MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF PREFERRED KENYAN MULTI-PURPOSE PUMPKIN (*Cucurbita moschata* DUCH.) CULTIVARS

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ABSTRACT
Pumpkin (*C. moschata*) is the most grown species, with a wide range of variability. Determining the degree of variability is the preliminary step in studying their genetic diversity. The objective of the present study was to characterize genotypically, compare the results with phenotypic data to establish correlations between their distances by classifying the accessions based on their dissimilarity. DNA extraction, polymerase chain reaction and Agarose Gel Electrophoresis (AGE) were done on 139 accessions using SSR and ISSR primers. Fluorescent capillary electrophoresis (CE) genotyping with labeled SSR was done on DNA samples of 96 selected accessions. Morphological characterization was done on-farm in a complete randomized design, replicated three times. Morphological data was subjected to analysis of variance using SAS. Means were separated at P=0.05. Chi square test (P=0.05) separated qualitative data. Unweighted Pair Group Method of arithmetic mean and Euclidean Genetic Distance constructed dendrograms using molecular and morphological data with XLstat. DNA quantity ranged from 70.02-2992 ng/µl and quality from 0.56-2.1 of 260/280 absorbance ratio. Molecular characterization with AGE revealed variations among accessions. Amplifications ranged 100-500 and 200-2000 bp, PIC 0.5 and 0.597, alleles number 526 and 509, polymorphism 21.3% and 74.01% in SSR and ISSR, respectively. CE revealed 23 alleles with a range of 181-326 bp. CE genotyping amplified 934 distinctive SSR DNA fragments. Mean PIC was 0.49, observed heterozygosity 0.5048, genotype number 6.8, gene diversity 0.5491 across the markers. Fluorescent SSRs had 98.54% mean polymorphism. CE revealed two unique alleles. Significant variations (P<0.05) resulted among 146 accessions morphologically with fruit ribbing being not significant. PCA provided 9 and 13 PCs for quantitative and qualitative data, respectively. Quantitative characters explained 82.37%, qualitative 71.54%, of total variation. Both morphological and molecular data revealed genetic diversity among accessions. The variation in Kenyan pumpkins is increasing hence there is need to conserve them to prevent genetic erosion through crossbreeding with exotic ones.
Keywords: Capillary, Dendrograms, Electrophoresis, Molecular, Morphological

INTRODUCTION
Pumpkin (*C. moschata*) has wide range of variability (Naik et al., 2015). Determining the degree of variability is the preliminary step in studying the genetic diversity (Ferriol et al., 2001). Characterization helps identify; provide basic information for classification and diagnostic features used in assessing relationships (Radford, 1986). Morphological characterization reflects variation of expressed regions of genome, and morphological characters as influenced by environment and plant development stage (Mladenovic et al., 2014). Molecular markers support detailed characterization of genetic diversity (El-Assal and Gaber, 2012), and they have the advantage that the DNA content of a cell is independent of environmental conditions, organ specificity and growth stage (Khanam et al*.,* 2012). Molecular genotyping takes advantage of variation in highly polymorphic genes (Gupta et al., 2010). Molecular characterization show higher levels of polymorphism indicating variation of all genome including expressed and non-expressed regions. Thus, morphological characterization should be complemented with molecular markers to achieve a reliable characterization of species diversity (Escribano et al., 1997; Ferriol et al., 2004). Different molecular markers reveal different classes of variation (El-Assal and Gaber, 2012). Microsatellites or Simple Sequence Repeats (SSRs) are di-, tri- or tetra- nucleotide repeats, show high level of polymorphism and average level of heterozygosity (Khanam, et al*.,* 2012), are useful for cultivar identification (Watcharawongpaiboon. and Chunwongse, 2007), and are co-dominantly inherited, allowing heterozygote in diploid genomes to be distinguished (Serra et al*.,* 2007). Inter Simple Sequence Repeats (ISSRs) are designed from SSR motifs, and are widely used in the analysis of genetic diversity and also in cultivar identification (Domyati et al., 2011, Behera et al., 2008). They detect genetic polymorphisms by generating large number of markers targeting multiple microsatellite loci distributed across the genome (Behera et al., 2008; Behera et al., 2012). They are dominant as the presence of homozygous fragment is not distinguishable from its heterozygote (Serra et al., 2007). Agarose gel electrophoresis is used in SSRs, ISSRs etc analyses (Wang et al., 2009), to discriminate, the sizes of

amplified products of genes based on migration patterns. Gel electrophoresis may not provide adequate discrimination of alleles (Gupta et al., 2010). They are not always amenable for accurately calculating the sizes of alleles and recording of data in an electronic format, which make downstream analysis problematic (Wang et al., 2009). Capillary Electrophoresis (CE) is widely used in SSR analyses because it increases test sensitivity and discriminatory power. DNA analyses with CE provide automated and accurate estimates of allele sizes. CE in combination with fluorescently labeled SSR primers provide high detection sensitivity of amplified DNA fragments (Wang et al., 2009), it has the ability to measure the size of Polymerase Chain Reaction (PCR) products with very high resolution (Gupta et al., 2010).

Statement of the Problem

Agricultural production has lost most of its genetic diversity; hence agricultural biodiversity in Kenya is under serious threat (Ekesa et al., 2009). African Indigenous Vegetables (AIVs) are threatened with extinction (Keding et al., 2007), because they are being replaced by modern varieties (Weinberger and Msuya, 2004). Limited information per species is available. Limited local surveys have been conducted to collect valuable information, occasioning pumpkin landraces to remain under-exploited and poorly documented (Hamisy et al., 2002). In Kenya, improvement of pumpkin is constrained by lack of characterization and selection for desirable traits. Consequently, naturalized pumpkins are threatened with extinction and erosion through cross pollination with introduced exotic ones (Adebooye et al., 2003). 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. The major constraints facing pumpkin production in Kenya include lack of information and documentation of the priority landraces, among others (Maundu et al., 1999; Onyango, 2002b). The study aimed at solving some of the constraints by characterizing the preferred pumpkins cultivars genotypically to provide valuable information on the cultivars, assessing their genetic diversity and relationships using molecular markers (SSR and ISSR), and genotype the DNA content of selected accessions using CE with fluorescence labeled SSR markers, and compare the results with morphological data, and classify the accessions into dissimilar groups using molecular and morphological data.

Research Justification

Pumpkins can be stored for up to 6 months, play an important role in poverty alleviation and maintenance of nutritional levels during long dry seasons, when other vegetables are not available. Primary agricultural production has neglected AIVs (Nyangito, 1998). Kenya is constantly confronted with food shortfalls (Ekesa et al., 2009). Food insecurity is a real issue due to recurrent seasons of failed or poor rains and sustained high food prices (Republic of Kenya, 2001). Pumpkins play a significant role in food security of the underprivileged (Weinberger and Msuya, 2004), and can contribute to reversing the trends, because they have a considerable potential as income earners (Onyango, 2002a), for the smallholders who account for over 65% of the total agricultural output. Enhanced knowledge of pumpkins could play a pivotal role in food and nutrition security (Schippers, 2000; Onyango, 2002a). Characterization and identification of accessions will provide breeders with considerable information concerning their value in production of new improved cultivars. Improvement, Conservation, utilization and cultivation will save local pumpkin from erosion and/or extinction for the benefit of future generations (Chweya, 1997).

General Objective

The general objective was to determine whether there is enetic erosion in preferred multi-purpose pumpkin (*Cucurbita moschata*) accessions from smallholder farmers in Kenya by genotypically characterizing and comparing the results with phenotypic characterization results.

Specific objectives

(i) Characterize genotypically pumpkin germplasm from Kakamega and Nyeri regions in Kenya.
(ii) Compare genotypic results with phenotypic characterization data
(iii) Classify accessions into dissimilar groups based on molecular and morphological data

Expected Outputs

(i) Multi-purpose pumpkin germplasm from Kakamega and Nyeri regions in Kenya genotypically characterized and the results compared with phenotypic data
(ii) Accessions grouped into dissimilar groups based on molecular and morphological data
(iii) Pumpkin characterization information documented for future improvement of local pumpkins

Beneficiaries of the outputs and outcomes

(i) Extension officer's capacity will be enhanced by availing pumpkin information for use in promotion of production and conservation.
(ii) Biotechnologists and future researchers will utilize the characterization information to make, modify, improve or develop pumpkins for specific uses.

MATERIALS AND METHODS

DNA Extraction and Quantification

Germplasm from Kakamega and Nyeri regions of Kenya, were planted at the Chuka University (CU), Ndagani research farm to provide material for morphological and molecular characterization. Molecular characterization was done at the Jomo Kenyatta University of Agriculture and Technology (JKUAT), Institute Biotechnology Research (IBR) and International Livestock Research Institute (ILRI) laboratories. DNA was extracted using the method described by Doyle and Doyle, (1987) and quantified using the NanoDrop-1000 spectrophotometer (Beauman, 2007). CTAB DNA Extraction Protocol was used for DNA extraction. The presence of DNA strands from the sample extract was confirmed with agarose gel electrophoresis (AGE). The gels were visualized with UV light and then photographed with a photo documentation camera. DNA samples were labeled and stored in a tightly sealed eppendorf tubes at 4^0c for Polymerase Chain Reaction (PCR) amplification with SSR and ISSR primers in AGE, for further concentration and quality determination with Optical Density (OD) reading on NanoDrop (ND-8000) spectrophotometer (ND Technologies, Inc., Wilmington, DE) following the manufacturer's instructions, and genotyping with capillary electrophoresis at ILRI research labs.

Agarose Gel Electrophoresis

PCR amplifications were performed with 5 SSR pairs and 7 ISSRs with 2 accessions to optimize the amplification protocol and test the performance of primers,. Fluorescence labeled SSRs were optimized by running different ratios of PCR products and then choosing the one giving the best signal profile i.e. signal/noise ratio and Relative Fluorescent Units (RFU). There after DNA of 139 accessions was subjected to PCR with SSR and ISSR primers with a Gene-Amp PCR system 9700 (Applied Biosystems). PCR reactions were performed with a stock solution containing 30ng/µl of DNA, 10X PCR buffer, 2.5 mM dNTP, 10 mM $MgCl_2$, 5 U/µl of Taq polymerase, and 5.0 pmoles /µl each of forward and reverse SSR pairs, and 5.0 pmoles /µl of ISSR. The final volume (10 µL) of PCR mixtures for each SSR and ISSR reaction contained 0.5µl of DNA, 0.5 µl PCR buffer, 0.4 µl dNTP, 0.4 µl $MgCl_2$, 4.2 µl of Taq polymerase, 0.5µl each of forward and reverse SSR pairs and ISSR, 3.0µl H_2O for SSR, 3.5µl H_2O for ISSR. Thermo cycling reactions were programmed in an initial denaturing at 94°C for 3 minutes, 30 (SSR) and 35 (ISSR) cycles of 30 seconds at 94°C, 1minute for 55°C (SSR) and 47°C (ISSR) , 2 minutes at 72°C, elongation at 72°C for 20 minutes and a final hold at 4°C for SSR and ISSR respectively. The PCR products were loaded onto 1.5% (W/V) agarose gel stained with 1 ug/ul ethidium bromide, run at 100 v. for 60 minutes and photographed under U.V. light trans-illuminator. Allele scoring for presence (1) and absence (0) was conducted; sizes of the amplified fragments were estimated using 1.5% agarose gel 5 ul per lane, 1 Kb DNA ladder (Bioline).

Capillary Electrophoresis

Quality and quantity of DNA from 96 selected accessions was checked before embarking on a genetic diversity analysis. Concentration of DNA in ng/µl was measured using NanoDrop spectrophotometer (ND-8000; USA). DNA purity was calculated at 260/280 nm wavelengths. The realized concentrations

guided the normalization of each DNA sample to a concentration of 30 ng/µl. Five SSR pairs Five fluorescently labeled with 6-FAM (PKCT-47), VIC (PKCT-62), NED (PKCT-111), PET (PKCT-122) and 6-FAM (PKCT-133) were used to amplify DNA of 96 selected accessions, for screening by CE on ABI prism and 3730 genetic analyzer (Applied Biosystems). PCR amplifications were performed with a Gene-Amp PCR system 9700 (Applied Biosystems). PCR reactions stock solution contained 30 ng/µl of DNA, 10X PCR buffer, 2.5 mM dNTP, 10 mM $MgCl_2$, 5 U/µl of Taq polymerase, and 5.0 pmoles /µl each of forward and reverse SSR pairs. The reaction per PCR cycle was 1µl of DNA, 1 µl PCR buffer, 0.8 µl dNTP, 0.8 µl $MgCl_2$, and 1µl each of forward and reverse SSRs, 0.1 µl of Taq polymerase and 4.3 µl H_2O with a final volume of 10 µl. The micro-tubes were placed in a thermal cycler (a Gene-Amp PCR system 9700 (Applied Biosystems) and the thermo cycling reactions programmed in an initial denaturing at 94°C for 3 minutes, 30 cycles of 30 seconds at 94°C, 1minute for 55°C, and 2 minutes at 72°C, elongation at 72°C for 20 minutes and a final hold at 4°C.

Purification of PCR products

PCR products were purified with Qiagen kit (QIAquick PCR purification kit) to remove any remaining dNTPs, primers, Taq, and Mg ion. Protocol used was as described in the QIAquick ® Spin Handbook of November, 2006. Purified PCR products were co-loaded with an electrophoresis cocktail prepared by pipetting 1.0 ml of HIDI into a 1.5ml eppendorf tube and 12.0 ul of LIZ-500 size standard added and mixed by vortexing. A 9.0 µl 0f the mixture was added into required number of wells of 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 in ice for 5 minutes then run on the ABI-3730 PCR machine. The DNA fragments were then size-fractioned using capillary electrophoresis on the ABI 3730. The GeneMapper v4.1 software (Applied Biosystems) was used to size peak patterns, using the internal GenescanTM-500LIZTM size standard and Genotyper 3730 for allele calling. Genotyping was done by capillary electrophoresis using the ABI prism 3730 (Applied Biosystems), 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

Amplified fragments were analyzed using GeneMapper v4.1 software (Applied Biosystems). Size calling, which include peak detection and fragment size matching were performed using GeneMapper v4.1software. Bins, which represent a fragment size or base pair range and dye color 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.

Morphological Characterization Data

Morphological data for comparison with molecular results was recorded from accessions planted on 23 may, 2012 at CU research farm. The data was based on IPGRI descriptors for Cucurbits. Measured characters were vegetative, stem, root, inflorescence, fruit and seed characters.

Data collection and analysis

Data generated by agarose gel electrophoresis was scored as presence (1) or absence (0) of SSR and ISSR bands. Polymorphism (%) and Polymorphic Information Content (PIC) were calculated from the data matrix. CE data was captured by genescan software (Applied biosytems). The resulting fragments of the alleles were scored with Genemapper software V 4.1 (Applied biosystems) to determine similarities and differences between the accessions. Any value greater than 1.0 was designated “1” and values less 1.0 were designated “0”. Total number alleles, number of common and private (Unique) alleles, PIC, Inbreeding Coefficient (F_{ST}), HS, and Analysis of Molecular Variance (AMOVA) were determined for

each SSR marker 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 Power Marker V 3.25 (Liu and Muse, 2005). Data contained in the electropherograms were analyzed by GeneMapper V 4.1 software (Applied Biosystems). To group the accessions based on molecular and morphological dissimilarity, cluster analysis was conducted on the Euclidean distance matrix (Rousseeuw and Kaufman, 1990), with unweighted pair group method based on arithmetic averages (UPGMA) (Hintze, 2001) using Xlstat. To compare molecular results with morphological data, analysis of variance was performed for all measured morphological traits in order to test the significance of variation among accessions using the Statistical Analysis System (SAS). SAS software generated modes, frequencies and means. Means were separated at P = 0.05. Significance level (P=0.05) of non parametric (nominal and ordinal) data was analyzed by Chi-square (χ^2) tests. Diversity index of qualitative morphological data and capillary electrophoresis data were determined with Shannon diversity index. Genetic relationships were displayed as dendrograms to infer relationships and compare the relatedness of accessions based on molecular results and morphological data.

RESULTS AND DISCUSSIONS

DNA Extraction and Agarose Gel Electrophoresis

All the 139 accessions confirmed presence of DNA bands. Concentration of DNA ranged from 70.02 - 2992 ng/µl with an absorbance ratio range 1.7 and 2.1 of 260/280 for most of the samples. Thirteen accessions had absorbance ratio below 1.7 with one accession having an absorbance ratio below 1 (0.56). SSR band size estimated with DNA ladder ranged 100 to 500 base pairs (bp). ISSRs were more polymorphic with band size range of 200-2000 bp. SSRs and ISSR loci detected 437 and 510 alleles, respectively (Table1).

Table 1: Primer allele size ranges and scores of amplified DNA bands by SSR and ISSR

SSR	Allele size range (bp)	Present (1)	Absent (0)	ISSR	Allele size range (bp	present (1)	Absent (0)
PKCT-47	300-500	85	54	ISSR 814A	500-1200	85	54
PKCT-62	100-500	79	60	ISSR 844A	200-1200	64	75
PKCT-111	100-400	86	53	ISSR 844B	200-1200	39	101
PKCT-122	300-500	95	44	ISSR 17898A	200-1200	88	51
PKCT-133	200-400	92	47	ISSR 17898B	200-2000	68	71
Total		437	258	ISSR 17899A	200-2000	82	57
				ISSR 17899B	200-1200	84	55
				Total		510	464

Polymorphism and polymorphic information content (PIC)

Alleles per locus ranged from 1 for PKCT-47, PKCT-62, PKCT-111 and PKCT-133, 2 for PKCT-111 and PKCT-122, 2 and 3 for PKCT-62, 3 to 4 for PKCT-47. PKCT-47 identified more fragments (4 alleles /primer) than any other SSR pair. PKCT-133 detected only monomorphic bands. Heterozygosity (2 bands) were detected by PKCT-47, 62, 111 and 122, and homozygosity (1 band) by PKCT-47, 62, 111 and 133. PIC was only in PKCT-47 and PKCT-62. A total of 526 polymorphic alleles were generated by SSRs with a polymorphism rate of 21.3% (Table 2).

Table 2: Polymorphic Information Content (PIC) of SSR and ISSR primers

Primers	TBA	NPB	P (%)	PIC	Primer	TBA	NPB	P (%)	PIC
PKCT-47	85	20	23.53	0.62	ISSR 814A	85	44	50.59	0.53
PKCT-62	79	4	5.06	0.38	ISSR 844A	63	53	84.38	0.72

PKCT-111	97	11	11.34	0	ISSR 844B	39	31	79.49	0.50
PKCT-122	173	78	45.09	0	ISSR 17898A	88	64	72.73	0.66
PKCT-133	92	0	0	0	ISSR 17898B	68	56	82.35	0.60
Total	526	113		1	ISSR 17899A	82	73	89.02	0.67
Mean	105.2	28.25	21`.26	0.5	ISSR 17899B	84	50	59.52	0.50
					Total	509	371		4.18
					Mean	72.7	53	74.01	0.597

***TBA**-Total number of bands amplified; **NPB**-Number of polymorphic bands; **P (%)-**Polymorphism %; **PIC-** Polymorphic information content*

PKCT-122 had the most number of polymorphic bands. The mean number of alleles amplified by SSRs was 105.2 and a mean PIC of 0.5. All ISSRs were polymorphic, and they generated 509 polymorphic alleles, with a polymorphism rate of 74.01%. The mean PIC was 0.597 for all ISSRs The number of fragments produced by each ISSR varied from 1 to 7 bands with ISSR 844B having the least (one band), ISSR 17898A the highest number of bands (7), with average mean of 72.7 of amplified alleles (Table 2).

Capillary Electrophoresis

Forward SSRs were labeled at the 5^{1}end of the oligonucleotide. The 5 labeled SSR pairs were all able to amplify scorable bands with CE. The band size range was from 181 to 326 bp (Table 3).

Table 3: Characteristics of labeled SSR markers indicating major allele frequency, genotype and allele number, gene diversity, observed heterozygosity, PIC and inbreeding coefficient

Marker	Allele size range (bp)	Major Allele Freq	Genotype No.	Allele No	Gene Diversity	Observed Heterozygosity (F)	Allele Freq	PIC	Genotype Freq	Polymorphism (%)
PKCT-47	205-269	0.7135	8.0000	5.0000	0.4510	0.3854	180	0.4101	96	100
PKCT-62	300-326	0.7895	4.0000	3.0000	0.3404	0.1684	190	0.2951	95	98.95
PKCT-111	187-210	0.4611	9.0000	5.0000	0.6884	0.5222	180	0.6402	90	93.75
PKCT-122	181-225	0.4375	6.0000	5.0000	0.6756	0.9271	192	0.6197	96	100
PKCT-133	201-251	0.5729	7.0000	5.0000	0.5902	0.5208	192	0.5334	96	100
Mean		0.5949	6.8000	4.6000	0.5491	0.5048	186.8	0.4997		

***PIC**- Polymorphic Information Content=**1-Σ(pi2)** (where Pi is the frequency of the i^{th} allele detected) and **F**-fixation index (inbreeding coefficient)*

Mean allelic analysis across 10 subcounties

Five SSR loci detected 23 alleles. Alleles per locus ranged from 5 for PKCT-47, 111, 122 and 133, 3 for PKCT-62 with allelic mean of 4.6. CE genotyping generated a total of 934 distinctive SSR DNA fragments with a mean of 186.8 across the markers. PIC mean was 0.49; observed heterozygosity 0.5048; mean genotype number 6.8 and gene diversity 0.5491 across the five labeled SSR loci. PKCT-47, 122 and 133 had 100% polymorphism, PKCT-62 and 111 had 98.95% and 93.75% polymorphism (Table 3).

Allelic patterns and frequency across loci and subcounties

Number of different alleles (Na) mean was 2.2, Khwisero and Nyeri Central accessions had the highest mean across all loci (Table 4).

Table 4: Mean values of allelic patterns across Sub Counties

Counties	KAKAMEGA					NYERI				
Subcounties (populations)	Butere	Kakamga Central	Kakamega East	Kakamega South	Khwisero	Mathira East	Mathira West	Nyeri Central	Nyeri South	Tetu
Allele Information	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE	Mean±SE
Na	2.6±0.245	3.2±0.490	3.0±0	2.8±0.37	3.4±0.51	3.2±0.49	2.8±0.37	3.4±0.4	3.2±0.37	2.2±0.2
Na Freq. >= 5%	2.6±0.25	3.2±0.490	2.2±0.37	2.4±0.4	2.8±0.37	3.2±0.49	2.8±0.37	3.0±0.45	3.2±0.37	2.2±0.2
Ne	2.294±0.15	2.565±0.44	1.876±0.21	1.847±0.24	2.208±0.41	2.495±0.31	2.466±0.38	2.711±0.38	2.093±0.19	1.786±0.09

I	0.869±0.08	0.984±0.15	0.727±0.10	0.682±0.15	0.869±0.19	0.969±0.14	0.913±0.15	1.035±0.14	0.885±0.11	0.654±0.05
Private Alleles	0.2±0.2	0.2±0.2	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
L Comm Alleles (<=25%)	0±0	0±0	0.2±0.2	0.2±0.2	0.2±0.2	0.2±0.2	0±0	0.2±0.2	0.2±0.2	0±0
L Comm Alleles (<=50%)	0.2±0.2	0.2±0.2	0.4±0.245	0.6±0.4	0.4±0.4	0.4±0.245	0.2±0.2	0.6±0.245	0.4±0.25	0.4±0.4
He	0.556±0.03	0.568±0.06	0.434±0.08	0.412±0.09	0.474±0.1	0.571±0.57	0.550±0.07	0.598±0.06	0.504±0.52	0.434±0.03
UHe	0.636±0.04	0.599±0.07	0.457±0.08	0.425±0.09	0.497±0.11	0.587±0.06	0.629±0.08	0.627±0.06	0.560±0.06	0.485±0.04
F_{IS}	0.272±0.22	0.230±0.17	-0.294±0.11	-0.341±0.11	-0.163±0.19	-0.240±0.15	-0.200±0.11	-0.341±0.20	-0.049±0.29	-0.430±0.19

Na- No of different alleles; **Na Freq. >= 5%-** No of different alleles with a frequency of ≥ 5%; **Ne-** No of effective alleles, **I-** Shannon Diversity index; **No. Private Alleles-**No. of alleles unique to a single population; **No. L Comm Alleles (<=25%)-** No of locally common alleles found in 25% or fewer populations; **No. L Comm Alleles (<=50%) -** No of locally common alleles found in 50% or fewer populations; **He=**Expected heterozygosity; **UHe=**Unbiased expected heterozygosity; **F**-fixation index (inbreeding coefficient)

Kakamega Central, Mathira East and Nyeri South accessions had the highest number of alleles with a frequency of ≥ 5% (Na (Freq ≥5 %). Number of effective alleles (Ne) was highest in Nyeri Central. Shannon's Information Index (I) was highest in Nyeri Central and lowest in Tetu. Private alleles (No of alleles unique to a single population) were found in Butere and Kakamega Central accessions, in two loci (PKCT-133 and 111) and in 2 accessions (KAPAP/CUC/JKK/KK -3 and 56). Number of local common alleles in 25%, or fewer (No. L Comm Alleles ≤ 25%) was found in Kakamega East, Kakamega South, Khwisero, Mathira East, Nyeri Central and Nyeri South, 50%, or fewer (No. L Comm Alleles ≤ 50%) was highest in Kakamega South and Nyeri Central, and lowest in Butere, Kakamega Central and Mathira West. Expected Heterozygosity was highest in Nyeri Central and lowest in Kakamega South (Table 4).

Inbreeding coefficient measures (F-statistics)

There was very little genetic differentiation in Nyeri South (F_{IS} = 0.049) accessions, Khwisero accessions were fairly similar with some differentiation ($F_{IS=}$0.163). The highest genetic differentiation was observed in Nyeri Central ($F_{IS=}$ -0.341), Butere (F_{IS} = -0.272), Mathira East (F_{IS} = -0.240) and Kakamega Central ($F_{IS=}$ -0.230) accessions. Positive values for F_{IS} means the amount of heterozygous offspring in the population will decrease, usually due to inbreeding. Kakamega East ($F_{IS=}$ -0.294), Kakamega South ($F_{IS=}$ -0.341), Mathira West (F_{IS} = -0.200) and Tetu ($F_{IS=}$ -0.430) accessions had negative values for F_{IS} (Table 4). Negative F_{IS} values indicate an increase in heterozygosity due to out breeding, when mating is more random and genes are more likely to be different. Therefore, individual accessions in these sub counties become less related. All the accessions in the sub-counties had moderate genetic differentiation (F_{ST} of 0.089). Mean F values within individual accession in the sub counties was F_{IS}=0.025 (mean inbreeding coefficient within individuals relative to the sub-counties) and F_{IT} = 0.111 (the mean inbreeding coefficient within individuals relative to the total accessions.)

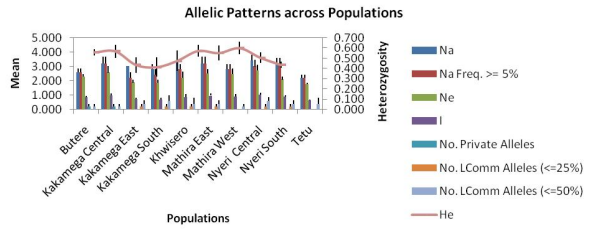

Figure 1: Allelic patterns across (Sub Counties

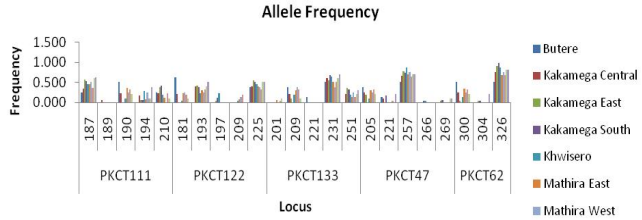

Figure 2: Allelic frequency across SSR loci

Allelic patterns of accessions across sub counties are illustrated in Figure 1. Allele frequency as displayed by the SSR ranged 0.029 to 0.969. The highest and lowest allele frequency was observed in PKCT-62 and 47 respectively in Kakamega South accessions. Total alleles observed was 23 with a size range between 181 to 326 (Figure 2).

Co-dominance of SSR markers

SSRs used displayed co-dominance in the accessions characterized. A single peak denoted homozygous genotypes while two peaks indicated heterozygous genotypes (Figure 3).

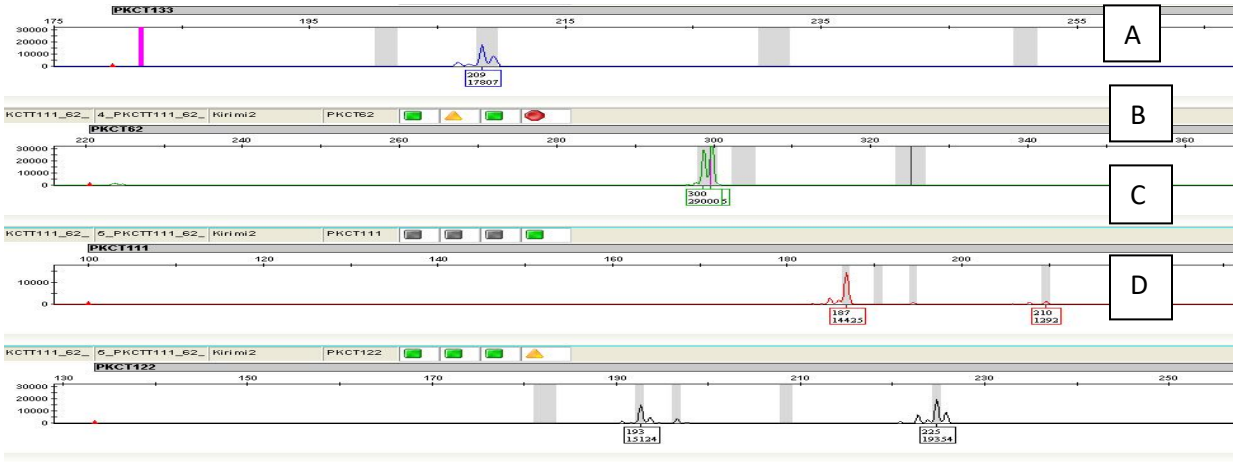

Figure 3: Electrophenogram displaying homozygous (A; B) and heterozygous (C; D) nature of PKCT-133, 62, 111 and 122. The X and Y-axis represent allele sizes and peak intensities, respectively.

Analysis of Molecular Variance (AMOVA)

AMOVA revealed that molecular diversity was highest within individuals (88%) as opposed to among individuals within in sub counties (9%) and among sub counties (3%). AMOVA Table 5 represents the accessions (individuals), and sub populations (sub counties) of Kakamega and Nyeri regions. It shows the degrees of freedom, sum of squares, mean square values, the estimated variation and cumulative variation for the data across all five SSR loci. There were significant differences among accessions from different sub counties at $P<0.026$, but no significant differences among individual accessions in the sub counties and within individual accessions at $P>0.093$ and $P>0.116$ respectively (Table 5).

Table 5: Analysis of molecular variance of SSR data across 10 Sub Counties (sub populations)

Source of Variation	df	SS	MS	Estimated variation	Variation (%)	*P*- value
Among subpopulations	9	19.594	2.177	0.036	3%	0.026
Among individuals in subpopulations	86	129.036	1.500	0.128	9%	0.093
Within individuals	96	119.500	1.245	1.245	88%	0.116
Total	191	268.130		1.409	100%	

Comparison of Molecular Results with Morphological Data

The methods described on molecular and morphological data are valid for this section, since it is a comparison between molecular and morphological results. Analysis of variance revealed significant differences among the accessions for most of the traits suggesting a high degree of phenotypic diversity. Only fruit ribbing that was not significantly different (P>0.05). Fruit skin glossiness showed a narrow range of phenotypic variation (Table 6). Morphological variation of quantitative traits was great with all characters showing high significant differences among the accessions (Table 7). Analysis of molecular variation also revealed genetic variation within individuals (Table 5).

Table 6: Chi-square analysis of observed versus expected frequency of some qualitative fruit characters

Descriptor	Score code	Descriptor status	Observed No.	Expected No.	Residual	χ^2	df	*P* value
Fruit skin glossiness	3	Dull	37	42.0	-5.0	6.143[a]	2	0.046*
	5	Intermediate	34	42.0	-8.0			
	7	Glossy	55	42.0	13.0			
Fruit ribbing	3	Superficial	50	42.0	8.0	2.476[a]	2	0.290**
	5	Intermediate	36	42.0	-6.0			
	7	Deep	40	42.0	-2.0			

a - Zero cells (0.0%) have expected frequencies less than 5. The minimum expected cell frequency is 42.0 for fruit skin glossiness and ribbing, *Characters were significant, **Characters were not significant

Table 7: Summary of some of the quantitative morphological traits

Traits	Min	Max	Sum	Mean	Variance	t- test	df	*P* value
Leaf Ratio	0.58	0.97	112.03	0.7673	0.004	-1.261	145	0.000
Peduncle Length	4.0	16.5	1044.6	8.290	5.550	-267.823	125	0.000
Fruit Flesh Thickness (mm)	10.5	42.6	3148.4	24.987	41.101	-69.182	125	0.000
Fruit L/W Ratio	0.6	3.0	144.6	1.148	0.156	-1.746	125	0.000
Maturation period	39	89	7171	56.91	145.65	-7.057	125	0.000
Total Fruit wt /Plant (kg)	0.25	19.25	493.75	3.919	8.263	-236.570	125	0.000
Stem Thickness (mm)	7.9	14.9	1554.3	10.65	2.152	-443.566	145	0.000

Genetic diversity of accessions

Shannon diversity index (HS) was used to determine the diversity among accessions based on qualitative morphological traits. Characters with high diversity had large HS index value. Conversely, characters with low diversity had low HS index. Shannon diversity index was 0.97 when all the accessions were combined (Kakamega and Nyeri), 0.91 and 1.05 for Kakamega and Nyeri accessions respectively for morphological traits (Data not shown). Capillary electrophoresis data revealed a diversity index of 0. 858 when all the accessions from Kakamega and Nyeri were combined, 0.826 and 0.890 for Kakamega and

Nyeri accessions respectively (Table 4). Both molecular and morphological results showed similar trends with slight deviations in values. Molecular data values were slightly lower than morphological values.

Phylogenetic analysis

For comparison between the dendrograms the number of accessions was reduced to 96 in order to obtain a uniform method of comparison, since some accessions used for morphological characterization were not used during molecular analysis due to failure of some accessions, missing data and lack of clear bands during agarose gel electrophoresis in others. New dendrograms were thus constructed without some of the accessions. The dendrograms were constructed using dissimilarity matrix by Euclidean distance and UPGMA clustering method using XLstat software.

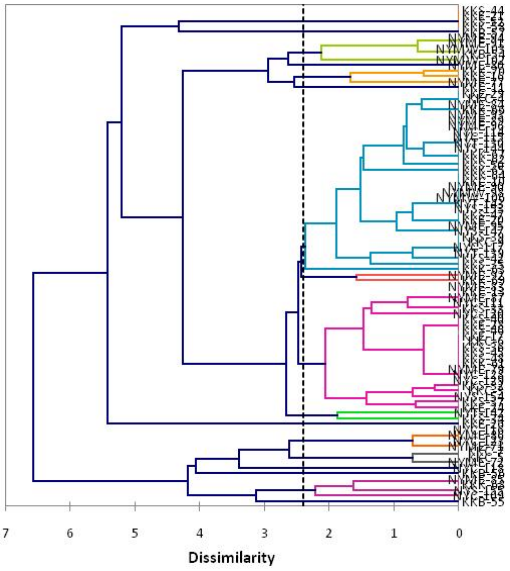

Figure 4: Capillary electrophoresis data dendrogram

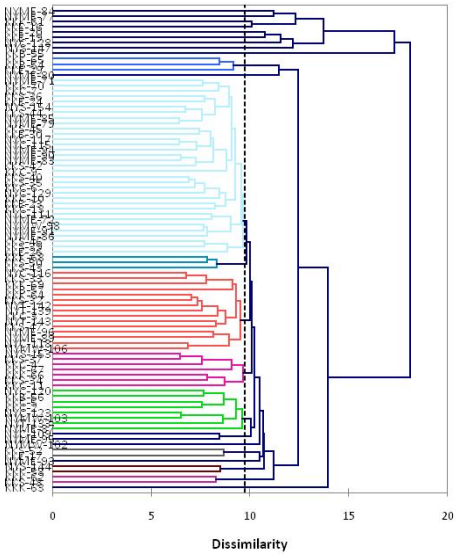

Figure 5: Morphological data dendrogram

Capillary electrophoresis data produced a dendrogram with 17 clusters whereas morphological had 23 clusters. Both dendrograms revealed that some of the cultivars clustered together (Figures 4 and 5). Local and green leaved accessions were clustered separately in the morphological (Figure 5), and together in capillary (Figure 4) dendrograms. Morphological data clustered all the green leaved accessions in simplicifolious, the variegated in bifolious, trifolius and more accessions. Accession KKB-55 and NYME-80 were clustered in simplicifolious in both morphological and capillary dendrograms

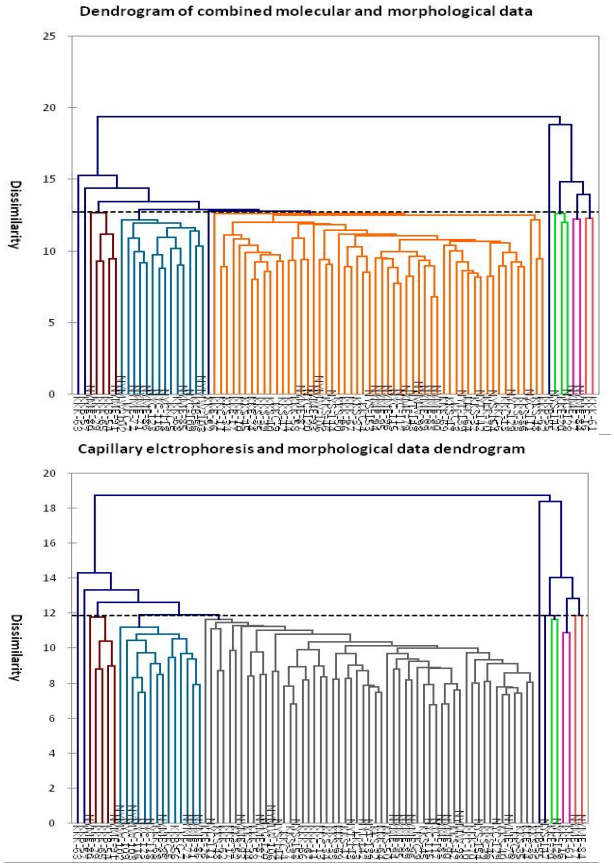

Figure 6: Combined capillary, SSR, ISSR and data morphological data dendrogram

Figure 7: Combined capilary and morphological dendrogram

Cluster analysis of molecular (SSR, ISSR on agarose gel and labeled SSR on capillary), and morphological (qualitative and quantitative) data pooled together, produced 10 clusters. The dendrogram clustered green leaved KKB-55 in simplicifolious, KKE-16, KKK-61 and NYME-77 and 84 in bifolious, KKE-20, NYC-128 and NYS-147 in trifolius. Variegated accessions KKB-57, KKK-63 and KKS-52 were clusterd in simplicifolious (Figure 6). Morphological data pooled together with SSR, ISSR separately, and combined SSR and ISSR data on agarose gel produced 4 clusters each, and they all clustered green leaved (KKB-55) in simplicifolious. Other accessions (variegated and green leaved) were clustered together. Capillary and morphological data pooled together also produced 10 clusters (Figure 7). Green leaved KKB-55 and NYS-147; variegated KKB-57 and KKK-63 accessions were clustered in simplicifolious. Other green leaved accessions were clustered in bifolious. The clustering pattern of all molecular data (SSR, ISSR and labeled SSR) and morphological data, and capillary and morphological data pooled together produced similar cluster groups with only two accessions clustered differently (KKS-52 and NYS-147).

DISCUSSIONS

Pumpkin germplasm presents great genetic variability (Santos et al., 2012). Genetic diversity can be revealed by morphological and molecular characterization (Mussane, 2010). Local landraces are repository of important genes for drought and pests resistance. Imported seeds of narrow genetic basis endanger the existing variability and genetic diversity of pumpkins (Santos et al*.,* 2012). Farmers have selected pumpkin germplasm for many generations, resulting in cultivars adapted to the local conditions (Santos et al., 2012). Human selection has increased inter-population diversity (El-Assal and Gaber,

2012). Characterizations helps in identifying desirable traits, adaptation zones, and give better understanding and use of relationships and genotypes within and among species to improve the pumpkin (Obilana et al*.,* 1994). Morphology depend on geographic origin (Yildiz., et al., 2014) and is manifested in genes different plants carry (Ferriol et al*.,* 2003). Characterization based on morphological descriptors and molecular markers, is very useful in genetic diversity analysis (Santos et al*.,* 2012). Molecular markers monitor variability at the DNA level of plants (Gaafar and Saker, 2006). Morphological characterization compliments the new molecular techniques (Mussane, 2010), in assessing the genetic diversity of plants.

Genetic diversity of accessions was evaluated by scoring the presence (1) or absence (0) of SSR and ISSR markers amplified PCR products on agarose gel electrophoresis. PIC is the value of a marker for detecting polymorphism within a population. It measures the usefulness of a marker and informativeness in specific families. It depends on the number of detectable alleles and the distribution of their frequency. SSR PKCT-47 identified 4 alleles. PKCT-122 and 111 detected 2 alleles. PKCT 133 detected only monomorphic bands. Only primers that detected 3 to 4 alleles (PKCT 47 and PKCT 62) were able to reveal PIC (Table 2). Other studies by Watcharawongpaiboon and Chunwongse (2007) using similar markers, polymorphisms were detected in all sets of pumpkins tested with PKCT 122, 3 sets by PKCT 111. Only one set in PKCT 62, PKCT 133 and 47 detected monomorphic bands in all sets. The number of fragments produced by each ISSR primer varied from 1 to 7. All the ISSRs used in the current study were 74.01% polymorphic and were able differentiate one accession from the other by the presence of unique fragments within 200-2000 bp range. ISSR separated accessions at different fragment size ranges. Studies by Heikal et al (2008) with similar ISSR primers showed 92.4% polymorphism. The difference in polymorphism can be explained by the difference in number of accessions used. In the current study 139 accessions were assessed with seven primers compared to 14 in the later study with the same number of primers. All the five fluorescently labeled SSR markers with capillary electrophoresis showed PIC values (Table 3). The mean diversity index per SSR locus was 0.54 within individuals, 0.86 among the accessions in the sub counties, which allowed for discrimination of each pumpkin accession. All the labeled SSR loci were polymorphic contrary to earlier findings with agarose gel electrophoresis, and previous studies by Watcharawongpaiboon and Chunwongse (2007) using similar SSR loci. The average number of alleles per locus identified by capillary was higher in individuals (4.6) and lower among the accessions in the sub counties (2.98). Fragment sizes obtained by capillary electrophoresis were of narrower range, the number of observed alleles for most of the SSR loci was higher than what was previously reported on agarose gel electrophoresis for the same SSR loci.

In the study, unlabeled SSR and ISSR markers polymorphism were screened on agarose gel electrophoresis system, which was less costly and more widely available (Beyene et al., 2005). The polymorphismpercentage was low compared to detected polymorphism on capillary electrophoresis. The differences could have been occasioned by marker screening systems (agarose gels for SSR and ISSR, and capillary electrophoresis for labeled SSR), and data collection procedures (automated for labeled SSR and manual scoring of alleles for SSR and ISSR) (Beyene et al., 2005). Labeled SSR markers on capillary electrophoresis detected many polymorphic bands and were considered an efficient method for diversity studies of pumpkins. A major disadvantage of SSRs on agarose gels was their low level of automation, which made them cost and time inefficient. The cost was further raised by the number of fluorescently labeled SSR primers needed for capillary electrophoresis. The price of labeled SSR primers was ten times higher compared to the unlabelled ones (Tsoney et al., 2013). In this study labeled SSR markers on capillary electrophoresis were used as a last minute solution after some SSRs and ISSRs markers on agarose gel failed to detect polymorphism and to adequately separate relationships among the accessions. Lack of proper separation led to clustering all the 139 accessions in 3 clusters, and there was no congruence between SSR and ISSR with morphological clustering.

Inbreeding coefficient (F_{ST}) within sub populations relative to the total population is the proportion of total genetic diversity (heterozygosity) distributed among the sub populations and it provides a measure of the genetic differentiation between sub populations and is almost always greater than (or equal to zero). If all individuals within the sub-populations mate completely randomly with each other and have constant allele frequencies, the F_{ST} value will be equal to 0 (Wright, 1978). F_{IS} is the deficiency or excess of average heterozygotes in each population. Within individual accessions (F_{IS} = 0.026) was observed in the sub counties. This could be due to consequence of self-fertilization and selection of pumpkin planting material exercised by farmers in the collection areas (Ghebru et al., 2002). Tetu sub county had substantially higher value (F_{IS} = -0.43) than any other sub county (Table 4). The large F_{IS} value illustrates large degree of relatedness among individuals in Tetu Sub County. Wright (1978) suggested that 0.0 to 0.05 indicated little genetic differentiation, the range 0.05 to 0.15 moderate genetic differentiation, the range 0.15 to 0.25 great genetic differentiation and values of F_{ST} above 0.25 indicated very great genetic differentiation. 'Differentiation' refers to polymorphic differences between populations at different levels of structure (populations and individuals). There was moderate genetic differentiation among the accessions in the 10 sub counties of Kakamega and Nyeri. Inbreeding (F_{ST} = 0.09) among all sub counties, ranged 0.003 to 0.173. The F_{ST}=0.09 value illustrates a moderate degree of differentiation, allele fixation and a moderate genetic divergence. This could be due to farmers practice of selection, which result in reduction of effective population sizes, thereby increasing the opportunity for fixation of alleles (Ghebru et al., 2002). Analysis of Molecular Variance (AMOVA) confirmed the later results; where only 3% of the total variation was only among the accessions in the sub counties (Table 5).

All labeled SSR loci detected variability within the pumpkin accessions. Private alleles (no. of alleles unique to a single population) were found in two sub populations of Butere and Kakamega Central with a mean of 0.2 for each, in two loci (PKCT-133 and 111), and in two accessions (KKC-3 and KKB-56). The two unique alleles were not present in any other accession. Occurrence of private (unique) alleles indicate that the two samples have unique allele patterns for the two markers (PKCT-133 and 111), and they can be distinguished from each other and from the rest of the samples (Arias et al., 2011). Results obtained by Esteras et al (2008) in *Cucurbita pepo,* on Mexican accession (M-8009) presented two unique alleles that were not present in any other accession. They concluded variability of the origin area still remained in some of the selected accessions. Arias et al (2011) reported a large number of markers showing unique alleles in isolates collected from pumpkin. Arias et al (2011) stated that members of the *Cucurbitaceae* have several unique traits which include a lianous structure of the plant body, the development of fleshy fruits, and a mode of sex determination that is not found in other model plants. Elucidation of the functions of these specific genes contribute to knowledge of unique *Cucurbitaceae* traits and allow the application of the information for *Cucurbitaceae* improvement (Ezura and Fukino, 2009).

The accessions showed a wide range of diversity in quantitative and qualitative characters except for fruit ribbing (Table 6 and 7). Ratio of female/male flowers, sex type and seed coat pattern were constant for all accessions. These results were consistent with McCormack, (2005) findings that major domesticated cucurbit species were all monoecious. The ratio of female/male flowers was mostly male (Maynard, 2007). There were variations in fruit and seed characters. Du et al., (2011) stated that genetic diversities of pumpkin manifest in fruits. Studies by Mladenovic et al., (2012) and Balkaya et al., (2010b) reported variations in the fruit and seed characters. Paris (1986) reported that fruit shape was a highly variable trait in *Cucurbita.* Similar reports by Gichimu (2009) stated that *cucurbits* have high genetic diversity for fruit shape and other fruit characteristics. Mladenovic et al, (2012), found that fruit morphological traits exhibited more extensive variance. Predominant fruit skin colour at maturity ranged from green to orange, secondary fruit skin colour pattern from speckled to striped, fruit skin texture from smooth to warty, and flesh colour from white to orange. Ahamed et al. (2011), reported fruit color ranges from green, yellow to brown and the flesh color ranges from whitish to greenish and orange and deep orange. Elliptic seeds were more common. Previous studies found widely elliptic seeds to be most common (Balkaya, et al.,

2010a). The predominant seed coat colours showed variability. Findings by Balkaya et al, (2010a), reported variability in seed colour.

Clustering analysis indicate the level of similarity at which accessions joined a cluster. Horizontal axis of the dendrograms represented the distance or dissimilarity between clusters. The vertical axis represented the accessions and cluster groups (Holland, 2006). In the current study, ISSR, unlabeled and labeled SSR markers and morphological traits were used to characterize 139 accessions collected from Kakamega and Nyeri counties. There were significant agreements between the SSR and ISSR data separately or combined when pooled together with morphological data, Combined SSR, ISSR, Labeled SSR and morphological data, and Labeled SSR and morphological data dendrograms. This congruence indicates that the techniques are equally suited for the analysis of genetic diversity in pumpkin (Beyene et al*.,* 2005). Different markers and morphological traits distinguish different groups ranging from 3 to 23 clusters. The fact that more than 3 clusters were observed was an indication of a high diversity, but it should be noted that the accessions were not all from the same Genera. Genera that clustered together may be a basis of possible introgressions between the genera in the field, the pumpkins were normally grown side by side (Xolisa, 2002). The existence of morphological and genetic diversity among the pumpkin accessions was further substantiated by shannon diversity index in both molecular and morphological data, which indicated that the variation was within the accessions themselves and among the accessions across the sub counties. SSR and ISSR on agarose gel, labeled SSR on capillary and morphological data as well as labeled SSR on capillary and morphological data pooled together showed some congruence in clustering with morphological data clustering. The three techniques were able to distinguish two groups of pumpkin accessions, with distinctive genetic and morphological traits. The first group constituted the variegated local accessions; the second group included green leaved accessions which are exotic in nature.

CONCLUSIONS

Biodiversity is the richness and variety of genetic information present in species or a population. Biodiversity at specific level is described as genetic variability existing among individuals, and genetic variability of a species can be defined as the number of different alleles per locus (allelic variability) or as the number of combinations of different alleles per genome (genotypic variability) (Tsonev et al., 2013). There was considerable amount of genetic diversity among the Kenyan pumpkins as indicated by the high number of alleles and clusters generated. The accessions were clustered on the bases of their genetic diversity and not on their geographic origin. The genetic diversity found in the Kenyan pumpkins can be used to modify or breed improved cultivars. Morphological traits are useful for preliminary evaluation of germplasm and can be used in assessing genetic diversity among morphologically distinguishable accessions. The results suggest that labeled SSR markers on capillary electrophoresis can be used to compliment morphological data in genetic diversity studies of pumpkins. Quantifying genetic variability and evaluating its distribution among and within the populations of a species permit to infer the best ways to preserve diversity of populations, to find propagation material of good quality, to find mother plants for seeds collections. Hence, sampling many accessions from all agro ecologies would be an effective way of capturing genetic variation for future collections before the existing diversity is lost as result of the introduction of high yielding and uniform varieties. Conservation of genetic diversity is very crucial in ensuring survival of local pumpkin species.

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