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CHARACTERIZATION OF THREE *CITRULLUS LANATUS* (THUNB.) MATSUM. & NAKAI VARIETIES BY FLUORESCENT CHROMOSOME STAINING AND RAPD ANALYSIS

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Abstract

Three commercial *Citrullus lanatus* varieties, namely Sonya, Kanya and Sweet black-2, were investigated with cyto-molecular techniques for characterization. Despite having similar somatic chromosome numbers and karyotype formulae (2n = 22), these three varieties had considerable diversities in Chromomycin A_3 (CMA)- and 4′, 6-diamidino-2-phenylindole (DAPI)-banding patterns. The total somatic chromosomal length was longest in Sweet black-2 (71.44 ± 2.94 µm) and shortest in Sonya (42.31 ± 2.45 µm). Each variety's unique GC- and AT-rich chromatin composition was represented by a variety of numbers, percentages, and patterns of distribution. Eleven random primers were employed to evaluate the genetic diversity among three varieties. Each variety showed a distinct fingerprinting pattern with 88.72% polymorphism while having 10 of the common RAPD bands.

Introduction

Watermelon (*Citrullus lanatus*) is an important cucurbit crop grown worldwide, but mostly in tropical and sub-tropical areas. In Bangladesh, this species is cultivated throughout the country mainly in Char (river basin) areas. The large edible watermelon fruits are popular with consumers throughout the world.

It is reported that the cultivated watermelon varieties were diploid and possessed 2n = 22 chromosomes (Májovsky 1978, Kumar and Subramaniam 1987, Zhao *et al.* 2000, Renner *et al.* 2017). In addition, few other somatic chromosome records were also available in previous literature, such as 2n = 14, 24 and 44 (Májovsky 1978, Kumar and Subramaniam 1987). With the fluorescent *in situ* hybridization (FISH) technique, the chromosomal locations of the 5S and 45S rDNA loci in three *C. lanatus* subspecies have been labeled by Guo *et al.* (2013). Furthermore, the distribution of rDNA has also been examined in *C. lanatus* var. *lanatus*, *C. lanatus* var. *citroides*, *C. colocynthis* and *C. rehmii* (Reddy *et al.* 2013). Li *et al.* (2016) described the pattern of distribution of the 5S and 45S rDNA sites in some *Citrullus* species namely, *C. lanatus* subsp. *vulgaris*, *C. lanatus* subsp. *mucosospermus*, *C. lanatus* subsp. *lanatus*, *C. colocynthis*, *C. ecirrhosus* and *C. rehmii* and also labeled the chromosomes with labeled rDNA sites using synthesized oligonucleotides probes of watermelon. Utilizing the banding pattern generated by PCR using RAPD markers, it is possible to estimate the degree of polymorphism among the different *C. lanatus* germplasm. Using RAPD markers, Li *et al.* (2016) estimated the genetic diversity among watermelon cultivars and created the preliminary genetic linkage map of watermelon. SSR markers were employed by Jarret *et al.* (1997) to identify genetic differences

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between PI accessions of *C. lanatus* var. *citroides*, *C. lanatus* var. *lanatus* and the wild species *C. colocynthis*. Mujaju *et al.* (2010) used both RAPD and SSR markers for the identification of genetic polymorphism among 10 watermelon accessions. Additionally, significant difference between sweet watermelons and cow-melon has been also reported by Levi *et al.* (2000, 2001a, 2001b) using RAPD, Levi *et al.* (2005) using RAPD and ISSR, Jarret *et al.* (1997) using SSR, and Navot and Zamir (1987) using isozymes.

Cytogenetical analysis with karyological parameters is essential for basic biological research and crop improvement. Understanding the genetic variety of various cultivars or subspecies or varieties is essential for the effective management and crop improvement initiatives of the species and information on genetic diversity would be a significant development in that direction. It is widely acknowledged that karyotype analysis typically plays a key role in determining a taxon's taxonomic status when taxonomic features are lacking, because karyotype represents a generally stable attribute that is unique to each specimen. The problem arises when different species share the same chromosomal number and virtually similar karyotype features. Staining using DNA base-specific fluorochromes such as CMA and DAPI can add a new level for characterization in such circumstances (Alam and Kondo 1995, Dash *et al.* 2017). The vast amount of watermelon genetic variability that is still unexplored in Bangladesh can be revealed through cytogenetic and RAPD analysis. Thus in the present study, the fluorescent banding karyotype as well as the RAPD profile of three common cultivated varieties, Sonya, Kanya and Sweet black-2 were analyzed. This finding can be useful to breeders for future crop improvement initiatives.

Materials and Methods

Seeds of three varieties *i.e.*, Sonya, Kanya and Sweet black-2 of *Citrullus lanatus* were grown and maintained in the Botanic Garden, Department of Botany, University of Dhaka, Bangladesh. The collected root tips were soaked on a filter paper to remove surface water and pretreated with 8-hydroxyquinoline (0.002 M) for 3 hrs at 20-25 ºC. Root tips were fixed in 45% acetic acid for 15 min at 4 ºC. The pretreated root tips were hydrolyzed for 35 sec at 60 ºC in a mixture of 1N HCl and 45% acetic acid (2:1). Slides were prepared by orcein squash method and observed under Nikon eclipse 50i microscope. For CMA and DAPI staining, Alam and Kondo´s (1995) protocol was followed. Dry slides were prepared by squash method with 45% acetic acid. For 25 min, the air-dried slides were immersed in McIlvaine's buffer (pH 7.0). For 15 min, the slides were gently washed in McIlvaine's buffer with $MgSO_4$ (5 mM). One drop of CMA (0.1mg/ml) was introduced to the materials for 30 min in a humid chamber before being washed with McIlvaine's buffer containing $MgSO_4$ for 10 min. Slides were mounted in 50% glycerol and stored at 4 ºC overnight before observation. These were observed using a Nikon (Eclipse 50i) fluorescence microscope equipped with a blue-violet (BV) filter cassette. After 24 hrs of air drying, the slides were first incubated in Mcllvaine's buffer (pH 7.0) for 30 min before being immersed in DAPI solution (0.01 mg/ml) for 20 min and mounted with 50% glycerol. Images were captured with a Nikon (Eclipse 50i) fluorescence microscope equipped with a UV filter cassette.

Total genomic DNA was isolated from leaves using a modified CTAB technique (Doyle and Doyle 1987). The concentration of DNA was determined using a spectrophotometer (Analylikjena, Specord 50, Germany). The A 260/280 results for DNA samples ranged from 1.5 to 1.7. The PCR reaction mixture for 25 µl contains template DNA (25 ng) 2 µl, de-ionized distilled water 9.5 µl, Master mixture (Promega, USA) 12.5 µl and primer 1 µl. Polymerase chain reaction (PCR) amplification was done in an oil-free thermal cycler (2720 model of Applied biosystems, USA) for 35 cycles after initial denature at 95 ºC for 5 min, denature at 95 ºC for 45 sec, annealing at 32 ºC for 45 sec, extension at 72 ºC for 3 min and final extension at 72 ºC for 7 min. Eleven

random primers such as OPG-5 (5′-AGT CGT CCC C-3′), OPF-22 (5′-AAG ATC AAA GAC-3′), OPD-69 (5′-CGC TCC AAA TCA-3′), OPC-15 (5′-GAC GGA TCA G-3′), OPC-14 (5′-TGC GTG CTT G-3′), OPC-10 (5′-TGT CTG GGT G-3′), OPAB-5 (5′-CCC GAA GCG A-3′), OPAB-6 (5′-GTG GCT TGG A-3′), OPN-4 (5′-GAC CA CCC A-3′), OPB-9 (5′-TGG GGG ACT C-3′) and OPA-5 (5′-AGG GGT CTT G-3′) were used in the present study for screening.

On 1.5% agarose gel, the amplified products were separated electrophoretically. The gel was made with 1.5 g of agarose powder, 8 µl of ethidium bromide (10 mg/ml), and 100 ml of 1 × TAE buffer. For 45 min, agarose gel electrophoresis was performed in 1 × TAE buffer at 50 volts and 100 mA. As a marker, a DNA ladder (100 bp and 1 kb) was electrophoresed alongside the RAPD reactions. DNA bands were photographed using a gel documentation system and examined on a UV-transilluminator. After gel electrophoresis, the PCR results were examined. The images were evaluated critically, and the RAPD analysis scores acquired using all primers were merged to create a single data matrix. The computer application "popgene32" (Version 1.31) was utilized to estimate polymorphic loci, Nei's (1972) genetic diversity, genetic distance (D) and create a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among the samples.

Results and Discussion

2n = 22 chromosomes were observed in three *Citrullus lanatus* varieties (Fig. 1a-1c, 1j-1l, Table 1) as like other cultivated watermelon varieties (Májovsky 1978, Kumar and Subramaniam 1987, Zhao *et al.* 2000). In addition, few other previous records were also available for this species, such as 2n = 14, 24 and 44 (Májovsky 1978, Kumar and Subramaniam 1987). The basic chromosome number of this family ranges from n = 5 (*Thladiantha*) to n = 20 (*Cucurbita*) and the most frequently reported number is n = 12 (38% of all studied species) and 11 (26% of all studied species) (Bhowmick and Jha 2022). According to previous and current reports, the basic number x = 11 has been confirmed for *Citrullus*. The highest length of total 2n chromosome complements was 71.44 ± 2.94 µm in Sweet black-2 and the lowest was 42.31 ± 2.45 µm in Sonya (Table 1). The range of chromosomal length was 1.34 ± 0.21 - 2.54 ± 0.32 µm in Sonya, 1.52 ± 0.15 - 3.36 ± 0.84 µm in Kanya and 2.97 ± 0.41 - 3.82 ± 0.76 µm in Sweet black-2 (Table 1).

These three varieties were similar in respect of centromeric formula (22m) and supported the previous finding of Kwon *et al.* (2008). Although in present investigation, no satellite was observed, a pair of satellites in chromosome pair II was reported by Kwon *et al.* (2008) in *C. lanatus*. The occurrence of all metacentric chromosomes and a narrower range of chromosomal length indicate the symmetric karyotype of these studied varieties. In evolutionary terms, symmetric karyotypes symbolize primitive nature (Stebbins 1971). Although information regarding chromosome morphology, size and karyotype is available in very few taxa, most of the studied species of this family showed symmetric karyotypes.

All the CMA- and DAPI-bands (except one entirely CMA banded chromosome in pair IX of Kanya) were observed at the terminal regions of respective chromosomes in three commercial *C. lanatus* varieties (Fig. 1d-1i, 1m-1r, Table 2). Three *C. lanatus* cultivars had unique CMA- and DAPI-banding patterns. The number, location, distribution, and percentage of CMA- and DAPI-bands varied throughout the varieties investigated (Fig. 1m-1r, Table 2). Sonya possessed seven CMA and five DAPI bands which covered 14.87% of GC-rich and 8.27% of AT-rich of the total chromatin length. The highest number of CMA (eight) and DAPI (six) bands were observed in Kanya, but a lower percentage of GC-rich (13.73%) and AT-rich (7.49%) than in Sonya. On the other hand, six CMA and three DAPI bands were found in Sweet black-2 that covered 9.55% GC-rich and 5.69% AT-rich of the total chromatin length (Table 2). A tendency of accumulating repetitive sequences in terminal regions was clearly observed in the karyotypes of these varieties

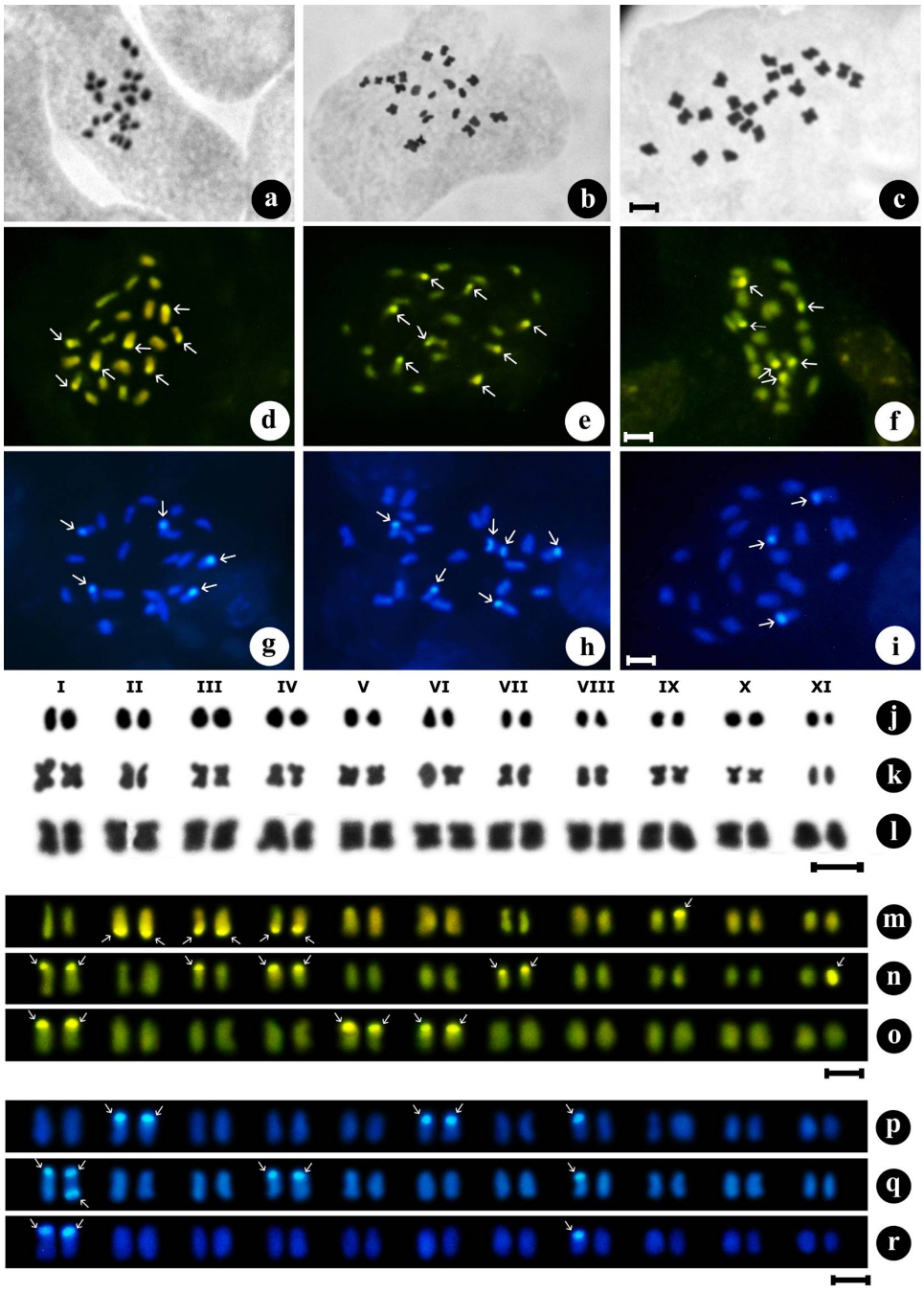

Fig. 1. Orcein, CMA and DAPI-stained metaphase chromosomes and karyotypes of three *Citrullus lanatus* varieties. (a) and (j) Orcein-stained, (d) and (m) CMA-stained, (g) and (p) DAPI-stained metaphase chromosomes and karyotypes of Sonya. (b) and (k) Orcein-stained, (e) and (n) CMA-stained, (h) and (q) DAPI-stained metaphase chromosomes and karyotypes of Kanya. (c) and (l) Orcein-stained, (f) and (o) CMA-stained, (i) and (r) DAPI-stained metaphase chromosomes and karyotypes of Sweet black-2 (arrow indicates CMA and DAPI band). Scale bar = 10 µm.

Table 1. Comparative orcein-stained karyotype analysis of three *Citrullus lanatus* varieties.

Varieties	2n	Total length of 2n chromosome complement (μm)	Range of chromosomal length (μm)	Centromeric formulae
Sonya	22	42.31 ± 2.45	1.34 ± 0.21 - 2.54 ± 0.32	22m
Kanya	22	48.87 ± 1.58	1.52 ± 0.15 - 3.36 ± 0.84	22m
Sweet black-2	22	71.44 ± 2.94	2.97 ± 0.41 - 3.82 ± 0.76	22m

m = metacentric chromosomes.

and revealed the equilocal distribution of heterochromatin at the chromosomal ends. For each specimen, karyotypic equations were created based on the number and placement of CMA- and DAPI-bands. The CMA-banded karyotype formulae were 6α + 1β + 15φ in Sonya, 7β + 1Ω + 14φ in Kanya and 6β + 16φ in Sweet black-2 whereas the DAPI-banded karyotype formulae were 5β + 17φ in Sonya, 4β + 1γ + 17φ in Kanya and 3β + 19φ in Sweet black-2 (Table 2). In Kanya, the entirely CMA fluoresced chromosomes indicated that GC-rich repeats were not restricted to the centromeric or terminal region but dispersed throughout the chromosome. Tandem amplification of GC-rich repeats over the length could explain these completely fluorescent chromosomes, as this kind of chromosome has been reported previously in other plant species (Dash *et al.* 2017). Heteromorphic CMA-banding patterns were observed in chromosome pair IX in Sonya and chromosome pair III and XI in Kanya (Fig. 1m-1o). In these heteromorphic pairs, one chromosome had a terminal CMA band whereas its homologue member did not show any band. DAPI-banding revealed similar types of heteromorphicity in chromosome pair VIII of all three varieties (Fig. 1p-1r). Chromosomal pair I of Kanya variety showed a different heteromorphic banding pattern (Fig. 1q). A chromosome in this pair had a terminal DAPI-positive band in the short arm, whereas its homologue had a terminal band in both arms, suggesting that this variation could have been a marker chromosome. The most likely cause of heteromorphic banding patterns is tandem amplification or deletion of GC- and AT-rich repeats in one of the respective chromosomes.

Three commercial *C. lanatus* varieties were studied with eleven oligonucleotide primers for determining the pattern of genetic variation among them. These eleven primers generated 61 reproducible bands which were used for estimating the genetic similarity among these three varieties (Fig. 2). Ten of the 61 bands were common DNA fragments. In this study, 88.72% polymorphism was identified, demonstrating that these three commercial varieties have a large range of polymorphism. Higher levels of genetic diversity have been found within the germplasm of *C. lanatus* var. *citroides* than in *C. lanatus* var. *lanatus*, according to earlier research (Navot and Zamir 1987, Jarret *et al.* 1997). Ten RAPD primers were employed to characterize eight accessions (five accessions of *C. lanatus* var. *citroides* and three of *C. lanatus* var. *lanatus*) of watermelon from Zimbabwe by Mujaju *et al.* (2010) to generate 138 bands, 122 of which were polymorphic. They proposed that *C. lanatus* var. *lanatus* accessions appear to possess diversity of the same magnitude as the *C. lanatus* var. *citroides* (Mujaju *et al.* 2010). In this investigation, the values of pair-wise Nei's (1972) genetic distances among varieties were calculated using computer software "popgene32" and combined data from eleven RAPD primers. The genetic distance ranged from 0.6769 to 0.9328. Sonya and Kanya had the greatest genetic distance (0.9328), whereas Sonya and Sweet black-2 had the shortest genetic distance (0.6769) (Fig. 3). The lowest genetic distance values among the three varieties were probably found due to similarities in their genetic constituent.

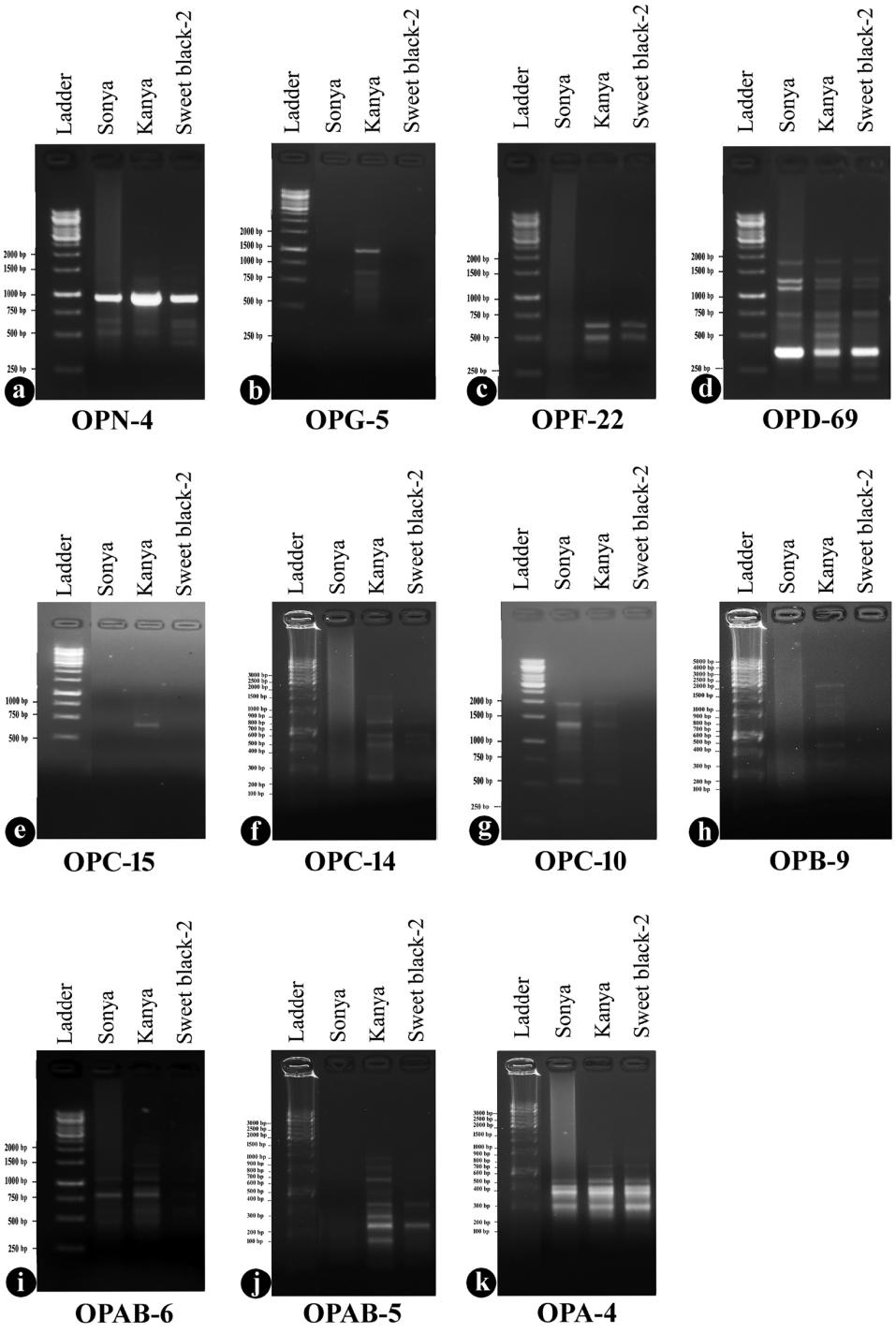

Fig. 2. RAPD analysis of three *Citrullus lanatus* varieties with eleven random primers (1 kb DNA ladder).

Table 2. Fluorescent banding patterns of three *Citrullus lanatus* varieties.

Varieties	CMA-banding				DAPI-banding			
	No. of CMA-bands	GC-rich chromatin length (μm)	% of GC-rich chromatin	CMA-banded karyotype formulae	No. of DAPI-bands	AT-rich chromatin length (μm)	% of AT-rich chromatin	DAPI-banded karyotype formulae
Sonya	7	6.29 ± 1.25	14.87	6α + 1β + 15φ	5	3.50 ± 0.77	8.27	5β + 17φ
Kanya	8	6.71 ± 1.31	13.73	7β + 1Ω + 14φ	6	3.66 ± 1.01	7.49	4β + 1γ + 17φ
Sweet black-2	6	4.67 ± 0.84	9.55	6β + 16φ	3	2.78 ± 0.89	5.69	3β + 19φ

α = Band in terminal region of long arm, β = Band in terminal region of short arm, γ = Band in terminal region of both arm, Ω = Band in whole chromosome, φ = No band.

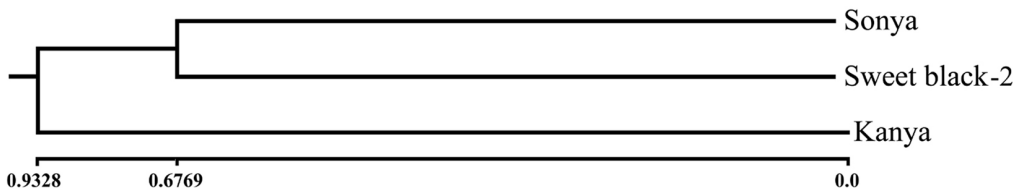

Fig. 3. UPGMA dendrogram based on Nei's (1972) genetic distance summarizing the data on differentiation of three *Citrullus lanatus* varieties by RAPD analysis.

Although the three *C. lanatus* varieties exhibited comparable karyotypic traits, each of them has distinct CMA/DAPI and RAPD-fingerprinting banding patterns. As a result, using cytogenetic and RAPD analyses, it was possible to effectively characterize each variety, which could be useful in future crop improvement programs.

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