

Numerical Taxonomic Study of *Marrubium* L. (Lamiaceae) in Egypt

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ABSTRACT

A numerical taxonomic study, using 137 macro- and micromorphological characters, SDS-PAGE and RAPD analyses of *Marrubium* (*M. alysson* and *M. vulgare*) has been achieved. The OTU's of *M. vulgare* are discriminated through various attributes, besides four biotypes within *M. alysson*. Throughout the usage of RAPD analysis, both *M. vulgare* and OTU 31 of *M. alysson* are segregated together in the dendrogram. Therefore, the present study recommended the allocation of OTU 31 of *M. alysson* at higher rank than biotype. In the intervening time, *Marrubium* species and its biotypes must be listed as endangered taxa and efforts must be intensified for their protection.

Key words: *M. alysson*, *M. vulgare*, SDS-PAGE, RAPD, biotypes



INTRODUCTION

Isolation of populations and the increment in human activities may decrease the size of populations which lead to the rising of genetic drift and consequently reduces the ability of the populations to face environmental changes (Mutegi *et al.*, 2015). The survival of these populations depends on their adaptive potential, which is determined by their genetic diversity level. The high genetic structure among populations and their low level of genetic heterogeneity are indicative of their recent genetic isolation, which could progressively lead to local adaptation.

Consequently, there is a need to develop the empirical information about the extent and distribution of genetic diversity in species that are targeted in order to develop improvement programs and conceive appropriate conservation strategies (Verma *et al.*, 2007; Boulila *et al.*, 2010). The destruction of genetic resources is particularly evident in the Middle East, and the need for conservation of these resources is urgent. Generally, the determination of these genetic variations can be assessed primarily through morphological and anatomical attributes which are helpful at all levels of the taxonomic hierarchy and can be used in the segregation of some related genera (Dinç & Doğu, 2012). On the other hand, both seed storage proteins and RAPD are good markers for assessing taxonomic and phylogenetic relationships at various levels and in the detection of genetic variation within and among populations (Marzouk & El-Darier, 2008; Tamkoc & Arslan, 2011; Sonboli *et al.*, 2011; Britto *et al.*, 2012; Tharachand *et al.*, 2012).

Marrubium comprises around 40 species indigenous in Europe, the Mediterranean and Asia and reported in the literature to be used in folk medicine (Meyre-Silva & Cechinel-Filho, 2010). In Egypt, *Marrubium* is represented by two species; *M. alysson* and *M. vulgare* (Boulos, 2002 & 2009). The existence of great environmental changes and high intensities of human pressure on natural ecosystems of the west Mediterranean coastal region accelerated their severe

degradation. Consequently the current study considered *M. vulgare* as one of the threatened species, while, *M. alysson* regarded as an indicator for high disturbance practices.

The present study aims to assess the taxonomic affinity and the inter- and intraspecific variation among the Egyptian *Marrubium* populations on the basis of an array of macro- and micromorphological characters and both seed protein electrophoresis and RAPD as molecular markers in order to provide basic information for evaluating their genetic resources.

MATERIALS AND METHODS

Thirty four OTU's representing *M. alysson* L. and *M. vulgare* L. were collected, in the spring of 2011, from 11 sites along the western Mediterranean coastal desert of Egypt along 463 Km (Plate 1, Table 1). The studied OTU's were deposited as voucher specimens at Alexandria University Herbarium (ALEX).

For macro- and micromorphological investigations, three replicas belonging to each OTU were examined and the terminology of De Vogel (1987) was adopted. For stem trichome inquiry, 10 microtome sections of each OTU were examined and the terminology of both Navarro & EL Oualidi (2000) and Osman (2012) were implemented.

The pollen grains were prepared according to Erdtman (1952) and 15 pollen grains for each OTU were estimated using the light microscope. Both pollen grains and nutlets were examined with SEM, the samples were transferred directly to a stub with double-sided adhesive tape and coated with gold in a polaron JFC-1100E coating unit, then were examined and photographed with JEOL JSM-5300 SEM. The number of perforation holes of pollen grains is counted in 4x4 µm and the terminology was based on Punt *et al.* (2007). However, descriptive terminology of Salmaki *et al.* (2008) was used for nutlet.

A total of 137 macro- and micromorphological attributes from the thirty four OTU's were subjected to numerical analyses using PAST program for mixed data

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set (Appendix 1). The OTU's were clustered through "Bray-Curtis" coefficient and the UPGMA method of sorting (Hammer *et al.*, 2001).

The seed protein electrophoresis was performed on the 8 morphologically discriminated groups through SDS-PAGE method (Sambrook *et al.*, 1989). Different molecular weights were determined using UVP Doc-It@LS Image Analysis Software and the output clustering (dendrogram) was performed using Jaccard coefficient and UPGMA method of sorting.

The five groups distinguished from the previous cluster analysis were subjected to RAPD examination. Each pooled sample from young leaves were sterilized for 5 minutes and rinsed well with distilled water, then stored with liquid nitrogen at -20°C till DNA isolation. Total genomic DNAs were extracted with GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific) following the manufacturer's protocol. Six decamer oligonucleotide arbitrary primers from Operon Technologies were tested for the amplification of DNA; their sequences are illustrated in Table 2. The PCR reactions were carried out in 50µl reaction volume containing 50 ng/µl DNA template, 1.0µl of 20 pmoles primer, 25µl DreamTaq Green PCR Master Mix (Thermo Scientific) and 23µl nuclease free water.

The amplification was conducted in a thermo cycler (Crecon 1002, Netherland) and programmed to the following conditions: the initial denaturation step at 94°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, primer annealing at 38°C for 1 minute, and extension at 75°C for 2 minutes. Final extension was carried out at 72°C for 10 minutes and the product was stored at -20°C. The PCR products were resolved on 1% agarose gel stained with 5µl ethidium bromide (10 mg/ml) in 0.5× TBE (Tris-Borate-EDTA) buffer (pH=8.3) at 75 volt for 45 minutes. Molecular weights were estimated using a 100 bp DNA ladder (peggold low molecular weight ladder).

Gel with amplified fragments was visualized under UV light and photographed by using (Gel documentation system, Digi-doc, UVP Company, England). The RAPD discrete bands were scored as binary character and UVP Doc-It@LS Image Analysis Software was used for molecular weights determination. The cluster analysis based on genetic distances was constructed using Jaccard coefficient and UPGMA method of sorting through PAST program for mixed data set (Hammer *et al.*, 2001).

RESULTS AND DISCUSSION

The agglomerative cluster analysis of the 34 OTU's of *Marrubium* based on morphological characters was exemplified by the dendrogram in Figure (1). It is remarkable that all the specimens that are assumed to represent one species and often one location are grouped together. The average taxonomic distance of the studied OTU's is 0.858, at which two major groups "I" and "II" are distinguished. The major group "I" comprises

OTU's from 1 to 3 and from 7 to 9 which represented *M. vulgare*, while *M. alysson* OTU's are segregated below the major group "II".

M. vulgare is discriminated with Palinactinodromous leaf venation (Fig. 2) and quadrangular uppermost internode with characteristic undulate sides and bulging corners (Plate 2). This is in accordance with Dinç and Doğu (2012) that the characters of stem anatomy have diagnostic value in separation of related species.

The species achieves more frequent glandular trichomes than the non-glandular ones and the presence of both un-branched non-glandular multicellular with equal cells and unbranched glandular bicellular-stalked with multicellular head cells (Figures 3 and 4). According to Navarro and EL Oualidi (2000), Jurišić Grubešić *et al.* (2007), El Beyrouthy *et al.* (2009), Mayekiso *et al.* (2009) and Xiang *et al.* (2010), the trichomes can be used for distinction at either subgeneric or subspecific level. The specimens of *M. vulgare* are also specified with 9 to 14 flowers per inflorescence and each flower achieves from 10 to 15 hooked teeth with laciniate margin. However, *M. alysson* exhibits from 3 to 10 flowers and 5-spiny tipped calyx teeth with entire margin. The unequal lobes of ovary are intermediated by appendages in *M. vulgare* (Fig. 5). That is in agreement with Täckhlohm (1974) and Boulos (2002) that the number of calyx teeth considered as a distinctive character between the two *Marrubium* species.

The pollen grains of *M. vulgare* are subprolate with perforate sculpture and less than 40 holes in 4x4 µm² area, while psilate-perforate ornamentation at the pole. Although, Akgül *et al.* (2008) recorded that the pollen grains of the Turkish *M. vulgare* were oblate-spheroidal, in the current study, both acetolized and non-acetolized pollen grains of *M. vulgare* achieved two different shapes; oblate spheroidal and subprolate, respectively (Plate 3). Harley *et al.* (2004) remarked that pollen grains of Lamiaceae are affected by the standard acetolysis procedures which destroys the colpous membranes and effectively collapses the natural shape of the pollen grain. Consequently, the present study recommends the subprolate shape, the non-acetolized form, as the true pollen grain shape of *M. vulgare*.

In the present investigation, the nutlet of *Marrubium* is brilliant dark brown to black, asymmetric, elliptic and rounded-trigonous in transverse section with truncate apex. The ventral side is roof-like with winged borders; the abscission scar is concave with obscure hilum localized at the base of the ventral line. This is congruent with the description of Mosquero & Pastor (2007) for the nutlet of the Spanish *Marrubium*. The nutlet characters are successfully applied in the discrimination between both *M. vulgare* and *M. alysson* especially those correlated to surface ornamentation. The nutlet sculpture of *M. vulgare* is tuberculate with smooth periclinal wall, while the nutlet apex attains low density of oil glands and rugose external periclinal wall of convex cell relief and anticlinal wall as channels.

The outlet base is verrucate with obscure external periclinal wall sculpture and the anticlinal wall is represented by channels. On the other hand, the outlet sculpture of *M. alysson* is pitted, while the apex of obscure external periclinal wall with concave cell relief

and the anticlinal wall of raised and striate sculpture cells.

The outlet sculpture at base is reticulate with depressed external periclinal wall and the anticlinal wall of cells is raised (Plate 4).

Plate (1): Map of the western Mediterranean desert of Egypt indicating the localities of the sampling sites (📍).

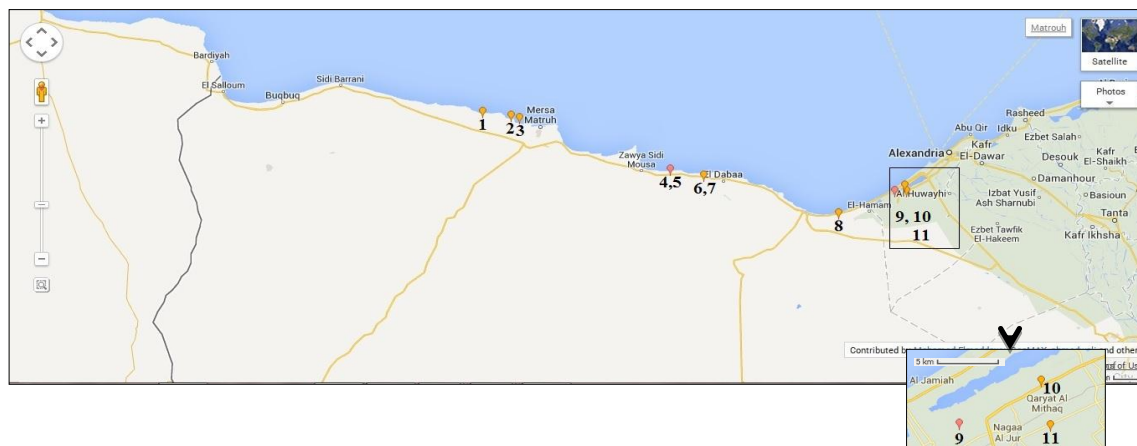


Table (1): *Marrubium* L. OTU's, their locations and the suggested clusters used for both SDS-PAGE and RAPD analyses.

OTU	Species	Location	Coordination	Clusters selected for SDS-PAGE analysis	Clusters selected for RAPD analysis
1-3	<i>M. vulgare</i>	Wadi Halazein (45 Km) West Matruh road	N 31° 24' 43'' E 26° 51' 31''	1	1
4-6	<i>M. alysson</i>	Wadi Um- El Rakhm (2 Km) South El- Kasr road	N 31° 23' 16'' E 27° 2' 34''	6	4
7-9	<i>M. vulgare</i>	Wadi Habis (5 Km) El- Kasr road	N 31° 22' 29'' E 27° 5' 54''	1	1
10-13		El-Gophera village (180 Km) "flat area between inter-ridges" Alexandria- Matruh road	N 31° 3' 22'' E 28° 5' 19''	5	3
14-15		El-Gophera village (180 Km) "foot of inland rocky ridge" Alexandria- Matruh road	N 31° 18' 0'' E 30° 16' 47''		
16-18		Hamema Village (El-Dabaa coastal side) "flat and stretched depression" 150 Km west Alexandria- Matruh road	N 31° 1' 7'' E 28° 18' 26''	8	5
19					
20-22		Hamema Village (El-Dabaa coastal side) foot of coastal ridge" 150 Km west Alexandria- Matruh road	N 31° 23' 11'' E 26° 51' 42''	3	4
23-25	<i>M. alysson</i>	Omayed railway station (80 Km) Alexandria Matruh road	N 30° 47' 22'' E 29° 11' 35''	8	
26-27		Fayed village (42 Km) (foot of rocky ridge) Alexandria- Matruh road	N 30° 55' 35'' E 29° 33' 33''	5	5
28					
29		Mithak village (42 Km) Alexandria- Matruh road	N 30° 57' 32'' E 29° 37' 35''	4	4
30					
31					5
32				2	2
33		Fayed village (42 Km) "lower slope of limestone rocky ridge" Alexandria- Matruh road	N 30° 55' 30'' E 29° 38' 3''	8	5
34					

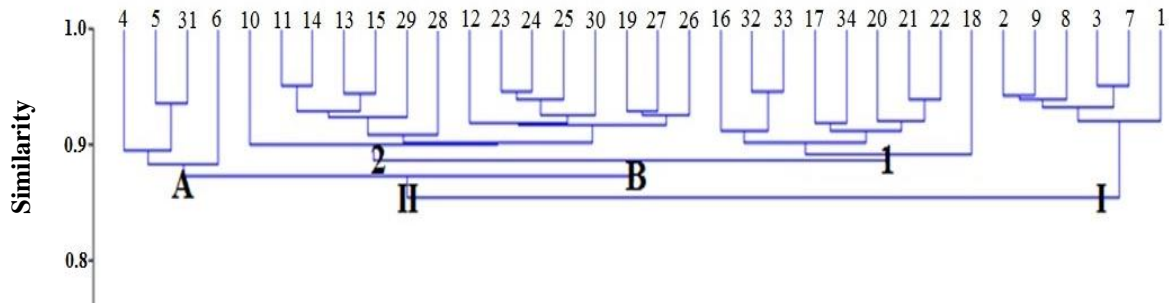


Figure (1): Dendrogram resulting from UPGMA method of sorting of the 34 OTU's of *Marrubium* L. using macro- and micromorphological characters. For detailed information see Table 1.



Figure (2): Asymmetrical leaf of *Marrubium* L. **a.** Uppermost leaf of *M. alysson* L. with rounded apex, acute base, obovate shape and basal marginal perfect actinodromous venation; **b.** Uppermost leaf of *M. vulgare* L. with crenate margin with appendages, rounded apex with appendage, acute base, orbicular shape and palinactinodromous venation. Scale bars 0.1 cm.

Plate (2): Transverse section of the uppermost internode of *Marrubium* L. **a.** Quadrangular-shaped in *M. alysson* L.; **b.** Quadrangular-shaped with bulging corners in *M. vulgare* L. Note the more frequent glandular hairs in *M. vulgare* than *M. alysson*.

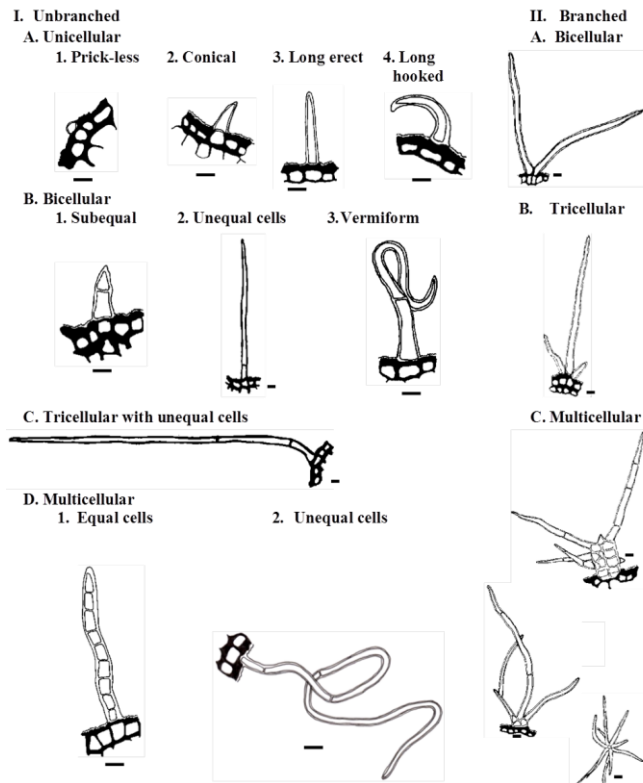


Figure (3): Types of non-glandular trichomes of *Marrubium* L. Scale bars 0.01 mm

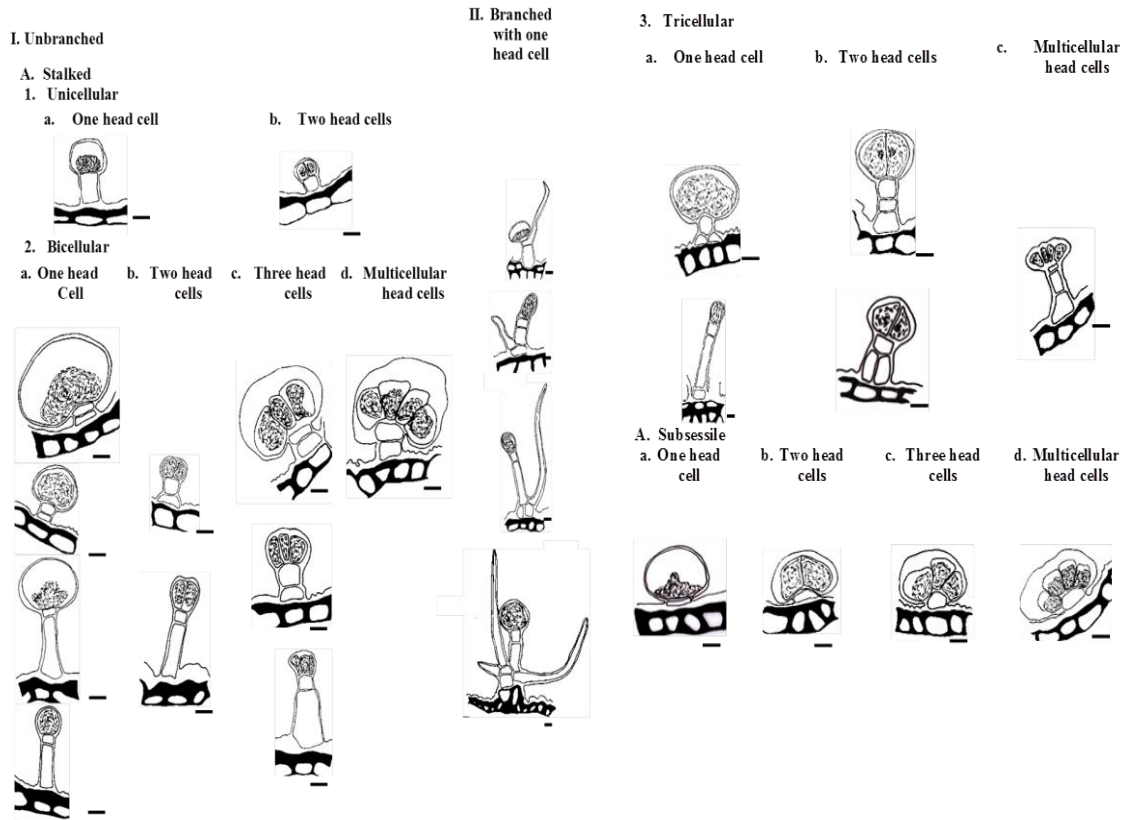


Figure (4):

Types of glandular trichomes of *Marrubium* L. Scale bars 0.01 mm.

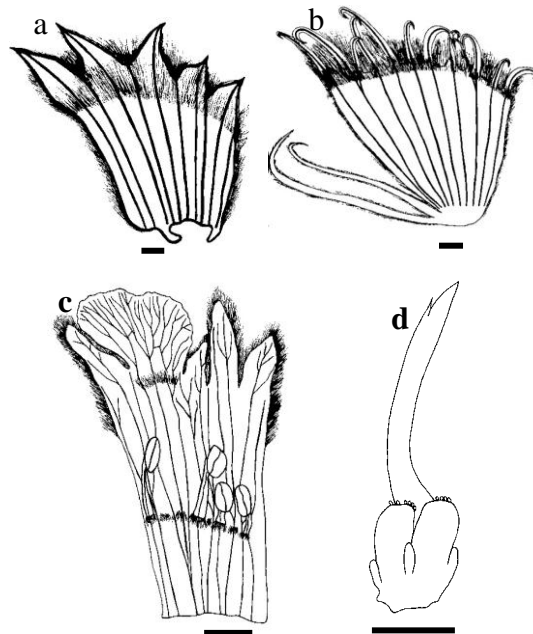


Figure (5): Flower morphology of *Marrubium* L. a. Calyx of *M. alysson* L. showing the obvious bilabiate, entire sepal margin, five spiny-tipped teeth with glabrous adaxial surface except a ring of hirsute hairs at the calyx throat; b. Calyx of *M. vulgare* L. showing the obscure bilabiate, lacinate sepal margin, 10-15 hooked teeth and glabrous adaxial surface except a ring of hirsute hairs at the calyx throat; c. Bilabiate corolla of *Marrubium* the glabrous adaxial surface except a prominent ring of hairs at the union between corolla and filament; d. Gynoecium of *M. vulgare*, showing quadrangular ovary with appendages among its lobes and gynobasic style with unequal bifid stigmas. Scale bars 1 mm

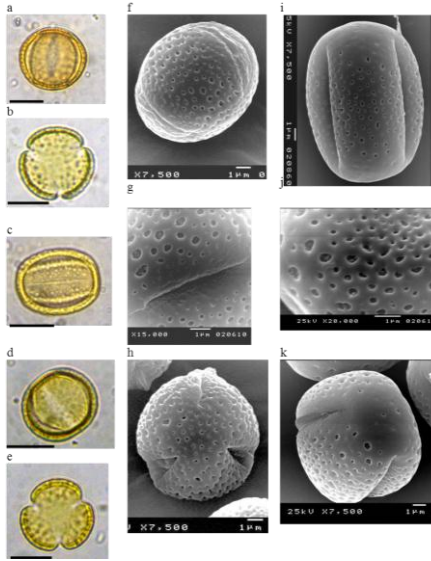


Plate (3): Light microscope and scanning electron microscope (SEM) micrographs of *Marrubium* L. pollen grains. a. Oblate spheroidal shape; b. Polar view; c. Subprolate shape; d. oblate spheroidal shape; e. Polar view. Scale bars 10 μ m. f. Oblate spheroidal with perforate sculpture; g. Perforation holes with granules; h. Perforate sculpture at pole; i. Subprolate shape; j. Perforation holes with granules; k. Psilate-perforate sculpture at pole. a, b, f-h, *M. alysson* L. and c-e, i-k, *M. vulgare* L.

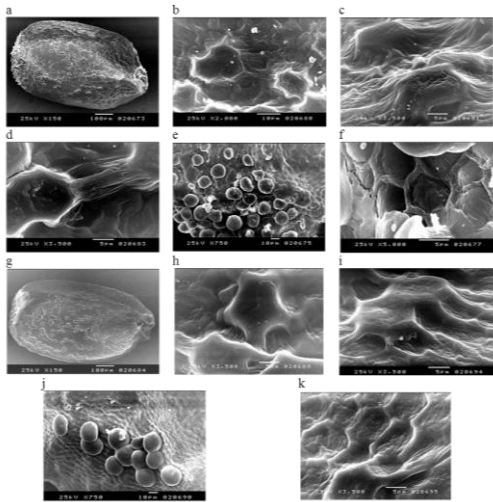


Plate (4): Scanning electron microscope (SEM) micrographs showing nutlet of *Marrubium*. *M. alysson* L. (a-f); a. Ventral surface with characteristic wings; b. Tuberculate surface sculpture with polygonal-shaped tubercles with flat apex; c. Pitted sculpture of external periclinal walls of tubercles; d. Striate sculpture of anticlinal walls of tubercles; e. Oil glands near apex; and f. Among oil glands obscure sculpture of external periclinal walls, with concave cell relief and raised anticlinal walls with striate sculpture near apex. *M. vulgare* L. (g-k); g. Ventral surface with characteristic wings; h. Tuberculate surface sculpture with polygonal-shaped tubercles with flat apex and with smooth external periclinal walls of tubercles; i. Striate anticlinal walls of tubercles; j. Reticulate sculpture near apex and oil gland; k. Among oil glands rugose sculpture of external periclinal walls and convex cell relief and channels representing anticlinal walls

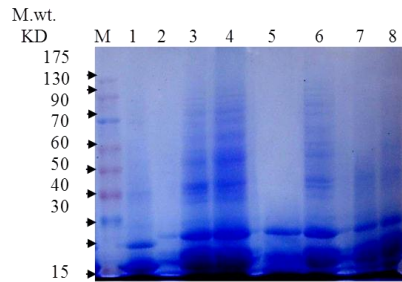


Plate (5): SDS-PAGE pattern of 8 *Marrubium* L. samples. For detailed information see Table 1.

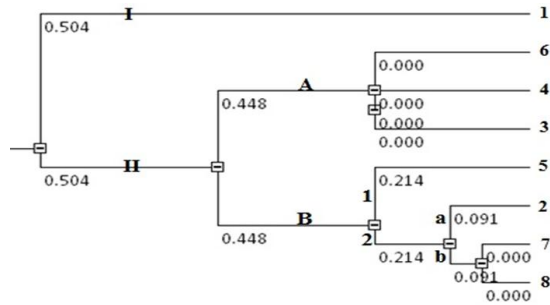


Figure (6): Dendrogram resulting from UPGMA method of sorting of 8 *Marrubium* L. samples using SDS-PAGE. For detailed information see Table 1

In the constructed dendrogram the OTU's of *M. alysson*, group "II", is further divided into two subgroups "A" and "B", at 0.874 similarity level. The subgroup "A" includes OTU's from 4 to 6 and 31, which discriminates through collenchyma thickness, inflorescence dimensions, number of flowers, corolla tube length, polar axis, equatorial diameter, colpus length and width, mesocolpium length and apocolpium diameter. Regardless, the similarities among the OTU's of subgroup "A" the OTU 31 is characterized with particular lengths of leaf and inflorescence. The subgroup "B" is further segregated into two clusters "1" and "2", the cluster "1" contains OTU's from 16 to 18, from 20 to 22 and from 32 to 34. This cluster distinguishes through stem length, lower and medium leaf dimension, petiole length, the ratio between petiole and leaf length, inflorescence dimension, calyx tube length, corolla length and middle lobe length as well as the dimensions of both third and fourth nutlets.

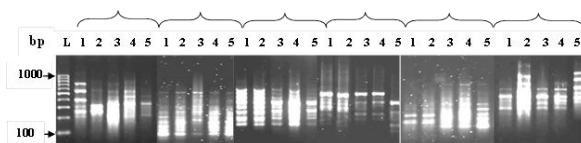
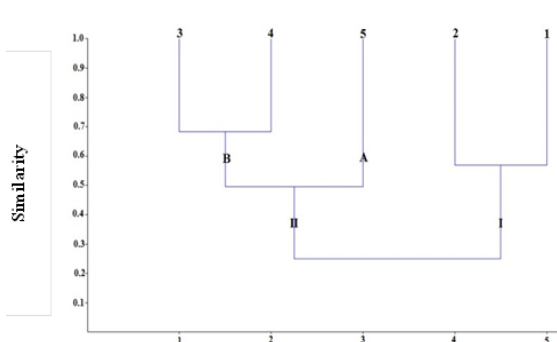
Within the cluster "2", OTU's from 10 to 15 are remarkable by long stem, low percentage of cortex and vascular tissue/radius, larger values of inflorescence dimensions and both colpus and nutlets. Notwithstanding, the OTU's from 23 to 30 are grouped together, the OTU's 28 and 29 are separated apart and specifies with short stem, large leaves, absence of glandular unbranched subsessile hair with one head cell, wide inflorescence, short corolla tube, low measurements of pollen grains besides the variation in nutlets dimension. While, OTU 19 is grouped together with OTU's from 23 to 27 and 30 due to the relatively short stem, short lowermost leaf, long calyx tube, long posterior stamen, short colpus and nutlet dimensions.

Table (2): Sequences of the 6 decamer oligonucleotide arbitrary primers selected for the study.

Primer Code	Sequence (5'... 3')	% G+C Content	Primer Code	Sequence (5'... 3')	% G+C Content
OPJ06	TCGTTCCGCA	60	OPJ10	AAGCCCGAGG	70
OPJ07	CCTCTCGACA	60	OPE-01	CCCAAGGTCC	70
OPJ08	CATACCGTGG	60	OPO-10	TCAGAGCGCC	70

Table (3): DNA banding patterns obtained from 6 operon decamer primers for the five *Marrubium* L. samples. T.N.B., Total Number of Bands for each primer; F.B., Number of Fingerprinting Bands; P.B., Number of Polymorphic Bands for each primer and %P.B., Percentage of Polymorphic Bands. For detailed information see Table 1.

Primer Type	1				2				3				4				5			
	T.N.B.	F.B.	P.B.	%P.B.	T.N.B.	F.B.	P.B.	%P.B.	T.N.B.	F.B.	P.B.	%P.B.	T.N.B.	F.B.	P.B.	%P.B.	T.N.B.	F.B.	P.B.	%P.B.
OPJ06	8	1	7	50	6	0	5	36	7	0	6	43	7	0	6	43	6	1	5	36
OPJ07	9	1	5	36	10	0	6	43	8	1	4	29	10	0	6	43	10	0	6	43
OPJ08	9	0	7	41	9	3	7	41	9	2	7	41	10	1	7	41	6	0	4	24
OPJ10	4	0	4	27	4	2	4	27	7	0	7	47	7	0	7	47	7	4	7	47
OPE-01	5	1	5	31	4	0	4	25	11	1	11	69	10	0	10	63	9	0	9	56
OPO-10	5	0	3	25	7	2	5	42	5	0	3	25	6	0	4	33	6	4	4	33
Mean	34.97				35.51				42.17				44.90				39.73			
Total	40	3	31		40	7	31		47	4	38		50	1	40		44	9	35	

**Plate (6):** RAPD pattern of the five *Marrubium* L. samples. L, DNA ladder (bp). For detailed information see Table 1**Figure (7):** Dendrogram resulting from UPGMA method of sorting of the 5 *Marrubium* L. samples based on RAPD data. For detailed information see Table 1

The detection of the taxonomic variation through previous dendrogram points out some problematical affinities among the studied OTU's; therefore the SDS-

PAGE is performed for valid circumscriptions of these groups. That in concordance with Britto *et al.* (2012) that the classification based primarily on morphological attributes may be unstable and influenced by environmental conditions. Seed storage proteins are widely applicable markers for various systematic problems and a valid alternative and/or improved approach to varietal identification, which currently is based on morphological traits recorded in the field (Marzouk, 2006; Torkpo *et al.*, 2006, Tamkoc & Arslan, 2011). In the present study, 8 pooled specimens are determined to cover the main morphological variations among the examined OTU's (Table 1). These specimens reveal 4 common genus-specific bands at 15, 16.9, 35.93 and 50 KD (Plate 5), while the dendrogram discriminates two major groups "I" and "II", at 0.504 dissimilarity level (Fig. 6). The major group "I" comprises sample 1 of *M. vulgare* which exhibits 1 species-specific band at 24.07 KD. However, *M. alysson* samples (major group "II") acquire 4 species-specific at 20, 29.14, 47.55 and 52.13 KD. The major group "II" is further divided into two subgroups, "A" and "B", at 0.448 dissimilarity level. The subgroup "A" includes samples 3, sample 4 and sample 6. The three samples achieve 19 bands each with the highest percentage of polymorphism 75 % and share in 6

common bands at 65.34, 67.96, 74.35, 83.88, 120.12 and 140.91 KD. On the other hand, the subgroup “B” is additionally separated into two clusters “1” and “2”, at 0.214 dissimilarity level. The cluster “1” contains sample 5 which generates the lowest number of bands (8 bands) and consequently the lowest percentage of polymorphism 20%. The cluster “2” is further isolated into two subclusters, “a” and “b”, at 0.091 dissimilarity level. The subcluster “a” contains sample “2” and the subcluster “b” contains samples 7 and 8; the two subclusters generate 10 11 bands, respectively.

As mentioned by Martián & Bermejo (2000), DNA fingerprinting methods are useful tools with a wide range of applications in plant population studies. In addition, the assessment of genetic diversity, through the use of molecular markers is crucial as it provides a repository of adaptability to environmental and other changes (Mondini *et al.*, 2009). Random amplified polymorphic DNA (RAPD) markers have proved to be a very useful tool providing a convenient and rapid assessment of the genetic differences among genotypes (Marzouk & El-Bakatoushi, 2011). As a result, for complete taxonomic declaration among the studied OTU's, five pooled samples are tested (Table 1). The six primers produce 221 bands, of which 175 bands are polymorphic (Plate 6). The primer OPJ06 produces the highest number of shared bands (4 bands); consequently, the present study recommends the use of this primer for specifying the two studied species. The primer OPE-01 achieves high percentages of polymorphism in samples 3, 4 and 5 (69%, 63% and 56%, respectively). The resulted dendrogram classifies two major groups “I” and “II”, at 0.26 similarity level (Fig. 7). The major group “I” comprises two samples; sample 1 (*M. vulgare*) and sample 2 (OTU 31 of *M. alysson*). These two samples share 11 common bands and exhibit low percentage of polymorphism, 34.97% and 35.51%, respectively. In spite of these similarities, sample 1 and 2 are specialized each 3 and 7 fingerprinting bands, respectively. In the major group “II” sample 5 is segregated from the two samples 3 and 4 at similarity level 0.5. The sample 5 performs the highest number of fingerprinting bands (9 bands), besides 2 specific bands at 839 and 1111 bp. On the other hand, samples 3 and 4 generate 6 common bands and reveal the high percentages of polymorphism.

In conclusion, the current study pointed out the distinction of four biotypes below *M. alysson* and (Table 1), besides the allocation of OTU 31 at a comparable rank to both *M. alysson* and *M. vulgare*. For the conclusive opinion about the taxonomic ranks of these biotypes, more informative types of molecular markers as RFLP or microsatellites or Simple Sequence Repeats (SSR) should be carried out. In the intervening time, *Marrubium* species and its suggested biotypes must be listed as endangered taxa and efforts must be intensified for their protection.

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دراسة تصنيفية عديدة علي جنس الروبيا (الفصيلة الشفوية) بمصر

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الملخص العربي

تم إجراء دراسة تصنيفية عديدة باستخدام ١٣٧ صفة مورفولوجية عامة ودقيقة واستخدام بعض طرق التحليل الجزيئي، مثل: الفصل الكهربائي لبروتين البذور، وتقنية "RAPD" علي نوعي الروبيا *Marrubium alysson* L. و *Marrubium vulgare* L. وقد تميزت العينات النباتية لنوع *M. vulgare* بصفات عديدة، بالإضافة إلي تحديد أربعة أنماط حيوية تحت النوع *M. alysson*. ومن خلال استخدام تقنية "RAPD" فقد تجمع معا كلا من النوع *M. vulgare* والعينة النباتية رقم ٣١ من النوع *M. alysson*. وتوصي الدراسة بوضع العينة النباتية رقم ٣١ في تصنيف أعلي من النمط الحيوي وبإدراج جنس الروبيا بنوعيه وبأنماطه الحيوية تحت الأنواع المهددة بالإنقراض مع تكثيف الجهود لحمايتها.

Appendix 1. List of characters and their character states recorded comparatively for the two *Marrubium* species

I. Stem macro- and micromorphological characters

1, Stem length (cm); 2, Stem radius (mm); 3, Epidermis thickness (mm); 4, Number of collenchyma layers in corners; 5, Collenchyma thickness in corners (mm); 6, Number of collenchyma layers among corners; 7, Collenchyma thickness among corners (mm); 8, Cortex thickness (mm); 9, Percentage of vascular tissue/radius; 10, Phloem thickness (mm); 11, Xylem thickness (mm); 12, Percentage of cortex and vascular tissue/radius; 13, Glandular hairs/ non-glandular hairs ratio (0- <1/1- >1); 14, Unbranched unicellular prick-less non-glandular hairs (0- absent/1- present); 15, Unbranched unicellular short conical non-glandular hair (0- absent/1- present); 16, Unbranched unicellular long erect non-glandular hair (0-absent/1- present); 17, Unbranched unicellular long hooked non-glandular hair (0- absent/1-present); 18, Unbranched bicellular erect non-glandular hair with subequal cells (0- absent/1- present); 19, Unbranched bicellular erect non-glandular hair with unequal cells (0- absent/1- present); 20, Unbranched bicellular vermiform non-glandular hair (0- absent/1- present); 21, Unbranched tricellular non-glandular hair with unequal cells (0- absent/1-present); 22, Unbranched multicellular non-glandular hair with equal cells (0-absent/1- present); 23, Unbranched multicellular non-glandular hair with unequal cells (0- absent/1-present); 24, Branched bicellular non-glandular hair (0-absent/1- present); 25, Branched tricellular non-glandular hair (0- absent/1- present); 26, Unbranched unicellular-stalked glandular hair with one head cell (0- absent/1- present); 27, Unbranched unicellular-stalked glandular hair with two head cells (0- absent/1-present); 28, Unbranched bicellular-stalked glandular hair with two head cells (0- absent/1-present); 29, Unbranched bicellular-stalked glandular hair with three head cells (0- absent/1- present); 30, Unbranched bicellular-stalked glandular hair with multicellular head cells (0- absent/1- present); 31, Unbranched tricellular-stalked glandular hair with one head cell (0- absent/1- present); 32, Unbranched tricellular-stalked glandular hair with two head cells (0- absent/1- present); 33, Unbranched tricellular-stalked glandular hair with multicellular head cells (0- absent/1-present); 34, Unbranched sessile glandular hair with one head cell (0- absent/1- present); 35, Unbranched sessile glandular hair with two head cells (0- absent/1- present); 36, Unbranched sessile glandular hair with three head cells (0- absent/1-present); 37, Unbranched sessile glandular hair with multicellular head cells (0- absent/1- present); 38, Branched glandular hair with one head cell (0- absent/1- present).

II. Leaf macromorphological characters

39, Leaf apex (0- Obtuse/1- Rounded); 40, Leaf venation (0- Basal marginal perfect actinodromous/1- Palinaetinodromous); Lowermost leaf: 41, Petiole length (cm); 42, Leaf length (cm); 43, Leaf width (cm); 44, Leaf length/leaf width; 45, Leaf shape (1- Ovate/2- Orbicular/3- Obovate); 46, Leaf base (0- Acute/1-Rounded); 47, Percentage of petiole length/leaf length; Medium leaf: 48, Petiole length (cm); 49, Leaf length (cm); 50, Leaf width (cm); 51, Leaf length/leaf width; 52, Leaf shape (1- Ovate/2- Orbicular/3-Obovate); 53, Percentage of petiole length/leaf length; Uppermost leaf: 54, Petiole length (cm); 55, Leaf length (cm); 56, Leaf width (cm); 57, Leaf length/leaf width; 58, Leaf shape (1- Ovate/2- Orbicular/3-Obovate); 59, Leaf margin (0- Crenate/1- Crenate with appendage); 60, Leaf apex appendage (0-Absent/1- Present); 61, Percentage of petiole length/leaf length.

III. Floral Morphology

62, Number of flowers per inflorescence; 63, Inflorescence length (cm); 64, Inflorescence width (cm); 65, Inflorescence length/ inflorescence width; 66, Bilabiate degree of calyx (0- Obscure/1- Obvious); 67, Sepal margin (0- Entire/1- Laciniate); 68, Number of calyx teeth (0- Five/1- From 10 to 15); 69, Sepal apex (0- Spiny-tipped/1- Hooked); 70, Calyx length (mm); Upper lip: 71, Longest sepal length (mm); 72, Medium sepal length (mm); 73, Shortest sepal length (mm); Lower lip: 74, Longest sepal length (mm); 75, Shortest sepal length (mm); 76, Calyx tube length (mm); 77, Percentage of calyx tube length/calyx length; 78, Petal color (0- White/1- Purple); 79, Corolla length (mm); Upper lip: 80, Longest petal length (mm); 81, Shortest petal length (mm); Lower lip: 82, Longest lateral petal length (mm); 83, Shortest lateral petal length (mm); 84, Middle lobe length (mm); 85, Corolla tube length (mm); 86, Percentage of corolla tube length/corolla length; Posterior stamens (short): 87, Mean of the two stamens length (mm); 88, Mean of the two filaments length (mm); 89, Mean of the two anthers length (mm); 90, Percentage of the two anthers mean length/the two filaments mean length; Anterior stamens (long): 91, Mean of the two stamens length (mm); 92, Mean of the two filaments length (mm); 93, Mean of the two anthers length (mm); 94, Percentage of the two anthers mean length/the two filaments mean length; 95, Gynoecium length (mm); 96, Ovary length (mm); 97, Ovary width (mm); 98, Ovary length/ovary width; 99, Percentage of ovary length/gynoecium length; 100, Style length (mm); 101, Posterior stigma

length (mm); 102, Anterior stigma length (mm); 103, Percentage of anterior stigma length/style length; 104, Appendages among ovary lobes (0- absent/1- present)

IV Pollen Grain Morphology

105, Polar axis (μm); 106, Equatorial diameter (μm); 107, Polar axis/Equatorial diameter (P/E); 108, Pollen shape (0- Oblate Spheroidal/1- Subprolate); 109, Apocolpium diameter (μm); 110, Apocolpium index; 111, Colpus length (μm); 112, Colpus width (μm); 113, Colpus length/colpus width; 114, Percentage of colpus length/polar axis; 115, Mesocolpium length (μm); 116, Exine thickness (μm); 117, Sculpture at poles (0- Perforate/1- Psilate-Perforate); 118, Number of perforation holes (0- <40/1- >40)

V. Nutlet Morphology

First nutlet (largest): 119, Nutlet length (mm); 120, Nutlet width (mm); 121, Nutlet length/nutlet width; Second nutlet: 122, Nutlet length (mm); 123, Nutlet width (mm); 124, Nutlet length/nutlet width; Third nutlet: 125, Nutlet length (mm); 126, Nutlet width (mm); 127, Nutlet length/nutlet width; Fourth nutlet (smallest): 128, Nutlet length (mm); 129, Nutlet width (mm); 130, Nutlet length/nutlet width; 131, Density of oil glands (0- Moderate/1- Dense); 132, Sculpture of external periclinal wall of tubercle (0-Smooth/1- Pitted); 133, Sculpture of external periclinal wall of cells at nutlet apex (0- Obscure/1-Rugose); 134, Relief of external periclinal wall of cells at nutlet apex (0- Concave/1- Convex); 135, Anticlinal wall of the cells at nutlet apex (0- Represented by channels/1- Raised and striate); 136, Sculpture at nutlet base (0- Reticulate/1- Verrucate); 137, Anticlinal wall of the cells at nutlet base (0-Depressed/1- Raised)