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The analogy of simple and inter simple sequence repeat markers in the assessment of genetic diversity of pumpkin accessions in Kenya

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Abstract

Pumpkin is found growing in many parts of Kenya although its genetic variation has not been determined using available molecular markers. This study compared SSR and ISSR efficacy in assessing diversity of 139 pumpkin accessions using the multiplex ratio (MR), polymorphic information content (PIC), effective multiplex ratio (EMR), marker index (MI), different (*Na*) and effective (*Ne*) alleles, Shannon index (I), expected (*He*) and unbiased expected heterozygosity (*UHe*), analysis of molecular variance (AMOVA), clusters and mantel correspondence. DNA ranged from 27-2992ng/µl and 0.45-2.1 of 260/280nm. SSR detected 23 total alleles and 4.6 average alleles of 100-700bp. ISSR detected 152 total alleles and 21.7 average alleles of 200-2000bp. Amplified and polymorphic DNA bands were 437 and 117 for SSR, 512 and 391 for ISSR, respectively. Total and polymorphic bands MR was 87.4 and 29.4 for SSR, 73.1 and 55.9 for ISSR, respectively. PIC, EMR and MI for ISSR were higher than for SSR. Markers with high polymorphism portrayed high EMR and MI. SSR PKCT-122 and ISSR 17899A had the highest polymorphism, PIC, EMR and MI. *Ne*, I, *He* and *UHe* was high for SSR, while *Na* was high for ISSR. AMOVA revealed significant (P=0.01; P=0.02) differentiation. Genetic diversity was 14% and 7% among, 86% and 93% within accessions for SSR and ISSR, respectively. Three clusters independent of geographic origin were revealed. SSR and ISSR Euclidean matrices showed positive significant (r=0.272, P=0.0001) correlation, which implied they reflected the same genetic diversity. Hence, the genetic diversity of pumpkins can be assessed effectively using either SSR or ISSR markers.

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Introduction

Pumpkin (*Cucurbita moschata* Duch.) is grown in many parts of the world where it has become adapted, naturalized and even diversified (Ntuli *et al.*, 2013). Naturalized pumpkins are highly polymorphic with different classifications based on fruit shape, geographic origin, number of chromosomes and other characteristics (Hazra *et al.,* 2007; Kiramana *et al.*, 2017). They portray complexities that make it difficult to distinguish different species due to similar growth habits (Paris, 2000). In Kenya, pumpkins are an integral part of food production systems (Kiramana and Isutsa, 2019). However, human activities have pushed pumpkins to extreme distortion and closer to extinction (Greenbaum *et al.,* 2015). Pumpkin reproductive success, biological diversity and species persistence are further threatened by the narrow genetic base of imported hybrid seeds (Kiramana and Isutsa, 2019). Conservation and improvement of naturalized pumpkin are thus becoming a major concern (Greenbaum *et al.,* 2015).

Genetic variability can be quantified through characterization and identification (Valgimigli, 2005; Datta *et al.,* 2010). It is presented as alleles per locus (allelic variability) or allele combination per genome (genotypic variability) (Valgimigli, 2005). Divergence in genome size and sequence manifested through phenotypic and genotypic differences can be measured by polymorphic genes, alleles and heterozygosity loci proportion (Hazra *et al.,* 2007; Mondini, *et al.,* 2009; Zhang *et al.,* 2018). Hence, DNA is the only basis for genetic differentiation, gene mutation, chromosome number and polyploidy, base-pair substitutions, insertions, deletions, and other alterations used to distinguish species (Hazra *et al.,* 2007; Zhang *et al.,* 2018).

Molecular markers possess different genetic qualities, genomic abundance, polymorphism, locus specificity, reproducibility, technical requirements and cost (Mondini *et al.,* 2009). They detect chromosome attributes and flanking regions at the 3' and 5' ends through a probe or specific starters (Mondini *et al.,* 2009). They complement morphological traits in accurately ascertaining genetic heritage and open ways to specify inter- and intra-species relationships (El-Assal and Gaber, 2012). They highlight polymorphisms between individuals by taking advantage of highly polymorphic genes based on the primary nucleic sequence of the DNA (Mondini *et al.,* 2009). They indicate the variation of expressed and non-expressed regions of the genome (Mladenovic *et al.*, 2014). As such, they have been used in many plant species to assess the genetic diversity of closely related species (Singh *et al.*, 2011). Simple Sequences Repeat (SSR) and Inter Simple Sequence Repeat (ISSR) molecular markers have short bits of DNA that identify specific sections of plant genomes and reveal variation (El-Assal and Gaber, 2012). They have been used to identify genetic variation and cultivars, as well as discriminate and catalogue different plant species (Hazra *et al.,* 2007). Specifically, SSR markers are based on di-, tri- or tetra-nucleotides (Khanam *et al.*, 2012), with variable tandem repeats of 1 to 6 nucleotides that are highly reproducible and abundantly distributed throughout the plant genome (Watcharawongpaiboon and Chunwongse, 2007). SSR shows high polymorphism, hyper-variability and ease detection (Singh *et al.*, 2011; Khanam *et al.*, 2012). They are analytically simple, readily transferable and amenable to automated allele detection and sizing (Khanam *et al.*, 2012).

ISSR markers are designed from SSR motifs and based on di-, tetra- or penta-nucleotides (Domyati *et al.*, 2011). They possess 16 to 25-bp-long microsatellites that target multiple genomic loci (Khanam *et al.*, 2012). ISSR can be used in any plant species with a sufficient number and distribution of SSR motifs in the genome (Domyati *et al.*, 2011). They amplify DNA at an amplifiable distance between two identical SSR repeats oriented in opposite directions to detect polymorphism (Behera *et al.*, 2008). ISSR is reproducible and requires small amounts of DNA and no prior genetic information of the DNA sequence of plants (Domyati *et al.*, 2011; Khanam *et al.*, 2012).

In Kenya, molecular markers studies have concentrated on staple crops and no adequate

extension has been done on under-utilized crops such as pumpkins (Kiramana *et al.,* 2017). Consequently, conservation and improvement of naturalized pumpkin are constrained by limited information on genetic diversity. Information on comparative analysis of SSR and ISSR markers efficacy in assessing the genetic diversity of naturalized pumpkin is lacking or is insufficient and needs to be enhanced (Datta *et al.,* 2010). Hence, the present study was undertaken to compare the efficacy of SSR and ISSR markers in assessing the genetic diversity of naturalized pumpkin accessions collected from two regions in Kenya. The present paper reports findings of the study whose purpose was to enhance the utilization of SSR and ISSR markers in the characterization of naturalized pumpkins to pave way for conservation and improvement to suit consumer demands in Kenya (Kiramana *et al.,* 2017).

Materials and methods

DNA extraction

Accessions for genetic diversity study were planted in Chuka University farm on 10th January 2013 in a completely randomized design 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 using the method described by Doyle and Doyle (1987). The presence or absence of DNA bands was determined using 0.8% w/v agarose gel electrophoresis. DNA bands were visualized with ultra-violet (UV) light and documented using a camera. DNA extraction and electrophoresis were repeated once the DNA bands were not visualized. Extracted DNA samples were stored at 4°C before analysis.

DNA quantification, qualification and normalization

DNA quality (260/280 nm) and quantity (µg/mL) were determined using Eppendorf Biophotometer. The yield of DNA in ng/µL and purity (260/280 nm) in wavelength was calculated. A ratio of 1.7-2.0 was considered good quality DNA.

The DNA concentration from extracts was used to guide the normalization of each sample to 50 ng/µL.

Testing and optimization of molecular markers

Testing and optimization of SSR and ISSR markers were conducted using six randomly selected pumpkin accessions. The temperature profile and several cycles influencing the Polymerase Chain Reaction (PCR) outcome were changed until the optimal protocol for each SSR and ISSR marker was obtained.

Polymerase chain reaction

DNA samples were subjected to PCR using SSR and ISSR. SSR with sequences, length and annealing temperatures as shown in Table 1. PCR was performed using a Gene-Am system 9700 (Applied Biosystems) in 10-µL volume containing reaction mixtures (Table 2). For SSR, PCR cycle had initial denaturing at 94°C for 3 minutes, 30 cycles for 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. For ISSR, PCR programming had initial denaturing at 94°C for 3 minutes, 35 cycles for 30 seconds at 94°C, 1 minute for 47°C, and 2 minutes at 72°C, elongation at 72°C for 20 minutes and a final hold at 4°C.

Agarose gel electrophoresis

The PCR products were loaded onto 1.5% w/v agarose gel, stained with 1 ug/uL ethidium bromide in 1 X TAE buffer, and then run at 100v for 60 minutes, visualized and photographed under UV light trans-illuminator. Band sizes were estimated using 1.5% w/v agarose gel, 5 uL DNA per lane, using 1 Kb DNA ladder (Bioline).

DNA band scoring and analysis

Sharp and precise DNA bands were used to generate data that was scored as presence (1) or absence (0) of DNA bands. Measures of genetic diversity including the total number of amplified bands, polymorphic bands, and percentage polymorphism were calculated. Multiplex ratio (MR) was estimated by dividing the total number of DNA bands with a total number of SSR or ISSR markers (Tonk *et al.*, 2014). Polymorphic information content (PIC), effective multiplex ratio (EMR) and marker index (MI) measured the usefulness and informativeness of each

SSR or ISSR marker in assessing genetic diversity (Najaphy *et al.,* 2011). Calculations were done as follows:

$$PIC_i = 1-\sum_{j=1}^{n} Pij2 \quad \text{Equation 1}$$

Where PIC_i = Polymorphic information content of marker I, and Pij = Frequency of the jth pattern for marker i and the summation extend over n patterns.

$$EMR = n\beta = np \times (np/n) \quad \text{Equation 2}$$

Where np = Total No. of polymorphic bands per loci, and β= Total number of polymorphic bands (np)/Total number of all amplified bands per loci (n)

$$MI = PIC \times EMR \quad \text{Equation 3}$$

Where MI = Marker index, PIC = Polymorphic information content, and EMR = Effective multiplex ratio.

Measures of genetic variability including the total number of different alleles, effective alleles, Shannon index, expected and unbiased expected heterozygosity, and AMOVA were estimated using GenAlEx 6.5 software (Peakall and Smouse, 2012). Heterozygosity and alleles, within and among genetic variability were generated by PowerMarker V 3.25 (Liu and Muse, 2005).

Cluster analysis grouped accessions in several traits (Kiramana *et al.,* 2017). Genetic distance matrices were generated by the Euclidean method using XLstat 2014 software (Rousseeuw and Kaufman, 1990). Wards method produced desirable compact clusters, while the unweighted pair group method of arithmetic averages (UPGMA) minimized within-cluster variance (Hintze, 2001). Cluster analysis results were presented as dendrograms. Data matrices for SSR and ISSR markers were analyzed using the Mantel correspondence test. A standardized Euclidean distance matrix was used to generate dissimilarity indices. Correlation of data matrices was done by Mantel (1967) test based on 10,000 permutations using XLstat 2014 software at P=0.05.

Results

DNA extraction, quantity and Quality

All 139 pumpkin accessions yielded DNA bands. The concentration (quantity) ranged from 27 to 2992 ng/µL, with absorbance (quality) ratio ranging from 0.45 to 2.1 of 260/280 nm. The absorbance ratio ranging from 1.7 to 2.1 was considered to reflect pure, good quality DNA.

Table 1. Markers, sequences, length and annealing temperatures.

SSR Marker	Marker sequence (5' to 3')	Length	Annealing temp (Tm °C)
PKCT-47	Forward: GGT CCC AAT AAT AGC AAC CAA	21	53.0
	Reverse: GTG GGA CAC ATC TTG AGC A	19	50.9
PKCT-62	Forward: GAA GTT CGT GGT CTG TGC AAG TC	23	57.4
	Reverse: CCT GAG TAA CCT CCG TGC TTC C	22	58.6
PKCT-111	Forward: GTT GCA GCG ACC GTT CTT CTT C	22	59.7
	Reverse: GCA TCT GAA GAC GAT GCG TCG T	22	61.0
PKCT-122	Forward: CTA AAC AGG ATG CCT CTG ACA C	22	52.8
	Reverse: CGG GAT TTC CGA AAC AAC GT	20	58.3
PKCT-133	Forward: TCG GAA TCG TCT TCA GCA ATA GTC	24	58.3
	Reverse: TCC TCT TCC ATT CCA CTT TCT CCT	24	58.2
	ISSR marker		
ISSR 814A	5'-CTC TCT CTC TCT CTC TTG-3'	18	35.7
ISSR 844A	5'-CTC TCT CTC TCT CTC TAC-3'	18	31.4
ISSR 844B	5'-CTC TCT CTC TCT CTC TGC-3'	18	38.8
ISSR 17898A	5'- CAC ACA CAC ACA AC -3'	14	42.0
ISSR 17898B	5'- CAC ACA CAC ACA GT -3'	14	42.0
ISSR 17899A	5'- CAC ACA CAC ACA AG-3'	14	42.0
ISSR 17899B	5'- CAC ACA CAC ACA GG-3'	14	44.0

DNA band analysis, polymorphism and multiplex ratio (MR)

SSR markers amplified 437 DNA bands, while ISSR amplified 512 bands (Table 3). SSR PKCT-122 and ISSR-17898A amplified the highest, while SSR PKCT-62 and ISSR 844B amplified the least DNA bands. The total number of polymorphic bands was 117 and 391 for SSR and ISSR, respectively. The multiplex ratio (MR) of total bands was 87.4 and 73.1 for SSR and ISSR, respectively. The MR for polymorphic bands was 29.3 and 55.9 for SSR and ISSR, respectively. The SSR PKCT-122 and ISSR 17899A had the highest, while SSR PKCT-62 and ISSR 814A had the least number of polymorphic bands. The SSR PKCT-133 yielded monomorphic bands only, while ISSR 814A yielded the highest monomorphic bands.

Polymorphism ranged from 0 to 83.7%, with a mean of 26.8% across SSR markers, and from 54.1 to 91.5%, with a mean of 76.4% across ISSR markers (Table 3).

Table 2. PCR components, stock solution concentration and reaction conditions for each marker.

Component	Stock concentration	Single reaction SSR	Single reaction ISSR
Buffer	10 X	0.5 µL	0.5 µL
$MgCl_2$	10 mM	0.4 µL	0.4 µL
dNTPs	2.5 mM	0.4 µL	0.4 µL
Taq polymerase	5 U/µL	4.2 µL	4.2 µL
H_2O	Nil	3.0 µL	3.0 µL
Genomic DNA	30 ng/µL	0.5 µL	0.5 µL
Forward (SSR)	5.0 pmoles/µL	0.5 µL	Nil
Reverse (SSR)	5.0 pmoles/µL	0.5 µL	Nil
ISSR	5.0 pmoles/µL	Nil	1µL
Final volume		10.0 µL	10.0 µL

Polymorphic information content, effective multiple ratio and marker index

Mean PIC was 0.25 and 0.72 across SSR and ISSR markers, respectively. High PIC was observed in SSR PKCT-47, ISSR-844A and ISSR-17898B, and least PIC in SSR PKCT-62 and ISSR-814A. MI ranged from 0.02 to 18.5 and16.2 to 50.1 across SSR and ISSR, respectively. High MI was observed in SSR PKCT-122 and ISSR-17899A, while the least MI in SSR PKCT-62 and ISSR-814A. EMR ranged from 0.20 to 68.6 across SSR, and 24.9 to 68.6 across ISSR. PIC, EMR and MI were high across and among ISSR markers. SSR PKCT-133 had no PIC, EMR and MI. The markers with the highest polymorphism (Table 3) portrayed high EMR and MI (Table 4). MI and PIC were not positively correlated for SSR (r=0.33, $P>0.58$) and ISSR (r=0.64, $P>0.12$). Significant correlation was found between EMR and number of polymorphic bands across SSR (r=0.99, $P<0.002$) and ISSR (r=0.91, $P<0.004$).

Table 3. DNA band analysis, polymorphism and multiplex ratio across SSR and ISSR markers.

Marker	DNA Band size (bp)	Total present	Total absent	Monomorphic bands (no.)	Polymorphic bands (no.)	Polymorphism (%)
SSR PKCT-47	100 - 700	85	54	65	20	23.53
SSR PKCT-62	100 - 400	79	60	75	4	5.06
SSR PKCT-111	100 - 350	85	54	74	11	12.94
SSR PKCT-122	300 - 400	98	41	16	82	83.67
SSR PKCT-133	200 - 400	90	49	90	0	0
Total		437	258	320	117	
Mean		87.4^{MR}			29.3^{MR}	26.77
ISSR 814A	500 - 1200	85	54	39	46	54.1
ISSR 844A	200 - 1200	63	76	9	54	85.7
ISSR 844B	200 - 1200	39	100	6	33	84.6
ISSR 17898A	200 - 1200	90	49	21	69	76.7
ISSR 17898B	200 - 2000	69	70	15	54	78.3
ISSR 17899A	200 - 2000	82	57	7	75	91.5
ISSR 17899B	200 - 1200	84	55	24	60	71.4
Total		512	461	121	391	
Mean		73.1^{MR}			55.9^{MR}	76.4

Allele size, Number and Frequency

Allele sizes ranged from 100 - 700 bp, and 200 - 2000 bp across SSR and ISSR markers, respectively. A total of 23 alleles were yielded by SSR, and 152 alleles by ISSR (Table 5). The number of alleles per locus ranged from 1-4 for SSR and 1-7 for ISSR markers. Alleles identified were 1 by SSR PKCT-133, 1-2 by SSR PKCT-111 and SSR PKCT-122, 1-3 by SSR PKCT-62, 1-4 by SSR PKCT-47, 1-4 by ISSR-814A, 1-6 by ISSR-844A and ISSR-17899B, 1-7 by ISSR-844B and ISSR-17898A, 1-5 by ISSR-17898B, and 7 by ISSR-17899A. The average number of alleles was 4.6 and 21.7 across SSR and ISSR, respectively. SSR had higher average *Ne*, I, *He* and *UHe*, while ISSR had high *Na* (Table 5). SSR PKCT-111, SSR PKCT-122, and ISSR 844B had the least *Na*. *Ne* was least in SSR PKCT-62, while I, *He* and *UHe* were least in SSR PKCT-111. ISSR-844B had the least *Ne*, I, *He* and *UHe*. SSR PKCT-133, ISSR-17899A and 17899B had the highest *Ne*, I, *He* and *UHe* (Table 5).

Table 4. Polymorphic information content, effective multiple ratio and marker index.

Marker	Polymorphic information content	Effective multiple ratio	Marker index
SSR PKCT-47	0.40	4.71	1.88
SSR PKCT-62	0.10	0.20	0.02
SSR PKCT-111	0.23	1.42	0.33
SSR PKCT-122	0.27	68.6	18.5
SSR PKCT-133	0	0	0
Total	1	74.93	20.73
Mean	0.25	18.7	5.2
ISSR 814A	0.65	24.9	16.2
ISSR 844A	0.77	46.3	35.7
ISSR 844B	0.69	27.9	19.3
ISSR 17898A	0.72	52.9	38.1
ISSR 17898B	0.77	42.3	32.6
ISSR 17899A	0.73	68.6	50.1
ISSR 17899B	0.70	42.9	30.0
Total	5.03	305.8	221.9
Mean	0.72	43.7	31.7

Analysis of molecular variance

ISSR AMOVA revealed 93% and 7% genetic variation within and among sub-counties, respectively. SSR AMOVA revealed 86% and 14% genetic variation within and among sub-counties, respectively. The AMOVA showed significant differences ($P<0.05$) within and among accessions in SSR and ISSR markers (Table 6).

Cluster analysis

The analysis produced three clusters for SSR and ISSR dendrograms. The variance within clusters I, II and III was 0.66, 0.60 and 0.56 for SSR and 1.184, 0.810 and 1.333 for ISSR, respectively. Euclidean dissimilarity coefficient ranged from 0 - 3.8 for SSR, and 0 - 4.3 for ISSR (**Fig**. 1 and 2). SSR largest cluster grouped 89 accessions, the second 41 and the third 9. ISSR largest cluster grouped 87 accessions, the second 49 and the third 3 accessions. The variance within clusters was 0.62 (52.7%) and between clusters was 0.55 (47.3%) for SSR, while it was 1.056 (63.5%) within clusters and 0.607 (36.5%) between clusters for ISSR. SSR dendrogram grouped in cluster I, II and III accessions from 10, 9 and 7 sub-counties, respectively. ISSR dendrogram grouped in clusters I, II and III accessions from 10, 10 and 2 sub-counties, respectively. The outlier accessions in SSR dendrogram were KK-1 and NY-119 and NY-132 in clusters II and III, respectively. Outlier accessions in ISSR dendrogram were KK-66, KK-37, NY-115 and NY-142 in cluster I, and KK-63 and NY-130 in cluster II, and KK-67 in cluster III.

Table 5. Allele size, number and frequency across SSR and ISSR markers expressed as mean ± SE.

Marker	Allele size (bp)	Alleles	*Na*	*Ne*	I	*He*	*UHe*
SSR PKCT-47	100-700	10	2.0±0.00	1.59±0.13	0.53±0.06	0.35±0.05	0.38±0.06
SSR PKCT-62	100-400	6	2.0±0.00	1.49±0.06	0.50±0.03	0.32±0.03	0.34±0.04
SSR PKCT-111	100-350	3	1.6±0.40	1.52±0.15	0.46±0.12	0.31±0.08	0.32±0.09
SSR PKCT-122	300-400	3	1.8±0.20	1.70±0.18	0.53±0.13	0.37±0.09	0.39±0.10
SSR PKCT-133	200-400	1	2.0±0.00	1.77±0.08	0.62±0.03	0.43±0.03	0.46±0.04
Mean		4.6	1.94±0.04	1.74±0.04	0.59±0.02	0.41±0.02	0.43±0.02
ISSR 814A	500-1200	10	2.00±0.00	1.76±0.07	0.61±0.03	0.42±0.03	0.45±0.03
ISSR 844A	200-1200	21	2.00±0.00	1.61±0.07	0.56±0.03	0.37±0.03	0.39±0.03
ISSR 844B	200-1200	28	1.71±0.29	1.40±0.11	0.40±0.08	0.26±0.06	0.27±0.06
ISSR 17898A	200-1200	28	2.00±0.00	1.74±0.06	0.61±0.02	0.42±0.02	0.44±0.03
ISSR 17898B	200-2000	22	2.00±0.00	1.61±0.07	0.56±0.03	0.37±0.03	0.39±0.03
ISSR 17899A	200-2000	22	2.00±0.00	1.79±0.05	0.63±0.02	0.44±0.02	0.46±0.02
ISSR 17899B	200-1200	21	2.00±0.00	1.80±0.07	0.63±0.03	0.44±0.02	0.46±0.03
Mean		21.7	1.97±0.03	1.68±0.03	0.57±0.02	0.39±0.01	0.41±0.01

Na = Number of different alleles; *Ne* = Number of effective alleles; I= Shannon information index; *He* = Expected heterozygosity; *UHe* = Unbiased expected heterozygosity.

Mantel correspondence test

Euclidean dissimilarity coefficients for SSR and ISSR matrices were 2.89 and 3.52, respectively. The null hypothesis of no correlation between different Euclidean matrices was tested using the Mantel correspondence test. The results showed a significant and positive correlation between SSR and ISSR matrices (r=0.272; *P*=0.0001).

Discussion

Genetic diversity provides an important basis for enhancing food security and sustainable development. Genetic variation once considered unlimited, is fast-eroding as traditional, naturalized and wild relatives of cultivated species are being replaced or distorted by exotic cultivars (Kiramana and Isutsa, 2019). Selecting the right molecular marker for assessment of genetic diversity depends on the specific application, presumed level of polymorphism, presence of sufficient technical facilities, time and financial limitations, among others (Sethi *et al.,* 2016). A good molecular marker can generate high genetic variability from multi-locus data in the genome under study (Ng and Tan, 2015). The utility of two or more markers has proved to be better than a single marker (Sethi *et al.,* 2016), and hence SSR and ISSR results were compared.

Quantity and quality of DNA

In the present study, DNA absorbance (quality) ranged from 0.45 to 2.1 of A260/280 ratio and thus the concentration ranged from small to large quantities. DNA with high purity, molecular weight and quality is difficult to isolate from plants (Abdel‐Latif and Osman, 2017; Kiramana *et al.,* 2017; Aboul-Maaty and Oraby, 2019). Small quantities are often lost through clotting, adsorption, chemical or enzymatic degradation, and contamination with impurities that come in contact with it. However, high purity DNA is required in PCR. Contaminants prevent the use of DNA in PCR by inhibiting the action of polymerases. High molecular weight and large quantities interfere with enzymatic reactions, resulting in false priming and poor synthesis. DNA quality is affected by polysaccharides and polyphenols in plants. Polysaccharides make DNA slimy and difficult to handle by inhibiting restriction, enzymatic and *Taq* polymerase activity, and co-precipitating with DNA. Polyphenols oxidize and covalently bind DNA, giving a brown color, and reduce maintenance time, which makes the DNA useless for molecular use.

Marker polymorphism

In the present study, detected DNA bands were scored as either present or absent. DNA bands are

scored to establish systematic and evolutionary relationships of species. The number of polymorphic bands was high in ISSR compared to SSR markers in the present study. Heikal *et al.*, (2008) reported similar results. High polymorphism percentage was also recorded in ISSR compared to SSR markers. Previously, polymorphism has been used to assess genetic diversity and relationships of *Cucurbita* species (Esmailnia *et al.*, 2015). However, the mechanism of detecting polymorphism differs for different markers (Kiramana *et al.*, 2017). The ISSR markers' 1-3 additional arbitrary nucleotides at the 5' or 3' end act as anchors against reverting strand slippage during amplification.

Table 6. Analysis of molecular variance.

Source of variation	df	SS	MS	Estimated variation	Variation (%)	*P*-value
Among SSR	9	29.293	3.255	0.164	14%	0.010
Within SSR	129	131.081	1.016	1.016	86%	
Total	138	160.374		1.180	100%	
Among ISSR	9	27.802	3.089	0.112	7%	0.020
Within ISSR	129	201.061	1.559	1.559	93%	
Total	138	228.863		1.671	100%	

The anchoring facilitates attachment of ISSR to specific inter-microsatellite regions, resulting in diverse banding patterns from identical repeat sequences with varying anchoring nucleotides. ISSR also target sequences that are abundant throughout the genome and evolving rapidly to reveal higher polymorphic fragments (Ansari *et al.*, 2012). Hemalatha and Shanmugasundaram (2010) observed that ISSR was superior to SSR in generating polymorphism. Heikal *et al.*, (2008) reported 92.4% polymorphism using the same sequence of ISSR as the present study. Previous studies by Domyati *et al.,* (2011) reported that ISSR has wide utility in the analysis of genetic diversity and detection of high and low levels of polymorphism in closely related individuals. ISSR amplify areas that are potentially highly polymorphic in microsatellite and inter-microsatellite loci (Datta *et al.*, 2010; Ansari *et al.*, 2012).

The differences observed in the polymorphism of SSR markers were attributed to variation in repeat units due to elevated mutation rates (Guichoux *et al.*, 2011). Stift *et al.*, (2004) observed high polymorphism in SSR contrary to the present findings. Wang *et al.,* (2007) found SSR polymorphism to be associated with the number and type of repeat units, genomic region, database source for marker development and materials used for detection. The difference in the number of repeat units in SSR is due to mutations that lead to slipped-strand mispairing during DNA synthesis. This results in the gain or loss of one, or more repeat units, depending on whether the newly synthesized DNA chain loops out or loops in. The propensity for the chain to loop out depends partly on the sequence of the SSR, and whether the event occurs on the leading or lagging strand (continuous or discontinuous DNA synthesis) (Semagn *et al.,* 2006). The low polymorphism of SSR was attributed to the high monomorphic bands, few alleles, and tri-nucleotide repeat units. SSR with di-nucleotide repeats are more prone to mutation and polymorphism than SSR with tri-nucleotide repeats. High polymorphism was found in SSR PKCT-122, while SSR PKCT-133 detected only monomorphic bands. These results were similar to those of Watcharawongpaiboon and Chunwongse (2007). The SSR and ISSR markers that revealed high polymorphism were considered useful in genetic differentiation of pumpkins (Verma *et al.*, 2017).

Informativeness of markers

High MR was recorded in SSR for total amplified bands, and in ISSR for polymorphic bands. The fewer SSR used and large polymorphic bands detected by ISSR resulted in high MR, respectively. Effective

multiplex ratio (EMR) and marker index (MI) evaluate the discriminatory power and the usefulness of markers (Najaphy *et al*., 2011; Tonk *et al*., 2014). EMR was derived from the product of the fraction and number of polymorphic bands and determines the most effective method for detecting genetic variation (Rostami *et al*., 2015; Yousefi *et al.,* 2015). High polymorphism results in high EMR (Najaphy *et al*., 2011). In the present study, ISSR had higher EMR than SSR markers, while EMR and polymorphic bands showed a positive correlation (Adawy *et al*., 2005).

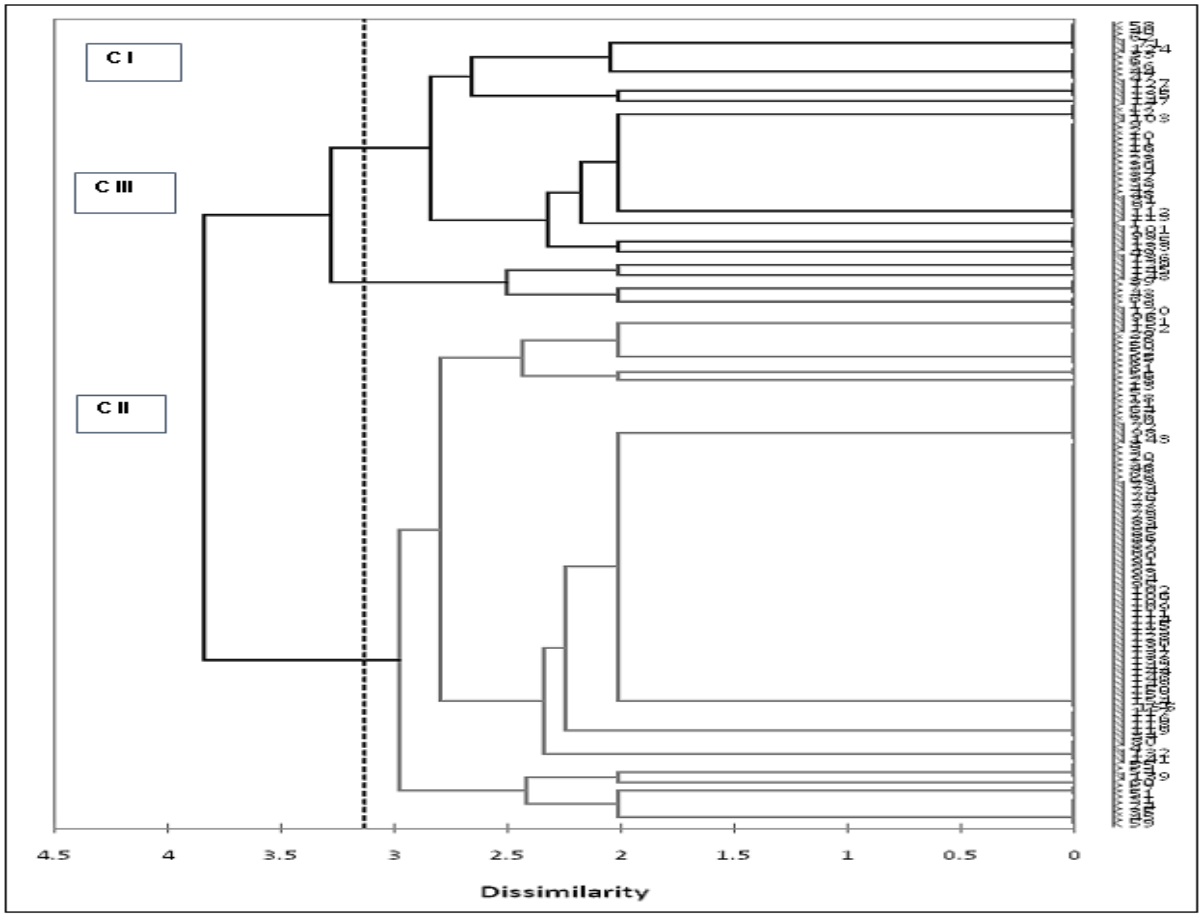

Fig. 1. Phylogenetic relationships of pumpkin accessions using SSR markers.

Marker index (MI), which is the product of PIC and EMR, shows the level of polymorphism for estimating efficiency and informativeness (Hemalatha and Shanmugasundaram, 2010; Najaphy *et al*., 2011; Rostami *et al*., 2015). The high MI of ISSR was linked to high EMR and PIC values, arising from high polymorphism in ISSR loci, compared to few polymorphisms in SSR loci (Myskow *et al.,* 2010). PIC ranged from 0 to 1, denoting only one and infinite alleles had been detected, respectively. PICs greater than 0.7 and 0.4 denotes highly and moderately informative, respectively. In the present study, PICs was high in ISSR, compared to SSR. Inan *et al.,* (2012) reported PICs from 0.60 to 0.90 with alleles from 4 to 15, using ISSR markers in *Cucurbita*. The low PICs was attributed to a few markers used (Noormohammadi *et al*., 2012). Gong *et al*., (2008) SSR revealed PIC of 0.62 with alleles ranging from 2 to 9 in *Cucurbita pepo* L. genotypes.

The SSR PKCT-122 and ISSR-17899A that had the highest EMR and MI were considered very vital in genetic diversity studies of pumpkins. The MR, MI, EMR and PIC portrayed ISSR markers as more informative and powerful in determining genetic variation and identifying and characterizing pumpkin genotypes (Noormohammadi *et al*., 2012; Rostami *et al*., 2015; Verma *et al*., 2017).

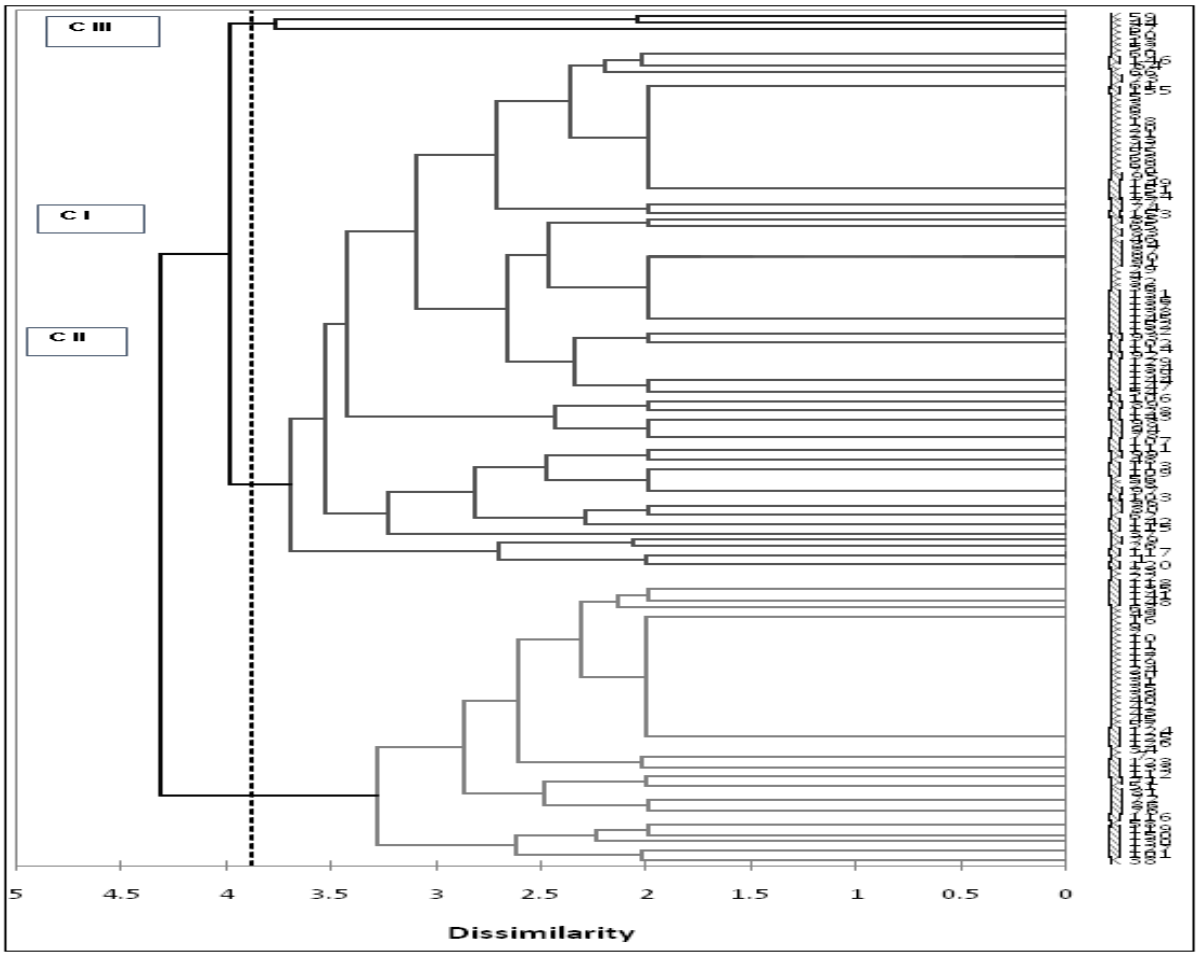

Fig. 2. Phylogenetic relationships of pumpkin accessions using ISSR markers.

Analysis of alleles and molecular variance

In the present study, ISSR detected a higher number, mean, and total alleles than SSR. Gyorgy *et al*., (2012) also obtained the same.

The high number of alleles was due to the ability of ISSR to determine intra- and inter-genetic diversity and reveal variations within unique regions at multiple loci in a single reaction (Serra *et al*., 2007). Kiramana *et al.,* (2017) reported 3-5 alleles per SSR locus, mean of 4.6 and 24 total alleles, using the same fluorescent SSR markers. Stift *et al*., (2004) reported that SSR was multi-allelic. Barzegar *et al.,* (2013) reported 94 alleles using 14 pairs of SSR loci on *Cucurbita pepo* L. Marilene *et al.,* (2012) obtained 137 alleles, using 15 ISSR on *Cucurbita* species. Genetic diversity measured using allele number is more sensitive to differences in population size and number (Greenbaum *et al.,* 2015). Allele size for SSR and ISSR markers in the present study revealed distant relationships in naturalized pumpkin (Kiramana *et al.,* 2017). Allele sizes were greater in ISSR compared to SSR. Kiramana *et al*., (2017) reported 181 to 326 bp, while Watcharawongpaiboon and Chunwongse (2007) obtained 100 to 413 bp in *C. moschata* L., using the same SSR. Stift *et al*., (2004) and Esteras *et al.,* (2012) reported over 500 bp and 100 to 350 bp in *Cucurbita* species, using SSR. Hardy *et al*., (2003) found that high mutation of SSR causes stepwise changes to the repeats, resulting in different sizes of alleles. Thus, the higher the mutation events, the larger the difference in allele sizes. Heikal *et al*., (2008) using the same sequence ISSR reported 50 and 1268 bp within and among *Cucurbita* species, respectively. Xanthopoulou *et al*., (2015) reported 600 bp to 4.0 kb in *C. pepo*, and Behera *et al*., (2008) from 150 to 2700 bp in bitter gourd, using ISSR. ISSR is designed from SSR motifs and amplifies DNA bands by locating regions within an amplifiable distance on DNA strands to generate alleles of a

particular size (Heikal *et al*., 2008; Domyati *et al.,* 2011). The *Na* number and frequency contribute to survival, reproduction, diversity and variability (Gyorgy *et al*., 2012; Noormohammadi *et al*., 2012; Kiramana *et al.,* 2017).

In the present study, ISSR *Na* was higher than SSR *Na*. ISSR is locus-unspecific and more randomly distributed throughout the genome than SSR. This abundance compensates for ISSR bi-allelic nature and enables them to detect more different alleles (Sethi *et al.,* 2016) than SSR. In the present study, SSR had high *Ne,* I, *He* and *UHe*, compared to ISSR. Sethi *et al.,* (2016) reported that SSR outperforms ISSR in terms of *Ne,* I and *He,* using *Gossypium arboreum*. Bilska and Szczecinska, (2016) reported that SSR markers are ubiquitous and characterized by high alleles per locus. Effective alleles indicate variation in allele frequencies and detect overestimates in marker polymorphism (Kiramana *et al*., 2017).

The markers with high *Ne* portrayed high *He,* I and *UHe*. The *Ne* were less than the *Na* in SSR and ISSR used, indicating unequal allele frequencies. If effective alleles are smaller than observed alleles, then actual allele frequencies are unequal and have large variations. If effective alleles are larger than observed alleles, then observed alleles overestimate marker polymorphism because they exceed the maximum expected polymorphism (Kiramana *et al*., 2017).

In the present study, SSR and ISSR markers did not overestimate polymorphism and did not exceed the maximum expected polymorphism. Shannon index (I) showed high (>0.5) values in 80% SSR and over 85% ISSR markers. SSR markers had a slightly higher I, compared to ISSR. SSR and ISSR Shannon indices in the present study indicated the presence of high genetic variability of naturalized pumpkins (Noormohammadi *et al.,* 2012). Shannon index is important in the conservation and management of populations (Greenbaum *et al.,* 2015). It characterizes species and indicates both richness and evenness in a population (Sethi *et al.*, 2016). In the present study, mean *He* and *UHe* were high for SSR, compared to ISSR. The high *He* and *UHe* of SSR markers were attributed to their co-dominant nature (Sethi *et al*., 2016). The *He*, also known as gene diversity (GD) of a locus, shows the probability that two gametes, randomly chosen from a gene pool, are of different alleles (Greenbaum *et al.,* 2015; Luo *et al*., 2019). It describes the expected proportion of heterozygous genotypes under Hardy-Weinberg equilibrium. *He* is influenced by alleles of intermediate or high frequency, sensitive to allele frequencies rather than to number, and insensitive to changes in the frequency of rare alleles lost from populations via drift or a species as populations are extirpated (Greenbaum *et al.,* 2015). The *UHe* estimates for a sample without related or inbred individuals composed of n allele copies (Harris and DeGiorgio, 2017). The multi-allelic nature, hyper-variability and high information content give SSR advantage as co-dominant markers to detect homozygotes and heterozygotes more efficiently (Datta *et al*., 2010; Kiramana *et al*., 2017). ISSR is dominantly inherited, which makes it hard to distinguish homozygotes from heterozygotes (Serra *et al*., 2007).

Genetic structure was inferred by AMOVA, which revealed high and low genetic differentiation within and among sub-county accessions, respectively, regardless of the marker used. Li *et al*., (2014) reported low inter-population and high intra-population genetic differentiation in apricots using SSR and ISSR markers. The high ISSR genetic differentiation within sub-county accessions compared to SSR was attributed to differences in molecular content and high polymorphism of ISSR markers (Sheidai *et al*., 2012). Serra *et al*., (2007) found that more polymorphic markers detected higher levels of genetic differentiation.

Phylogenetic relationships

Phylogenetic relationships showed that high genetic diversity existed within, as opposed to among accessions in SSR and ISSR dendrograms (Greenbaum *et al.,* 2015). Kiramana *et al*., (2017) found high genetic diversity within sub-county

accessions using principal coordinates, F_{IS} and F_{IT} analysis, and high genetic diversity in individual, within and among sub-county accessions.

The variance within clusters was high in both SSR and ISSR dendrograms and attributed to the allogamous nature of pumpkins (Grisales *et al.,* 2009). Accessions within a cluster were more similar than others that joined at a much higher level. Greater branch lengths reflected greater differences (Holland, 2006).

This study yielded three clusters regardless of markers used. Dendrograms horizontal clades represented distance of accessions, while vertical clades represented dissimilarity within accessions (Kiramana *et al.*, 2017). Cluster joining was represented by splitting of vertical lines into horizontal lines. The vertical split was shown by the short or long bar and depicted the dissimilarity of clusters (Holland, 2006). Two clusters revealed closely related accessions and one cluster distantly related accessions in the SSR dendrogram. All the three clusters revealed distantly related accessions in the ISSR dendrogram.

The ISSR and SSR dendrograms did not cluster accessions based on geographical origin. There was no distinct separation between variegated and green-leafed accessions. ISSR had more outlier accessions joining arbitrarily at a much higher distance than SSR (Holland, 2006). SSR and ISSR dendrograms' Euclidean distance matrices showed a positive significant correlation similar to Li *et al.* (2014) while working on buffalo grass.

The positive significant correlations between the SSR and ISSR distance matrices indicated that the markers reflected the same pattern of genetic diversity. These showed that SSR and ISSR markers could complement each other to draw more accurate conclusions (Adawy *et al.*, 2005).

Conclusion

Although ISSR has higher average polymorphism, MR, and PIC, EMR, MI and AMOVA, SSR has higher *Ne,* I, *He* and *UHe* in pumpkin. Indices in SSR and ISSR that yield almost similar values include phylogenetic relationships, I, *He* and *UHe* in pumpkin. Mantel test's significant and positive correlation proves that SSR and ISSR markers reflect the same genetic diversity in pumpkin, and hence their combination would be highly efficient in detecting genetic diversity of pumpkins. SSR PKCT-122 detecting high polymorphism, heterozygotic alleles, MR, EMR and MI, and hence is recommended for use in assessing the genetic diversity of pumpkins. The highly species-specific SSR markers and ISSR that does not show much divergence are all recommended for use in the assessment of genetic diversity of pumpkins to develop improved cultivars that can face evolving and limiting environmental challenges.

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