Genetic variability of *Nepeta septemcrenata* Benth. (Lamiaceae) Assessed by RAPD Markers

M.A. Elkholy[1], M. Mansour[1], K. Omar [2]

[1.] Botany Department, Faculty of Science (Boys branch), Al-Azhar University, Cairo, Egypt.
[2.] Saint Katherine Protectorate, the Nature Conservation Sector (NCS), Egyptian Environmental Affairs Agency(EEAA).
kariem_npe@yahoo.com

Abstract: Genetic variability in *N.septemcrenata* populations from six locations in Saint Katherine Protectorate (SKP), using ten primers has been evaluated by RAPD-PCR analysis. A total of 122 DNA bands were detected, 54 bands were polymorphic, 44 were monomorphic and 24 were unique. The percentage of polymorphic bands ranged from 20% to 73.3% with an average of 40.69%. The amplified DNA bands ranges in size between 176 to 1874 bp. Number of unique bands ranged from 1 to 9 with average 2.4 per primer, it was observed that most of unique bands scored at location 6 (Wadi Elfaraa). Genetic distance between populations ranged from 0.0 to 0.38. Cluster analysis based on the presence or absence of bands was performed by dice similarity coefficient. Results show that there's genetic distance between population 1 and population 6 this results explained as the effect of environmental condition varies from location to another and we find that the two locations are similar in their climate but varies in edaphic factors which may be the reason for this variation.
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1. Introduction

The genus *Nepeta* belongs to family Lamiaceae, it comprises approximately 250 species of annual or perennial herbs distributed in temperate Europe, Asia and Africa (Mabberley, 1997). Species of the genus contain up to 1% of essential oil as well as nepetalactone, iridoid, bitter principles, tannins and minerals. *Nepeta* essential oil is mainly composed of citral, citronellal, geraniol, carvacol, nepetol, thymol, pulegon, actinidine and monoterpene alkaloid (Nowiński, 1983; Mackú and Krejcá, 1989; Bown, 1999& Senderski, 2004). *Nepeta septemcrenata* is the only species of the genus *Nepeta* in Egypt (Täckholm, 1974, Boulos 2002).

The preservation of genetic diversity is important, because it provides long-term evolutionary potential for changing environmental conditions (Dobson, 2000). Several reports have indicated losses of botanical diversity. At some point losses will affect local communities, and at a higher level, they will affect global stability.

As biodiversity loss accelerates, ecologists have devoted increasing effort to understand how these declines will affect ecosystem functioning (Chapin et al. 1997; Tilman 1999; Loreau *et al.* 2001). Most effort has focused on plant biodiversity (Balvanera *et al*. 2006), and how loss of plant species or functional groups impacts processes such as ecosystem productivity (Hector *et al.* 1999; Tilman *et al.* 2006a; Cardinale *et al.* 2007), nutrient cycling (Hooper & Vitousek 1998), and ecosystem stability (Tilman *et al.* 2006b). What is less understood is if and how the loss of diversity at the producer level impacts associated consumer species (Haddad *et al.* 2001; Balvanera *et al.* 2006; Crutsinger *et al.* 2006; Johnson *et al.* 2006), and whether the effects of plant species extinctions are dampened or magnified across trophic levels (Cardinale et al. 2006; Duffy et al. 2007).

In recent years, molecular markers derived from DNA using electrophortic techniques have provided powerful markers for the study of several aspects in all biological fields including systematic and genetic relationships of plant species and sub-specific ranks. Currently, the technique of choice is the RAPD (Random Amplified DNA) based on Polymerase Chain Reaction (PCR). This approach is based on using the PCR as proposed by Williams *et al.* (1990) to amplify DNA sequences with single short (9-10 bp) primers of arbitrary nucleotide sequence. It requires small amounts of DNA, easy to perform and reveals dominant molecular markers of ultimate potentialities in several fields of plant science including systematic and evolution (Witkus *et al.*, 1994). The RAPD method also provided useful evidence for gene mapping (Barua *et al.* 1993, Komatsuda *et al.* 1997) and genetic diversity (Baum *et al.*, 1997 and Mohamed 2004).

RAPD markers have been widely used to identify and characterize sub specific categories in many plant species. At sub-specific categories,

RAPD has been applied to reveal genetic diversity among wild barley (*Hordeum spontaneum*) in the near east (Baum *et al.*, 1997), eight sweet sorghum (*Sorghum bicolor*) cultivars (Abdel-Tawab *et al.*, 2001) and different accessions of *Linum usitatissimum* (Fu *et al.*, 2001). RAPD has been also used for the determination of genetic relationships among cultivars of barely from different parts of the world (Kuczynska *et al.* 2001; Yu *et al.* 2002; El-Shazly and El Metairi, 2006) and turf grass (Al-Khalifa *et al.*, 2005).

Many authors have also utilized the RAPD-PCR approach to study genetic diversity and species relationships in some plant genera. Examples include *Morus* (Awasthi *et al.*, 2004), *Vigna* (Elkholy, 2005) and *Crotolaria* (Elkholy *et al.*, 2006). The applications of RAPD in plant biodiversity also included investigation of genetic diversity in wild accessions of two *Artemisia* species in Egypt (Mohamed, 2004).

The aim of the present work is to reassess the genetic diversity among 6 accessions of *Nepeta septemcrenata species* in SKP based on DNA fingerprints as revealed by RAPD-PCR polymorphism.

2. Material and Methods

- ***Study area:***

Six locations were sampled for this study, the selection of these locations was based on the following criteria: (1) the area must be isolated, (2) the plant can be collected from this area, (3) number of plant individuals within each location must be sufficient for collection because our goal to conserve this plant not to consume the gene bank. The selected locations are Shak Mosa, Shak Abo Hamman, Wadi Elrotk, Farsh Elloza, Elgabal Elahmar and Wadi Elfaraa. These locations are illustrated on the map of the study areas (Fig. 1).

- ***Plant materials:***

Nepeta septemcrenata dry seed were collected from the six isolated locations in Saint Katherine Protectorate during the fruiting season (August, 2009). At each location numbers of ecological variables were studied such as altitudinal range, aspect, microhabitat, soil characteristics, climatic conditions, grazing pressure and morphological aspects of the plant.

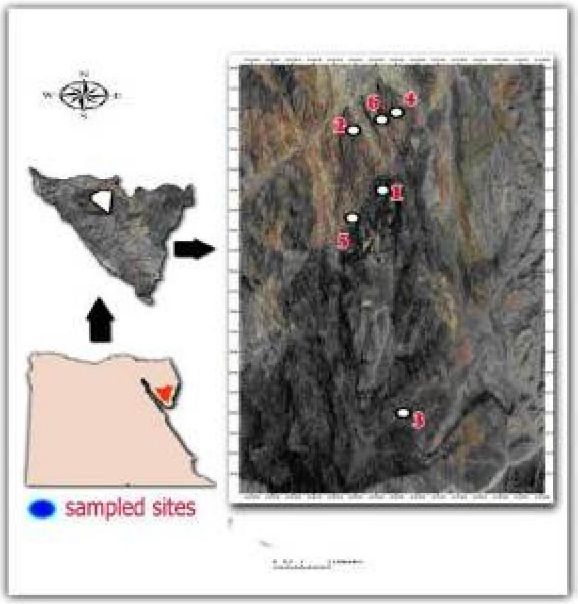

Fig. 1 Location map for RAPD PCR Sample Sites. 1= Shak Mosa, 2= Shak Abo Hamman, 3= Wadi Elrotk, 4= Farsh Elloza, 5= Elgabal Elahmar, 6= Wadi Elfaraa.

- ***Genomic DNA isolation:***

In order to extract the DNA, seeds of the six locations were germinated on cotton pads in Petri dishes in growth chambers under a regime of 15°C/5°C day and night temperature and 12 h/12 h light for 7–10 days, after that each accessions was represented by a bulk consisting of young leaves. DNA was isolated from bulked fresh leaf tissue from each population according to Kang & Yang, (2004). About 0.5 gm *N.septemcrenata* leaves were put in a 1.5 ml Eppendorof tube, the leaf tissue was homogenized in liquid nitrogen and added 50 µl DNA extraction buffer (500 mM NaCl, 100 mM Tris-HCl pH 7.5 and 50 mM EDTA pH 7.5). After an initial homogenization, another 150 µl of DNA extraction buffer was added and homogenized for 20 sec. Then 20 µl of 20% SDS were added and vortexed for 30 sec. The samples were incubated at 65ºC for 10 min. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the samples, mixed by vortexing for 30 sec. and then centrifuged at 10.000 g for 3 min. at 4ºC. The supernatant was transferred to a fresh tube and extracted once more time with phenol/chloroform/isoamyl alcohol (25:24:1). The supernatant was transferred to a fresh tube and a double volume of ethanol was added to each tube, mixed well, and the tubes were incubated at -20ºC for 30 min. The samples were then centrifuged at 10.000 g for 10 min. at 4ºC. The pellet was washed with 70% ethanol, dried and resuspended in sterile dH2O containing 20 µg /ml DNase- free RNase A. The

concentration and purity were determined from the A260/A280 ratio using a double beam spectrophotomete.

- ***Random amplified polymorphism DNA (RAPD) technique:***

A set of ten random 10-mer primers (Table 1) was used in the detection of polymorphism among the six *N.septemcrenata* accessions. RAPD is amplification of parts of genomic DNA using at least one short oligonucleotide primer. The following components were added to a sterile Eppendorof tube on ice as followed: 2 µl 25 mM MgCl2; 2.5 µl 2 mM dNTPs; 0.3 µl Taq DNA polymerase (5 unit/µl); 2 µl 0.4 uM 10-mer primer (from Bioneer, New technology). The volume was completed to 25 µl dH2O. Thirty ng of each DNA extracted sample were used for amplification reaction 5 µl of the 10-mers random primer (15 ng /ml) were added to Gene pack PCR tubes kits. The total volume was completed to 25 µl using sterile dH2O water. The primer code and sequence was illustrated in Table 1.

Table 1. Names, sequance and GC% of primers that gave rise to reliable and stable products in the examined *Nepeta* accessions.

Primer	Sequence (5′-3′)	G+C %
A-11	5′CAATCGCCGT 3′	50
A-14	5′TCTGTGCTGG 3′	60
A-16	5′AGCCAGCGAA 3′	60
B-10	5′CTGCTGGGAC 3′	70
B-14	5′TCCGCTCTGG 3′	70
C-01	5′TTCGAGCCAG 3′	60
C-18	5′TGAGTGGGTG 3′	60
G-02	5′GGCACTGAGG 3′	70
G-05	5′CTGAGACGGA 3′	60
G-07	5′GAACCTGCGG 3′	70

- ***Visualization and analysis of RAPD-PCR products:***

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts. PCR products were visualized on UV light and photographed using a Polaroid camera. The gels were scanned for molecular weight (bp). The different molecular weights of bands were determined against DNA ladder from BioRoN. Amplified products were visually examined and the presence or absence of each size class was scored as 1 or 0, respectively.

- ***Data Analysis:***

The bands revealed by the primers are polymorphic bands detected as the presence of amplified bands in some lanes but not all lanes of the gel containing bands while monomorphic bands present in all lanes and unique bands found in only one lane but not in all lanes. The banding patterns generated by RAPD-PCR markers analyses were compared to determine the genetic variation among the 6 *N.septemcrenata* accessions. Clear and distinct amplification products were scored as '1' for presence and '0' for absence of bands. Bands of the same mobility were scored as identical. The genetic similarity coefficient (GS) between two genotypes was estimated according to Dice coefficient (Sneath and Sokal, 1973). The similarity matrix was used in the cluster analysis. The cluster analysis was employed to organize the observed data into meaningful structures to develop taxonomies. At the first step, when each accession represents its own cluster, the distances between these accessions are defined by the chosen distance measure (Dice coefficient). However, once several accessions have been linked together, the distance between two clusters is calculated as the average distance between all pairs of accessions in the two different clusters. This method is called Unweighted Pair Group Method using Arithmetic Average (UPGMA) (Sneath and Sokal, 1973). The genetic distance of each band to all others calculated by the simple equation:

$$D = 1 - \frac{N_{xy}}{(N_x + N_y - N_{xy})}$$

where:

D_{xy} = the genetic distance between plant "x" and plant "y"

N_{xy} = the number of bands shared by plant "x" and plant "y"

N_x = the number of bands in plant "x"

N_y = the number of bands in plant "y"

3. Results and Discussion

All primers detected polymorphism among different locations except primer A-16. In total, 122 bands were produced, 54 of which were polymorphic, 44 were monomorphic and 24 were unique (Fig. 2). Primers A-11 and G-7 produced the highest, while primer A-16 not produced polymorphic bands. The percentage of polymorphic bands ranged from 20% to 73.3% with an average of 40.69%. The average number of polymorphic bands produced was 5.4 per primer. Only the amplified DNA fragments ranging in size between 176 to 1874 bp were used for statistical analyses. Number of unique bands range from 1 to 9 with average 2.4 per primer, it was observed that most of unique bands scored at location 6 (Wadi Elfaraa). Genetic distance between locations ranging from 0.0 to 0.38. Cluster analysis based on the presence or absence of bands was performed by dice similarity coefficient, based on Unweighted Pair Group Method with Arithmetic Averages (UPGMA).

Numbers, type of bands with its size range within this study are illustrated in Table 2.

Table 2. Number and type of bands and their size range revealed by used primers in the examined populations.

Primer	Size range Of Products (bp)	No. of polymorphic bands	No. of monomorphic bands	No. of unique bands	Total no. Of alleles	Polymorphism (%)	Monomorphism %	Uniqueness %
A-11	203-1847	11	3	1	15	73.33	20	6.67
A-14	309-1282	4	3	1	8	50	37.5	12.5
A-16	390-991	0	5	1	6	0	83.33	16.67
B-10	285-1304	4	5	3	12	33.33	41.67	25
B-14	263-1221	3	10	2	15	20	66.67	13.33
C-1	299-1703	6	6	3	15	40	40	20
C-18	464-1392	3	7	0	10	30	70	0
G-2	251-959	3	4	0	7	42.86	57.14	0
G-5	344-1775	7	1	4	12	58.33	8.33	33.33
G-7	176-1546	13	0	9	22	59.09	0	40.91
Total bands scored		54	44	24	122	44.26	36.07	19.67

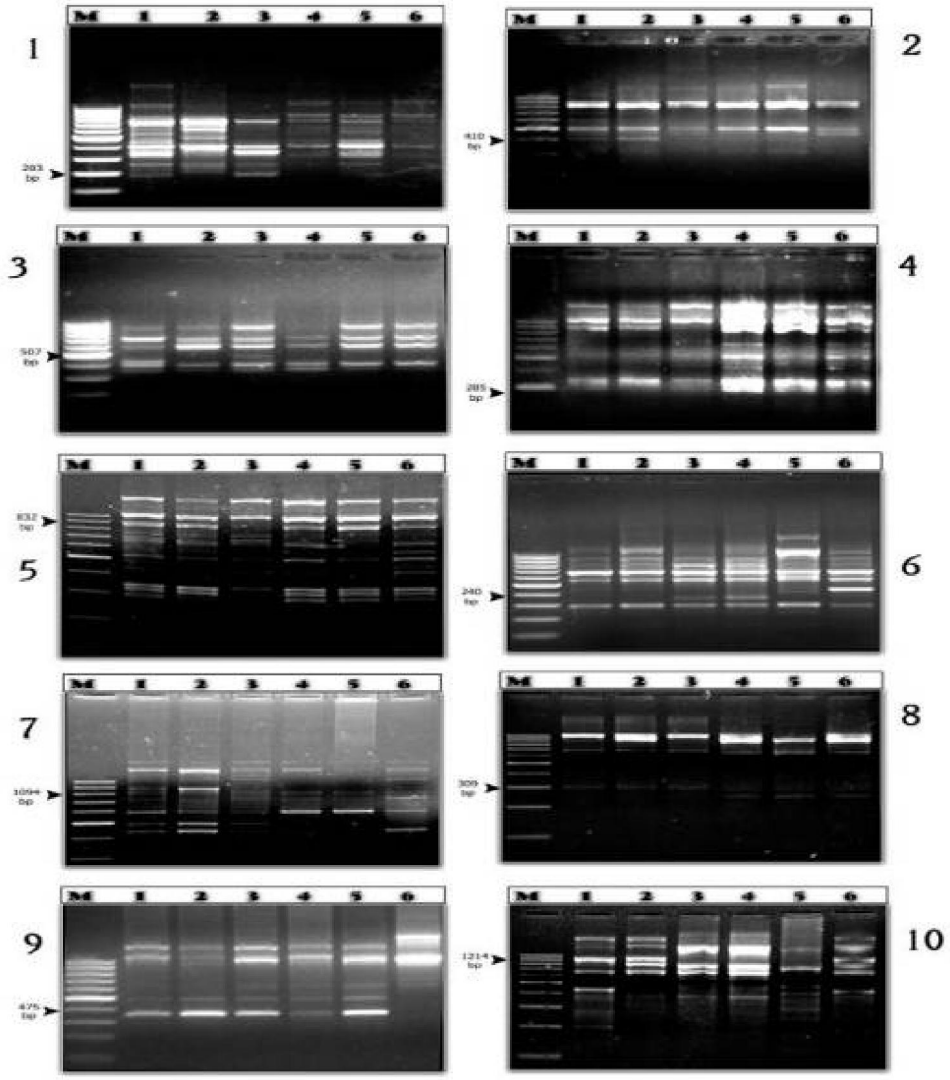

Fig 2. RAPD markers as revealed by the ten primers in the six *N. septemcrenata* populations from the Saint Katherine Protectorate. 1= A-11, 2= A-14. 3= A-16, 4= B-10, 5= B-14, 6= C-01, 7= C-18, 8= G-02, 9= G-05 and 10= G-07.

Genetic similarity ranged between 0.69 to 0.93. Similarity matrix show that there's a great variation between location specially between location 1 (Shak Mosa) and location 6 (Wadi Elfaraa).

Table 3. Similarity among different locations using Dice Coefficient method

	Wadi Elfaraa	Farsh Elloza	Shak Abo Hamman	Wadi Elrotk	Elgabal Elahmar	Shak Mosa
Wadi Elfaraa	**100**					
Farsh Elloza	74	**100**				
Shak Abo Hamman	71	77.1	**100**			
Wadi Elrotk	70.7	83.8	80.5	**100**		
Elgabal Elahmar	70.5	85.7	71	77.3	**100**	
Shak Mosa	69.6	74.4	93	80.3	72.2	**100**

Dendogram illustrating high similarity between location 1 (Shak Mosa) and location 2 (Shak Abo Hamman) show fig. 3.

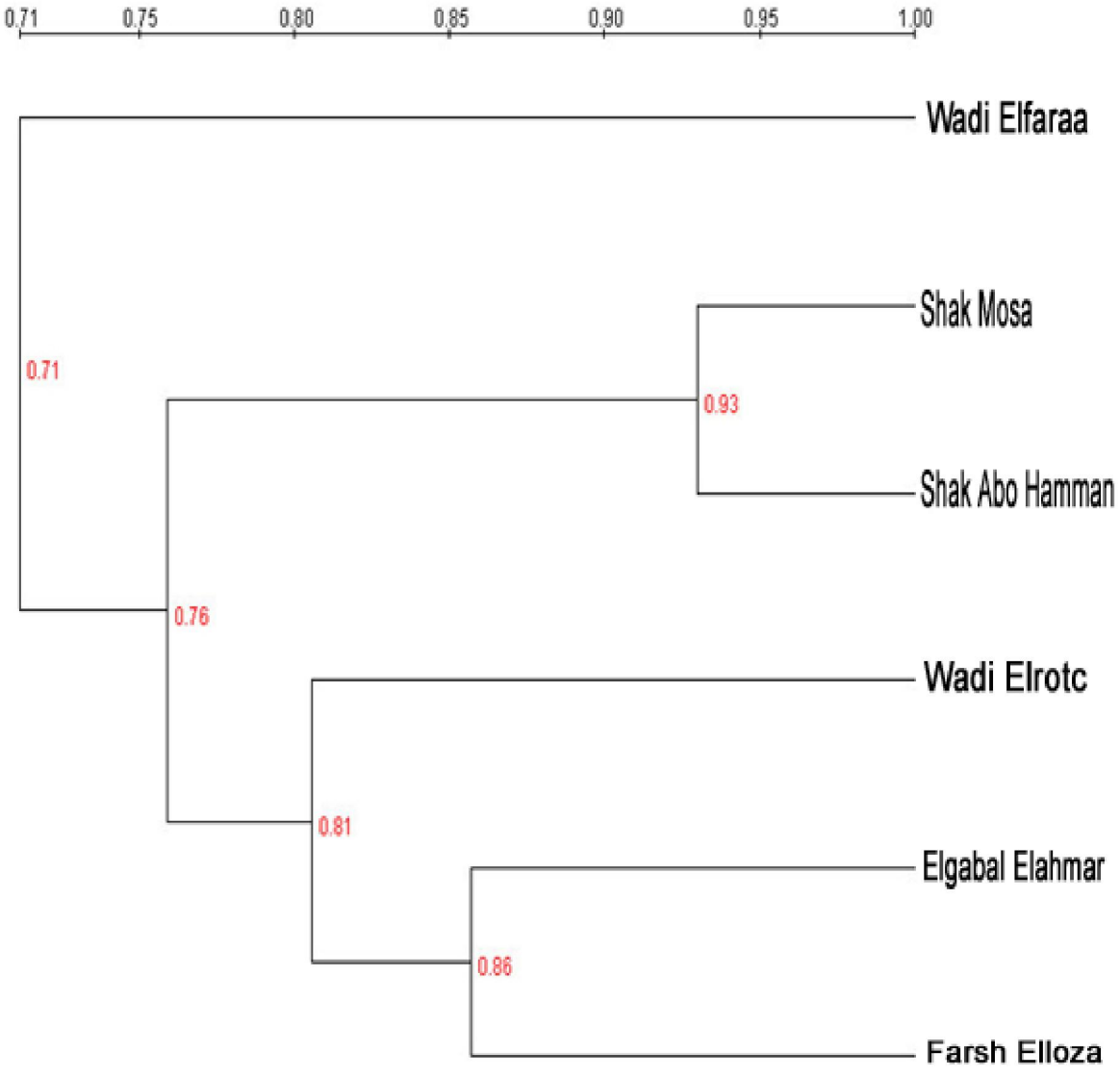

Fig 3. Dendogram obtained by cluster analysis based on presence /absence matrix.

Table 4. Morphological variations observed among the six studied accessions of *N. septemcrenata*

Location code	Location	Mean Branch No.	Mean No of Leaf/branch	Mean Internode Lenghth (cm)	Leaf length (cm)	Leaf Width (cm)	Leaf Area (cm2)	Plant Width (cm)	Plant Height (cm)	Plant Size Index (cm)	Shape index of leaf (cm)
1	Shak Mosa	40	10	5	1.367	0.833	0.895	64	64	64	1.64
2	Shak Abo Hamman	68	20	2.667	1.547	1.02	1.24	47.667	37.292	42.479	1.516
3	Wadi Elrotk	80	22	4.9	2.8	1.833	4.033	59.741	51.667	55.704	1.527
4	Farsh Eloza	25	12	5.533	1.625	1.268	1.619	53.6	50.333	51.967	1.282
5	Elgabal Elahmar	300	12	7	1.267	1.033	1.028	77.6	65	71.3	1.226
6	Wadi Elfaraa	56	14	2.5	1.167	0.767	0.703	24	35	29.5	1.522

Table 5. Variation of eco-geographical variables among the six studied locations

Eco-geographical variables	1	2	3	4	5	6
Topography						
Altitude	1930	1890	1740	2036	2181	1850
Aspect	North west	North east	North	North west	South west	West
Climatic Variables						
Annual minimum temp.	8.83	9.93	8.45	10.25	8.09	8.83
Annual maximum temp.	20.13	21.19	19.82	21.47	19.46	20.13
Precipitation	7.92	5.67	8.83	5.33	9.25	7.92
Edaphic Variables						
Micro-Habitat	Gorge	Gorge	Wadi bed	Wadi bed	Gorge	Wadi bed
Soil texture	loamy sand	loamy sand	loamy sand	sandy	sandy	sandy loamy
water content%	0.45	0.45	4.80	0.45	0.78	0.08
PH	7.10	8.40	8.70	8.40	8.40	8.30
T.D.S PPm	238.00	59.00	110.00	62.00	99.00	73.00
EC µs/ cm	395.00	272.00	191.00	128.00	166.00	132.00
Org.matter%	3.39	0.57	3.28	2.26	3.28	0.23
CaCO3%	14.50	17.50	13.50	15.50	16.00	15.00
Ca++meq/L	9.00	2.00	4.00	2.00	2.50	2.50
Mg++ meq/L	6.50	1.00	2.00	1.50	1.00	1.00
Na+ PPM	38.52	43.90	17.49	15.35	16.28	32.90
K+PPM	38.52	49.52	17.49	15.35	16.28	39.89
HCO3- meq/L	11.50	11.00	11.00	6.00	10.00	9.50
Cl-meq/L	5.75	5.00	4.25	3.00	4.00	3.75
SO4-- meq/l	42.00	38.00	28.50	23.00	23.00	23.50

It was observed that there's a great variation in morphological aspects among different locations this variation at phenotype may result from change in environmental factors which may be lead to change in genotype of *N. septemcrenata*. Table 5 Show that there's no difference at climatic variables between location 1 & 6, so we eliminate this variable from

explanation of the genetic variability between these two locations while edaphic factors show great variation between these locations that may be the reason for this genetic variation.

Differences in polymorphism among restricted species may be related to the extent to which their locations occur in heterogeneous habitats in agreement with (Babbel and Selander 1974; Van Valen 1965). Several studies on this subject agree that the best approach to sampling to assume an association of genetic diversity with diversity in ecogeographical patterns (Antonovics, 1971; Ferguson *et al.*, 1998; Loveless and Hamrick, 1984; Moeller and Schaal, 1999). Linhart and Grant (1996) conclude that the experimental evidence overwhelmingly supports the generalization that natural selection tailors the genetics of plant populations to their environment. Variability detected among locations is essential for their ability to survive and successfully respond to environmental changes and this agrees with (Ryman *et al.*, 1995).

In our study locations which are close to each other tend to be uniform because genetic differentiation is often prevented by gene flow and this recorded by (Fahima *et al.*, 1999; Gallois *et al.*, 1998) and Loveless and Hamrick (1984). The genetic variability between Shak Mosa and Wadi Elfaraa may be caused by the great variation in water content between two locations and this agrees with (Crater, Arizona. Mitton *et al*., 1998). A significant association of genetic diversity with eco-geographical variables was detected in *N.septemcrenata* locations the same results recorded by (**Del Rio, 2001).** The highest genetic distance recorded within study was 0.38 and this are small, we can explain this as species with restricted distributions maintain less **genetic** diversity than more widespread species in agreement with (Hamrick and Godt 1989; Frankel and Soulé 1981; Ledig 1986 and Soulé 1980). Drury, (1974) noted that although many researchers predicted low levels of **genetic** polymorphism in plant species with limited ranges and small numbers of individuals, few had measured **genetic variation** in such taxa. The two factors thought to be responsible for the deletion of **genetic variation** are: (1) change in allelic frequencies due to **genetic** drift, which may lead to fixation (Carson 1983; Nei *et al*. 1975), and (2) strongly directional selection toward **genetic** uniformity in a limited array of environments (Van Valen, 1965). Loss of **genetic variation** due to these factors is more likely to occur in geographically restricted species, with few individuals, than in widespread species, with many individuals (Babbel and Selander 1974; Frankel and Soulé 1981).

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Corresponding Author:
Mr. Kariem Abd Elhay Omar
Environmental resercher
Saint Katherine Protectorate, the Nature Conservation Sector (NCS), Egyptian Environmental Affairs Agency(EEAA) Egypt.
E-mail: kariem_npe@yahoo.com

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