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

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ABSTRACT

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

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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,