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Allozymic and Morphologic Differentiation between three Ballota Species (Labiatae) growing in Southern Sinai, Egypt

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ABSTRACT

Ballota undulate (rare), *B. Saxatilis* (endangered), and *B. Kaiseri* (extremely endangered and endemic) are three of Mediterranean origin species grow in southern Sinai Mountains. They are very close morphologically and have been confused and mixed in their natural habitats. The present study aimed to provide genetical and morphological differentiation among the three *Ballota*s peceis using allozyme, PCA, and discriminant analyses for plants growing in a common environment. Electroporetic analysis of enzymes coded by 21 gene loci demonstrated that there is a high degree of genetic similarity among the three *Ballota* species suggesting that speciation apparently has occurred in this genus with little divergence at genes coding for allozymes. Twelve quantitative and nine qualitative characters were selected for the study of phenotypic similarity and morphological variation in a sample of 147 plants, served as OUT's. The analyses could differentiate between *B. undulata* as one group and *B. kaiseri* and *B. saxatilis* as another group, but could not separate *B. saxatilis* from *B. kaiseri*. The incomplete isolation of *B. undulata*, *B. kaiseri*, and *B. saxatilis*, in combination with their high genetic identities and their very similar morphology, raise the question as to whether they should be retained as separate species.
Key words: Allozymic divergence, *Ballota*, PCA, discriminant analysis, Morphologic differentiation, Sinai.

INTRODUCTION

Sinai's unique location of being the meeting place of Asia, Africa, and Europe, is reflected in its unique flora that combines elements from the three continents, while its main elements are Saharo-Arabian, Irano-Turanian, Mediterranean, and Sudanian. Labiatae (Lamiaceae) which is a family typically representative of Mediterranean and Irano-Turanian regions is one of the four dominant families (Compositae, Labiatae, Leguminosae, and Cruciferae) in Southern Sinai flora. Genus *Ballota* consists of about 33 species and belongs to Labiatae. Some of *Ballota* species including *B. undulata* and *B. saxatilis* are well-known in folk medicine in Egypt, Turkey and Europe (Svensson and Wigren, 1984; Meriçli *et al*., 1988; Çitoglu *et al*., 1998; Wichtl and Anton, 1999; Bader *et al*., 2003).

In Egypt, five *Ballota* species were recorded by El-Husseiny (1989) who provided a key for morphological differentiation. Four of them; *B. damascene* Boiss., *B. Undulate* (Sieb. Ex Fres.) Benth, *B. Saxatilis* (Sieb. Ex J. et C. Presl), and *B. Kaiseri* Täckh. were recorded from the southern Sinai. However in the last twenty years just three (*B. undulata*, *B. saxatilis*, and *B. kaiseri*) were recorded in the floristic and ecological surveys of the area. Also, during these surveys there were often confusing individuals which were difficult to be identified to the species level.

Numerical taxonomy and allozyme analyses are powerful tools for elucidating systematic relationships, discriminating morphologically cryptic taxa, as well as confirming the taxonomic status of taxa (e.g. Soltis, 1985; Bruederle and Fairbrothers, 1986; Ford and Ball, 1989; Arntzen and Olgun, 2000).

The present study aimed to provide (1) genetic (allozyme) differentiation among populations of *Ballota undulata*, *B. kaiseri*, and *B. saxatilis* utilizing starch gel electropohoresis, and to determine whether allozyme data could clarify relationships among these species within the *Ballota* complex, and (2) morphological differentiation between the three *Ballota* species from plants growing in a common environment.

MATERIALS AND METHODS

Study species

Ballota plants are perennial or under shrub and obtusely tetragonal. Leaves are crenate to dentate and petiolate. Flowers are in axillary whorls. Bracts are longer or shorter than whorls. Bracteoles are many and linear. Verticillasters are many-flowered. Calyx is funnel-shaped, 10-veined and sulcate with a dilated spreading limb; 10 or more dentate or irregularly crenate or dentate, and lobes sometimes mucronate. Corolla is bi-labiates; upper lip erect, more or less concave, oblong-ovate, retuse, densely hairy; lower lip spreading 3-lobed, middle lobe broad, retuse; tube included or slightly exerted, annulate hairy inside. Stamens are 4, didynamous, ascending under the upper lip; filaments papillose; anther-cells diverging. Style is exerted; stigma bifid, branches equal and subulate. Nutlets are obovoid and rounded at the tip.

The study targeted three *Ballota* species: *B. undulata*, *B. saxatilis*, and *B. kaiseri* grow in southern Sinai Mountains. *Ballota kaiseri* is an extremely endangered and endemic species that is restricted to crevices in outcrops of smooth-faced granite, to elongated gaps, -

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and to narrow ravines in such rocky terrain (Danin, 1986). *B. saxatilis* is an endangered species that has a discontinuous distribution and may be recorded as a relict of Mediterranean origin (Danin, 1986). *B. undulata* (rare) is also Mediterranean but has a more continuous distribution in a wider range of relatively wet microhabitats (Danin, 1986).

The distribution of the three *Ballota* is affected positively by elevation. While *B. undulata* prefer lower pH values (-ve relation), *B. kaiseri* and *B. saxatilis* share the same microhabitats favoring high soil clay and silt percent and organic matter content and consequently low sand percent (-ve relation) which means a low pH also and high soil moisture content (Zaghloul, 2003).

Population sampling, seed collection, and Germination

Open-pollinated seeds were collected from plants at seventeen sites in St. Catherine area of south central Sinai, representing sixteen populations of *B. undulata*, seven *B. kaiseri*, five *B. saxatilis*, and one confusing *Ballota* plant (Fig. 1).

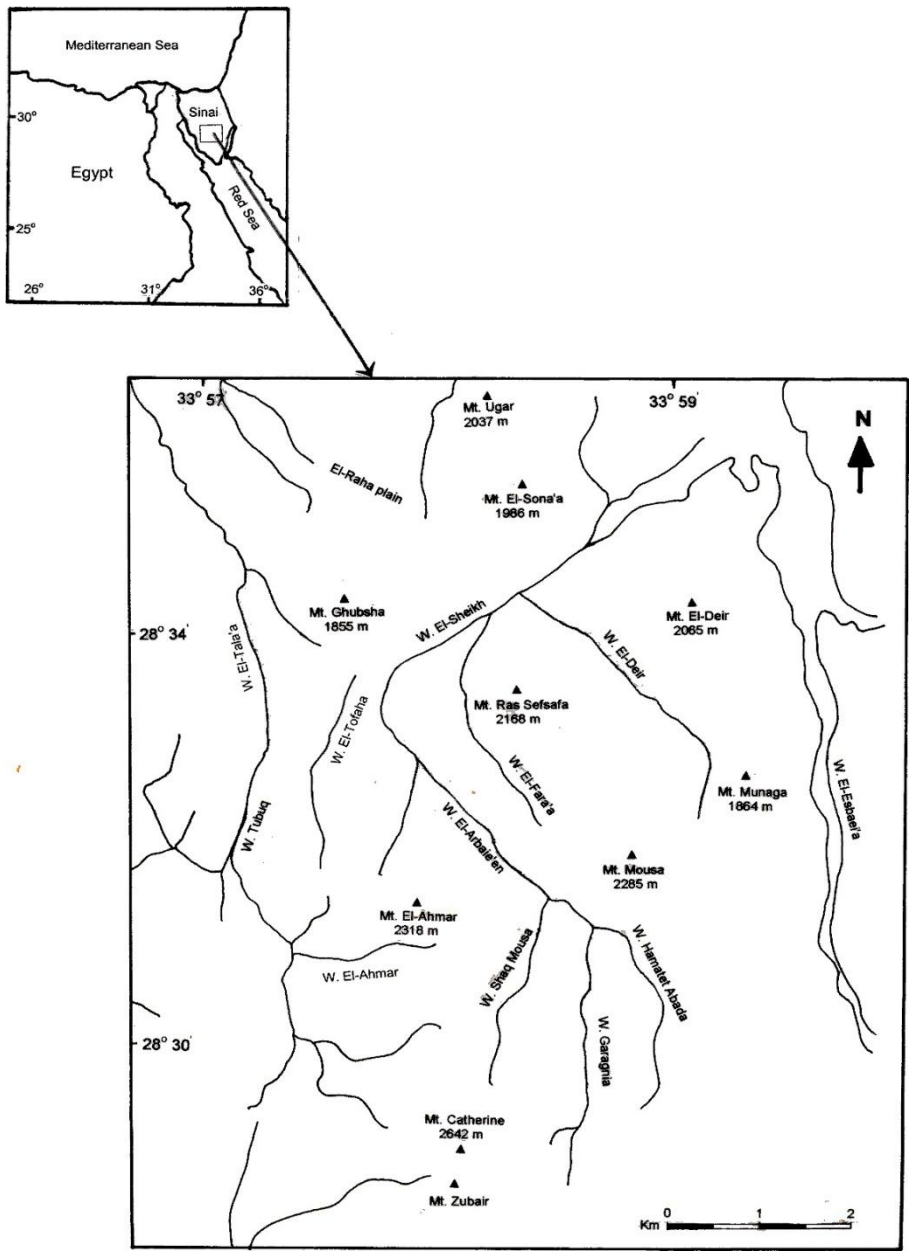


Figure (1): Locations of sampled *Ballota* populations. Sites beginning with a U indicate *B. undulata*, with a K = *B. kaiseri* and S = *B. saxatilis*.

As *Ballota* populations are generally small and sometimes very small (just few individuals), a complete sample of all observed seeding individuals was obtained from each population. Seeds were maintained in dry conditions until they had subsequently been germinated in growth chamber and grown in greenhouse. Germination procedure is discussed in details in Zaghloul *et al.* (2006). Because they routinely provide the clearest banding patterns, young whole seedling plants (5-10 cm) were used for electrophoretic analysis. Others were left to grow until flowering to measure the morphological parameters serving as Operational Taxonomic Units (OTUs).

Allozyme divergence

The whole plants were crushed with mortar and pestle using the extraction buffer of Wendel and Parks (1982). Following extraction, sample wicks onto which the extracts had been absorbed were frozen at -70^{o}C until used for electrophoresis. Twelve enzyme systems were scored using five gel and electrode buffer systems: system 4 of Soltis *et al*. (1983) for Aco; system 11 of Soltis *et al*. (1983) for Idh; a tris-citrate (34:40 Poulik, a modification of Soltis *et al*., 1983) system (Brown and Allard, 1970) for Acp, Pgi, and Pgm; system 8- (a modification of system 8 of Soltis *et al*., 1983) for Fe, Tpi, Mnr, Aat, and Dia; and morphiline citrate (Clayton and Tretiak 1972) system for Mdh, and 6-Pgd. Stain recipes are from Soltis *et al*. (1983) with the exception of diaphorase (Dia) and menadione reductase (Mnr) which come from Cheliak and Pitel (1984).

Populations belonging to each of the three species were pooled to examine genetic diversity within the *Ballota* complex and were subjected to analysis using the (LYNSPROG) computer program developed by M. D. Loveless and A. Schnabel. The following genetic diversity statistics were obtained: allele frequencies, number of alleles per polymorphic locus (A_p), proportion of polymorphic loci (*P*), observed heterozygosity (H_o), Nei's genetic identity (1972), and the gene diversity statistics (H_T, H_S, and G_{ST}) of Nei (1973). A matrix of genetic distances was used to generate a phenogram by the unweighted pair-group method (UPGMA; Sneath and Sokal, 1973) using POPGENE program version 1.31 program (Yeh *et al*., 1999) and Nei's (1978) unbiased genetic distances to alleviate any bias by small sample sizes.

Morphologic differentiation

A sample of 147 plants (128 *Ballota undulata*, 6 *B. kaiseri*, and 13 *B. saxatilis*) where randomly chosen from F_1 offspring of the sampled populations to serve as OUT's. They were examined for phenotypic similarity and morphological variation using PCA and statistical methods.

The chosen plants encompassed the geographical and morphological range of each *Ballota* species. The number of both *B. kaiseri* and *B. saxatilis* were very low comparable to *B. undulata* but represent the actual ratio of the three species in the field and in collected and germinated sampls.

Twelve quantitative and nine qualitative characters were selected for morphological study among all OTUs in *Ballota*. The twenty one vegetative, flower, and fruit characters chosen were reported to be variable among taxa or had been used in previous taxonomic treatments (Table 1). A minimum of 5 measurements was obtained to calculate the mean for each quantitative character for OTUs, except in few cases when living plant material was limited. Means and ranges of the quantitative characters are given in Table (1).

Table (1): Lineament density (L/A) ratio and *Acacia tortilis* frequency of the different hydrographic basins at Wadi Feiran area.

Basin	Area (A) Km^2	No. of Lineaments (L)	Lineament Density L/A (km^{-2})	Acacia *tortilis* Frequency
WadiFeiran	284.72	328	1.15	227
WadiNysrien	211.03	232	1.09	26
Wadi Ager	183.47	93	0.51	36
Total		653		289

Principal Components Analysis (PCA) was used to construct two dimensional models of morphological variation. A correlation matrix of characters was constructed to calculate eigenvectors for the first three components. One-way analysis of variance (ANOVA) was used to explore differences in group means in the ten quantitative characters.

The ANOVA procedure depends on data being at least a reasonably close approximation to a normal distribution. The Anderson-Darling test was used to allow the possible conclusion that the departures from normality are detectable or not. Bartlett's and Levene's tests were used to test for variance homogeneity. Kruskal-Wallis may come to the rescue, as an alternative to ANOVA, with data sets that failed that test for homogeneity of variance.

Teukey's was used to compare all the groups (species) with each other and indicates which pair of species is different using the significant characters resulted from ANOVA and Kruskal-Wallis tests. The resulted seven significant quantitative characters were subjected to discriminant analysis using MINITAB release 13 computer program to determine which combination of characters provided maximal discrimination between species.

In discriminant analysis we fit a linear equation of the type:

$$\text{Group} = a + b_1*x_1 + b_2*x_2 + \ldots + b_m*x_m$$

where *a* is a constant and b_1 through b_m are regression coefficients.

Those variables with the largest (standardized) regression coefficients are the ones that contribute most to the discrimination between groups (species).

RESULTS

Genetic diversity

Twenty polymorphic loci (Aco-1, Aco-2, Idh-1, Idh-2, Fe-1, Tpi-1, Tpi-2, Dia-1, Mnr-1, Aat-1, Aat-2, Pgm-2, Pgm-3, Pgi-1, Pgi-2, Acp-1, Mdh-1, Mdh-2, 6Pgd-1, and 6Pgd-2) and one monomorphic locus (Pgm-1) were scored and used in the allozyme analysis. The percentage of polymorphic loci was 95.2% for the *Ballota* complex and there were 3.7 alleles per polymorphic locus. The expected heterozygosity (H_e) was 0.266. Little genetic differentiation was found between different *Ballota* species. The proportion of total genetic diversity found between species (G_{ST}) was 6.97%. Values for the proportion of polymorphic loci (*P*), mean expected heterozygosity (H_e), and mean number of alleles per polymorphic locus (*AP*) in the three species were 95.2, 0.195, and 3.5 for *B. undulate*; 95.2, 0.297, and 2.75 for *B. kaiseri*; and 90.5, 0.317, and 2.89 for *B. saxatilis*.

No alleles were found that consistently differentiates the three species (Table 2). Twelve alleles were exclusively found in one of the three taxa; eleven in *B. undulata* (Idh-1,4; Idh-2,1; Tpi-1,4; Tpi-2,1; Tpi-2,4; Dia-1,3; Aat-1,3; Aat-2.1; Pgi-2,1; Mdh-2,1; 6-Pgd-1,3; and 6-Pgd-1,4), and one in *B. saxatilis* (Aat-2,4), but their frequencies tended to be very low (mean = 0.0058). Despite the lack of diagnostic markers, the greatest discrimination of the taxa was provided by the striking differences in allele frequencies occurred at six loci (Fe-1, Dia-1, Aat-1, Aat-2, Pgm-2, and Mdh-1) where one of the rare alleles in *B. undulata* populations became the common one in *B. saxatilis*. *B. kaiseri* had intermediate allele frequency at all these loci and for other six loci (Aco-1, Aco-2, Pgm-3, Pgi-2, Acp-1, and 6Pgd-2) that had less extreme differences in allele frequencies for *B. undulata* and *B. Saxatilis* (Table 2). Also, the common allele at Tpi-2 in populations of both *B. undulata* and *B. kaiseri* has become fixed in all *B. saxatilis* populations. In all other loci, the three species share the same common allele with relatively similar frequency (Table 5).

Genetic identity and distance measures

Nei's (1972) measures of genetic distance (*D*) and identity (*I*) were computed and presented for interspecific and intraspecific pair wise comparisons (Table 3). Genetic identity between the three taxa ranged from 0.879 (between *B. saxatilis* and *B. undulata*), 0.954 (between *B. saxatilis* and *B. kaiseri*), to 0.974 (between *B. kaiseri* and *B. undulata*). The average identity of pair wise comparison for each species was 0.944 for *B. undulate*, 0.967 for *B. kaiseri*, and 0.91 for *B. saxatilis*. These values are higher than the congeneric means and within the range commonly found for conspecific populations (Crawford, 1983). The intraspecific genetic idendity ranged from 0.925 to 0.997 with a mean of 0.977 for *B. undulata*, from 0.744 to 0.972 with a mean of 0.903 for *B. kaiseri*, and from 0.717 to 0.972 with a mean of 0.879 for *B. saxatilis*. The confused family was revealed to be *B. undulata* as its average genetic identity and distance were 0.95 and 0.052, respectively, almost the same as *B. undulata*. This closeness was reflected in the UPMGA phenogram (Fig. 2). UPGMA distance phenograms derived from Nei's (1978) unbiased genetic distances of pair wise comparisons of the three taxa is shown in Figs. 2 and 3. Due to the low genetic divergence between species, populations of the three taxa did not fall into three distinct groups as expected for three species. Also, populations of *B. kaiseri* and *B. saxatilis* at Kahf El-Ghola (KKaGh and SKaGh) were grouped together with *B. saxatilis* population at Ramadan Garden in W. El-Arbaie'en (SArRa) forming the most distinct group.

Table (2): Summary table for the overall mean ± SE for altitude and slope at different study localities in south Sinai. ANOVA (*F* ratio and its significant) was calculated for each variable between 13 localities.

ID	Locality	Altitude (m)		Drainage gradient (m/km)	
		Mean	± SE	Mean	± SE
1	**Nysrien**	389.00	11.65	5.48	.622
2	**Rumana**	480.43	3.88	3.53	.297
3	**Feiran**	630.50	20.97	5.01	.534
4	**Qusier**	559.60	15.43	9.78	.551
5	**El-Tar**	524.00	1.00	11.25	1.25
6	**Surief**	533.00	1.00	16.05	.350
7	**Nefuz**	615.80	8.91	7.40	.366
8	**Agala**	723.00	17.97	17.38	.745
9	**Alyat**	665.67	24.92	8.28	.370
10	**Nakhla**	763.00	1.00	24.55	1.15
11	**Gohaier**	908.00	1.00	20.00	1.50
12	**Sebah**	780.00	2.08	5.80	.520
13	**Akhbar**	793.67	3.76	3.17	.437
	Total			8.48	.744
	F	46.836		84.250	
	P	<.0001		<.0001	

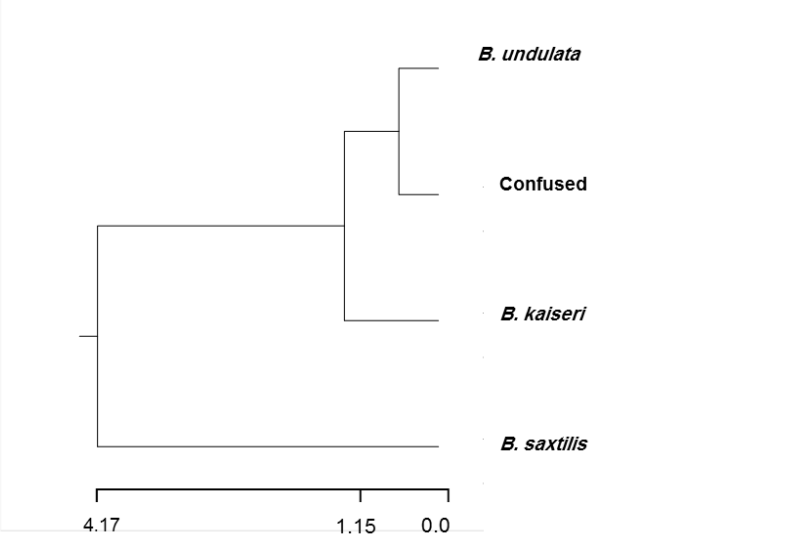


Figure (2): Distance phenogram (UPGMA) derived from Nei's genetic distances of all pair wise comparisons of three taxa of *Ballota* as well as the confused one generated using POPGENE.

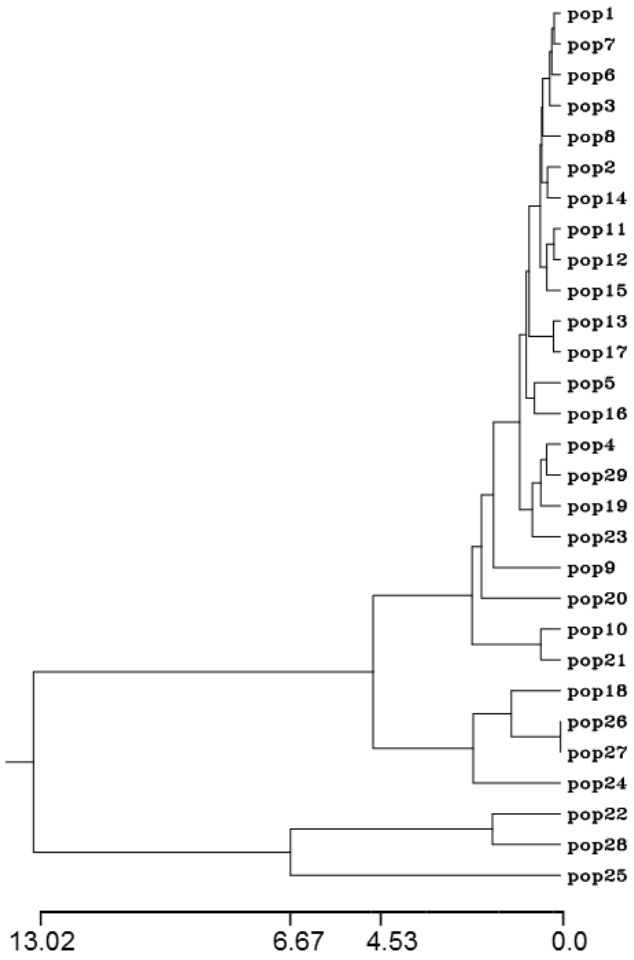


Figure (3): Dendrogram of 29 populations using unbiased Nei's genetic distance.

Table (3): Drainage gradient (slope), and catchment's area of the hydrographic sub-basins in the 13 localities.

Hydrographic major basins	Hydrographic sub-basins	Slope (m/km)	Catchment area (Km^2)
W. Nysrien	Nysrien	5.48	35.25
W. El-Ager	Rumana	3.53	29.7
W. Feiran Down stream Major basin	Feiran	5.01	27.31
	Qusier	9.78	7.26
	El-Tar	11.25	4.89
	Surief	16.05	2.99
	Nefuz	7.40	10.71
	Agala	17.38	14.66
	Alyat	8.28	16.02
	Nakhla	24.55	2.94
	Gohaier	20.00	3.55
	Sebah	5.80	5.92
	Akhbar	3.17	5.75

Morphologic differentiation

Results of ANOVA analysis showed highly significant variation (P = 0.000) among the three *Ballota* species for the quantitative characters 6, 14, 15, 16, 17, and 20 (Fig. 3); and significant variation for character 5 (P = 0.047). Anderson-Darling's test detected departures from normal distribution in *B. undulata* OTU's for the quantitative characters 2, 15, 17, and 20. Bartlett's and Levene's tests for variance homogeneity detected highly significant variance non-homogeneity in only one quantitative character, 5. ANOVA results for the quantitative characters 2, 5, 15, 17, and 20 were invalidated as they are violating the assumption of normality and/or variance homogeneity. Kruskal-Wallis's test showed highly significant variation (P = 0.000, the same as ANOVA) among the three *Ballota* species for three of these quantitative characters 15, 17, and 20, while it resulted in non-significant variation in characters 2 and 5 (Table 4).

Tukey's test was applied for significant quantitative characters 5, 6, 14, 15, 16, 17, and 20 to determine out which group (species) differs from which. A nonparametric (Steel-Dwass) test should be applied instead of Tukey's test for ANOVA-invalidated characters but out of these characters, two (2 and 5) have non-significant variation according to Kruskal-Wallis's test and the other three characters (15, 17, and 20) have very highly significant variation (p = 0.000) in both ANOVA and Kruskal-Wallis's test. As a result it was not necessity to apply Steel-Dwass's nonparametric test. The Tukey's pairwise comparisons revealed that in five characters (14, 15, 16, 17, and 20) out of the seven, *B. undulata* significantly differs from both *B. kaiseri* and *B. saxatilis*, and in one character (6) *B. undulata* significantly differs from *B. saxatilis* only. No difference in any of the seven chosen quantitative characters was detected between *B. kaiseri*, and *B. saxatilis*.

Although ANOVA detected a significant variation of quantitative character 5 between *Ballota* species, Tukey's test resulted in no difference between any two of the three species.

The most useful quantitative characters (from the seven significant ones) for differentiating among groups (species) using discriminant analysis (Tables 6 and 7) were: leaf width: length ratio (17), leaf clasping ratio (20), leaf length (15), anther length (14), calyx limb width (6), and calyx tube length (5). Leaf width (16) was the least differentiating character.

Using these characters, three linear discriminant functions were produced:

B. undulata = -679.04 + 9.83 * calyx tube length + 8.2 * calyx limb width + 13.58 * anther length + 42.78 * leaf length – 34.1 * leaf width + 793.4 * leaf width:length ratio + 62.92 * leaf clasping ratio.

B. kaiseri = -725.9 + 10.85 * calyx tube length + 7.28 * calyx limb width + 14.77 * anther length + 43.76 * leaf length – 34.55 * leaf width + 806.72 * leaf width:length ratio + 74.6 * leaf clasping ratio.

B. saxatilis = -709.67 + 9.71 * calyx tube length + 6.35 * calyx limb width + 14.42 * anther length + 44.61 * leaf length – 35.21 * leaf width + 811.31 * leaf width:length ratio + 63.78 * leaf clasping ratio.

Table (4): Pearson correlation test between altitude, slope, catchment area, and nature of soil surface in the study area. South Sinai (N = 57).

		Altitude	Slope	Catchment area	Nature of soil surface %				
					Fine fraction	Gravel	Cobble	Stone	Boulders
Altitude		1							
Slope		.404(**)	1						
Catchment area		-.627(*)	-.632(*)	1					
Nature of soil surface %	**Fine fraction**	-.407	-.551	.031	1				
	Gravel	-.582(*)	-.494	.155	.503	1			
	Cobble	-.209	.189	.275	-.575(*)	-.082	1		
	Stone	.540	.589(*)	-.102	-.807(**)	-.861(**)	.296	1	
	Boulders	.613(*)	.615(*)	-.156	-.897(**)	-.799(**)	.313	.912(**)	1

** Correlation is significant at the 0.01 level (2-tailed), * Correlation is significant at the 0.05 level (2-tailed).

Table (5): Summary table for the overall mean and standard deviation for vegetation parameters of *Acacia* trees at different study localities in south Sinai. ANOVA (*F* ratio and its significant) was calculated for each variable between 13 localities.

ID	Locality	N	Height (m)		Crown cover (m^2)		Trunk circumference (m)				Vitality	
							(CAG)		(CBH)			
			Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
1	**Nysrien**	26	8.923	2.200	9.854	2.493	1.655	0.461	1.503	0.455	2.000	0.938
2	**Rumana**	36	10.681	2.361	10.992	2.158	1.577	0.325	1.422	0.329	1.944	0.860
3	**Feiran**	38	12.674	3.481	13.084	3.261	2.667	0.688	2.520	0.670	3.447	0.602
4	**Qusier**	25	5.668	1.014	6.113	1.059	0.706	0.118	0.608	0.113	0.880	0.726
5	**El-Tar**	8	7.225	1.594	7.785	2.422	1.0463	0.197	0.931	0.199	1.500	1.069
6	**Surief**	9	6.233	1.537	6.792	1.914	0.836	0.199	0.740	0.206	1.444	0.726
7	**Nefuz**	23	7.896	1.799	6.580	1.481	1.261	0.404	1.137	0.393	1.652	0.775
8	**Agala**	23	9.161	2.614	7.374	1.913	1.420	0.532	1.277	0.519	2.391	0.656
9	**Alyat**	43	9.133	1.857	8.726	1.964	1.853	0.475	1.712	0.451	2.651	0.783
10	**Nakhla**	17	9.594	2.203	8.843	2.542	1.835	0.598	1.672	0.577	1.765	0.831
11	**Gohaier**	12	11.125	2.631	11.019	2.521	2.135	0.374	2.135	0.359	3.000	0.739
12	**Sebah**	18	9.311	1.948	8.670	1.848	1.740	0.523	1.601	0.512	1.389	0.979
13	**Akhbar**	11	8.318	1.549	7.971	1.362	1.660	0.342	1.493	0.330	1.455	0.820
	Total	289	9.323	2.939	9.162	3.041	1.676	0.704	1.533	0.683	2.125	1.076
	F		16.876		22.384		31.805		31.579		20.916	
	P		<.0001		<.0001		<.0001		<.0001		<.0001	

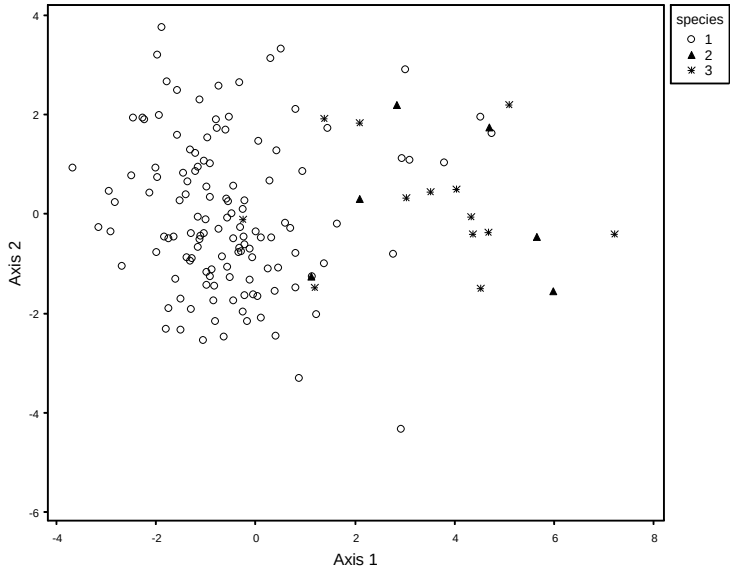
Table (6): Pearson correlation test of altitude, slope, and catchment area and vegetation parameters of *Acacia tortilis* including tree height, crown cover, trunk circumference at ground level (CAG) and at breast height (CBH), and vitality at different study localities, South Sinai.

	Altitude	Slope	Catchment area	*Acacia* height	Crown cover	CAG	CBH	Vitality
Altitude	1							
Slope	.404(**)	1						
Catchment area	-.627(*)	-.632(*)	1					
***Acacia* tree height**	.257	-.183	.464	1				
Crown cover	.002	-.307	.566(*)	.918(**)	1			
DAG	.357	-.209	.362	.938(**)	.866(**)	1		
DBH	.384	-.173	.329	.937(**)	.869(**)	.997(**)	1	
Vitality	.213	-.035	.416	.834(**)	.772(**)	.822(**)	.839(**)	1

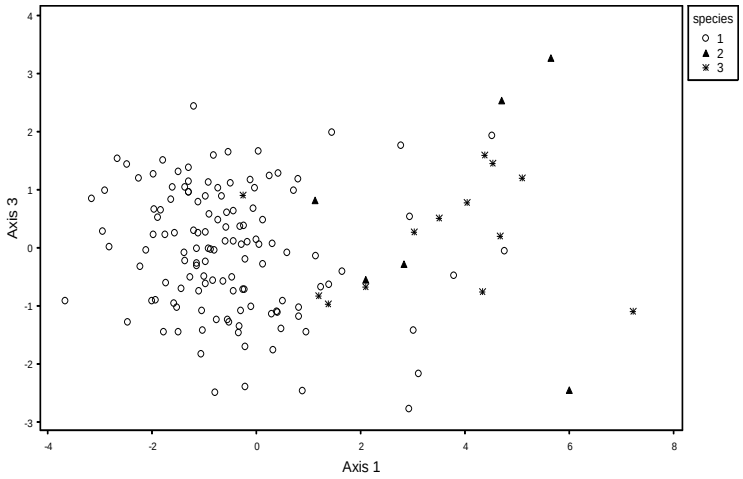
** Correlation is significant at the 0.01 level (2-tailed), * Correlation is significant at the 0.05 level (2-tailed).

Table (7): Pearson correlation test of grazing intensity, urbanization and vegetation parameters of *Acacia tortilis* including tree height, crown cover, trunk circumference at ground level (CAG) and at breast height (CBH), and vitality at different study localities, South Sinai.

	Grazing intensity	Urban-ization	*Acacia* height	Crown cover	CAG	CBH	Vitality
Grazing intensity	1						
Urbanization	.672(**)	1					
Acacia height	-.473	-.391	1				
Crown cover	-.517	-.517	.918(**)	1			
CAG	-.311	-.191	.938(**)	.866(**)	1		
CBH	-.347	-.220	.937(**)	.869(**)	.997(**)	1	
Vitality	-.570(*)	-.422	.834(**)	.772(**)	.822(**)	.839(**)	1



Figure(4): Principal components analysis (axis 1 and 2) based on the correlation matrix of 21 morphologic characters for individuals of *B. undulata*(1), *B. kaiseri*(2), and *B. saxatilis*(3).



Figure(5): Principal components analysis (axis 1 and 3) based on the correlation matrix of 21 morphologic characters for individuals of *B. undulata*(1), *B. kaiseri*(2), and *B. saxatilis*(3).

A plot of the first three principal components derived from the morphologic data matrix is presented in Fig. 4 and 5. The first three components (axes) explain 63.4% of the total variance; 34.5%, 18.5%, and 10.4%, respectively. Examination of the character coefficients for the first principal component revealed that characters 6, 10, 11, 14, 15, 16, 17, and 20 were primarily responsible for the component scores. Characters 2, 2, and 3 were most heavily weighted in principal component 2, while Character 5 was most heavily weighted in principal component 3. Individuals of *B. undulata* are separated from *B. kaiseri*, and *B. Saxatilis* along axis 1. Individuals of *B. kaiseri* and *B. saxatilis* could not be separated along any of the first three axes. *B. undulata* usually lacks calyx teeth (characters 10 and 11); have wide calyx limb (character 6), shorter anthers (character 14), smaller leaves with width more than length (characters 15, 16, and 17) and hence lower leaf clasping ratio (character 20); which partially explains its separation from the other two species along component 1.

DISCUSSION

Ballota undulata, *B. kaiseri*, and *B. saxatilis* are three species that are very close morphologically, beenconfu--sed, and/or mixed in their natural habitats as well as herbarium specimens. El-Husseiny (1989) provided a key to distinguish between different species of *Ballota* in the Egyptian flora, in which *B. undulata* has been distinguished from *B. kaiseri* and *B. saxatilis* by having multicrenate to dentate calyx limb (character 7a), and *B. kaiseri* has been separated from *B. saxatilis* by having bracts shorter than verticellasters and obtuse calyx teeth (character 9b) while *B. saxatilis* has bracts longer than verticillasters and triangular-mucronate teeth (character 9a). Although the character of having bracts shorter or longer than verticillasters combined with leaf characters (e.g. characters 18, 19, and 21) allow for the differentiation of *B. kaiseri* and *B. saxatilis* in natura l habitats, they were completely unreliable for plants grown in the greenhouse (common garden). The large range of variation in floral characters (e.g. flower position, 4; calyx tube length, 5; calyx limb width, 6; calyx limb shape, 7; and corolla color, 12) within each species specially *B. undulata* makes the situation even worse, and it becomes more and more difficult when we consider that the three species occur in almost the same natural microhabitats (Zaghloul, 2003), flower simultaneously in late spring, and are subjected to the same stresses, i.e. they are probably incompletely isolated in nature. This situation was the motivation for the current study.

Electrophoretic analyses of enzymes coded by 21 gene loci demonstrated that there is a high degree of allozymic similarity among *Ballota undulata*, *B. kaiseri*, and *B. saxatilis*. The lowest genetic identity value for any two populations compared in this study was 0.712. The mean genetic identity value for comparison of 0.914 for all populations of the three species investigated is similar to genetic identity values calculated among populations of individual species in other plant groups (Gottlieb, 1981; Crawford, 1983). However, the genetic identity values for *Ballota* species are similar to results observed in several genera of annuals including *Gaura* (Gottlieb and Pilz, 1976), *Clarkia* (Gottlieb, 1974), *Coreopsis* (Crawford and Smith, 1982), *Lycopersicon* (Rick *et al*., 1976), and *Stephanomeria* (Gottlieb, 1973), and some perennials like *Heuchera* (Soltis, 1985), and *Sullivantia* (Soltis, 1982).

Allozyme data for *Ballota* suggest that speciation apparently has occurred in this genus with little divergence at genes coding for allozymes. The three *Ballota* species do not have any fixed differences at genes coding enzymes, and exclusively all of the unshared alleles are of very low frequency. The trend of change of allele frequencies at Fe-1, Dia-1, Aat-1, Aat-2, Pgm-2, and Mdh-1 loci where one of the rare alleles in *B. undulata* become the common one in *B. saxatilis* with intermediate values for both alleles in *B. kaiseri*, supports the conclusion that *B. kaiseri* being originated from hybridization between *B. undulata* and *B. saxatilis* (Danin, 1986).

PCA, statistical, and discriminant analyses, based on morphological characters differentiated only between *B. undulata* and both *B. kaiseri* and *B. saxatilis*, but could not separate *B. saxatilis* from *B. kaiseri*. The incomplete isolation of *B. undulata*, *B. kaiseri*, and *B. saxatilis*, in combination with their high genetic identities and their very similar morphology, raise the question as to whether they should be retained as separate species.

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Appendix (1): List of plant species recordred in the study area. Identification and nomenclature are according to Boulos (1999, 2000, 2002, and 2005).

Family	Plant species	Life form
Amaranthaceae	*Aerva javanica* (Burm. F.) Juss. ex. Schult.	Sub-shrub
Anacardiaceae	*Pistacia khinjuk* Stocks var. *glaberrima* Schweinf. ex Boiss. L.	Tree
Asclipeiadaceae	*Calotropis procera* (Aiton) W. T. Aiton	Shrub
Boraginaceae	*Alkanna orientalis* (L.) Boiss.	Per. Herb
	Heliotropium aegyptiacum Lehm.	Per. Herb
Capparaceae	*Capparis sinaica* Veill.	Shrub
	Capparis spinosa L.	Shrub
Caryophylaceae	*Silene leucophylla* Boiss.	Per. Herb
Chenopodiaceae	*Anabasis articulate* (Forssk.) Moq.	Shrub
Cleomaceae	*Cleome dorserifolia* (Forssk.) Delile	Per. Herb
Compositae	*Achillea fragrantissima* (Forssk.) Sch. Bip.	Sub-shrub
	Artemisia judaica L.	Sub-shrub
	Artemisia herba-alba Asso.	Sub-shrub
	Centaurea aegyptiaca L.	Per. herb
	Echinops spinosus L.	Per. herb
	Iphiona scabra DC.	Per. herb
	Pulicaria crispa (Forssk.) Oliv.	Sub-shrub
Cruciferae	*Diplotaxis harra* (Forssk.) Boiss.	Per. herb
	Launaea angustifolia (Desf.) Kuntze	Per. herb
	Launaea spinosa (Forssk.) Sch. Bip. ex Kuntze	Per. herb
	Zilla spinosa (L.) Prantl	Per. herb
Cucurbitaceae	*Citrullus colocynthis* (L.) Schrad.	Per. herb
Dipsacaceae	*Pterocephalus sanctus* Decne.	Ann.
Ephedraceae	*Ephedra aphylla* Forssk.	Shrub
Globulariaceae	*Globularia arabica* Jaub. & Spach	Per. herb
Graminae	*Cynodon dactylon* (L.) Pers.	Per. herb
	Poa sinaica Steud.	Per. herb
	Polypogon viridis (Gouan) Breistr.	Per. herb
Juncaceae	*Juncus rigidus* Desf.	Per. herb
Labiatae	*Ballota undulata* (Fresen.) Benth.	Per. herb
	Lavandula coronopifolia Poir.	Per. herb
	Lavandula pubescens Decne.	Per. herb
	Mentha longifolia (L.) Huds.	Per. herb
	Phlomis aurea Decne.	Per. herb
	Origanum syriacum L.	Per. herb
	Salvia aegyptiaca L.	Per. herb

	Stachys aegyptiaca Pers.	Per. herb
	Teucrium polium L.	Per. herb
Leguminoase	*Acacia tortilis* (Forssk.) Hayne subsp. *raddiana* (Savi) Brenan	Tree
	Astragalus spinosus (Forssk.) Muschl.	Per. herb
	Retama raetam (Forssk.) Webb & Berthel	Shrub
Moraceae	*Ficus palmata* Forssk.	Shrub
Moringaceae	*Moringa peregrina* (Forssk.) Fiori	Tree
Palmae	*Phoenix dactylifera* L.	Tree
Papaveraceae	*Glaucium arabicum* Fresen.	Per. herb
Resedaceae	*Caylusea hexagyna* (Forssk.) M. L. Green	Ann.
	Ochradenus baccatus Delile.	Per. herb
	Reseda arabica Boiss.	Ann.
Rosaceae	*Cotoneaster orbicularis* Schltdl	Shrub
Rubiaceae	*Galium sinaicum* (Delile ex Decne.) Boiss.	Per. herb
	Kickxia macilenta (Decne.) Danin	Sub-shrub
	Verbascum sinaiticum Benth.	Per. herb
Scrophulariaceae	*Anarrhinum pubescence* Fresen.	Sub-shrub
Solanaceae	*Hyoscyamus muticus* L.	Per. herb
	Lycium shawii Roem. & Schult.	Shrub
	Solanum nigrum L.	Per. herb
Umbilliferae	*Deverra triradiata* Hochst. ex Boiss.	Shrub
Zygophyllaceae	*Fagonia arabica* L.	Sub-shrub
	Fagonia mollis Delile	Sub-shrub