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Genetic Differentiation of *Citrullus colocynthis* (L.) Populations Depending on Allozyme Diversity

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CITRULLUS colocynthis (L.) is a desert plant having a large history in pharmaceutical industries as an important medicinal plant. Allozyme variation and population genetic structure of *C. colocynthis* in 6 natural populations from western region, Saudi Arabia, were investigated by 6 enzyme systems. The genetic differentiation within and among *C. colocynthis* populations has been revealed by allozyme analysis. Ten loci were monomorphic in all populations, whereas seven loci having two alleles (α-EST-1, β-EST-1, MDH-1, ADH-4, ADH-5, ALO-4 and PRX-2) were polymorphic. The mean allozyme variability indices within *C. colocynthis* populations (P = 31.17%; A = 1.21) were lower than those for insect-pollinated species (P = 34%; A = 2.67; H_e = 0.205) except H_e value (0.415) was higher. The observed mean heterozygosity (H_o= 0.757) was higher than the expected mean heterozygosity (H_e= 0.415) indicating the existence of natural selection against homozygosity. The Inbreeding coefficient values (F) of polymorphic loci were negative suggesting a significant excess of heterozygosity in the studied populations. The UPGMA dendrogram of 84 individuals of *C. colocynthis* confirmed the notable variability within populations. Differentiation among-population (F_{ST}) recorded 16.2% of the total variation. Low F_{ST} and high genetic distance (D) values suggested a low differentiation among *C. colocynthis* populations. Little gene flow (N_m) was deduced in all populations except ME based on the allozyme data. We suggest that population ME can be recommended for both genetic conservation and breeding programs.

Keywords: *Citrullus,* Allozyme, Population, Heterozygosity, Gene flow.

Introduction

Citrullus colocynthis L. (Cucurbitaceae), is a drought-resistant wild perennial herb that widely found in Africa and Asia. *C. colocynthis* has many medicinal advantages against various diseases such as diabetes II and breast cancer (Grossman et al*.,* 2007 and Huseini et al., 2009). *C. colocynthis* grows fast in the sandy soil regions of Saudi Arabia under changeful environmental conditions (Chaudhary & AL-jowaid, 1999). Although the wide distribution of *C. colocynthis*, our knowledge about its genetic diversity is very poor. Loss of genetic variation decreases the ability of wild species to survive fluctuations in climate, diseases, pollutants and natural enemies (Frankham, 1995). Genetic diversity and good management of its natural populations are needed for more adaptation and improvement in future. Isozymes are useful in the field of biochemical genetics and population genetics as biochemical markers (Wendel & Weeden, 1989). Isozymes examined genetic variation and phylogenetic relationships in several *Citrullus* spp. by Navot & Zamir (1987), Biles et al. (1989) and Navot et al. (1990), but most of them were monomorphic. However they will be useful and accurate when we examine more accessions and individuals than some DNA markers (Klaas, 1998; Levi et al*.,* 2001b; Ritschel et al*.,* 2004; Solmaz et al*.,* 2010 and Minsart et al*.,* 2011).

Few studies described the genetic variability and population structure in *C. colocynthis* using isozymes. The aim of this work is to estimate the genetic differentiation within and among six natural populations of *C. colocynthis* from western region of Saudi Arabia, based on isozyme polymorphism. This could be useful for conservation and breeding purposes.

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Materials And Methods

Plant materials

Leaves of eighty four individuals of *C. colocynthis* (14 individuals in each of six populations studied) were collected for assessment of genetic variability. The six populations were collected from six sites from the western region of Saudi Arabia (Table 1).

Isozymes

The examined isozymes were: Alcohol dehydrogenase (ADH); (E.C. 1.1.1.1), aldehyde oxidase (ALO); (E.C.1.2.3.1), α-and β-esterases (EST); (E.C.3.1.1.1), peroxidase (PRX); (E.C.1.11.1.7) and malate dehydrogenase (MDH); (E.C.1.1.1.37). Extraction of isozyme was carried out by homogenizing 1g of fresh leaves in 1ml extraction buffer (1M Tris-HCl, pH 8.8) then centrifuged at 10000rpm and the supernatant was separated using native-polyacrylamide gel electrophoresis method (Stegemann et al*.,* 1985). Protocols of Scandalios (1964) for α-and β-EST, Wendel & Weeden (1989) for ALO, Weeden & Wendel (1990) for ADH, Jonathan & Wendell (1990) for MDH and Heldt (1997) for PRX were used for isozyme staining.

Data analysis

Parameters for detecting the within-populations genetic variation and the inbreeding coefficient; Wright's *F* [$F = (1 - H_o/H_e)$], were estimated as described by Hamrick & Godt (1989) and Nei (1973) (Tables 3 and 4). Negative *F* indices refers to tendency to heterozygosity whereas positive values indicate an inbreeding system of mating. Levels of significance among populations for each parameter were determined by t-test (Varghese et al., 1999). Parameters of populations genetic structure; F_{IS}, F_{IT} and $F_{ST;}$ were investigated by Wright's *F*-statistics (Wright,1965). Gene flow; *Nm*, was calculated by Wright's (1951) equation with modification by Crow & Aloki (1984): $F_{ST}=1/(4N_m\alpha + 1)$ where $\alpha = [n/(n-1)]^2$ and n is the populations number. Isozyme frequency data were used to calculate genetic distance estimates according to Nei (1973). To discern the genetic variability within populations, each band of 84 individuals was coded as 1 for presence or 0 for absence. Clustering was performed using UPGMA procedure and represented in a phenogram by using SAHN and TREE modules, respectively, and it was also performed for studying the relationships among populations. The computer program NTSYS-pc 2.2 was used in all previous operations (Rohlf, 1998).

Results

Allele diversity

Polymorphisms were observed in all the six systems analyzed. A total of 31 alleles were detected among the 24 loci from 6 enzyme systems evaluated. The frequencies of them are demonstrated in Table 2. Ten loci (α-EST-2, α-EST-3, α-EST-4, α-EST-5, β-EST-4, β-EST-5, MDH-2, ADH-2, ADH-3and PRX-3) were monomorphic in all populations, whereas seven loci having two alleles (α-EST-1, β-EST-1, MDH-1, ADH-4, ADH-5, ALO-4 and PRX-2) were polymorphic in at least one population. The two loci; β-EST-3 and PRX-1 were only detected in populations TA and JE, respectively.

TABLE 1. Code, sample size (N) and sites of six natural populations of *C. colocynthis* from western region of Saudi Arabia.

Population	Pop. code	N	Geographic origin	Latitude North	Longitude East	Altitude (m)	Rainfall (mm/year)
Taif	TA	14	Taif city	21°16′N	40°24′E	1672	119
Jeddah	JE	14	170 km W of Taif	21°32′N	39°11′E	21	53.5
Mecca	ME	14	63 km W of Taif	21°25'N	39°49'E	300	111.8
Rabigh	RA	14	222 km N W of Taif	22°47'N	39°2'E	13	44
Al Khurmah	KH	14	120 km E of Taif	21°54''N	42°1'E	1020	390
Al Bahah	BA	14	250 km S of Taif	20° 24' N	41° 57'E	2300	138.1

TABLE 2. Estimated allele frequencies at 24 loci in six population of *C. colocynthis*.

Locus	Alleles	Population					
		JE	TA	ME	RA	KH	BA
α-EST-1	a	0.50	0.00	0.50	0.00	0.50	0.50
	b	0.50	0.00	0.50	0.00	0.50	0.50
α-EST-2	a	1.00	1.00	1.00	1.00	1.00	1.00
α-EST-3	a	1.00	1.00	1.00	1.00	1.00	1.00
α-EST-4	a	1.00	1.00	1.00	1.00	1.00	1.00
α-EST-5	a	1.00	1.00	1.00	1.00	1.00	1.00
β-EST-1	a	0.50	0.50	1.00	0.50	0.50	1.00
	b	0.50	0.50	0.00	0.50	0.50	0.00
β-EST-2	a	1.00	1.00	0.00	0.00	0.00	0.00
β-EST-3	a	0.00	1.00	0.00	0.00	0.00	0.00
β-EST-4	a	1.00	1.00	1.00	1.00	1.00	1.00
β-EST-5	a	1.00	1.00	1.00	1.00	1.00	1.00
MDH-1	a	0.50	0.50	0.50	0.63	0.67	0.67
	b	0.50	0.50	0.50	0.37	0.33	0.33
MDH-2	a	1.00	1.00	1.00	1.00	1.00	1.00
ADH-1	a	0.00	0.00	1.00	1.00	0.00	1.00
ADH-2	a	1.00	1.00	1.00	1.00	1.00	1.00
ADH-3	a	1.00	1.00	1.00	1.00	1.00	1.00
ADH-4	a	0.50	0.50	0.50	0.50	0.50	0.50
	b	0.50	0.50	0.50	0.50	0.50	0.50
ADH-5	a	0.36	0.00	0.63	0.86	0.93	1.00
	b	0.64	1.00	0.37	0.14	0.07	0.00
ALO-1	a	1.00	1.00	1.00	1.00	1.00	0.00
ALO-2	a	1.00	1.00	1.00	1.00	1.00	0.00
ALO-3	a	1.00	1.00	1.00	1.00	1.00	0.00
ALO-4	a	0.71	0.93	1.00	0.93	1.00	0.89
	b	0.29	0.07	0.00	0.07	0.00	0.11
PRX-1	a	1.00	0.00	0.00	0.00	0.00	0.00
PRX-2	a	0.79	0.00	1.00	0.00	0.96	0.00
	b	0.21	0.00	0.00	0.00	0.04	0.00
PRX-3	a	1.00	1.00	1.00	1.00	1.00	1.00

Genetic variation within populations

Parameters of genetic variability showed considerable level of genetic variation within populations (Table 3). *A* ranged from 1.14 in population ME to 1.30 in population KH with a mean of 1.21 alleles per locus, whereas the mean of A_p was 1.72, ranging from 1.56 in populations KH to 1.97 in population ME. *P* ranged from 25.0% in population TA to 35.3% in population BA with a mean of 31.17%. H_o ranged from 0.636 in population RA to 0.938 in population ME, whereas the range of H_e was from 0.358 in population KH to 0.492 in population ME. The observed heterozygosities were higher than those expected in all populations. Obviously population ME possessed the highest level of genetic variation H_e, while populations RA and KH had rather lower levels of genetic variation. The mean observed heterozygosity was higher than that expected, with values of 0.757 and 0.415, respectively. The UPGMA dendrogram (Fig. 1) obtained using the 33 isozyme bands scored in the 84 individuals, showed a notable variability within populations examined. At coefficient 0.59, ten groups were formed, five of them; G1, G3, G4, G5 and G6, corresponded to populations JE, RA, BA, ME and KH, respectively. Most of the individuals of the same population grouped together, except population TA, which presented lower similarity values among individuals, representing the higher genetic diversity within population.

The *F* values of all loci were lower than zero. One exception; locus PRX-2 in population KH was greater than zero reflecting a deficiency of heterozygosity (Table 4). The Inbreeding coefficient ranged from – 0.902 in population ME to –0.578 in population RA with mean value of –0.602. Obviously, *C. colocynthis* populations revealed an excess of heterozygosity.

Genetic variation among populations

F_{IS} and F_{IT} estimates were generally negative. F_{IS} ranged from -0.958 for population ME to -0.60 for population RA, and averaged -0.788, indicating significant excess of heterozygosity in the populations, which was in accordance with the values of *F* mentioned above. F_{ST} ranged from 0.008 for population ME to 0.306 for population KH, with a mean of 0.162, indicating that 16.2% of the total genetic variation exists among populations.

TABLE 3. Genetic variability estimates within *C. colocynthis* populations.

Population	P	A	A_P	H_o	H_e
JE	31.8	1.27	1.85	0.816	0.458
TA	25.0	1.20	1.69	0.785	0.408
ME	33.3	1.14	1.97	0.938	0.492
RA	26.3	1.16	1.58	0.636	0.367
KH	35.0	1.30	1.56	0.647	0.358
BA	35.3	1.18	1.69	0.720	0.409
Among-population mean	31.17	1.21	1.72	0.757	0.415
SE	1.81	0.03	0.06	0.047	0.021
t-value	17.2***	46.7***	26.6***	16.2***	19.6***

-P (<0.99) percentage of polymorphic loci; *A* average number of alleles per locus; A_p average effective number of alleles per locus; H_o observed heterozygosity; H_e expected heterozygosity under Hardy–Weinberg equilibrium; *F* Fixation Index (Inbreeding Coefficient); SE the standard error.
-ns P >0.05; ** P <0.01; *** P <0.001.

TABLE 4. Inbreeding coefficient values (F) for polymorphic loci in *C. colocynthis* populations.

Locus	JE	TA	ME	RA	KH	BA
α-EST-1	-1	-	-1	-	-1	-1
β-EST-1	-1	-1	-	-1	-1	-
MDH-1	-1	-1	-1	-0.609	-0.516	-0.516
ADH-4	-1	-1	-1	-1	-1	-1
ADH-5	-0.540	-	-0.609	-0.203	-0.077	-
ALO-4	-0.383	-0.077	-	-0.077	-	-0.007
PRX-2	-0.295	-	-	-	0.079	-
Mean	-0.745	-0.769	-0.902	-0.578	-0.586	-0.631

Average genetic distance (*D*) values ranged from 0.240 to 0.468 with a mean of 0.325 (Table 5). Consequently, Nei's genetic distances (*D*) and geographic distances were estimated among pairs of *C. colocynthis* populations as illustrated in Table 6. On the basis of these genetic distance values, UPGMA cluster analysis showed the genetic relationship of the populations studied through a dendrogram (Fig. 2). Two principal groups were found in the cluster analysis. The first cluster was made up of population JE, TA and BA and the second was formed by the other populations. It appeared that populations of closest geographic proximity could not be clustered together. This was also confirmed statistically by correlation analysis on Nei's genetic distance and geographic distance (r = -0.013; p = 0.980). The gene flow (N_m) values ranged between 0.57 and 2.35 in all populations except that of population ME (Table 5). The genetic uniqueness of each population was estimated by F_{ST}, D and N_m (Table 5). Lower values of F_{ST}, D and higher N_m were obtained for the analyses of population ME (0.008, 0.243, 31.0) than those for all 6 populations (0.162, 0.325, 6.83), respectively. It could be concluded that population ME had some unique genetic features and being suitable for conservation process.

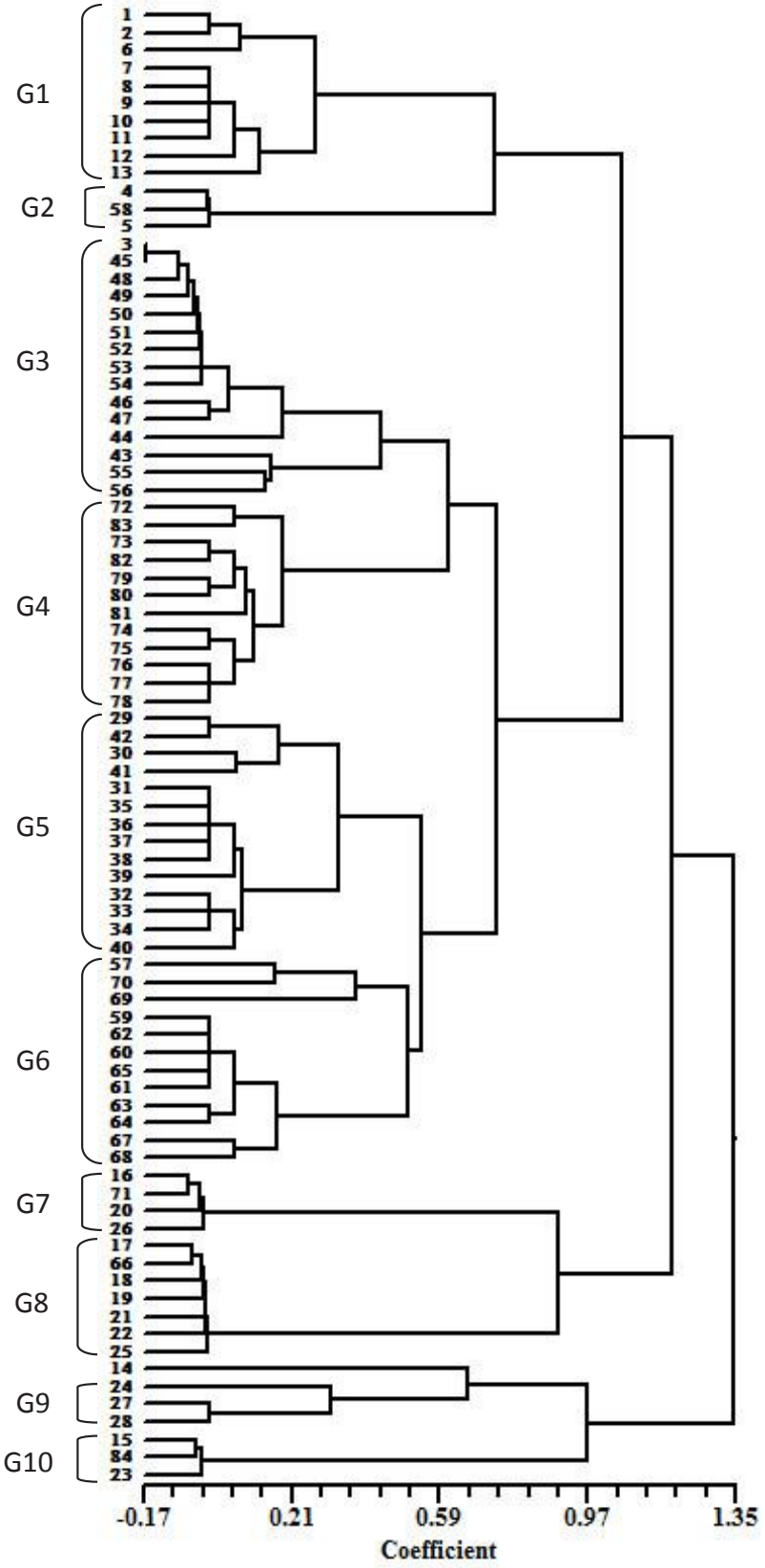

Fig. 1. The dendrogram of 84 individuals of *C. colocynthis* generated by UPGMA analysis.

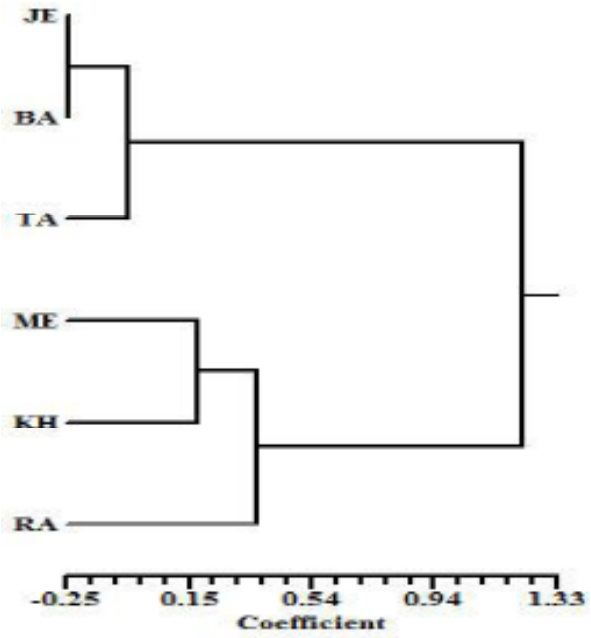

Fig. 2. The UPGMA dendrogram of the six populations of *C. colocynthis* based on the Nei's genetic distances.

TABLE 5. F-statistics, average Nei's genetic distance and gene flow values in *C. colocynthis* populations.

	F-statistics						Genetic distance	Gene flow
Pop.	**H_I**	**H_S**	**H_T**	**F_{IS}**	**F_{IT}**	**F_{ST}**	**D**	**N_m**
JE	0.753	0.443	0.490	-0.699	-0.537	0.096	0.354	2.35
TA	0.754	0.394	0.500	-0.914	-0.508	0.212	0.404	0.93
ME	0.971	0.496	0.500	-0.958	-0.942	0.008	0.243	31.0
RA	0.544	0.340	0.426	-0.600	-0.277	0.202	0.240	0.99
KH	0.540	0.293	0.422	-0.843	-0.279	0.306	0.242	0.57
BA	0.638	0.372	0.436	-0.715	-0.463	0.147	0.468	1.45
Mean	0.700	0.389	0.462	-0.788	-0.501	0.162	0.325	6.21
SE	0.067	0.029	0.016	0.057	0.099	0.042	0.040	4.96
t-test	10.52***	13.16***	29.76***	-13.904***	-5.038**	3.845*	8.098***	1.252^{ns}

-F_{IS} inbreeding coefficient within population; F_{IT} total inbreeding; F_{ST} genetic diversity among populations; *D* average genetic distance; N_m gene flow calculated by Wright's model based on F_{ST}; SE the standard error.

-ns P >0.05; ** P <0.01; *** P <0.001.

TABLE 6. Geographical distances (km) (above diagonal) and Nei's genetic distances (below diagonal) among *C. colocynthis* populations.

	JE	**TA**	**ME**	**RA**	**KH**	**BA**
JE		133	65	169	297	272
TA	0.241		68	215	166	187
ME	0.279	0.405		178	232	227
RA	0.354	0.297	0.133		324	400
KH	0.205	0.360	0.108	0.155		220
BA	0.692	0.715	0.288	0.262	0.381	

Discussion

Considerable genetic variation in the six populations of *C. colocynthis* was detected using allozymes, although many researchers predicted low levels of genetic diversity in species having small numbers of individuals and limited ranges (Drury, 1974). The mean allozyme variability indices of *C. colocynthis* within populations (*P*=31.17%; *A*=1.21) were lower than those of Hamrick & Godt (1997) for insect-pollinated species (*P*=34%; *A*=2.67; H_e=0.205) except H_e value (0.415) was higher. The two *P* and *A* estimates were also much lower than those of the four cucurbits; *Cucurbita argyrosperma* ssp. *sororia* (100% - 2.08), *Cucurbita argyrosperma* ssp. *argyrosperma* (93% - 2.5), *Cucurbita moschata* (97% – 2.06) and *Cucurbita pepo* (92% – 2.08) (Montes-hernandez & Eguiarte, 2002). Whereas, our *P*, *A* and H_e values were higher than those of *Cucurbita pepo* and *Cucurbita maxima* (Decker-walters et al., 1990), *Cucumeropsis mannii* and *Langenaria siceraria* (Koffi et al., 2008 and 2009). In all populations the values of H_o were higher than H_e reflecting excess in heterozygosity that was also confirmed by negative inbreeding coefficient (*F*) values for polymorphic loci. These results conflicted the hypothesis of Biles et al. (1989) and Levi et al. (2001a) that the genus *Citrullus* had a narrow genetic basis, but they were in consistent with Mujaju & Nybom (2011) and Shaik et al. (2015) who found that the diversity was higher in the wild species *C. colocynthis* than *C. lanatus*. This outcome confirmed the hypothesis that the species widely distributed, as *C. colocynthis,* have a higher genetic diversity percentage (H_e) than geographically limited species (Hamrick et al., 1992). The greater allelic diversity within populations suggesting elimination of homozygotes from *C. colocynthis* populations by natural selection and possessing either mating systems characterized by a mixture of self-fertilization and outcrossing (Schoen & Clegg, 1984) or a mode of insect-mediated crosspollination which promoted random mating (Wright, 1951). Previous investigations showed that some species of family Cucurbitaceae had mostly an outcrossing mating system that maintained genetic variation within their populations (Costich & Meagher, 1992 and Montes-hernandez & Eguiarte, 2002).

The F_{IS} and F_{IT} values were negative and revealed an excess of heterozygosity for all populations. The genetic differentiation among *C. colocynthis* populations (F_{ST}=0.162) was similar to that detected for the animal-pollinated plants (F_{ST}= 0.187) (Hamrick, 1989), but lower than the averages for insect-pollinated species (F_{ST}=0.245) and wind-pollinated species (F_{ST}=0.238) (Crawford et al., 2001). Similar results were obtained by Koffi et al. (2008 and 2009) who recoded low level of genetic variation among accessions and cultivars of *Cucumeropsis mannii* and *Lagenaria siceraria* compared to the genetic diversity within them. This was in harmony with Hamrick & Godt (1996) and Nybom (2004) who mentioned that species with outcrossing mating system have lower genetic differentiation among populations compared with systems of selfing mating. There was no significant correlation between geographic distance and genetic distance (*D*). Estimates of gene flow indicated low levels of migration among *C. colocynthis* populations except for population ME. Estimate of *Nm*>4 suggests that gene flow in population ME was enough to face the effects of genetic drift (Kang & Chung, 1997). The extensive gene flow could be attributed to its biological properties, especially its seed dispersal by insects (Dane et al., 2007). However, it appeared that genetic drift might have occurred in other populations which recorded fewer estimates of *Nm*.

Conclusions

Allozyme markers were powerful to reveal variability in *C. colocynthis*. The mean estimate of F_{ST} (16.2%) indicated that up to 83% of the total genetic variation existed within *C. colocynthis* populations. Therefore, we suggest fewer populations but more individuals within populations for its breeding work. The lack of significant correlation between genetic distance and geographic distance suggested that when sampling populations, it is not necessary to arrange sites identically within the area under study and the population must be selected only if it has high genetic variation. Furthermore, analyses of genetic uniqueness demonstrated that population ME is genetically unique and should also be paid additional interest for conservation purposes.

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التمايز الوراثي لعشائر السيترولوس كولوسينثيس (الحنظل) اعتمادا على تنوع أليلات المشابهات الانزيمية

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سيترولوس كولوسينثيس (الحنظل) هو نبات صحراوى له تاريخ كبير في الصناعات الدوائية باعتباره أحد النباتات الطبية الهامة. وقد تم بحث اختلاف الأليلات المنتجة للمشابهات الإنزيمية وكذلك التركيب الوراثي العشائرى داخل نبات الحنظل من خلال 6 عشائر طبيعية تم تجميعها من المنطقة الغربية بالمملكة العربية السعودية بواسطة 6 نظم إنزيمية. وقد اظهرت النتائج وجود تمايز جيني واضح داخل وبين عشائر نبات الحنظل. ظهرت عشرة مواقع جينية كمواقع وحيدة المظهر فى كل العشائر، في حين اعتبرت سبعة أخرى (α-EST-1, β-EST-1, MDH-1, ADH-4, ADH-5, ALO-4 and PRX-2) كمواقع متعددة الأشكال. وكانت متوسطات مؤشرات تباين الأليلات داخل العشائر (P = 31.17%، A = 1.21) أقل من تلك الخاصة بالأنواع المعتمدة على التلقيح الحشري (P =34%؛ A = 2.67، He = 0.205). وكان متوسط التغاير الزيجوتى الملحوظ (0.757) أعلى من متوسط التغاير الزيجوتى المتوقع (0.415) مما دل على وجود انتقاء طبيعي ضد التماثل الزيجوتى. وكانت قيم معامل تزاوج الأقارب (F) للمواقع الجينية متعددة الأشكال سلبية مما يشير إلى وجود زيادة كبيرة في التوجه نحو التغاير الزيجوتى داخل عشائر الحنظل. وقد أكد مخطط الشكل الشجرى للقرابة المعتمد على 84 فردا من عشائر الحنظل ذلك التباين السابق ذكره داخل العشائر. وعلى الجانب الأخر سجل التمايز بين العشائر 16.2% من مجموع التباين الكلى، وهذة القيمه المنخفضة مع قيم المسافات الوراثية العالية (D) أظهرت أن التمايز بين العشائر كان منخفضا على العكس من التباين داخلها. وبالإضافة لكل ما سبق ومع تقدير تدفق منخفض للجينات في جميع العشائر باستثناء تلك المجمعه من مدينة مكة، نوصى بصلاحية نباتات الحنظل الموجودة بمكة- من خلال نتائجنا- لبرامج التربية وحفظ الأصول الوراثية.

وتخلص الدراسة إلى أهمية البيئة الصخرية القاحلة في سيناء كمصدر متميز للأكتينوبكتريا ذات قدرات واعدة لإنتاج المضادات الحيوية.