

**Assessment of the Genetic Diversity among Wild Colocynth
(*Citrullus colocynthis* (L.)Schrad.) Ecotypes Collected from Different
Regions of Sudan¹**

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Abstract: Better utilization and conservation of the medicinal and aromatic plants (MAP), most of which are collected from the wild, necessitate their screening to identify potential genotypes for both production and utilization in interbreeding to develop superior genotypes. The objective of this research, therefore, was the assessment of the genetic relationship among wild colocynth *Citrullus colocynthis* (L.)Schrad. ecotypes. Seeds of twelve colocynth plants (SF, K10, K10₁, K114, ABD, SH, G, GD, GD1, ND, SD and NK) were collected from wild plants growing in twelve diverse ecologies of Sudan's States. Commercial watermelon (Wm) seeds were obtained from the seed store and tested for comparison. Randomly Amplified Polymorphic DNA (RAPD) technology was used to explore the genetic relatedness of the ecotypes. Ten RAPD primers were used to amplify CTAB-extracted DNA from leaves of each ecotype. Data were used to create similarity matrices using the PAST 3.01 software package and a dendrogram was constructed based on Jaccard's similarity coefficients. A total of 321 DNA fragments

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were detected by RAPD markers for the 13 tested plants giving an average of 24.69 alleles per ecotype. The number of fragments detected for each ecotype ranged from 11 (GD) to 64 detected for K10; ten fragments were detected for watermelon. Hundred percent polymorphism was recorded for the tested primers with high Polymorphism Information Content (PIC) values in the range of 0.97-1.00. Similarity matrix constructed from data generated indicated that the closest ecotypes, with 67% similarity, were ABD and K114, while the most distant ones (no similarity) were SD and GD as well as GD and Wm. The constructed dendrogram divided the 12 colocynth ecotypes and the watermelon cultivar into three major clusters; the first includes watermelon cultivar as an out-group, the second includes all ecotypes except K10 which is grouped in a separate cluster. These results conclude that the ecological zone does not affect the genetic relatedness of the tested ecotypes despite their high genetic polymorphism. The variability between the ecotypes points out that Sudan's colocynth germplasm possesses high potentiality for future improvement through crossing and selection.

Key words: Medicinal plants, genetic relatedness, RAPD markers, ecotypes, *Citrullus colocynthis* L.

INTRODUCTION

Medicinal and aromatic plants (MAPs) have been an important resource for human healthcare from prehistoric times to the present day. Between 50000 and 70000 plant species are known to be used in traditional and modern medicinal systems throughout the world (Lange and Schippmann 1997). The great majority of MAPs are still being provided by collection from the wild vegetation (Srivastova *et al.* 1996).

The genus *Citrullus* includes several diploid ($2n = 22$) species, including *C. lanatus*, which gave rise to the red-fleshed sweet dessert watermelon (Shimotsuma 1963). Colocynth [*Citrullus colocynthis*(L.) Schrad.] is an annual and/or perennial cucurbit species, known as the “bitter apple,” indigenous to desert soils throughout northern Africa, including Sudan, the Middle East, and southwestern Asia (Paris 2015). *Citrullus colocynthis* has various ethnobotanical and medicinal uses as an anti-inflammatory,

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antimicrobial and anticancer agent (Memon *et al.* 2003). It is used as a cathartic, purgative and vermifuge, and for the treatment of fever, amenorrhea, jaundice and rheumatism (Batanouny 1999). In addition, *C. colocynthis* has also gained increasing attention as a natural insecticide due to its deterrent, antifeedant, growth regulating and fertility reducing properties on insects (Torkey *et al.* 2009). *Citrullus colocynthis* has been reported as a useful source of genes for enhancing biotic and abiotic stress resistance in the common watermelon cultivars (Levi *et al.* 2016).

In Sudan, colocynth grows as a wild plant, widely distributed in northern, eastern, western and central parts of Sudan in sandy and clay soils in hot deserts. The pulp of the fruit is extracted and exported because it has powerful cathartic properties and is used in medicines. A tar is processed from the fruit and used in dressing hides to make water skin and in treating some skin diseases of camels. Reports indicated that colocynth contains cucurbitacin E in form of glycoside which has been shown to have tumour-necrosing activity (Evans 1989; Hatamet *et al.* 1989). Hussein (2012) prepared biofuel from colocynth seeds' oil and determined its physical properties, concluding that such oil can be used as biofuel.

Better utilization and conservation of colocynth, as medicinal plants, necessitate their screening to identify potential accessions/lines for both cultivation and utilization in breeding to develop superior genotypes. Yet, very little work has been reported in literature regarding genetic diversity of colocynth growing wildly all over the country. This study has, therefore, aimed at determining the genetic diversity among twelve wild ecotypes, using RAPD molecular markers technique.

MATERIALS AND METHODS

This research was carried out at the Department of Horticulture and in the molecular biology laboratory of the Department of Botany, Faculty of Science, University of Khartoum, Sudan.

Plant material:

Seeds of twelve colocynth were collected from wild plants growing in different ecological zones of Sudan. These were: White Nile Bank, Khartoum State (SF); 10 Km West of Omdorman (Yellow [K10] and brown [K10₁] seeds); 114 Km North West Omdorman (K114); Goz Abu Dolo, 66 Km north west Omdorman(ABD); Shendi upper terrace soil, River Nile State (SH); Algadarif State, eastern Sudan(G); Gash delta (brown seed GD and yellow seed GD₁); Mileat, North Darfur (ND); Dreab Elreah, South Darfur (SD) and North Kordofan State (NK). For comparison and authentication of the genetic relatedness, watermelon (Wm), cultivar Congo, was also included in the analysis.

Genomic DNA extraction:

Seedlings for leaves' sap extraction were raised in the nursery in 15 cm pots filled with sandy loam soil. Genomic DNA of each ecotype was isolated by sap-extraction method (CIMMYT 2000) as follows:

Young leaves(3-4) were collected from 15 days old plants, washed well with 70 % ethanol then distilled water and placed in a blender, mixed with 5ml of CTAB buffer (50 mMTris-HCl; 0.25 mM EDTA; 1M NaCl, 2 % CTAB and 0.15 % 2-mercaptoethanol). The extracted sap was collected in 15 ml falcon tube, incubated at 60^oc for one hour and left to cool at room temperature for 10 minutes. Equal volume of freshly prepared chloroform/isoamyl alcohol (24:1) were added and mixed thoroughly by inverting the tube gently, left for 30 minutes then centrifuged at 12000 rpm for 10 minutes. The supernatant was transferred to a new tube then cold isopropanol (3ml) was added to precipitate the DNA and the mixture was kept at 4^oc for 24 hours. The sample was then centrifuged at 4000 rpm for 10 minutes and the supernatant was carefully discarded. The pellets formed were then washed by ethanol and centrifuged at 3000 rpm for 5 minutes. One ml of Tris-EDTA (TE) buffer (10 mMTris-HCl, 1.0 mM EDTA, pH 8.0) was added, then the tube was placed in water bath at 65^oc for 5 minutes then 1/10 volume (200 μ l) of 8M ammonium acetate and 2 volumes (2ml) of cold ethanol (95-100 %) were added, mixed by inversion and left to set for 30 minutes. The contents were centrifuged at 2000 rpm for 10 minutes; DNA pellets were air-dried at room

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temperature for an hour and then dissolved in 300 μ l of TE buffer and kept at 4°C until used. The purified total DNA was quantified by gel electrophoresis.

Polymerase Chain Reaction (PCR):

Ten RAPD primers(Gene Link, Inc. and Operon Tech., NY 10532, USA) were used to amplify colocynth genomic DNA. The code number and sequences of RAPD primers are shown in Table 1. Reactions of PCR amplification were carried out in a total volume of 20 μ l. The PCR mixtures contained *i.e.* final concentration:5X FIRE POL PCR master Mix (ready to load), 5X reaction buffer (0.4 M Tris-HCl,0.1M (NH₄)SO₄, 0.1 % w/v Tween 20), 12.5 mM dNTPs,50 ng of the primer under test,1U Taqpolymerase and 20 ng template DNA.

The amplification procedure used consisted of one cycle at 94°C for one minute, annealing at 32°C for 3 minutes, extension at72°C for 2 minutes and final extension step at72°C for 10 minutes. Amplified DNAs by RAPD markers were electrophoresed in 1.5 % agarose gel.

Agarose gel electrophoresis of the PCR products

PCR products obtained by each primer were electrophoresed in 1.5 % agarose gel (0.75 g agarose dissolved in 50ml of 1x TBE buffer (0.089 mol/l Tris-borate, and 0.002mM EDTA, pH 8.0). Then 2 μ l of ethidium bromide (10mg/ml) were added. The comb was adjusted and the gel was poured (making sure that there were no bubbles formed) and left to solidify. DNA mixtures were prepared for electrophoresis as follows: DNA samples were transferred to a clean Eppendorf tube and 3 μ l of loading dye (bromophenol blue dye) was added to each DNA with gentle back and forth motion and the gel was then immersed in 1X TBE buffer. The buffer was added until it reached a level approximately 3-5 mm above the gel surface. The sample mixtures were loaded into the wells using plastic -tipped micropipettes. 1Kb ladder (invtrrogen) was used as a molecular weight marker. The apparatus (Habaib, UK, 9H310083) was closed and the power was turned on; the voltage was adjusted to 75V (400 mA). The running was continued without cooling for 20 minutes after which the gel was visualized under trans illumination cabinet (ModelTM-10E, Uvitec Product) and images were captured, photographed and analyzed.

Data analysis:

DNA fragments/bands obtained by both marker types were scored as present (1) or absent (0). PIC values were calculated as described by Anderson *et al.* (1993), as follows:

$$PIC = 1 - \sum P_{ij}^2$$

Where P_{ij}^2 is the relative frequency of the j^{th} allele of the i^{th} locus, summed over all alleles for individual marker locus over all lines. A marker with a PIC value of more than 0.5 is considered as highly informative, between 0.25 and 0.5 as informative and less than 0.25 as slightly informative (Bolstein *et al.* 1980). Similarity between the ecotypes was analyzed on the basis of their scores. Data were used to create similarity matrices using the PAST 3.01 software package. Similarity matrix and dendrogram were reconstructed based on Jaccard's similarity coefficient (Jaccard 1908).

Table 1. Codes and sequences of RAPD primers used for characterization of colocynth ecotypes

Primer	Sequences (5'-3')
1. OPB-10	CTGCTGGGAC
2. OPC-9	CTCACCCCTCC
3. OPC-17	CCTGGGCCTC
4. OPG- 5	CTGAGACGGA
5. OPK-9	CCCTACCGAC
6. OPK-16	GAGCGTCGAA
7. OPY-1	GTGGCATCTC
8. OPY-2	CATGCCGCA
9. OPY-7	AGAGCCGTCA
10. OPY-17	GACGTGGTGA

RESULTS AND DISCUSSION

A total of 321 DNA fragments (bands) were detected for the 12 ecotypes and the watermelon cultivar, giving an average of 24.69 alleles per plant. The number of fragments detected for each plant ranged from 11 in GD to 64 in

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K10, while 10 fragments were detected for the watermelon cultivar Congo. The molecular size of the amplified fragments by all primers ranged from 200 to 3000 bp. The number of different fragments generated by each primer ranged from 9 detected for OPY-7 to 60 for OPY-2 with an average of 32.1 for each primer (Table 2; Fig. 1). Hundred percent polymorphism was recorded for each of the primers tested with high PIC ranging from 0.97 to 1.00.

Results in Table 3 show that 1-46 unique fragments with different sizes were detected for a particular ecotype but not for others. Fragments of these kinds are normally referred to as positive markers. In this respect, ecotype K10 showed 46 unique fragments with 9 of the 10 primers tested, indicating a great variation. Similarly, each of ecotypes SD, SF, GD1 and NK produced 4 unique fragments that were not detected for the other ecotypes. Two unique fragments were detected for ecotype G while one fragment was detected for ND, GD and SH. Watermelon cultivar scored three unique fragments.

Table 2. Polymorphism percentages calculated for each RAPD primer against 12 colocynth ecotypes

Primer code	Total No. of bands	No. of polymorphic bands	Polymorphism %	PIC
OPB-10	37	37	100	0.99
OPC-9	28	28	100	0.99
OPC-17	25	25	100	0.99
OPG-5	27	27	100	0.99
OPK-9	32	32	100	0.99
OPK-16	18	18	100	1.00
OPY-1	31	31	100	0.99
OPY-2	60	60	100	0.97
OPY-7	9	9	100	1.00
OPY17	54	54	100	0.97

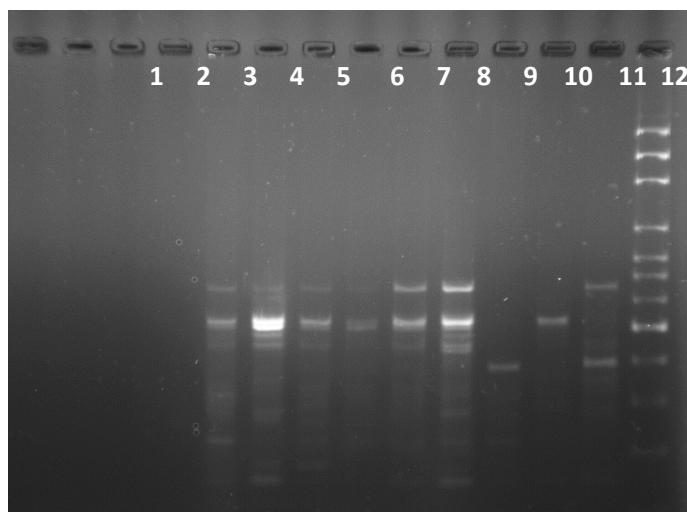


Fig. 1. Banding profile of 13 colocynth genotypes' DNA amplified by OPY-17 RAPD primer (M= 1.5 Kb DNA ladder; Lanes 1-13 represent the 13 genotypes)

For reproducibility problems associated with RAPD primers, the use of more than one unique band may be recommended for the characterization and identification of a specific ecotype. Pair-wise genetic similarities based on RAPD markers between ecotype using Jaccard's genetic similarity coefficients, indicated that the closest ecotypes with genetic similarity of 67 % were ABD and K114, followed by 66 % for K10 and ABD and K10 and K114. No similarity was observed between SD and GD nor between GD and Wm (Table 4). A dendrogram showing the grouping pattern of ecotypes consisted of three major clusters was constructed and is shown in Fig. 2. The first cluster included watermelon as an out group; while the second included all colocynth ecotypes, except K10, which was grouped in the third cluster separately. Similar results were also reported by Levi *et al.* (2001) who analyzed accessions of *Citrullus lanatus* and *C. colocynthis* with 30 RAPD primers; the primers grouped the two species in different clusters. The high

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percentage of polymorphism based on RAPD markers reported among ecotypes in this study, is in agreement with the high polymorphism found in *C. colocynthis* populations using ISSR markers (Badr *et al.* 2018). Mujaju *et al.* (2010) reported successful differentiation between two major groups of watermelon (*Citrullus lanatus*) landraces from Zimbabwe using RAPD and ISSR markers. Another study using AFLP markers indicated that genetic distance between watermelon cultivars (*C. lanatus* var. *lanatus*) and the wild *C. colocynthis* was very high (Hwang *et al.*, 2011). Gichimu *et al.* (2009) were able to document the genetic diversity of some wild and cultivated watermelon (*Citrullus* sp.) accessions in Kenya including *C. colocynthis* based on morphological features; the wild accessions were separated easily from cultivated ones.

Table 3. Specific fragments (bands) for each colocynth ecotype generated by different RAPD primers

Colocynth ecotype	Primer (OP-)	Specific fragment size (bp)
SD	B-10	960
	C-17	500
	C-9	520 + 600
ND	C-9	460
	C-17	480
G	Y-17	650
	B-10	550 + 920
SF	C-17	380 + 740
	ABD	No unique band was detected
K114	K114	No unique band was detected
	Y-7	500
	B-10	580 + 950 + 1100 + 2000 + 2500
	G-5	550 + 1000 + 1600 + 2000
	K-9	350 + 550 + 800 + 1200
	K-16	580 + 600 + 650 + 850 + 900 + 950
	Y-1	600 + 650 + 800 + 820 + 880 + 1000 + 1200 + 1500 + 2000
	Y-17	600 + 800 + 900 + 950
	C-9	550 + 650 + 900 + 950 + 1200
K10 1		No unique band was detected
GD	G-5	520
GD 1	B-10	250 + 300 + 760
	Y-2	750
NK	B-10	880
	K-9	520 + 980 + 1100
SH	Y-17	720
*Wm	G-5	350 + 400
	Y-2	500

*Wm = watermelon cultivar Congo used for comparison

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Table 4. Genetic similarity indices of RAPD markers for different colocynth ecotypes

	SD	ND	G	SF	ABD	K114	K10	1	GD	GD 1	NK	SH	K10	Wm*
SD	1.00													
ND	0.30	1.00												
G	0.06	0.11	1.00											
SF	0.15	0.25	0.24	1.00										
ABD	0.16	0.24	0.44	0.54	1.00									
K114	0.13	0.23	0.39	0.29	0.67	1.00								
K10	0.03	0.04	0.09	0.10	0.11	0.05	1.00							
K10														
1	0.11	0.18	0.42	0.36	0.66	0.66	0.10	1.00						
GD	0.00	0.05	0.11	0.07	0.11	0.15	0.03	0.22	1.00					
GD 1	0.12	0.12	0.29	0.24	0.39	0.46	0.07	0.48	0.18	1.00				
NK	0.05	0.11	0.26	0.17	0.28	0.32	0.07	0.41	0.17	0.36	1.00			
SH	0.07	0.12	0.40	0.23	0.44	0.50	0.07	0.42	0.08	0.28	0.28	1.00		
Wm	0.09	0.10	0.07	0.08	0.05	0.07	0.03	0.06	0.00	0.13	0.08	0.08	1.00	

Wm = watermelon cultivar Congo used for comparison

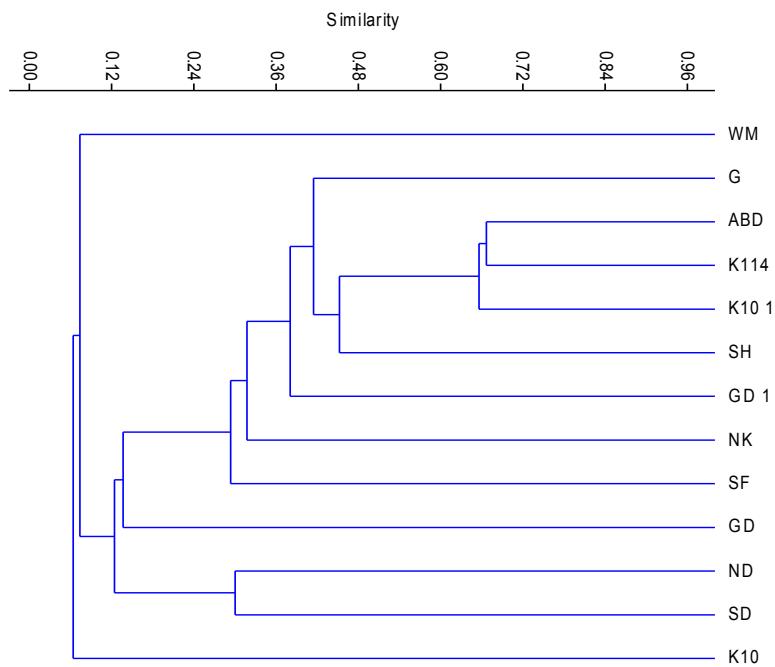


Fig. 2. A dendrogram of 12 colocynth ecotypes and a watermelon cultivar (WM) based on data generated from RAPD markers. Values along x-axis correspond to Jaccard's coefficient of similarity.

CONCLUSION

The study was the first report comparing the Sudan's wild *C. colocynthis* ecotypes. The study assessed the genetic diversity existing between these ecotypes, however, more samples and more markers are to be included. Selection of ecotypes that acquire the desirable biotic and abiotic resistance genes has priority for the future breeding programs.

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تقييم التباين الوراثي ضمن طرز بيئية بريئة لنبات الحنظل (*Citrullus colocynthis* (L.) Schrad.) تم جمعها من مناطق مختلفة من السودان²

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المستخلاص: تتطلب المحافظة على الاستخدام الأمثل للنباتات الطبية والعلمية، والتي غالباً ما تجمع من البرية، مسحها للتعرف على الأنواع الجينية الواحدة للإنتاج والاستخدام في التهجين لاستبطاط أنواع جينية أفضل. الهدف من هذه الدراسة، وبالتالي، هو تقويم العلاقة الوراثية فيما بين طرز بيئية بريئة من نبات الحنظل(*Citrullus colocynthis* (L.) Schrad). جمعت بذور 12 نبات حنظل (SF, K10, K10₁, K114, ABD, SH, G, GD, GD1, ND, SD and NK) من نباتات تنمو بريأة في 12 بيئه متباعدة في ولايات السودان، بالإضافة إلى بذور نبات البطيخ (Wm) من متجر للبذور وتم إختبار المقارنة. استخدمت تقنية تضخيم الحمض النووي العشوائي متباين الأشكال (RAPD) لاستكشاف علاقة القربي بين الطرز البيئية. تم استخدام عشرة بادئات تضخيم عشوائي لتضخيم الحمض النووي المستخلص بطريقة بروميد السينتايلاكريلامايد ثلاثي الميثيل (CTAB) من أورق كل نوع بيئي. استخدمت البيانات للحصول على مصفوفة التشابه بحزمة برامجيات PAST 3.01 وأنشئت شجرة التنوع الوراثي (dendrogram) على أساس معامل جاكارد للتشابه. تم الحصول على 321 حزمة حمض نووي ببادئات التضخيم العشوائي للنباتات الثلاثة عشر

المختبرة معطية متوسط 24.69 أليل للنوع البيئي. تراوح عدد الحزم المكتشفة لكل نوع بين 11 (GD) إلى 64 أكتشفت النوع K10، بينما أكتشفت عشرة حزم للبطيخ. تم تسجيل نسبة 100 % من التباين للبادئات المختبرة مع قيم محتوى معلومات تعدد الأشكال (PIC) في المدى من 0.97 - 1.00. أوضحت مصفوفة النشابة المصممة من البيانات أن أقرب الطرز البيئية لبعضها، بنسبة تشابه 67 %، هما ABD و K114، بينما كان أبعدها عن بعضها (لا تشابه) هما SD و GD وكذلك ABD و Wm. قسمت شجرة التباين الوراثي طرز الحنظل البيئية الإناثا عشر وصنف البطيخ لثلاث عناقيد مجموعات. ضمن العقد المجموعي الأول صنف البطيخ كمجموعة خارجية، وضم الثاني كل الطرز البيئية ما عدا الطراز K10 الذي وضع في مجموعة منفصلة. خلصت النتائج إلى أن النطاق البيئي لم يؤثر على التقارب الوراثي للطرز البيئية المختبرة بالرغم من التعدد العالياً لأشكالها الوراثية. أوضح التباين بين الطرز البيئية أن المصادر الوراثية للحنظل في السودان تمتلك إمكانيات عالية للتحسين مستقبلاً عن طريق التهجين والإختيار.