable number of repeat units within the SSRs (Semagn *et al.,* 2006). The SSRs have motifs of 1-6 nucleotides repeated several times with a characteristic mutational behaviour (Guichoux *et al*., 2011). Mutational process causes changes to the number of repeats and allele size (Hardy *et al*., 2003). Fluorescent SSR markers can detect alleles with as little as 2 bp size difference (Wang *et al*., 2009), which makes them effective in cultivar identification. Capillary electrophoresis reduces sizing error, achieves better fragment separation, greater accuracy and consistent allele sizing (Vemireddy *et al*., 2007; Wang *et al*., 2009).

The number, mean, and total alleles were high across the five fluorescent SSR markers. The composition of alleles determines the limit to selection (Neel and Cummings, 2003). Stift *et al*. (2004) reported 2 to 6 alleles per locus with an average of 4.4, using SSR pairs on a set of *Cucurbita* genotypes. Mao *et al*. (2014) reported a total of 53 alleles in *C. moschata* cultivars detected by EST SSR pairs. Allele and genotype frequencies were high across the markers. These showed that the accessions were dynamic and changing. Allele frequencies show the genetic diversity of a species population or the richness of its gene pool. In the absence of selection, mutation and migration in a large random-mating population, the frequencies of alleles and genotypes remain constant from generation to generation. The present results agreed with the concept of Hardy-Weinberg equilibrium, which serves as a baseline from which populations can be compared to see if any evolutionary processes are occurring. It determines whether genotypes in a given population are in equilibrium or if any of the assumptions are causing allele and genotype frequencies to shift with each successive generation (Onovo, 2009).

The high observed heterozygosity and genetic diversity revealed across fluorescent SSR markers was attributed to SSR co-dominance or multi-allelic nature, hyper-variability, high information content and amenability to automation (Noormohammadi *et al*., 2012). Genetic diversity analysis requires high resolution fragment separation and great accuracy (Wang *et al*., 2009). In the present study, three SSR loci had PIC values exceeding 0.5, hence highly polymorphic. Gong *et al*. (2008) reported an average PIC value of 0.62 in *C. pepo* L. genotypes. Katzir *et al.* (1996) reported PIC values between 0.49 and 0.75 in melon, and 0.18-0.64 in cucumber for each SSR. The SSR PKCT-111 marker showed the greatest genetic diversity of accessions. The marker was considered the most informative, while SSR PKCT-62 marker was less informative. PIC describes genetic diversity within accessions and characterizes the degree of polymorphism for each SSR locus. PIC values less than 0.25 indicate low polymorphism, values between 0.25 and 0.5 indicate average polymorphism, and values higher than 0.5 indicate high polymorphic locus (Liu *et al*., 2007). The level of polymorphism created using the five fluorescent SSR markers was high at 98.5%, which showed that the markers effectively detected genetic diversity of pumpkins (Jamalirad *et al*., 2012). Ntuli *et al. (*2015) reported average polymorphism of 67.86% on *C. pepo,* and a range from 55.6% to 88.9% among watermelon landraces using SSR markers. Polymorphism is produced by variations in the number of tandem repeats within DNA microsatellites at certain loci (Zhou *et al*., 2009). Different markers differ in the mechanism of detecting polymorphism, but they could complement each other to draw more accurate conclusions (Adawy *et al*., 2005).

Allelic Pattern Distribution across Subcounties

The high average number of different alleles in Khwisero and Nyeri Central, and frequency of ≥5% in Kakamega Central, Mathira East and Nyeri South suggested high genetic variation within accessions in these subcounties. The number and frequency contributes to the variation, survival and reproduction. The low number and frequency of different alleles with a frequency of ≥5% in Tetu and Kakamega East accessions can result in decrease of accession survival and adaptation to changing environment. The number of different alleles is sensitive to differences in size and number of populations and is affected as populations decline in size or extirpate (Neel and Cummings, 2003). The accessions from Nyeri Central had the highest number of effective alleles, which indicates the variation in allele frequency and detects overestimates of marker polymorphism. In the present study, the number of effective alleles was less than the actual number of alleles in all subcounty accessions which indicated unequal allele frequencies. If the effective number of alleles is much smaller than the observed number of alleles, then the actual allele frequencies are unequal and have large variations. If they are larger than the observed number of alleles, then the observed number of alleles overestimates the marker polymorphism because it exceeds the maximum expected polymorphism (VanRaden *et al*., 1999).

Shannon information index was high among accessions in Kakamega Central, Mathira East and Nyeri Central. The index ranges from 0 to 1. It tends to 1 when populations have equal proportion or a high number of populations are present. A low index means a population is dominated by one genotype (Ramezani, 2012). The index showed that genetic diversity of accessions within all subcounties existed. Private alleles were detected in accessions KK-3 and KK-56 for SSR PKCT-133 and PKCT-111 markers.

Esteras *et al.* (2008) reported two unique alleles in *C. pepo* that were not present in any other accession. Arias *et al.* (2011) reported a large number of markers showing unique alleles in isolates collected from pumpkin. The number of private (unique) alleles measures genetic distinctiveness (Kalinowski, 2004). The alleles are found only in a single population among a broader collection of populations, and they indicate gene flow and the mean number of germplasm exchanged between populations (Szpiech and Rosenberg, 2011). Occurrence of private alleles presents a good source of genetic variability (Kimani *et al*., 2014). Members of the cucurbitaceae have several unique traits which include a lianous structure of the plant body, development of fleshy fruits, and a mode of sex determination that are not found in other plants (Arias *et al.,* 2011). Elucidation of unique traits contributes knowledge and allows application to improve cucurbitaceae plants (Ezura and Fukino, 2009).

Kakamega Central, Butere, Mathira West and Tetu accessions had no locally common alleles found in 25% or fewer populations. These could only mean that the accessions were cultivated for a very short period of time or the accessions were introductions from other places. Locally common alleles found in 50% or fewer populations were found in accessions within all subcounties. The accessions that had no locally common alleles in 25% or fewer populations, recorded low, whereas those that had low locally common alleles in 25% or fewer populations, recorded high locally common alleles in 50% or fewer populations, respectively. Locally common alleles are frequent only in one to several populations (Maguire *et al.,* 2002). They occur in high frequency in a limited area and indicate a long history of selection and local adaptation to specific environment (Frankel *et al.,* 1995; Van Zonneveld *et al*., 2012). Accessions with high locally common alleles should be given a priority when conservation measures are undertaken (Van Zonneveld *et al*., 2012). Although accessions within Kakamega Central and Butere had low locally common alleles found in 25% and 50% or fewer populations, the allelic composition in accessions KK-3 and KK-56 was different due to private alleles. The expected and unbiased expected heterozygosity were high among accessions within the Kakamega Central and Butere subcounties. Thus, to maintain the unique composition and genetic diversity, accessions within the two subcounties should be conserved (Jalonen *et al*. 2012). The expected and unbiased expected heterozygosity were high among accessions from Kakamega Central, Mathira East and west, Nyeri Central and South and Butere, and were low among accessions from Kakamega East and South, Khwisero and Tetu Subcounties. The Subcounties located near major towns had high expected and unbiased expected heterozygosity because of nearness of farmers to major towns. The farmers easily accessed seeds of new cultivars from the towns and markets nearby which they introduced into their farms. Pumpkin being a monoecious plant, cross-pollinated by honeybees gets new genes through pollen grains from flowers of new cultivars introduced, resulting in increase in heterozygosity of local accessions. Farmers intercropped the local landraces with introduced cultivars on the same farm making gene exchange easy. The expected and unbiased expected heterozygosity was low in Subcounties that were far from major towns, because farmers deliberately selected pumpkin landraces for specific traits and recycled pumpkin seeds every season with little or no new seed introduction (Balkanya, 2009).

Inbreeding within and among Accessions

The inbreeding coefficient value for F_{IS} was negative with a mean of -0.316 among accessions within Kakamega East and South, Mathira West and Tetu subcounties. The F_{IS} values measures the extent of inbreeding of individual accessions. It ranges from -1.0 (all heterozygous) to +1.0 (no heterozygote's) (Zaki *et al.*, 2012). Thus, the negative F_{IS} value observed in the study reflected heterozygosity and genetic variability of individual accessions within the four subcounties. This was attributed to outcrossing between local and exotic green-leafed cultivars intercropped by farmers in the four Subcounties. The inbreeding coefficient value for F_{IS} was positive among accessions within Kakamega Central, Butere, Khwisero, Mathira East, Nyeri Central and Tetu Subcounties. The positive F_{IS} value in the six Subcounties was attributed to continuous selection and recycling of pumpkin seeds exercised by farmers (Ghebru *et al*., 2002). The F_{IS} value can be very useful to the aspiring germplasm collectors because it reflects the differences in the prospecting areas for germplasm collection (Zaki *et al.,* 2012). The inbreeding coefficient (F_{IT}) of individual accessions with regard to the total accessions was estimated at a mean of 0.116, meaning that only 11.6% of the total genetic variation was explained by differences in individual accessions and most of the genetic diversity of 88.4 % corresponded to differences of all accessions among subcounties. The high genetic variation was attributed to the natural cross-pollination but self-compatibility of *C. moschata*. Inbreeding causes little loss of vigour and a considerable degree of robustness in given traits (Grubben and Chigumira, 2004).

The mean inbreeding coefficient value F_{ST} was 0.026, meaning that only 2.6% of the total genetic variation was explained by differences among accessions within subcounties, and most of the genetic diversity of 97.4 % corresponded to the differences of accessions among subcounties (Barbarosa *et al.*, 2014). F_{ST} value gives the proportion of the total genetic diversity or heterozygosity that is distributed to accessions among subcounties (Gamar *et al.,* 2013). It is greater than or equal to 0 if all individuals within sub-populations mate completely randomly with each other and have constant allele frequencies. F_{ST} value range of 0.0 to 0.05 indicates little, 0.05 to 0.15 moderate, 0.15 to 0.25 great and above 0.25 very great genetic differentiation (Gamar *et al.,* 2013). The low F_{ST} value in the present study showed little genetic differentiation among accessions in the subcounties. This was attributed to practice by farmers for selecting and recycling seeds every season over the years. The practice reduces the effective population size and increases the opportunity for fixation of alleles (Ghebru *et al*., 2002). Farmers maintain more than one distinct landrace as varieties which are carried over from one generation to the next (Kimani *et al*., 2014). They fix favourable genotypes of interest, separate favourable genotypes, and reduce the

percentage of heterozygotes unknowingly through inbreeding. Grisales *et al.* (2009) reported that inbreeding reduces cultivar fitness in the long-run, increases genetic variance between families, and reduces it within families, with a progressive increase in the additive variance at the expense of dominance in totally homozygote lines.

The mean inbreeding coefficient value F_{ST} was 0.089 across the five fluorescent SSR markers, meaning that the total genetic variation explained by differences of the five fluorescent SSR markers was 8.9%, and most of the genetic diversity of 91.1 % corresponded to differences in each marker. The F_{ST} value across SSR loci indicated little genetic differentiation among accessions. The mean inbreeding coefficient value F_{IS} was -0.025 across the five fluorescent SSR markers. The negative F_{IS} value reflected heterozygosity and genetic variability of individual accessions. The inbreeding coefficient (F_{IT}) was 0.111 across the five fluorescent SSR markers, meaning that only 11.1% of the total genetic variation of individual accessions was explained by differences of the five fluorescent SSR markers, and most of the genetic diversity of 88.4 % corresponded to differences of accessions explained by each fluorescent SSR marker. The mean F_{IS} and F_{IT} values across the markers were low which reflected low differentiation and genetic variability of individual accessions. Fluorescent SSR PKCT-122 marker had negative F_{IS} and F_{IT} values, and high heterozygosity. These findings confirm earlier ones in the present study using non-labeled SSR markers on agarose gel electrophoresis, where this marker detected the most heterozygote alleles. Watcharawongpaiboon and Chunwongse (2007) reported polymorphisms on all pumpkin hybrid sets tested with SSR PKCT-122. Thus the marker is good for detecting heterozygotes in pumpkin cultivars.

Genetic Variation within and among Accessions

In the present study, partitioning of genetic diversity by AMOVA revealed that most of the variation existed within individual accessions. This was attributed to the fact that *C. moschata* is allogamous and monoecious. The staminate flowers are precocious and the pistillate flowers emerge later post-planting. This habit ensures cross pollination by bees, which reduces to a minimum the possibility of endogamy (Grisales *et al.*, 2009). In most cases, farmers occasionally maintain more than one distinct landrace as varieties which are carried over from one generation to the next (Kimani *et al*., 2014). The low variation of individual accessions relative to all accessions among subcounties was attributed to the drift in small populations, which farmers initially select from field to be used as seeds for the coming season (Gamar *et al*., 2013). The low divergence among subcounties was attributed to the traditional farming systems with agronomic, economic and cultural considerations, and also due to transit of pumpkin germplasm via human migration coupled with seed trade within subcounties (Kimani *et al.*, 2014).

Genetic relationships among accessions

Principal coordinate analysis showed genetic variability between and within accessions, with a higher genetic diversity of individual accessions within subcounties than of accessions among subcounties. However, there were groups of individual accessions presenting genetic similarities between them. These results agree with F_{IS} and F_{IT} values which showed high genetic diversity in individual accessions within subcounties. Cluster analysis indicated the level of dissimilarity among accessions was high. Genera that clustered together were a basis of a possible introgression between the genera in the field where many pumpkin varieties were normally intercropped (Xolisa, 2002). The varying lengths in the dendrogram branches showed genetic differences existed among accessions. The high variance between clusters was attributed to gene flow enhanced by seed exchanges between farmers from different subcounties (Ntuli *et al*., 2015).

CONCLUSION AND RECOMMENDATIONS

The accessions were not clustered on the basis of their geographic origin but on their genetic diversity. The high variation within accessions showed that there is considerable amount of genetic diversity among pumpkins in Kenya that could be used to modify and improve the local landraces. The high genetic variations revealed by fluorescent SSR markers and capillary electrophoresis showed their usefulness in genetic diversity studies of pumpkins. The inbreeding coefficient (F_{IS}) values could be useful when planning germplasm collection because they reflect the differences for the prospecting areas for germplasm collection. The F_{IS} will enable germplasm collectors to sample many accessions from many agro-ecological zones to effectively capture genetic variation in future collections before the existing diversity is lost. Conservation of genetic diversity within the local landraces needs to be expedited to ensure survival and adaption to changing environmental conditions.

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