MOLECULAR MARKERS ASSOCIATED WITH DROUGHT TOLERANCE IN *Citrullus colocynthis*

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Herbal remedy drugs production became so important, especially with flooding the pharmaceutical Egyptian market with a number of synthetic drugs of questionable efficacy associated with the increasing cost of such drugs. Therefore, the demand of high-yield and high-quality medicinal plants will continue to increase in the future. In addition, a growing concern with the awareness of the side effects of the drugs associated with regular exposure to synthetic chemicals has triggered a "back to nature" idea with an appeal of new discovery natural products to meet primary health care.

More than two thousand species are grown wild in Egypt with no complete inventory of medicinal and aromatic plants in each region and, in general, the list of medicinal plants in Egypt and the Arab countries is inexhaustible (Batanouny, 1999).

Citrullus colocynthis L. (*Cucurbitaceae*) is a widely grown desert plant with multi-use potential, is one of the native. Plants of the Middle East countries which is used in traditional medicine. It contains active substances such as saponins, alkaloids and glycosides (Abdel-Hassan *et al*., 2000) and is used in traditional medicine to inhibit the implantation of embryos. Also used to treat (constipation, rheumatism, cancer, oedema, bacterial infections and diabetes). The plant possesses of large amounts of phenolics and flavonoids, which prompted the need to evaluate its antioxidant (Sunil and Mamal, 2008). Moraver, it is also used as antidiabetic and immunostimulant and antioxidant (Bendjeddou *et al*., 2003). However, there have been some reports of its side effects which can induces infertility in both sexes (Chaturvedi *et al*., 2003).

Drought stress is a major environmental factor influencing plant growth and development. *Citrullus colocynthis* is a very drought tolerant cucurbit species with a deep root system (Si *et al*., 2008), it is a source of drought tolerance genes. Since this species is widely distributed in the desert areas and well adapted to drought stress (Dane *et al*., 2006).

DNA fingerprinting is a technology that has matured and is poised for very widespread practical application, such as the identification of plants in commerce, plant breeding and research. In addition, commercial applications include the pro-

tection of medicinal plant breeder's rights and patents, quality control in plant production, processing, and labeling of plant-derived drugs (Soltis *et al.*, 1992).

Molecular markers have been used to determine the association of drought tolerance and shown to be useful for diversity assessment in a number of plant species (Waugh and powell, 1992). These markers, based on the polymerase chain reaction (PCR) technique, are the most commonly used for these purposes, several different PCR-based techniques have been developed during the last decade, each with specific advantages and disadvantages. Inter-simple sequence repeat (ISSR) markers permit detection of polymorphisms in inter- microsatellite loci, using a primer designed from dinucleotide or trinucleotide simple sequence repeats.

The objectives of this study were to:

1. Identify some molecular markers based on ISSR primers associated with drought tolerance *in Citrullus colocynthis.*
2. Detect of some drought tolerance genes.
3. Sequence the successfully amplified drought tolerance genes related to.

MATERIALS AND METHODS

This study was carried out at the laboratories of Genetics Department, Faculty of Agriculture, Ain Shams University, Shoubra El-Kheima, Cairo, Egypt and Biotechnology Laboratory in North Sinai Station, Desert Research Center, Egypt.

1. Plant materials

Citrullus colocynthis L. (*Cucurbitaceae*) plants were collected from four different sites, Red sea coast (Elba Mountain), New Valley area, North Sinai, and Saint Katreen areas by Egyptian Desert Genebank.

2. Methods

2.1. Molecular studies

1-DNA preparation

Genomic DNA from each site was isolated according to the method of Junhans and Metzlatt (1990).

2- ISSR-PCR analysis

Inter-Simple sequence repeats (ISSRs) has been developed as an anonymous, RAPDs-like approach that accesses variation in the numerous microsatellite regions dispersed throughout the various genomes (particularly the nuclear genome) and circumvents the challenge of characterizing individual loci that other molecular approaches require. Microsatellites are very short (usually 10-20 base pair) stretches of DNA that are "hyper variable", expressed as different variants within populations and among different species.

2.1. Polymerase chain reaction

ISSR-PCR reactions were conducted using 14 primers, (Table 1). The

reaction conditions were optimized as follows:

dNTPs (8 mM mix)	2.5 µl
Taq DNA polymerase (5 U/µl)	0.3 µl
10 X buffer with 15 mM $MgCl_2$	3.0 µl
Primer (10 mM)	2.0 µl
Template DNA (50 ng/µl)	2.0 µl
H_2O (dd)	up to 30 µl

Amplification was carried out in Stratgene Robocycler Gradient 96 which was programmed as follows: Denaturation (one cycle) 94°C for 2 minutes, followed by 30 cycles; as follows Denaturation 94°C for 30 second, annealing 44°C for 45 secs, extention 72°C for 1 minute and 30 secs, and finally one extension cycle at 72°C for 20 minutes and 4°C (infinitive).

2.2. Statistical analysis

Data was subjected to statistical analysis of molecular variance (AMOVA) using performed GENALEX6 Genetic analysis in Excel, (Peakall and Smouse, 2006) to partition the total molecular variance between and within populations.

2.3. Detection of some drought tolerance genes

Genomic DNA based analysis of four *Citrullus colocynthis* plants from Elba Mountain, New Valley, North Sinai, and Saint Katreen areas were amplified by five primers of drought tolerance genes, namely (Dehydrin gene), (UB gene), (P5CS gene), (PEPKS gene) and (ACT gene) (Table 2) these primers were designed as degenerate primers based on genes conserved sequences and depending on the data bases of NCBI (National Center for Biotechnology Information).

2.4.1. Polymerase chain reaction

PCR reactions were optimized as follows:

dNTPs (8 mM mix)	2.5 µl
Taq DNA polymerase (5 U/µl)	0.3 µl
10 X buffer with 15 mM $MgCl_2$	3.0 µl
Primer (10 mM)	2.0 µl
Template DNA (50 ng/µl)	2.0 µl
H_2O (dd)	up to 30 µl
Primer (10 mM) reverse	1.0 µl
Template DNA (50 ng/µl)	2.0 µl
H_2O (dd)	up to 30 µl

Amplification was carried out in Stratgene Robocycler Gradient 96 which was programmed as follows: Denaturation (one cycle) 94°C for 5 minutes, followed by 30 cycles; as follows denaturation 94°C for30 second, annealing 50-60°C for 60 secs, extention 72°C for 30 secs, and finally one cycle extension at 72°C for 5 minutes.

2.4.2. Gel electrophoresis

PCR-Products of 15 µl were resolved in 1.5% agarose gel electrophoresis with 1 x TAE running buffer. The run was performed at 80 V for 180 min and the gel was stained with ethidium bromide. A marker of 1 Kb plus DNA Ladder 1 µg/µl (Invitrogen) that contains a total of twenty bands ranging from 12000 to 100 bp was used. Bands were detected on UV-transilluminator and photographed by Gel doc-

umentation system Biometra Bio Doc Analyze 2000.

2.4.3. DNA Sequencing

The automated DNA sequencing reactions were conducted for the fragments with specific forward primer using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE applied Biosystems, USA).

Data analysis was conducted using Blast programs from National Center for Biotechnology Information (NCBI), USA http://www.ncbi.nlm.nih. gov/BLAST/

RESULTS AND DISCUSSION

1- *Molecular markers associated with drought tolerance*

DNA-based molecular markers have proved their utility in field like taxonomy, physiology, embryology and genetics, etc. As the science of plant genetics progressed, researches have tried to explore these molecular techniques for their applications in food crops, horticultural plants, etc. and recently in pharmacognestic characterization of herbal medicine (Nissen *et al.*, 1995).

1.1. ISSR-PCR analysis

Inter simple sequence repeats (ISSR) are ideal as markers for molecular genetic studies because of their abundance and the high degree of polymorphism between individuals within a population of closely related genotypes.

ISSR patterns showed a total of 149 DNA bands detected across the fourteen ISSR primers, 99 of them were polymorphic (about 66%), (Table 3 and Fig. 1).

As high as 22 (ISSR-PCR markers) out of the 149 bands (about 15%) were found to be useful as specific markers. The largest number of ISSR specific markers was scored for primers HB12, HB14 and 844B (four markers for each), followed by primers HB11 and HB15 (three markers for each), while two markers were scored for primer (844A). One marker was scored for the primers (ISSR1 and 814), otherwise invariable results were shown by primers (HB8, HB9, HB10, HB13, ISSR2 and ISSR4), which revealed no specific markers as shown in (Table 4). Three ISSR markers characterized new valley region (1100, 1050 and 900 bp). While, Elba Mountain was characterized by three ISSR markers at (800, 700 and 350 bp) but North Sinai was characterized by four ISSR markers (1200, 670, 450 and 200 bp) while, Saint Katreen was characterized by 12 ISSR markers (1000, 700, 1500, 900, 600, 500, 350, 1200, 900 500, 400 and 250 bp). These results are in agreement with Wu *et al*. (2004) who suggested that ISSR has been used for genetic diversity analysis and obtaining high polymorphism and good molecular markers to evaluate and preserved the endangered wild species of rice, *Oryza granulate*.

Analysis of molecular variance (AMOVA) based on ISSR results

Analysis of molecular variance indicated that 100% of the genetic variation was attributed to differences within samples. No significant genetic variation was detected among samples, this mean that there is no difference among samples per sites. However, the sum squares was found to be 560.000, 1.957 and 0.720, for within samples, among sites and among samples per sites respectively. Detailed results from AMOVA are shown in Table (5). The estimated variance was 0.001, 0.232 and 0.0 for among sites, within samples and among samples per sites.

2.1. Detection of some drought tolerance genes in Citrullus colocynthis

2.1.1. Dehydrin Gene

Dehydrin gene is responsive to drought stress and caused accumulation of dehydrin-like proteins (Vinod *et al*., 2006).

Dehydrins (DHNs) compose a family of intrinsically unstructured proteins that have high water solubility and accumulate during late seed development, low temperature or water deficit conditions, They are thought to play a protective role in freezing and drought tolerance in plants and change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a water stimulus.

The PCR product using specific primer of Dehydrin drought tolerant gene showed the appearance of one band with fragment size of 190 bp, that might to be apart of Dehydrin genes (Fig. 2). This was in agreement with Hinniger and Victorea (2006), who isolated and characterized three Dehydrins genes, which were expressed during *Coffea canephora* (Robusta) in various tissues with molecular sizes of 240 bp.

3.1.2. PEPCS (phosphoenolpyruvate carboxylase) gene

PEPCS gene mode of action is the improvement of drought tolerance and increase efficiency of used water by over-expressing PEPC protein under water stress at reproductive stages with a strong impairment of photosynthesis and grain filling (Kazuo and Shinozak, 2005).

PEPC over expressing increase in intrinsic water use efficiency (WUE) under drought conditions. Opposite effects were observed for transgenic plants under-expressing the corresponding proteins.

The PCR product using specific primer of PEPCS drought tolerance gene indicted the appearance of PEPCS gene, with molecular size of 650 bp as shown in (Fig. 3). The same conclusion was reached by Sanchez and Flores (2006) who stated that *Arabidopsis* phospho-enolpyruvate carboxylase genes encode polypeptides and are differentially expressed in response to drought. Results showed that PEPC gene is part of the adaptation of the plant to drought tolerance with molecular size 670 bp.

3.1.3. UB gene

UB gene (Ubiquitin) is present in all eukaryotic species, it is a multifunctional protein. One of its main known functions is to tag proteins for selective degradation by the proteosome.

The PCR product using specific primer of UB drought tolerance gene indicted the appearance of UB gene, with molecular size of 450 bp, as shown in (Fig. 4). Our results agreed with those of Zhou and Chang (2010) who reported that ubiquitination plays important roles in plant abiotic stress responses and stated the over expression of soybean ubiquitin gene for enhancing drought stress tolerance through modulating abiotic stress in *Arabidopsis*. This UB gene fragment was with molecular size of 450 bp.

3.1.4. P5CS gene (1-pyrroline-5-carboxylate synthetase)

The mode of action of P5CS gene is the transcription of the amino acid proline which considerd one of the most accumulated osmolytes under salinity and water deficit conditions in plants. Transcription of the P5CS genes is differentially regulated by drought, salinity and abscisic acid, suggesting that these genes play specific roles in the control of proline biosynthesis.

The PCR product using specific primer of P5CS drought tolerant gene indicted the appearance of one fragment with size of 600 bp (Fig. 5).

The same results of P5CS gene was obtained by Hayati and Santoso (2001), who identified P5CS gene on sugarcane by PCR using heterologous primer. The product size was 640 bp.

3.1.5. ACT2 Gene

The mode of action of ACT2 gene is the activation of the process of root hair growth and development.

The PCR product using specific primer of ACT2 drought tolerance gene indicted that appearance of one band with a fragment size of 700 bp as shown in (Fig. 6). Similar result of ACT gene was obtained by Devaiah and Athmaram (2007), who identified 2 new ACT genes with 2 full lengths of 825 and 700 bp from drought tolerant peanut, which were up regulated in response to drought stress.

DNA Sequencing and BLAST analysis

The reactions of automated DNA sequencing were conducted for the five fragments with specific forward primer using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE applied Biosystems, USA), in conjunction with ABI PRISM (310 Genetic Analyzer). However, nucleotide sequence homology could be identified for only the one fragment (ACT2) as shown in Fig. (7), but there is no significant similarity for the other four fragments. BLAST analysis was done using National Center for Biotechnology Information (NCBI), USA (http://www. ncbi.nlm.nih.gov/BLAST/).

E-values or Expect value (E) is a parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size (Pearson and Lipman, 1988).

The ACT fragment was highly similar to act mRNA from *Brassica oleracea* (AF044573.1) with 100% similarity (E-value 2e-136) and *Arabidopsis thaliana* (AK318637.1, AF370302.1) with 89-91% similarity identified genes with E-value (6e-91,1e-98), respectively.

SAMMURY

In the present study *Citrullus colocynthis* L. (*Cucurbitaceae*) plants were collected from four different sites (Red sea coast (Elba Mountain), New Valley area, North Sinai, and Saint Katreen) by Egyptian Desert Genebank .Molecular markers associated with drought tolerance were studied by fourteen preselected (ISSR) primers exhibited polymorphism obtained from the DNAs of sixteen samples of *C. colocynthis* (C1-C4) from the four different sites.

Detection and sequencing of some drought tolerance genes in *Citrullus colocynthis* using the genomic DNA based on the four plants from different sites. The specific primers of those drought tolerant genes were (Dehydrin gene), (UB gene), (P5CS gene), (PEPKS gene) and (ACT gene), which were succeed to detect some drought tolerant genes among the four locations of *Citrullus colocynthis*.

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Table (1): ISSR primers names and their sequences.

Primer name	Sequence	Primer name	Sequence
844B	$(CT)_8$ GC	HB9	$(GT)_6$ GG
HB13	$(GAC)_3$ GC	HB10	$(GA)_6$ CC
814	$(CT)_8$ TG	HB11	$(GT)_6$ CC
844A	$(CT)_8$ AC	HB12	$(CAC)_3$ GC
HB8	$(GA)_6$ GG	HB14	$(CTC)_3$ GC
ISSR1	$(CAA)_5$	HB15	$(GTG)_3$ GC
ISSR2	$(CAG)_5$	ISSR4	$(GACA)_4$

Table (2): Primers of drought tolerance genes and their sequences.

Primers	Sequences
Dehydrin	F- AAC AAG GTA CGG TGG AAG R- ATC CTC CAG TAC CAG GAA GC
UB	F- GCA GCT CGA GGA TGG AAG R- CCA GCT GCT TAC CCG CAA AG
P5CS	F- GTT YAA RYT XGT XAG RGG XGC HTA R- CTC RTA XGC XCK XCK XAR XAR RTA
PEPKS	F- TGG CCC CAC TCA TCT TGC TAT TT R- GCC GCC TTG CTC GTG TCC AT
ACT2	F- ATT CAG ATG CCC AGA AGT CTT GTT R- GAA ACA TTT TCT GTG AAC GAT TCC T

Table (3): ISSR analysis from the DNAs of *C. colocynthis via* 14 ISSR primers.

Primer code	Total amplified fragments	Length range (bp)	Polymorphic fragments	% of poly-morphism
HB8	7	250-750	5	71
HB9	11	400-1800	9	82
HB10	13	150-2000	9	69
HB11	11	300-1400	10	91
HB12	15	200-2000	7	47
HB13	9	300-1550	1	11
HB14	15	250-2400	8	53
HB15	11	350-2000	9	82
ISSR1	11	450-3000	9	82
ISSR2	9	450-2500	6	67
ISSR4	10	350-3400	7	70
814	9	400-2200	7	78
844A	8	300-1500	4	50
844B	10	400-1600	8	80
Total	149	-	99	66

Table (4): Numbers and specific markers molecular weights for the *C. colocynthis* from four different sites resulting from ISSR-PCR.

Primers	New Valley		Elba Mountain		North Sinai		Sant Katreen		Total
	Numbers	MW (bp)	Numbers	MW (bp)	Numbers	MW (bp)	Numbers	MW (bp)	
HB8	-	-	-	-	-	-	-	-	-
HB9	-	-	-	-	-	-	-	-	-
HB10	-	-	-	-	-	-	-	-	-
HB11	-	-	1	350	-	-	2	400, 250	3
HB12	-	-	1	700	3	670, 450, 200	-	-	4
HB13	-	-	-	-	-	-	-	-	-
HB14	-	-	1	800	-	-	3	1200, 900, 500	4
HB15	-	-	-	-	-	-	3	600, 500, 350	3
ISSR1	1	1050	-	-	-	-	-	-	1
ISSR2	-	-	-	-	-	-	-	-	-
ISSR4	-	-	-	-	-	-	-	-	-
814	1	900	-	-	-	-	-	-	1
844A	-	-	-	-	-	-	2	1500, 900	2
844B	1	1100	-	-	1	1200	2	1000, 700	4
Total	3	-	3	-	4	-	12	-	22

Table (5): Analysis of molecular variance **(AMOVA)** of sixteen *C. colocynthis* of four different sites resulting from all ISSR-PCR data.

Source	DF	SS	MS	Est. Var.	%
Among sites	3	1.957	0.652	0.001	0.0%
Among samples/sites	12	0.720	0.060	0.000	0.0%
Within samples	2416	560.000	0.232	0.232	100.0%
Total	2431	562.678	0.944	0.232	

DF = Degrees of freedom SS = Sum of squares

MS = Mean square Est. Var. = Estimated Variation

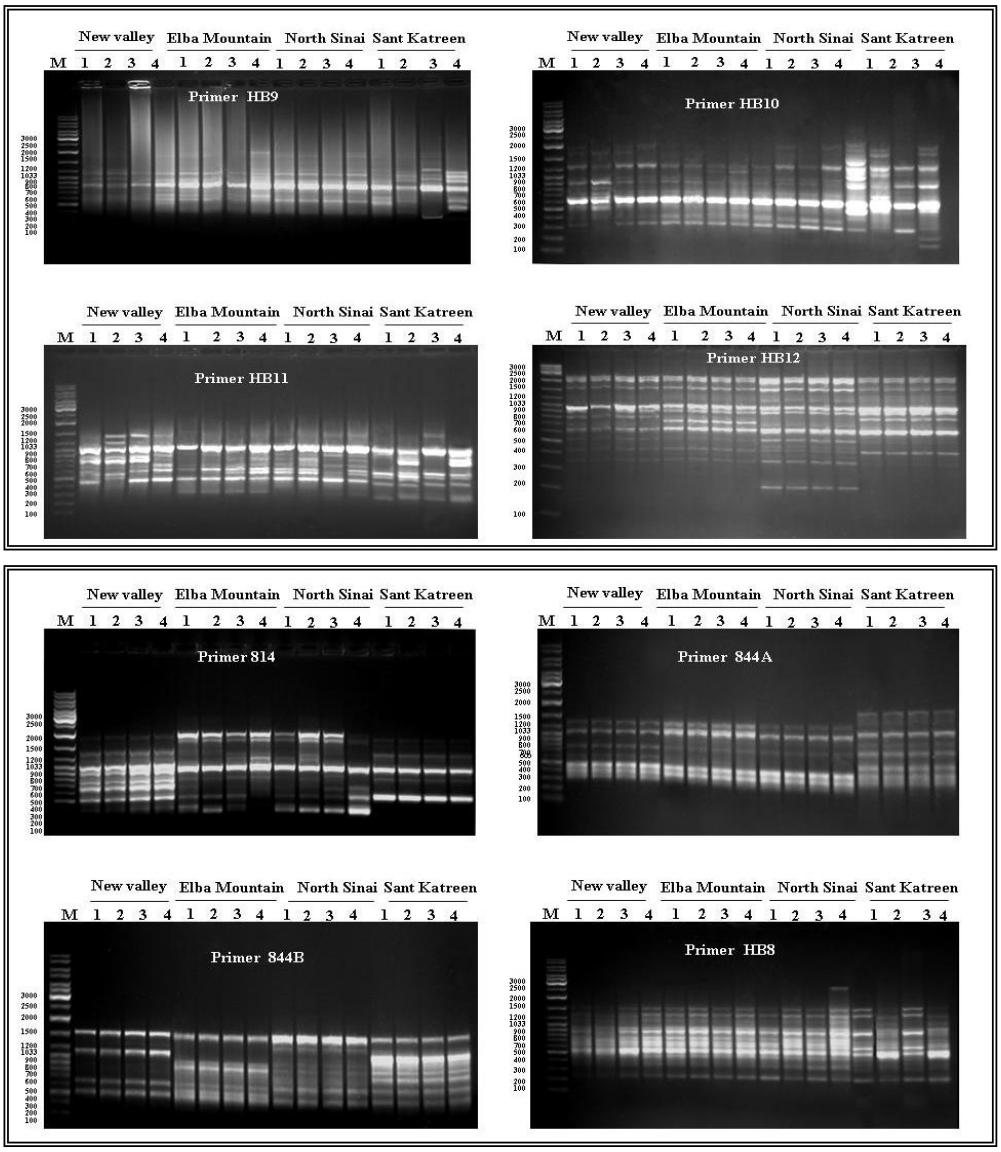

Fig. (1): ISSR-PCR of DNAs of *C. colocynthis*, New Valley (1-4), Elba Mountain (1-4), North Sinai (1-4) and Sant Katrren (1-4) via 13 random primers. M: 1 Kb DNA ladder (Stratagene®).

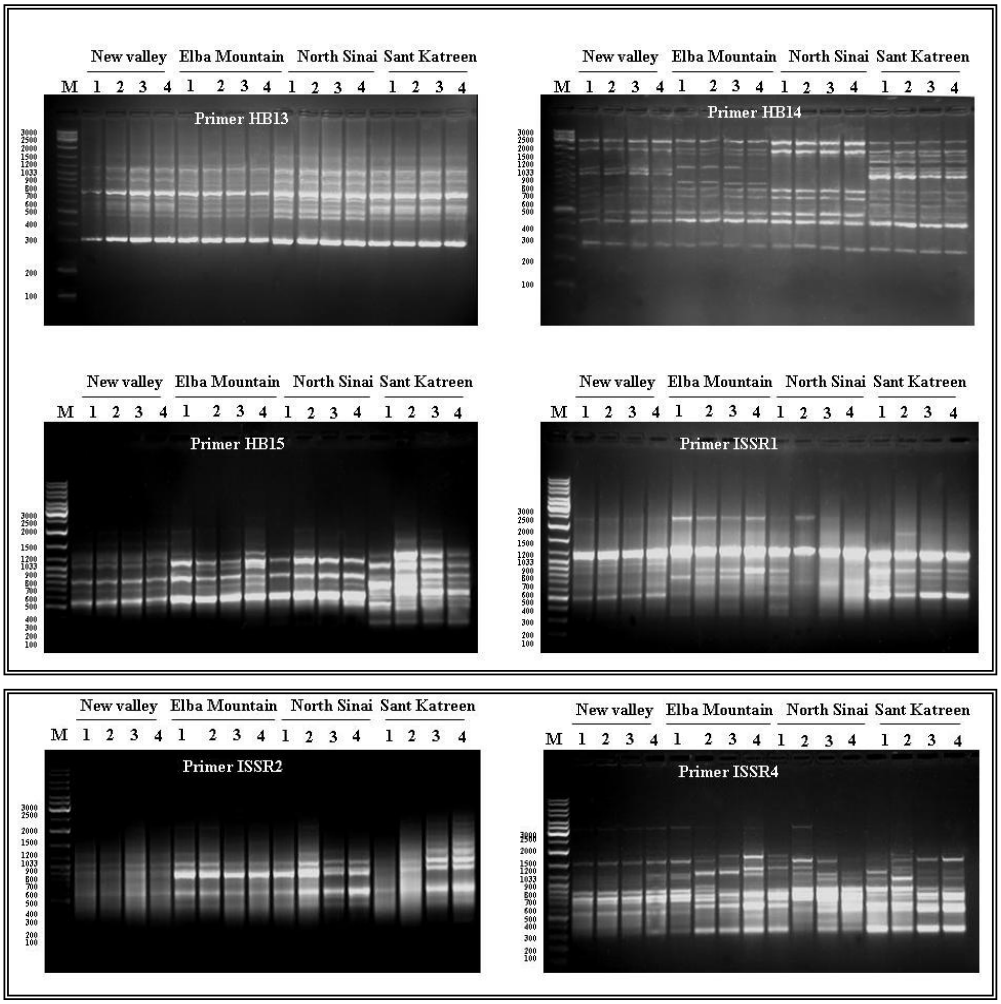

Fig. (1): Continued

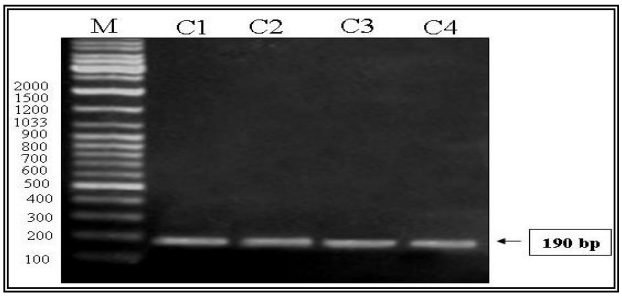

Fig. (2): PCR product of *C. colocynthis* Dehydrin Gene where: (C1) mean New Valley, (C2) Elba Mountain, (C3) North Sinai and (C4) Sant Katrren. M: 1 Kb DNA ladder.

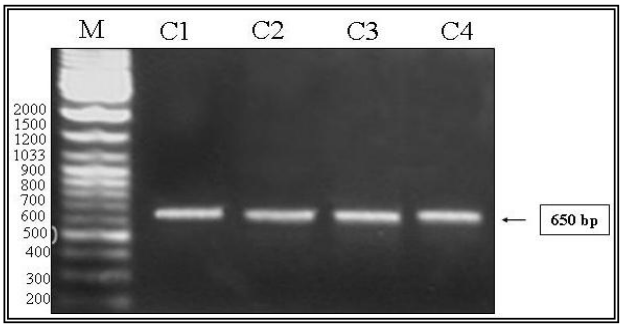

Fig. (3): PCR product of *C. colocynthis* PEPCS Gene where: (C1) mean New Valley, (C2) Elba Mountain, (C3) North Sinai and (C4) Sant Katrren. M: 1 Kb DNA ladder.

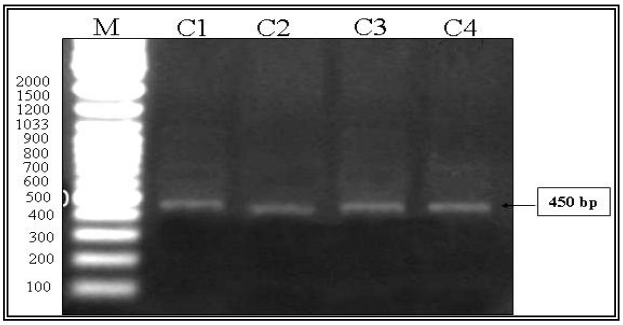

Fig. (4): PCR product *C. colocynthis* of UB Gene where: (C1) mean New valley, (C2) Elba Mountain, (C3) North Sinai and (C4) Sant Katrren. M: 1 Kb DNA ladder.

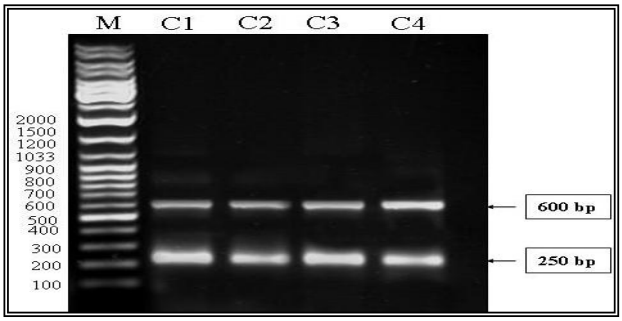

Fig. (5): PCR product of *C. colocynthis* P5CS Gene where: (C1) mean New Valley, (C2) Elba Mountain, (C3) North Sinai and (C4) Sant Katrren. M: 1 Kb DNA ladder.

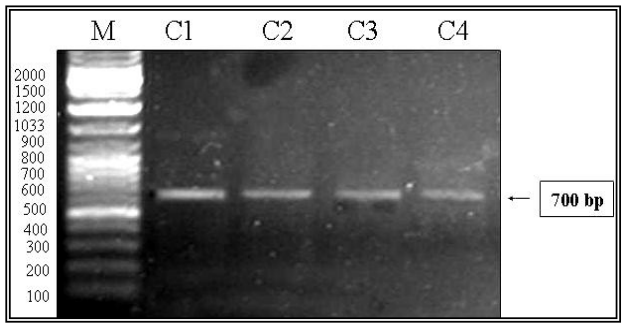

Fig. (6): PCR product of *C. colocynthis* ACT2 Gene where: (C1) mean New Valley, (C2) Elba Mountain, (C3) North Sinai and (C4) Sant Katrren. M: 1 Kb DNA ladder.

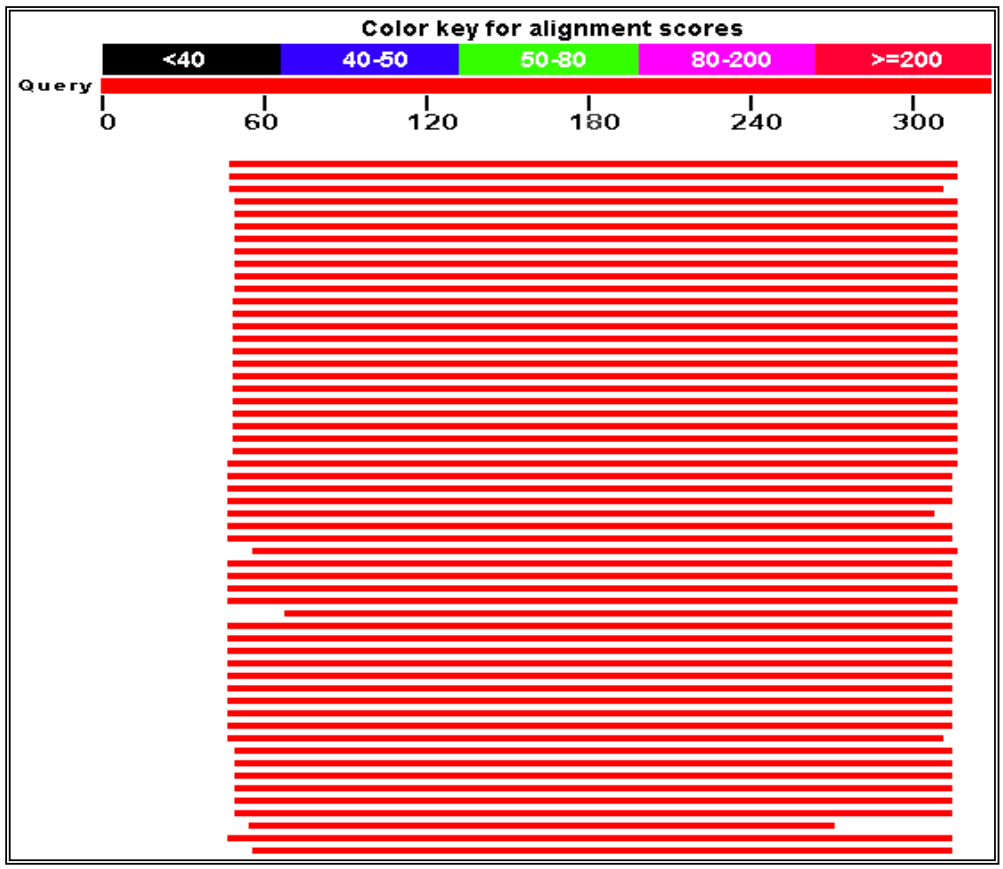

Fig. (7): Distribution of blast on query sequence of ACT2 Gene.