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Genetic diversity in *Cucurbita pepo* landraces revealed by RAPD and SSR markers

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A B S T R A C T

Cucurbita pepo landraces are important traditional leafy vegetable crops that are widely eaten in KwaZulu-Natal, South Africa, and elsewhere. The use of molecular markers is beneficial in the genetic study of landraces with different agro-ecological origins. In this study, genetic variation in seven selfed and unselfed *C. pepo* landraces from three districts in the KwaZulu-Natal Province was investigated using the random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers. Of the 36 and 55 primers tested, 9 RAPD and 10 SSR primers were selected for their reproducibility and high polymorphism. A total of 100 fragments were detected by RAPD, out of which 94 (94%) fragments were considered polymorphic. SSR markers revealed a total of 56 alleles, where 38 (68%) alleles were polymorphic. The sizes of fragments ranged from 75 to 1800 bp and from 124 to 251 bp in RAPD and SSR markers, respectively. The number of fragments per primer ranged between 9 and 14 and between 1 and 12, in RAPD and SSR, respectively. The genetic differentiation coefficient between populations (G_{ST}) ranged between 0.0022 and 0.0100 with RAPD marker and between zero and 0.0076 with SSR marker. Both markers revealed overwhelming averages of gene flow: 97.78 and 132.09 in RAPD and SSR markers, respectively. Both techniques discriminated the landraces very effectively, but only the RAPD marker was able to discriminate landraces according to fruit colour change at maturity as well as their agro-ecological origins. Dendrograms of both markers depicted the close relationship between landraces originating from the Umkhanyakude district (MNS and MS landraces). All genetic parameters indicated that there was plentiful genetic diversity in *C. pepo* landraces of northern KwaZulu-Natal, South Africa.

1. Introduction

The importance of *Cucurbita pepo* as a vegetable crop has been long recognized worldwide due to its high nutritional and economic value (Formisano et al., 2012; Ghobary and Ibrahim, 2010; Tsivelikas et al., 2009). In South Africa the shoot tips, leaves, flowers, fruits and seeds of *C. pepo* are cooked to prepare leafy vegetable dishes (Jansen van Rensburg et al., 2007; Voster et al., 2007). It has a wide range of genetic variability, both in vegetative and reproductive characteristics (Ferriol et al., 2003; Formisano et al., 2012; Kathiravan et al., 2006).

Landraces are diverse arrays of local varieties that are adapted to local environmental conditions and inputs, and have wide genetic diversity (Modi, 2004; Mujaju et al., 2010). Traditional small

scale farmers prefer to preserve their landraces through *in situ* conservation methods (Modi, 2004). Intercropping of different *Cucurbita* species is widely practiced by communities and small scale farmers in South Africa and other countries (Molebatsi et al., 2010; Mujaju et al., 2010; Torquebiau et al., 2010). Such intercropping enhances the geneflow among the cucurbit species due to random bee pollination (Cuevas-Marrero and Wessel-Beaver, 2008; Mujaju et al., 2010). Gene exchange among plant populations located in distant geographical areas can be influenced by the informal seed exchanges between farmers (Barboza et al., 2012; Du et al., 2011).

Cucurbits are naturally cross-pollinated, but self-pollination is generally practiced in them for inbreeding purposes (Ercan and Kurum, 2003). Selfing increases plant mean homozygosity, which is not the natural genetic state of cross-pollinated species and thus reduces the proportion of heterozygosity in the population (Cardoso, 2004; Ercan and Kurum, 2003). Characters studied by Ghobary and Ibrahim (2010) in selfed *C. pepo* showed that the

phenotypic expression of these traits were indicative of their genetic behaviour.

The potential applications of RAPD fingerprinting in molecular biology include: determination of taxonomic identities; detection of interspecific gene flow; assessment of kinship relationships; analysis of mixed genome samples; and production of specific probes and gene mutations (Hadrys et al., 1992; De Wolf et al., 2004). Despite some limitations, RAPD markers have been used extensively and successfully to analyze genetic diversity in cucurbits (Cuevas-Marrero and Wessel-Beaver, 2008; Dey et al., 2006; Hadia et al., 2008; Khan et al., 2009; Morimoto et al., 2006; Tsivelikas et al., 2009). These markers are also beneficial because they: can be applied to unknown genomes and to limited DNA quantities; are a simple technique; can produce a nearly unlimited number of markers; use a large set of primers which can screen the entire genome (Navajas and Fenton, 2000; Gajera et al., 2010; Khan et al., 2009).

Simple sequence repeat (SSR) or microsatellite markers occur frequently in most eukaryotic genomes and can be very informative, reliable (reproducible), codominant, multiallelic and highly polymorphic, making them well suited for detecting variation among closely related varieties (Formisano et al., 2012; Garcia et al., 2004). However, the application of SSR techniques to plants is only possible when the microsatellite markers suitable for that plant are available (Formisano et al., 2012; Garcia et al., 2004). These markers are available for *Cucurbita* species (Formisano et al., 2012; Gong et al., 2008).

Although genetic diversity among such nutritious and economically valuable *C. pepo* landraces or genotypes has been done earlier in other countries using RAPD (Hadia et al., 2008) and SSR (Formisano et al., 2012), none of these studies were conducted in South Africa. The aim of the present work was to analyze the polymorphism and genetic relationship among and within *C. pepo* landraces from the Umkhanyakude, uThungulu and Zululand districts of northern KwaZulu-Natal using the random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers. This work further analyzed the effect of self pollination in genetic variation of these landraces, as they are traditionally intercropped with other *Cucurbita* landraces, thus enhancing gene flow between different landraces.

2. Materials and methods

2.1. Plant material

The seeds of *C. pepo* collected from the uThungulu (Nkandla: 28°37′S, 31°25′E), Umkhanyakude (Mseleni: 27°38′S, 32°47′E) and Zululand (Ulundi: 28°32′S, 31°47′E) districts were grown at the Ethnobotany Garden and Agriculture Farm at the University of Zululand (28°51′S, 31°50′E), and were used as the source of plant material. Two sets of plants were used to harvest leaf material for DNA extraction. One set was from the seeds that were freshly from the communities of the three districts. Another set was from the seeds that were initially from the communities but the fruits were self-pollinated to ensure the fixing of traits, where the natural pollinators (bees) were suspected to mix some pollen as the communities intercrop different Cucurbitaceae species in their fields. Young (folded to semi-folded) leaves were picked, freeze-dried and stored at 4 °C for future use. The following *C. pepo* landraces' populations had their genetic diversity analyzed: Umkhanyakude unselfed (MNS); Umkhanyakude selfed (MS); Umkhanyakude green ripe fruits (CPSP); uThungulu unselfed (TNS); uThungulu selfed (TS); Zululand unselfed (ZNS); and Zululand selfed (ZS).

2.2. Self pollination procedure

The *C. pepo* plants from the Umkhanyakude, Uthungulu and Zululand districts were grown in different areas that were about two kilometers away from each other to prevent the incidence of pollen transference among plants from different districts. Both pistillate and staminate flowers that were to be selfed the following morning were covered with a light, fine-porous cloth (curtain fabric), mimicking a cheesecloth bag (used by Winsor et al., 2000), in the afternoon prior to flower anthesis. These flowers were recognized by the appearance of a slight touch of yellow or orange at the apex of the corolla tube or rather when the yellow/orange colour of the petals (corolla) was clearly seen or intensified from the outside, as described by Ercan and Kurum (2003). At flower anthesis, soon after dehiscence of pollen sacs (pollen anthesis), self pollination (pollen transference from the staminate flower to the stigma of the pistillate flower, in the same plant) was initiated from 04h00 until about 08h30 in the morning. The viability and germination potential of pollen grains was still high at these early hours of the day (Nepi and Pacini, 1993; Agbagwa et al., 2007) and both flower types were wide open.

During selfing, the staminate flowers were picked and had their corolla tube removed to expose the pollen-laden stamens and the pollen was gently rubbed on the stigma lobes of the pistillate flower in the same plant (Fike, 2011; Thralls and Treadwell, 2008). One male flower was used for each female recipient (Spencer and Snow, 2001) due to high levels of irregularities in anthesis of both staminate and pistillate flowers of one plant. To prevent uncontrolled bee pollination, after self pollination the pistillate flowers were recovered for the whole day. The cover was removed the following day, since the female flowers are receptive on the ovules for only one day (day of flower anthesis) (Nepi and Pacini, 1993; Agbagwa et al., 2007).

2.3. Genomic DNA extraction

The DNeasy Plant Mini Kit (from QIAGEN®, Valencia, CA, USA) was used to extract DNA from the leaves according to the manufacturer's instructions. Twenty plants per accession were used to source leaf material. The yield of DNA in ng/µl was measured using the Nano Drop ND-1000 Spectrophotometer (software ND-1000 V3.5.1; USA). The DNA purity was calculated at 260/280 nm wavelengths. The DNA with an absorbance ranging between 1.7 and 1.9 were considered pure and were used for Polymerase Chain Reactions (PCR).

2.4. RAPD analysis

Approximately 50 ng of DNA was amplified through the PCR using 25 µl reactions under the following conditions: 1× of GoTaq® Green Master Mix, 2× (Promega Corporation); 0.4 µM random 10-mer oligonucleotide primer (Inqaba Biotechnical Industries (Pty) Ltd), and Nuclease-Free Water (Promega Corporation).

Amplification was performed in a MJ Mini Personal Thermal Cycler (from BIO-RAD) programmed for an initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 36 °C for 30 s, and 72 °C for 1 min, and final extension of 72 °C for 4 min. Amplified products were separated in 1% agarose in 1× Tris-Borate-EDTA (TBE) buffer with 125 ng ethidium bromide per liter, using gel electrophoresis run at 70 V for 1 h. The nucleic acid markers 100 bp (Promega Corporation) and 1 kb (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd) were used to compare the amplification product sizes.

Of thirty six primers tested, nine primers producing distinct polymorphic bands were selected for further analysis (Table 1). Each primer producing constituent amplification of well defined,

Table 1
Sequence, produced band size range and polymorphism of different RAPD primers, as well as genetic variability within seven *Cucurbita pepo* populations.

RAPD primer	Sequence (5′–3′)	Band size range (bp)	N	A	P	H	I	H_S	G_{ST}	Nm
CB9	GGTGACGCAG	100–1300	10	9	90	0.1084	0.2201	0.1081	0.0027	183.3149
CB12	AGTCGACGCC	100–1300	9	8	89	0.1417	0.2707	0.1412	0.0031	160.5899
CB13	ACGCATCGGA	100–1100	10	10	100	0.1334	0.2584	0.1331	0.0022	223.7226
CB15	GGCTGGTTCC	75–1400	12	11	92	0.1783	0.3227	0.1772	0.0060	82.5414
CB17	GTAACCAGCC	100–1400	12	11	92	0.1781	0.3225	0.1774	0.0039	127.1654
CB19	GGTGCTCCGT	75–1400	14	14	100	0.1335	0.2585	0.1330	0.0035	143.1361
CB21	CAGCACTGAC	100–1800	12	12	100	0.1623	0.3004	0.1611	0.0070	70.7242
CB23	CTGGGCACGA	200–1400	11	9	82	0.1865	0.3340	0.1847	0.0100	49.4545
CB27	AAGTGCGACC	200–1300	10	10	100	0.1335	0.2586	0.1329	0.0047	106.6980
Total	–	–	100	94	94					
Average	–	–	11.11	10.44	94	0.1506	0.2829	0.1499	0.0051	97.7840

CB: Cucurbita; *N*: total number of bands; *A*: number of polymorphic bands; *P*: percentage of polymorphism; *H*: Nei's gene diversity; *I*: Shannon's information index; H_S: Genetic diversity index within populations; G_{ST}: Genetic differentiation coefficient between populations; Nm: Gene flow.

brightly staining bands was used in further amplification of DNA from all individuals. Amplification was repeated to ensure reproducibility of scored products. Random Amplified Polymorphic DNA markers were scored for presence or absence, and each marker was identified by primer and marker size.

The data was analysed using the Population Genetic Analysis (POPGENE version 1.31) (Yeh et al., 1999). The following genetic diversity parameters were determined: (1) the number of polymorphic bands (A) and the percentage of polymorphic bands (P); (2) gene diversity (H) and Shannon's information index (I); (3) Nei's genetic distances (D) and genetic identity (I_N), which were evaluated using the cluster analysis that was performed with the Unweighted Pair Group Method of Arithmetic Average (UPGMA); and 4) the coefficient gene differentiation among the populations within species, which was determined using Nei's gene diversity method. The formula was $G_{ST} = D_{ST}/H_T$, $H_T = H_S + D_{ST}$, where, H_T is the total gene diversity, H_S is the gene diversity within the population, and D_{ST} is the gene diversity between populations. The gene flow was determined as Nm = 0.5 $(1 - G_{ST})/G_{ST}$ (Yuan et al., 2007).

2.5. SSR analysis

Genotyping using SSR markers was conducted by INCOTEC South Africa (Pty) Ltd. Company at Pietermaritzburg, South Africa. All samples were used in bulked amplification, using DNA extracted from all the leaf samples of each entry submitted. Ten SSR markers were used (Table 4). Polymerase chain reaction products were fluorescently labeled and separated by capillary electrophoresis on an ABI 3130 automatic sequencer (Applied Biosystems, Johannesburg, South Africa). Analysis was performed using GeneMapper 4.1. The program GGT 2.0 (Van Berloo, 2008) was used to calculate the Euclidean distances between bulked samples. The matrix of the genetic distances was used to create an UPGMA dendrogram of the results. POPGENE was used to calculate: Nei's gene diversity (*H*); Shannon's information index (*I*); genetic diversity index within populations (H_S); genetic differentiation coefficient between populations (G_{ST}) and gene flow (Nm).

3. Results

3.1. RAPD and SSR polymorphism

The analysis of seven *C. pepo* populations with the nine RAPD primers that were polymorphic identified a total of 100 reproducible fragments (Table 1). Among them, 94 were polymorphic (94%), ranging in size from 75 to 1800 bp. A range between nine and 14 fragments were amplified per primer, with an average of 11.11 bands. The number of polymorphic fragments for each primer varied from eight (CB12) and 14 (CB19), with an average of 10.44 bands.

A total of 56 alleles were observed with a range from one (CUTC002746 and CUTC04645) to 12 (PKCT122) alleles per SSR marker (Table 2). Thirty-eight alleles were polymorphic, where zero (CUTC002746; CMPMC21 and CUTC04645) to 11 (PKCT122) were detected for each SSR marker. The SSR markers detected 67.86% polymorphism, which varied from 0% (CUTC002746; CMPMC21 and CUTC04645) to 100% (CUTC009607). The allele size ranged from 124 to 251 bp. Microsatellite markers: CUTC002746; CMPMC21 and CUTC04645 were found to be monomorphic primers by giving similar bands: 195 bp; 127 and 129 bp; and 195 bp, respectively, in all accessions.

3.2. Population genetic diversity, differentiation (G_{ST}), and gene flow (Nm)

In RAPD markers, the Nei's gene diversity index (H) varied from 0.1084 (CB9) to 0.1865 (CB23), with an average of 0.1506 ± 0.0267 (Table 1). The Shannon's information index (*I*) ranged from 0.2201 (CB9) to 0.3340 (CB23), with an average of 0.2829 ± 0.0386. The genetic diversity index within populations (H_S) varied from 0.1081 (CB9) to 0.1847 (CB23), with an average of 0.1499 ± 0.0007. The SSR markers CUT002746 and CUTC04645 had the lowest genetic variability (*H* = 0.0191; *I* = 0.0544; H_S = 0.0191), while marker PKCT122 had the highest (*H* = 0.1726; *I* = 0.3148; H_S = 0.1719); where the averages were: *H* = 0.0675; *I* = 0.1454 and H_S = 0.0672 (Table 3).

With RAPD markers, the genetic differentiation coefficient between populations (G_{ST}) ranged between 0.0022 (CB13) and 0.0100 (CB23), with an average of 0.0051 (Table 1). This showed that the genetic variation between populations accounted for between 0.22% and 1.00%, with an average of 0.51% of the total variation. The gene flow (Nm) ranged between 49.4545 (CB23) and 223.7226 (CB13), with an average of 97.7840. This indicated that there was a high genetic exchange between *C. pepo* populations. However, with SSR markers the G_{ST} ranged from zero (CUT002746; CMPMC21 and CUTC04645) to 0.0076 (CUTC009607) with an average of 0.0038 (Table 3). Gene flow varied between 65.4118 (CUTC009607) and 2000.00 (CUT002746; CMPMC21 and CUTC04645) with an average of 132.0931.

The Nei's gene diversity index (*H*) and Shannon's information index (*I*) were conducted using RAPD and SSR markers to further understand the genetic diversity among the selfed and unselfed populations of *C. pepo* originating from three different districts (Table 4). The RAPD markers showed that Nei's gene diversity index and Shannon's information index were the highest in ZS population (*H* = 0.1677; *I* = 0.3060) and the lowest in TNS population (*H* = 0.1301; *I* = 0.2518). With SSR markers, they were the highest in TNS population (*H* = 0.0853; *I* = 0.1742) and the lowest in TS population (*H* = 0.0564; *I* = 0.1260).

The RAPD markers showed that selfed populations of the uThungulu and Zululand districts had higher Nei's gene diversity index

Table 2
Polymorphism and sizes of alleles detected by ten SSR markers in seven *C. pepo* populations.

SSR marker	CMTP9	CMTP132	CUTC002746	CMPMC21	CUTC004399	CUTC04645	CMPTM39	CUTC009607	PKCT122	PKCT111	Total	Average
Size Range (bp)	155–195	115–140	190–201	122–135	170–190	185–206	165–185	150–175	210–255	199–213		
Detected Alleles	7	4	1	2	4	1	4	10	12	11	56	5.6
Polymorphic Alleles	3	2	0	0	3	0	2	10	11	7	38	3.8
Percentage Polymorphism	42.86	50.00	0	0	75.00	0	50.00	100	91.67	63.64	67.86	
Allele sizes per populations												
MNS	159, 162, 168, 171	124, 125, 136	195	127, 129	175, 181	195	174, 176	154, 156	208, 210, 233, 235, 241, 243, 245, 247, 249, 251	204, 206, 208, 210		
MS	159, 162, 168, 171	124, 125, 136	195	127, 129	175, 178, 179, 181	195	174, 176	154, 156	210, 212, 233, 235, 237, 239, 247, 249, 251	204, 206, 208, 210		
TNS	159, 162, 168, 171, 174	124, 125, 136	195	127, 129	175, 178, 179, 181	195	174, 176, 180, 182	154, 156, 158, 167, 169, 171	208, 210, 212, 233, 235, 237, 239, 243, 245, 247, 249, 251	204, 206, 208, 210, 219, 221, 223, 225		
TS	159, 162, 168, 171	124, 125	195	127, 129	175	195	174, 176	152, 154, 156, 158, 169, 171	210, 212, 243, 245, 247, 249, 251	204, 206, 208, 210		
ZNS	159, 162, 168, 171, 187	124, 125, 136	195	127, 129	175, 181	195	174, 176	154, 155, 156, 157, 160	208, 210, 212, 233, 235, 243, 245, 247, 249, 251	204, 206, 208, 210, 217, 219, 221, 223		
ZS	159, 162, 168, 171, 187	124, 125, 136	195	127, 129	175	195	174, 176	155, 157, 160	208, 210, 212, 233, 235, 237, 239	204, 206, 208, 210, 217, 219, 221, 223		
CPSP	151, 159, 162, 168, 171, 187	124, 125, 134, 136	195	127, 129	175, 181	195	174, 176	152, 154, 156, 157, 160, 169	208, 210, 212, 233, 235, 237, 239, 245, 247, 249, 251	200, 202, 204, 206, 208, 210		

Populations (district and population name): MNS, Umkhanyakude unselfed; MS, Umkhanyakude selfed; TNS, uThungulu unselfed; TS, uThungulu selfed; ZNS, Zululand unselfed; ZS, Zululand selfed; CPSP, Umkhanyakude *C. pepo* species with dark green mature fruits.

Table 3
Genetic variability within seven *C. pepo* populations based on SSR markers.

SSR marker	H	I	H_S	G_{ST}	Nm
CMTP9	0.0886	0.1879	0.0885	0.0011	442.4883
CMTP132	0.0569	0.1323	0.0568	0.0010	506.5375
CUTC002746	0.0191	0.0544	0.0191	0.0000	2000.0000
CMPMC21	0.0381	0.0958	0.0381	0.0000	2000.0000
CUTC004399	0.0436	0.1068	0.0433	0.0060	82.6283
CUTC04645	0.0191	0.0544	0.0191	0.0000	2000.0000
CMPTM39	0.0435	0.1067	0.0434	0.0022	225.8178
CUTC009607	0.0809	0.1750	0.0803	0.0076	65.4118
PKCT122	0.1726	0.3148	0.1719	0.0040	123.0468
PKCT111	0.1122	0.2260	0.1115	0.0064	77.7294
Average	0.0675	0.1454	0.0672	0.0038	132.0931

H: Nei's gene diversity; I: Shannon's information index; H_S: genetic diversity index within populations; G_{ST}: genetic differentiation coefficient between populations; Nm: gene flow.

and Shannon's information index than their analogous unselfed populations, while the opposite was evident in populations from the Umkhanyakude district. However, SSR markers revealed an opposite scenario.

3.3. Specific RAPD marker production per primer per landrace(s)

Specific RAPD markers for CPSP population only were produced by: primers CB9 and CB12 (700 bp); primers CB13, CB19 and CB21 (1000 bp); and primer CB27 (1100 bp). Also primers CB15 and CB17 produced exclusive markers 100 bp and 500 bp, respectively, in all populations except CPSP population. The CPSP population had fruits that did not change their colour to orange or yellow at maturity.

Primer CB9 also showed the effect of selfing by identifying a unique band of 400 bp in unselfed populations from all districts including CPSP population. Primer CB21 also identified marker 900 bp for only unselfed populations from all districts excluding CPSP population. Primer CB15 produced specific band 200 bp for only selfed populations from all districts, but included the CPSP population. Primer CB9 produced unique band 600 bp only in MS and ZS populations, which were both selfed but from different districts. Primer CB23 produced specific band 1000 bp in MNS and MS populations, both from one eco-geographic region, the Umkhanyakude district.

The following specific RAPD markers were only amplified in each of the following populations: MNS population [CB9 (800 bp), CB12 (900 bp), CB27 (800 bp)]; TNS population [CB23 (900 bp), CB27 (1000 bp)]; MS population [CB9 (1200 bp)] and TS population [CB13 (900 bp)].

3.4. Unique SSR alleles per population

Unique alleles specific for CPSP population were detected by SSR marker: CMTP9 (151 bp); CMTP132 (134 bp) and PKCT111 (200 bp and 202 bp) (Table 2). The following markers also revealed alleles that are unique to TNS population: CMTP9 (174 bp); CMPTM39 (180 bp and 182 bp); CUTC009607 (167 bp) and PKCT111 (225 bp). Marker CMTP9 (with 187 bp) and CUTC009607 (with 157 bp and 160 bp) revealed a relationship in ZNS, ZS and CPSP populations by the presence of alleles unique only to them. The CMTP132 revealed the uniqueness of TS population by the absence of allele 136 bp compared with other populations. The CUTC009607 marker revealed allele 169 bp as unique to uThungulu populations and CPSP population. The following SSR markers revealed alleles unique to populations from the uThungulu [CUTC009607 (158 bp and 171 bp)] and Zululand [CUTC009607 (155 bp) and PKCT111 (217 bp)] districts.

In populations from the Umkhanyakude district, the SSR marker CUTC004399 showed the presence of alleles 178 bp and 179 bp only in selfed population (Table 2). However, marker PKCT122 showed the presence of 208 bp, 241 bp, 243 bp and 245 bp in unselfed populations while selfed populations had 212 bp, 237 bp and 239 bp. In addition to unique alleles mentioned earlier in the TNS population, the following markers revealed alleles presented in brackets only in unselfed populations from the uThungulu district: CUTC004399 (alleles 178 bp, 179 bp and 181 bp); PKCT122 (alleles 208 bp, 233 bp, 235 bp, 237 bp and 239 bp) and PKCT111 (alleles 219 bp, 221 bp and 223 bp). Marker CUTC009607 showed allele 152 bp only in selfed populations from the same district. Differences found only in unselfed populations from the Zululand district were shown by markers: CUTC004399 (allele 181 bp); CUTC009607 (alleles 154 bp and 156 bp) and PKCT122 (alleles 243 bp, 245 bp, 247 bp, 249 bp and 251 bp). Primer PKCT122 also detected alleles 237 bp and 239 bp only in selfed populations from the same district.

3.5. Genetic identity and genetic distance between C. pepo populations, based on RAPD and SSR markers

The genetic identity (I_N) ranged from 0.9985 to 0.9996, while the genetic distance (D) varied from 0.0004 to 0.0015 with RAPD markers (Table 5). The TS and TNS populations were the highest in genetic identity ($I_N = 0.9996$) and the closest in the genetic distance ($D = 0.0004$). The CPSP and MNS populations as well as CPSP and TNS populations were the lowest in genetic identity ($I_N = 0.9985$) and the furthest in genetic distance ($D = 0.0015$).

With SSR markers, the genetic distance ranged from 0.2542 to 0.5157 (Table 6). The closest distance (highest degree of similarity) was observed between MNS and MS populations which are both

Table 4
Genetic variation among *C. pepo* populations based on RAPD and SSR analysis.

Markers population	RAPD		SSR	
	H	I	H	I
MNS	0.1643 ± 0.0523	0.3003 ± 0.0733	0.0580 ± 0.0478	0.1283 ± 0.0801
MS	0.1393 ± 0.0469	0.2645 ± 0.0681	0.0600 ± 0.0428	0.1329 ± 0.0737
TNS	0.1301 ± 0.0349	0.2518 ± 0.0512	0.0853 ± 0.0611	0.1742 ± 0.1002
TS	0.1461 ± 0.0302	0.2760 ± 0.0449	0.0564 ± 0.0399	0.1260 ± 0.0724
ZNS	0.1427 ± 0.0391	0.2704 ± 0.0556	0.0726 ± 0.0558	0.1528 ± 0.0942
ZS	0.1677 ± 0.0438	0.3060 ± 0.0613	0.0618 ± 0.0467	0.1348 ± 0.0823
CPSP	0.1587 ± 0.0318	0.2941 ± 0.0467	0.0762 ± 0.0575	0.1589 ± 0.0966

Values are mean ± standard deviation. *H*, Nei's gene diversity; *I*, Shannon's information index. Populations (district and population name): MNS, Umkhanyakude unselfed; MS, Umkhanyakude selfed; TNS, uThungulu unselfed; TS, uThungulu selfed; ZNS, Zululand unselfed; ZS, Zululand selfed; CPSP, Umkhanyakude *C. pepo* species with dark green mature fruits.

Table 5
Nei's original measure of genetic identity and genetic distance among seven *C. pepo* populations with RAPD markers.

Population ID	MNS	MS	TNS	TS	ZNS	ZS	CPSP
MNS	****	0.9995	0.9988	0.9992	0.9986	0.9989	0.9985
MS	0.0005	****	0.9988	0.9991	0.9987	0.9991	0.9992
TNS	0.0012	0.0012	****	0.9996	0.9994	0.9990	0.9985
TS	0.0008	0.0009	0.0004	****	0.9994	0.9990	0.9993
ZNS	0.0014	0.0013	0.0006	0.0006	****	0.9990	0.9988
ZS	0.0011	0.0009	0.0010	0.0010	0.0010	****	0.9989
CPSP	0.0015	0.0008	0.0015	0.0007	0.0012	0.0011	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Table 6
Pairwise Jaccard's genetic distances between *C. pepo* populations based on SSR markers.

Population ID	MNS	MS	TNS	TS	ZNS	ZS
MS	0.2542					
TNS	0.3901	0.4259				
TS	0.2750	0.2887	0.4509			
ZNS	0.3698	0.4173	0.4337	0.3736		
ZS	0.4810	0.5157	0.5127	0.3783	0.3838	
CPSP	0.3640	0.4078	0.4413	0.3687	0.4052	0.4556

Populations (district and population name): MNS, Umkhanyakude unselfed; MS, Umkhanyakude selfed; TNS, uThungulu unselfed; TS, uThungulu selfed; ZNS, Zululand unselfed; ZS, Zululand selfed; CPSP, Umkhanyakude *C. pepo* species with dark green mature fruits.

from the coastal region in the Umkhanyakude district. However, the furthest distance (lowest degree of similarity) was observed between MS and ZS populations, a selfed Umkhanyakude district and a selfed Zululand district (inland region) population, respectively.

3.6. Cluster and population structure analysis, based on RAPD and SSR markers

The phylogenetic relationship between populations was further illustrated by a dendrogram using the UPGMA algorithm based on Nei's genetic distance (1972). The dendrogram with RAPD markers grouped the populations into two main clusters (Fig. 1). Cluster two had a CPSP population (landrace with green and white fruit variegation at maturity) which was distant from a group with all other six populations. Cluster one had two sub-clusters with a clear grouping according to geographical origin. Sub-cluster one grouped the populations from the Umkhanyakude district (MNS and MS). Sub-cluster two had populations from the uThungulu district (TNS and TS) which were then assembled with populations from Zululand district (ZNS and ZS).

The UPGMA dendrogram with SSR markers also grouped populations into two main clusters (Fig. 2). Cluster one had the first sub-cluster which grouped populations according to their geographic origin (MNS and MS populations from the Umkhanyakude district). The second sub-cluster grouped selfed populations from different origins (TS and ZS populations from the uThungulu and Zululand districts, respectively). The unselfed population from the Zululand district (ZNS) and *C. pepo* population from the Umkhanyakude district which retains its green fruit colour at
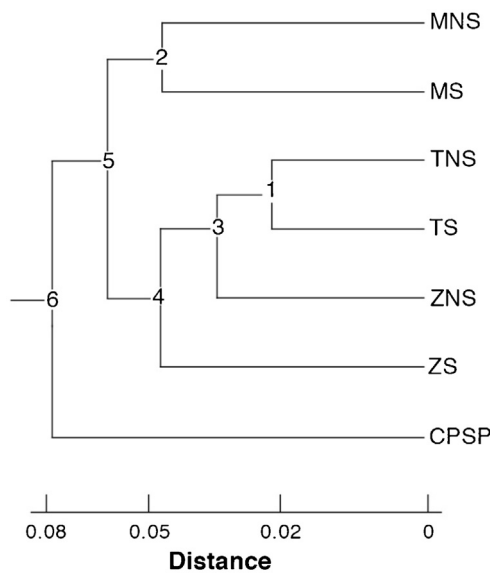

Fig. 1. Unweighted Pair Group Method of Arithmetic Mean (UPGMA) dendrogram based on Nei's (1972) genetic distance, summarizing data on differentiation in seven populations of *C. pepo* eco-geographical populations with RAPD markers. Populations (district and population name): MNS, Umkhanyakude unselfed; MS, Umkhanyakude selfed; TNS, uThungulu unselfed; TS, uThungulu selfed; ZNS, Zululand unselfed; ZS, Zululand selfed; CPSP, *C. pepo* species with dark green mature fruits.
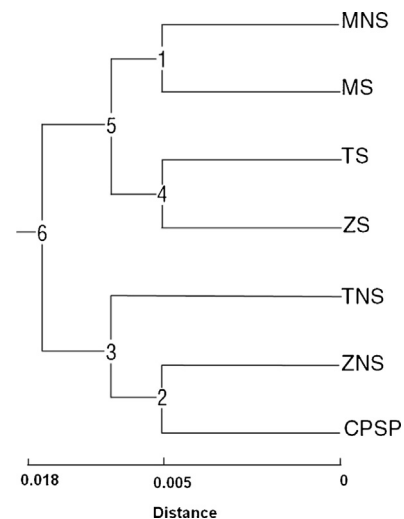

Fig. 2. Unweighted Pair Group Method of Arithmetic Mean (UPGMA) dendrogram based on Nei's (1972) genetic distance, summarizing data on differentiation in seven populations of *C. pepo* eco-geographical populations with SSR markers. Populations (district and population name): MNS, Umkhanyakude unselfed; MS, Umkhanyakude selfed; TNS, uThungulu unselfed; TS, uThungulu selfed; ZNS, Zululand unselfed; ZS, Zululand selfed; CPSP, *C. pepo* species with dark green mature fruits.

maturity formed cluster two and were assembled with unselfed populations from the uThungulu district (TNS).

4. Discussion

4.1. Polymorphism in C. pepo *based on RAPD and SSR analysis*

The level of polymorphism among the *C. pepo* population was relatively high with RAPD marker, ranging between 82% and 100% with an average of 94%. This high level of RAPD markers polymorphism in *C. pepo* genotypes is in accordance with the results of Hadia et al. (2008), Kathiravan et al. (2006), and Tsivelikas et al. (2009), who reported that *C. pepo* is a highly polymorphic species. The degree of RAPD polymorphism detected in the present study was slightly higher than that reported in other studies of *Cucurbita maxima* 84%, *Cucurbita moschata* 87% and *C. pepo* 89% (Hadia et al., 2008); *Trichosanthes dioica* 79% (Khan et al., 2009), and *Citrullus lanatus* 88% (Mujaju et al., 2010); *Momordica charantia* 37% (Behera et al., 2008; Dey et al., 2006), *Lagenaria siceraria* 30% (Morimoto et al., 2006), *Lagenaria sphaerica* 24% (Morimoto et al., 2006), and *Cucumis melo* 72% (Yildiz et al., 2011).

A total of 56 SSR alleles, with a size range from 124 bp to 251 bp, as detected by 10 SSR markers were found among *C. pepo* landraces of northern KwaZulu-Natal. In Zimbabwe, Mujaju et al. (2010) reported a total of 43 SSR alleles, with 123 bp to 285 bp size range, as revealed by nine SSR markers among *C. lanatus* landraces. The percentage polymorphic alleles varied from 0% to 100% with an average of 67.86%. Mujaju et al. (2010) reported a percentage polymorphic alleles range from 55.6% to 88.9% among watermelon landraces. The monomorphism of SSR markers: CUTC002746; CMPMC21 and CUTC04645 concurs with Tantasawat et al. (2010) who reported monomorphism in SSR marker VM 21 which gave a single band of 179 bp in all accessions of *Vigna* species.

4.2. Population genetic structure and geographic diversity of northern KwaZulu-Natal C. pepo *landraces*

Gene differentiation and gene flow are important indexes to evaluate the population genetic structure. The gene differentiation coefficients of *C. pepo* landraces in northern KwaZulu-Natal were 0.0051 and 0.0038, when analyzed with RAPD and SSR markers, respectively. Ferriol et al. (2003) reported the gene differentiation coefficients of *C. pepo* landraces, mainly from Spain, as 0.25 and 0.18, when analysed with SRAP and AFLP, respectively. Further, the gene differentiation coefficients of Spanish *C. moschata* landraces as determined by AFLP and SRAP analyses were 0.28 and 0.17, respectively (Ferriol et al., 2004b). The average values of G_{ST} were 0.0051 and 0.0038, based on the RAPD and SSR markers, respectively. This indicates that the gene differentiation was higher within populations (99.49% and 99.62%) than between populations (0.51% and 0.38%), respectively. Similar findings were reported by Yuan et al. (2007) among *Punica granatum* populations in China.

The average values of the gene flow were 97.7840 and 132.0931, based on the RAPD and SSR analysis, respectively. According to Han et al. (2007), this gene flow overwhelmed the effect of genetic drift. Gene flow among *Cucurbita* species is possible for long distances pollen transfer by bees ranging between 800 m and 1300 m (Montes-Hernandez and Eguiarte, 2002; Spencer and Snow, 2001). However, *C. pepo* distributed in different districts of KwaZulu-Natal with long geographical distance, had a very low possibility of the pollen spread by bees between populations. Therefore the main way of gene exchanges was probably enhanced by seed exchanges between farmers of different districts in KwaZulu-Natal, which agrees with the reports of Barboza et al. (2012); Du et al. (2011) and Mujaju et al. (2010).

The Nei's gene diversity index (*H* = 0.1506 and 0.0675), Shannon's information index (*I* = 0.2829 and 0.1454) and the genetic diversity index within populations (*H_S* = 0.1499 and 0.0672), as revealed by RAPD and SSR markers, respectively, also indicate molecular genetic diversity for the *C. pepo* populations studied herein. The gene diversity obtained in the present study by RAPD and SSR markers was lesser than that obtained by AFLP markers among different *C. moschata* accessions (Wu et al., 2011). It was also lesser than that obtained by RAPD markers among *T. dioica* accessions (Khan et al., 2009) as well as by ISSR, SRAP and RAPD markers among Turkish and Foreign *C. melo* genotypes (Yildiz et al., 2011).

The production of specific RAPD marker by primer CB23 (1000 bp) in both MNS and MS populations from the Umkhanyakude district as well as SSR alleles unique to populations from the uThungulu [CUTC009607 (158 bp and 171 bp)] and Zululand [CUTC009607 (155 bp) and PKCT111 (217 bp)] districts only, probably indicate the effect of eco-geographic differences in gene diversity of plants, where landraces from the same geographic area are closely related. A similar observation was reported among *Cucurbita* species studied in Spain (Ferriol et al., 2004a, 2004b) and in China (Du et al., 2011).

4.3. Genetic variation between unselfed and selfed C. pepo *populations*

The Nei's gene diversity index and Shannon's information index revealed by RAPD markers was higher in selfed populations from uThungulu and Zululand districts than their corresponding unselfed population. This probably means that selfing enabled an easier detection of rare alleles, which can increase their effectiveness of selection and the amount for genetic improvement in a breeding program, which concurs with the report by Ghobary and Ibrahim (2010). However, the detection of higher genetic diversity in unselfed populations from these districts than their analogous selfed populations, as shown by SSR markers, probably revealed the heterozygosity in unselfed than selfed populations. Cardoso (2004), Ercan and Kurum (2003) and Ferrari et al. (2006, 2007) reported that self-pollination enhances homozygosity while reducing the heterozygosity in plant genomes. The production of specific RAPD markers and SSR alleles for either selfed or unselfed populations only, also confirmed that selfing had changed the genetic state of these *C. pepo* landraces from different districts in northern KwaZulu-Natal. Ferrari et al. (2006, 2007) reported the change in a plant's genetic state with selfing.

4.4. Specific RAPD markers and unique SSR alleles per landrace(s)

With both RAPD and SSR markers, some fragments were uniquely amplified from single populations, indicating the genotype variation among *C. pepo* landraces in northern KwaZulu-Natal. Similar results were observed for some South American and Spanish *C. moschata* accessions using SRAP and AFLP markers (Ferriol et al., 2004a). Hadia et al. (2008) have identified the specific RAPD markers that showed genotype variation among *C. maxima*, *C. moschata* and *C. pepo* species, as well as those showing differences within their populations. Again, Barracosa et al. (2008) in their study of *Ceratonia siliqua*, made use of unique RAPD markers that were cultivar-specific to differentiate the Portuguese cultivars.

Six RAPD primers (CB9; CB12; CB13; CB19; CB21 and CB27) and three SSR markers (CMTP9; CMTP132 and PKCT111) produced unique bands for only the CPSP population. Two RAPD primers (CB15 and CB17) also produced unique bands for all other six populations except CPSP. The presence and absence of unique bands only in the CPSP population indicated that these primers were probably marking the genes or loci that are affecting fruit colour development or change at maturity among these landraces, where the

CPSP maintains its green fruit variegation at maturity. Paris (2000) reported 11 loci that have been identified as affecting developmental fruit colour in *C. pepo*. Of these, three genes of major effect – D, l-1, and l-2 – account for a considerable portion of the genetic variation in intensity of fruit colouration that is observed in this species. The developmental fruit colouration from light green fruits, several days past anthesis, except for some darkening of the main capillary veins, becoming blackish-green past anthesis, and then turning intense orange on ripening (Paris, 2000, 2009), as observed in other landraces, was conferred by genotype D/D l-1/l-1 L-2/L-2 (Paris, 2000). However, fruits of L-1/ – L-2/ – plants are intense green throughout development (Paris, 2000), as was the case with the CPSP landrace.

4.5. Genetic diversity and relationship among C. pepo populations

The range of genetic identity from 0.9985 to 0.9996 (with RAPD) and genetic distance range from 0.0004 to 0.0015 (with RAPD) and from 0.2542 to 0.5157 (with SSR) indicated the presence of variability among the seven populations of *C. pepo* in northern KwaZulu-Natal. With both RAPD and SSR markers, populations originating from the same districts (uThungulu and Umkhanyakude districts, respectively) were the closest in genetic distance, thus had the highest degree of similarity. However, those from distant districts were the farthest, probably because most of the farmers have maintained the production of these landraces for many years within each district. The same was reported among *P. granatum* cultivars (Yuan et al., 2007), *T. dioica* accessions (Khan et al., 2009) and *C. moschata* accessions (Barboza et al., 2012; Du et al., 2011). The lowest genetic identity and farthest genetic distance between CPSP and MNS populations, both from the Umkhanyakude district (determined with RAPD markers), was possibly because the CPSP population did not change fruit colour to orange or yellow at maturity whereas the MNS population did change. Therefore differences in genes that are responsible for fruit colour formation at maturity as explained by Paris (2000, 2009) might have influenced this high diversity between these populations. The same reason probably explains the grouping of populations with orange or yellow mature fruit colour, as demonstrated by dendrogram of RAPD markers.

The farthest distance between MS population from the Umkhanyakude district and ZS population from the Zululand district using SSR markers was probably the effect of geographic origins and ecotype variations of these populations, since the Umkhanyakude district is more coastal while the Zululand district is inland. Cortese et al. (2010) reported the same among *Panicum virgatum* populations using SSR markers.

The genetic diversity among studied *C. pepo* populations based on RAPD and SSR markers through dendrograms was very small. This was possibly due to the seed sourcing of these populations from three districts of northern KwaZulu-Natal (which is only one province), which was probably a small scale to show high genetic diversity. Earlier studies reported genetic diversity among *Cucurbita* species from different provinces of the same country (Tsivelikas et al., 2009) and also different countries (Ferriol et al., 2003, 2004a, 2004b). This low genetic diversity might also have resulted from the investigation of one *Cucurbita* species, the *C. pepo*. Hadia et al. (2008) in their research reported a higher genetic diversity among populations of *C. maxima*, *C. moschata* and *C. pepo*, which are three different species. Further, this low genetic diversity was probably due to the very high gene flow which was detected by these markers.

The dendrograms showed that populations from the Umkhanyakude district clustered together with both RAPD and SSR markers. Different factors could have led to this grouping. Probably there was no seed exchange between the farmers

from other investigated districts, in contrast to the reports by Barboza et al. (2012). Also, the deep sand coastal ecotype of the Umkhanyakude district (Jury et al., 2009) as compared to the uThungulu and Zululand districts might have changed the genetic make-up of its *C. pepo* populations. This clustering further showed that these populations are grouped according to their agro-ecological regions rather than selfing effect. This concurs with the findings of Ferriol et al. (2004a, 2004b) in their study of *C. maxima* and *C. moschata*, respectively, where accessions grouped according to their geographical origin with both SRAP and AFLP markers. Amadou et al. (2001) using RAPD markers also found the highest similarity among *Vigna subterranea* accessions that originated from the same country. Tsivelikas et al. (2009) also reported the grouping of *C. moschata* landraces according to their agro-climatic regions, with RAPD markers.

The grouping of populations from the uThungulu and Zululand districts with both markers was probably due to the existence of seed exchanges among farmers of these districts. The same was reported earlier by other researchers in cucurbits (Montes-Hernandez and Eguiarte, 2002; Barboza et al., 2012; Ferriol et al., 2004a, 2004b) and other species (Yuan et al., 2007). In populations from these districts, the SSR markers also revealed the effect of self pollination in changing the gene make-up (Ghobary and Ibrahim, 2010), where the selfed populations clustered separately from the unselfed populations.

5. Conclusion

Both RAPD and SSR markers have revealed high polymorphism and genetic diversity among the *C. pepo* landraces with different agro-ecological origins. They further revealed the effect of selfing on the change of genetic make-up in *C. pepo* landraces. RAPD markers revealed the genetic diversity in landraces with differences in fruit colour change at maturity to a higher extent. *C. pepo* landraces from the Umkhanyakude district (MNS and MS landraces) clustered together, with both RAPD and SSR markers.

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