BIODIVERSITY STUDY OF *Zilla spinosa* (L.) IN EGYPT

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Brassicaceae (Cruciferea) is one of the largest families of Angiosperms that includes a large variety of food, condiments, ornamental and fodder species that are of economic significance (Cheo *et al*. 1987 and Appel and Al-Shehbaz, 2003). *Brassicaceae* is one of the four largest families in Egypt, it follows Asteraceae, Orchidaceae, and Fabaceae. It is represented by 53 genera and 104 species. *Zilla* Forsk. is one of the genera of family *Brassicaceae*, tribe Brassiceae DC. It is represented by two species *Zilla macroptera* Coss (Morocco) and *Zilla spinosa* (L.) prantl with three subspecies subsp. *Costata* Maire and Weiller (Algeria, Chad, Libya, Mauritania, Morocco), subsp. *biparmata* (O.E. Schulz) Maire and Weiller (Egypt, Libya), and subsp. *Spinose* (L.) prantl (Egypt, Iraq, Israel, Jordan, Kuwait, Oman, Qatar, Saudi Arabia, Sudan, United Arab Emirates, Syria, Yemen) (https:// brassibase.cos.uni-heidelberg.de/).

Zilla spinosa is one the most popular plant species of the family Cruciferea, because of its essential uses in folk medicine. It is used as a drink against kidney or gall bladder stones (Heneidy and Bidak 2001). *Z. spinosa* phytochemicals have a broad range of biological activities, including antioxidant, antifungal, antifibrotic, hepatoprotective, and antiviral activities (Karawya *et al*. 1974; El-Menshawy *et al*. 1980; Omara *et al*. 2018 and Al-Qahtani *et al*. 2020). It is a perennial xerophyte in arid parts of Egypt, which grows as an evergreen plant that flowers during most of the year under favorable conditions (Boulos, 1999).

Zohary (1966) in Flora Palestina recorded two varities *Z. spinosa* var. *spinosa* with fruit 0.8-1 cm, ribbed and irregularly wrinkled, flowers 1-2 cm, and *Z. spinosa* var. *microcarpa* with fruit smoother than *Z. spinosa* var. *spinosa* 0.6-0.8 cm, flowers 0.6-1 cm. According to Boulos (1999), *Zilla* is a monospecific genus in the Egyptian Flora represented by *Z. spinosa* with two subspecies: subsp. *spinosa* in which silicula is subglobose, with inconspicuous outgrowths on the valves, and subsp. *biparmata* in which silicula conspicuously covered with shield-like outgrowths or wings on the valves. However, Täckholm (1974) recorded two different species in Egypt: *Zilla biparmata*, and *Zilla spinosa*. *Z. biparmata* with cube shaped pod under the spiny style with a deep grove surrounded by a corky margin on each of the 4 lateral faces. *Z. spinosa* in which the pod is globose and lack the corky margins, and divided one of the two species (*Z. spinosa*) into two varieties: var. *spinosa* in which the plant is typically 50-60 cm, with broad pod 8-10 mm, and var. microcarpa in which the plant is 10-20cm, with smaller pod. Our field observations of *Z. spinosa* showed the presence of high degree of morphological diversity with and within populations in Egypt.

The ability of start codon targeted markers (SCoT) to identify polymorphisms and determine genetic diversity across species is much greater than that of random primers, according to several studies (Zeng *et al*., 2014 and Tiwari *et al*., 2016). Another important method for resolving some taxonomic issues at the family *Brassicaceae*, generic, or specific level is the morphology of pollen grains (Gabr, 2018). Nearly 80% of the Brassiceae tribe's chromosome counts are known, and they range from *n*= 6 to 75 (Warwick and Anderson, 1993). The chromosome number of *Z. spinosa* was counted in previous studies (Murín and Chaudhri 1970; Harberd 1972; Amin 1973; Al-Shehbaz and Al-Omar 1982; Anderson and Warwick 1999 and Warwick and Al-Shehbaz 2006), but there was no record for *Z. spinosa* karyotype in any previous study.

The aim of this study is to revise the morphological and genetic variation among *Zilla spinosa* different populations, to resolve the taxonomic problem of this species in Egypt because of the confused infra-specific groupings, to determine the pollen grain characters using SEM, and to investigate the chromosome number and the karyotypes.

MATERIALS AND METHODS

The herbarium specimens deposited in the Herbarium of Cairo University (CAI) (dated back 95 years) were examined. In addition to the authentic specimens kept in the Royal Botanic Garden Herbarium at Kew (K), and other virtual herbaria available online (the JSTOR Global Plants database), acronyms follow Index Herbariorum (http:// sweetgum.nybg.org/ih/). Scientific name for both subspecies follows IPNI (http://www.ipni.org/). Field work was conducted in 2020-2022, 60 fresh

representative specimens of *zilla spinosa* belonging to 15 different populations (4 individuals / population, 3 populations / locality, the 5 localities marked with * in Table (1) were collected from two phytogeographical regions: The Mediterranean coastal strip and Eastern Desert (Table 1). All specimens were examined for morphological diversity in all distribution localities. Data of morphological investigation were outlined in Table (2). Different morphological criteria of stem, leaves, inflorescence, flower, and fruit were examined (55 different morphological characters). Voucher specimens were deposited in (CAI).

Molecular study

From the specimens collected from all localities, 5 specimens representing the two morphotypes were selected for molecular study, the first morphotype was represented by specimens given the accession numbers 1s, 2s, and 5s, while 3b and 4b specimens representing the second morphotype (1s was collected from Bahig, 2s was collected from Wadi Digla, and 5s from Wadi Hagool, while 3b was collected from Wadi Digla and 4b was collected from Wadi Hof).

DNA extraction and purification

Total DNA was extracted from five samples of *Z. spinosa* by DNeasy Plant Kit (QIAGEN, Germany). The extracted DNA concentration and quality were determined by NanoDrop.

SCoT "Start Codon Target"

Due to various advantages over other marker approaches, SCoT markers were utilized for the first time in *Z. spinosa*. These advantages include quicker production of species-specific primers than SSR, lower cost than AFLP (Jiang *et al*., 2014), and higher reproducibility than RAPD (Xiong *et al*., 2011). The findings of the current study demonstrated the effectiveness of SCoT markers in determining the genetic diversity among *Z. spinosa* morphotypes.

SCoT-PCR Reactions

Nine SCoT primers were used in the detection of polymorphism:

SCoT-1 (5'-ACGACATGGCGACCACGC-3')
SCoT-2 (5'-ACCATGGCTACCACCGGC-3'),
SCoT-3 (5'-ACGACATGGCGACCCACA-3'),
SCoT-4 (5'-ACCATGGCTACCACCGCA-3'),
SCoT-5 (5'-CAATGGCTACCACTAGCG-3'),
SCoT-7 (5'-ACAATGGCTACCACTGAC-3'),
SCoT-9 (5'-ACAATGGCTACCACTGCC-3'),
SCoT-11 (5'-ACAATGGCTACCACCAGC-3'),
SCoT-12 (5'-CAACAATGGCTACCACCG-3').

According to the procedure, the amplification reaction was conducted in 25 µl comprising 3 µl template DNA (10ng), 12.5 µl Master Mix (sigma), 2.5 µl primer (10pcmol), and 7 µl dH_2O, according to (Ibrahim *et al*. 2019a).

Thermocyling Profile PCR

PCR reaction was carried out in a Perkin-Elmer/GeneAmp® PCR System

9700 (PE Applied Biosystems) programmed as follow, after initial denaturation cycle that lasts for 5 minutes at 94ºC, 40 cycles were set to be completed, each cycle included a 45-second denaturation step at 94ºC, a 50ºC annealing step for 50s, and a 1-minute elongation step at 72ºC. In the last cycle, the primer extension phase was prolonged to 7 min at 72ºC.

a) Detection of the PCR Products

By electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TBE buffer at 95 volts, the amplification products were separated. PCR products were seen and photographed under UV light using a Gel Documentation System (BIO-RAD 2000).

Data analysis

For all samples were used in SCoT analysis, only distinguishable and obvious bands were visually assessed as either present (1) or absent (0). The final data sets also contained both monomorphic and polymorphic bands. A binary statistic matrix was subsequently created. The unweighted pair group method with arithmetic averages (UPGMA) was then used to calculate the coefficients of the Dice's similarity matrix between samples. Using the PAST software Version 1.91, a phylogenetic tree (dendrogram) was created from this matrix according to the Euclidean similarity index (Hammer *et al*., 2001).

Sample Preparation for Scanning Electron Microscope (SEM)

For pollen study, fresh anthers were collected from the floral buds placed through an ETOH dehydration series, then dried with a Denton DCP-1 Critical Point Drying apparatus, using CPD drying method which is utilizing liquid CO_2 (carbon dioxide). With this technique, liquid from tissues is removed without being affected by surface tension. Pollen samples were prepared and scanned on a JEOL 1200 EX (JEOL, Japan) SEM at 20 kv. Size measurements were obtained from the average of 25 randomly selected grains. The pollen terminology followed Punt *et al*. (1994).

Cytogenetic studies

Sample preparation

Collection of plant materials took place during 2020 and 2022. Twenty specimens representing the two morphotypes were collected (10 specimens from each morphotype collected from the 5 different localities, 2 specimens from each locality). The seeds from the specimens were soaked for one hour in distilled water, and then germinated at room temperature. To root tips that were cut about 1 cm long, colchicine (C22H25NO6, 0.025 percent) was added for two hours at room temperature before being rinsed with distilled water. The samples then were fixed in ethanol: glacial acetic acid (3:1, v/v). After properly cleaning the samples

with water, they were hydrolyzed with 1 N HCl for 5 minutes at 64ºC. The root tips were squashed with 45% acetic acid to make the slides and dyed with acetoorcein solution.

Microscope examination, karyotype and idiograming

Chromosomes examination has to do using a verticals fluorescence microscope (Leica DM2500) equipped with a cooled monochrome digital camera (Leica DFC340FX). Twenty cells with clearly observed and well spread where check and photographed at 100X magnification under oil immersion. Chromosome counting and karyotype has performed using the automated Karyotype and FISH software processing (Leica CW4000) system. Ideograms were constructed from complete chromosomes which showed the greatest possible banding pattern in at least ten different metaphase plates.

The lengths of each chromosome's short and long arms (p and q) were measured for all examined cells, and the total length was calculated (TL=p+q). The relative length (RL) of the chromosomes (TL / sum TL × 100) and the mean relative length (MRL) of each chromosome pair were then determined. The centromeric index (CI) was determined using the formula (P / TL x 100), and the mean centromeric index (MCI), which is an estimate of the centromeric index value of each chromosome pair, the chromosomes were classified in accordance with Ibrahim *et al.* (2019b).

RESULTS AND DISCUSSION

Taxonomy

***Zilla spinosa* (L.) prantl**, Nat. Pflanzenfam. 3(2): 175 (1891).

Basionym: *Bunias spinosa* L., Mant. Pl. 96 (1767).

Common name: Spiny zilla.

Thorny, perennial, desert shrubs vary from 50 cm to 2.2 m in height. Stems densely dichotomously branched. Basal leaves rosette, glabrous, fleshy, soon deciduous, spathulate, entire, dentate or lyrate-pennatifid, 2-10 x 2-5 cm, shortly petioled; cauline leaves entire, alternate, few, small, lanceolate-oblong, 1.5-3 x (0.2) 0.25-0.4 cm, shortly petioled; old stems leafless. Racemes few flowered, ebracteate; Flowers tetramerous, hermaphrodite, shortly pedicelled; pedicel 1-2.5 x 1 mm, thickened in fruit; receptacle 1-2 x 2 mm; Calyx erect, saccate, Sepals green, oblong-ovate, two wide (4.5-8 x 2.5 mm) and two narrow (4.5-8 x 1-1.5 mm). Petals pink to violet or white, oblong-spathulate, entire, acute or obtuse to retuse, long-clawed, 0.7-1.8 x 0.2- 0.35 cm. Stamens six, tetradynamous, 4 long with stamen filament 5-7 x 1 mm, and two short with stamen filament 4-6 x 1 mm; anthers yellow, ovoid-oblong, minute, 3-4 x1 mm. Ovary 1-2 x 1-2 mm, style 1.5-4 x 0.5-1 mm, stigma 0.5-1 x 0.5-1mm; Silicles 0.6-1.5 x 0.7-0.8 cm, on

2 mm long, thick pedicel, indehiscent, ovate-globular, ribbed with transverse wrinkles between ribs or ribs absent, tapering into a conical spiny beak, 2-celled; valves and septum woody; seeds ellipsoid, smooth, 1 per locule.

Our taxonomic revision of *Zilla spinosa* in Egypt was based on the examination of both herbarium specimens and fresh collected specimens and showed the presence of a significant degree of phenoplasticity in all analyzed populations (old and fresh). Basal leaves were Entire, dentate or pinnatifid, and the petals color varies from pink to violet or white. We determined two morphotypes that are similar in all morphological characters present in Table (2), but those morphotypes differ in fruit shape. The first morphotype in which silicula is smooth without outgrowths or ribs on the valves, and the second morphotype in which silicula ribbed and irregularly wrinkled (Fig. 1). The two morphotypes were tracked in mixed populations across the entire geographic range of the species (Table 1), indicating that environmental conditions have no effect on their distribution or phenotypic diversity.

Distribution

It is native to Algeria, Chad, Egypt, Iraq, Kuwait, Lebanon-Syria, Libya, Mauritania, Morocco, Oman, Palestine, Saudi Arabia, Sudan, Yemen (*Zilla spinosa* (L.) Prantl (Plants of the World Online, Kew Science).

SCoT analysis

The SCoT fingerprinting profiles generated by the nine primers targeting start codon in five samples are shown in Fig. 2. The polymorphism produced by nine SCoT primers is summarized in Table (3). These primers generated a total of 113 amplicons; 66 of these were polymorphic (56%). The number of total bands ranged from 7 for primer SCoT-05 to 17 for primer SCoT-03; the number of polymorphic bands also varied greatly between 3 and 11 in the cases of SCoT-01 and SCoT-03, SCoT-09 primers, respectively. The average number of polymorphic amplicons was 7.3 per primer. The Frequency between nine SCoT primers ranged from 0.55 SCoT-07 to 0.87 SCoT-01. The PIC values varied among the SCoT primers; it ranged from the lowest value of 0.20 for primer SCoT-01 to the highest value of 0.37 for primer SCoT-07.

Genetic similarity and cluster analysis based on SCoT marker

The resulting data of SCoT among five samples using the UPGMA and Dice coefficient assays, indicating the genetic similarity ranged from 0.70 to 0.85 (Table 4). The highest genetic similarity (0.85) was detected between samples (1S and 5S), while the genetic similarity between sample 1S and sample 2S (0.84), and the genetic similarity between sample 3b and 4b (0.83). The lowest genetic similarity (0.70) was detected between samples (4b and 5S). The SCoT marker data for 5 samples were used to produce a genetic

distance tree based on Dice's genetic similarity matrix (Fig. 3). In this tree, two main clusters are formed; the first cluster includes the three specimens representing the first morphotype (1S. 5S and 2S), and the second cluster includes the two specimens representing the second morphotype (3b and 4b). According to the molecular results, the phenotypic diversity between the two morphotypes was due to genetic factors, and we considered the two morphotypes as two subspecies: subsp. *spinosa* represents the first morphotype having silicula smooth without outgrowths or ribs, and subsp. *biparmata* represents the second morphotype with silicula ribbed and irregularly wrinkled.

Pollen Features Using SEM

The study of Pollen grains of Z*illa spinosa* specimens using SEM showed that pollen of the two subspecies were isopolar, radially symmetrical, tricolpate (Fig. 4). The studied pollen grains were medium in size in the two subspecies. The pollen grains of *Z. spinosa* subsp. *biparmata* have a polar axis length of 33.63 µm, an equatorial diameter of 18.1 µm. The pollen grains of *Z. spinosa* subsp. *spinosa* have a polar axis length of 33.25 µm, an equatorial diameter of 16.8 µm. The shape of pollen grains is prolate in the two subspecies. In subsp. *biparmata*, the P/E ratio was 1.86, while the P/E ratio in subsp. *spinosa* was 1.98 (Table 5). The colpi of the two subspecies are usually widest at the equator and gradually narrowing towards the poles, subsp. *spinosa* possessed longer colpus of 29.24 µm, but subsp. *biparmata* possessed colpus length of 28.53. The two subspecies had the same colpus width of 0.44 µm. The exine ornamentation in both subspecies is microreticulate (lumina less than 1 µm in diameter) to coarsely reticulate (lumina more than 2 µm in diameter), with straight or slightly sinuous muri. Due to uneven lumina size and shape ranging from polygonal to circular or indefinite shape, the reticulate exine appeared heterobrochate (Fig. 4). The lumina diameter was 0.7-2.98 µm in subsp. *biparmata,* and 0.7-2.8 µm in subsp. *spinosa.* The largest dimeter of lumina was near the equator and the dimeter gradually decreased towards poles. There was no inter luminal tissues in the two subspecies and the reticulum appeared open.

Cytogenetic analysis

Chromosome number

Chromosome count for the two subspecies of *Z. spinosa* was done in the mitotic metaphase (Fig. 5). The two subspecies recorded the same chromosome number. The base chromosome number that was recorded is x=16, 2n=32.

Karyotype analysis

The karyotyping data of the two subspecies are provided in Fig. (6), and Table 6. The retrieved results showed that the karyotype formula for *Z. spinosa* subsp. *spinosa* is 2n=32 = $\mathbf{L^{m}_{16} + M^{sm}_{10} + S^{sm}_{6}}$ **+ St chro. 13**, **15.** As 16

chromosomes are large, 10 chromosomes are medium, 6 chromosomes are small, in addition to chromosomes no 13 and 15 have satellite. The total genomic length is 85.93 µm. While the karyotype formula for *Z. spinosa* subsp. *biparmata* is 2n=32 = $\mathbf{L^{m}_{16} + M^{sm}_{10} + S^{sm}_{6}}$ **+ St chro. 15.** As 16 chromosomes are large, 10 chromosomes are medium, 6 chromosomes are small, in addition to only chromosome no 15 has satellite. The total genomic length is 116.85 µm.

The 16 chromosome pairs were grouped based on the centromere position into two types: metacentric and submetacentric (Table 6). The chromosome pairs from 1 to 8 are metacentric, the chromosome pairs from 9 to 16 are submetacentric. In addition, the chromosomes are variable in the two subspecies with respect to their mean relative length (MRL), as shown in Fig. (8). The chromosome pair 1 is the longest in the two subspecies, its length ranges from 6.83 µm in subsp. *spinosa* to 10.70 µm in subsp. *biparmata*, and its mean relative length (MRL) ranges from 7.95 % in subsp. *spinosa* to 9.16 % in subsp. *biparmata*. The length of the shortest chromosome pair 16 ranges from 4.45 in subsp. *spinosa* to 4.80 µm in subsp. *biparmata*, and its mean relative length (MRL) ranges from 4.11 % in subsp. *biparmata* to 5.18 % in subsp. *spinosa*. The idiogram showing the relative size of the chromosomes is represented in Fig. (7)

Our taxonomic revision of *Zilla spinosa* in Egypt revealed that all the studied populations (old and fresh) had a considerable degree of phenotypic diversity. We found two distinct morphotypes, based mainly on fruit traits. The first morphotype, in which the silicula is smooth and lack any outgrowths or ribs, and the second has ribbed silicule (Fig. 1).

Little concern has been paid to the study of *Z. spinosa* genetic diversity in Egypt. In this study, we attempt to clarify the contribution of genetic diversity to the phenoplasticity of Egyptian morphotypes. The achieved molecular results by using SCoT markers give the dendrogram that separated two main clusters; the first cluster includes the three specimens representing the first morphotype, and the second cluster includes the two specimens representing the second morphotype (Fig. 3). The retrieved molecular results confirmed that SCoT technique could be used for identification and differentiation of the two morphotypes and revealed that the phenotypic diversity within the Egyptian *Z. spinosa* populations is genetically controlled, this agrees with Khattab *et al*. (2014) who reported that *Zilla* plants have high genetic variation. According to molecular results we treated the two identified morphotypes from morphological study as two distinct subspecies: subsp. *spinosa* represent the first morphotype (silicula smooth), and subsp. *biparmata* represent the second morphotype (ribbed silicula), this agrees with Boulos (1999) who recorded two subspecies of *Z. spinosa* in Egypt: subsp. *spinosa* having silicula with inconspicuous outgrowths on the valves,

and subsp. *biparmata* having silicula conspicuously covered with shield-like outgrowths on the valves.

Pollen shape results were validated by studies of Brassicaceae pollen grains conducted by Rollins and Banerjee (1979), Anchev and Deneva (1997) and Arora and Modi (2011). Pollen grains of *Zilla spinosa* were medium in size in the two subspecies, having polar axis 31.85-35.4 µm. According to Erdtman (1969). medium grains have polar axis 25–50 µm.

Pollen grains in the two subspecies have prolate shape. In subsp. *biparmata*, the P/E ratio was 1.86, while the P/E ratio in subsp. *spinosa* was 1.98. According to Erdtman (1986), prolate shape having $1.33 \leq P/E \text{ ratio} < 2$.

Pollen grains of both subspecies were tricolpate with microreticulate to coarsely reticulate exine ornamentation. The tricolpate pollen grains of the Brassicaceae were demonstrated by Abdel Khalik *et al.* (2002), who additionally identified three forms of exine ornamentation (coarsely reticulate, reticulate, and microreticulate) based on lumina size.

Our cytological results agree with molecular results and confirm the genetic diversity between the two subspecies. The base chromosome number that was recorded in both subspecies of *Z. spinosa* is x=16, 2n=32, this agrees with Murín and Chaudhri (1970), Harberd (1972), Amin (1973), Al-Shehbaz and Al-Omar (1982), and Anderson and Warwick (1999).

Karyotype analyses of the two subspecies (Fig. 6 and Table 6) revealed that in subsp. *biparmata* only chromosome number 15 has a satellite, but subsp. *spinosa* has two chromosomes have satellites, chromosome number 13 and 15, and showed that there are 16 large, 10 medium, and 6 small chromosomes. The total genomic length ranges from 85.93 µm in subsp. *spinosa* to 116.85 µm in subsp. *biparmata*.

The chromosome pair 1 is the longest in the two subspecies, its length ranges from 6.83 µm in subsp. *spinosa* to 10.70 µm in subsp. *biparmata*. The length of the shortest chromosome pair 16 ranges from 4.45 in subsp. *spinosa* to 4.80 µm in subsp. *biparmata*. The mean relative length (MRL) is variable in the two subspecies (Fig. 8). MRL of the longest chromosome pair ranges from 7.95 % in subsp. *spinosa* to 9.16 % in subsp. *biparmata*. (MRL) of the shortest chromosome pair ranges from 4.11 % in subsp. *biparmata* to 5.18 % in subsp. *spinosa*. Based on the centromeric position, the 16 chromosomal pairs were divided into two categories: metacentric and submetacentric (Table 6). Chromosome pairs numbered 1 through 8 are metacentric, while those numbered 9 through 16 are submetacentric. Our results agree with Guerra (2008), who supposed that karyological data in taxonomy contribute to evaluating the genetic links across species or populations.

CONCLUSION

According to molecular and cytological results which confirmed the genetic diversity between the two identified *Z. spinosa* morphotypes. We treated these morphotypes as two distinct subspecies: subsp. *spinosa*, and subsp. *biparmata*.

SUMMARY

Zilla is a monospecific genus in the flora of Egypt represented by *Zilla spinosa*. The field observations of *Zilla spinosa* populations showed the presence of a high degree of morphological diversity. The taxonomic problem of this species is mainly related to its confused infra-specific treatments in different floras. Our taxonomic revision of *Z. spinosa* was carried out on 15 recently collected populations covered the geographical range of this species in Egypt, in addition to 33 old herbarium specimens from different localities dating to nine decades ago. Herbarium specimens and recently obtained populations were grouped together by morphological studies under two distinct morphotypes depending mainly on fruit characters. SCoT-PCR technique was used to study the genetic diversity of the two morphotypes and separate two main clusters, and according to molecular results we treated the morphotypes as two distinct subspecies: subsp. *spinosa*, and subsp. *biparmata*. Pollen grains characters of the two subspecies were studied for the first-time using SEM. Chromosome numbers of the two subspecies of *Z. spinosa* were counted. The chromosome lengths and centromeric positions as obtained from mitotic chromosomal preparations were used to establish the first report of the two examined subspecies karyotype.

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Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability Statement

All data generated or analyzed during this study are included in this published article.

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Table (1). The geographical distribution of the studied *Z. spinosa* specimens (arranged from North to South).

No.	***Locality***	**GR**	***Lat.***	***Long.***	***Date***	***Collector***
1	Burg el Arab	M	30°55'24"	29°32'16"	1.4.1960	El Batanouny s.n. (CAI)
2	Burg el Arab	M	30°55'23"	29°32'35"	25.1.1978	S. Abd El Wahab s.n. (CAI)
3	Bahig *	M	30°55'21"	29°35'33"	10.2.2022	Amany Sallam s.n. (CAI)
4	Bahig	M	30°55'20"	29°35'40"	9.3.1978	M. Muller et al. s.n. (CAI)
5	Behiera province, Abu el Matamir	M	30°54'13"	30°10'16"	20.3.1987	Alaa Amer 9911 (CAI)
6	Ismailia	ED	30°35'39"	32°16'54"	15.2.1976	Adel Gazzar s.n. (CAI)
7	sinai, 5 km N of El Hassana	ED	30°24'33"	33°45'21"	4.4.1988	H. Hosni et al. s.n. (CAI)
8	Wadi Nasuri	ED	30°00'15"	31° 33'56"	28.12.1953	Täckholm, Imam and Sisi s.n. (CAI)
9	Wadi Digla *	ED	29°57'39"	31°19'45"	7.12.2020	Amany Sallam s.n. (CAI)
10	Wadi Digla *	ED	29°57'37"	31°19'35"	13.2.2022	Amany Sallam s.n. (CAI)
11	Wadi Digla	ED	29°57'35"	31°20'38"	23.10.1955	Mustafa Imam s.n. (CAI)
12	Eastern desert, Wadi Digla	ED	29°57'12"	31°20'25"	25.3.1985	M. G. Shaded s.n. (CAI)
13	Eastern desert, Wadi Digla	ED	29°57'11"	31°20'29"	25.4.2006	M. Abdel Aleem s.n. (CAI)
14	Wadi Hof	ED	29°52'33"	31°25'14"	8.3.1974	Nabil, Mahdi, Sisi and Abdel Aziz
15	Wadi Hof	ED	29°52'17"	31°25'54"	15.4. 1950	Ibrahim Said Ibrahim s.n. (CAI)
16	Wadi Hof	ED	29°52'11"	31°25'34"	15.4.1927	Gunnar Täckholm s.n. (CAI)
17	Wadi Hof *	ED	29°52'10"	31°24'49"	5.12.2020	Amany Sallam s.n. (CAI)
18	Wadi Hof	ED	29°51'59"	31°25'43"	11.2.1978	Gamal Fahmy s.n. (CAI)
19	Entrance of wadi Hof	ED	29°51'43"	31°25'13"	30.10.1952	Fikry Ibrahim Franois s.n. (CAI)
20	Helwan, near the spring	ED	29°51'32"	31°29'07"	18.11.1951	M. El Kassas s.n. (CAI)
21	Wadi Hagool, Suez Road	ED	29°47'52"	32°19'16"	1.12.2004	M. A. El Ghani and Reham A. Fattah s.n. (CAI)

Table (1) : Cont’						
22	Wadi Hagool *	ED	29°47'50"	32°19'22"	15.2.2022	Amany Sallam s.n. (CAI)
23	Wadi Qiseib, Res Sea	ED	29°24'13"	32°25'43"	9.2.1956	N. El-Hadidi s.n. (CAI)
24	Wadi Qiseib, N. Galala	ED	29°24'07"	32°25'33"	9.2.1956	M. Imam and A. Abd El Fadeel s.n. (CAI)
25	Wadi Aber, Gebel Ataqa, Suez	ED	29°59'13"	32°22 43"	9.2.1966	V. Täckholm s.n. (CAI)
26	Wadi Garawi, Helwan Desert	ED	29 48'32"	31 25 24"	29.2.1960	V. Täckholm *et al*. s.n. (CAI)
27	Wadi Araba, Res Sea, 10 km west of the road	ED	28°22'34"	33°30'25"	7.2.1960	V. Täckholm *et al*. s.n. (CAI)
28	El Ghardaqa, Res Sea	ED	27°13'36"	33 49 19"	3.4.1966	Francois Daumas s.n. (CAI)
29	Along the road Edfu- Mersa Alam, 12 km from Edfu	ED	24°58'27"	32°52'39"	2.2.1961	V. Täckholm *et al*. s.n. (CAI)
30	Mouth of wadi Ghadir, Red Sea coast	ED	24°48'35"	34°53'14"	5.2.1961	V. *Täckholm et al*. s.n. (CAI)
31	Bir Om Enaba, Res Sea	ED	24°21'34"	35°12'36"	11.2.1960	V. *Täckholm et al.* s.n. (CAI)
32	Wadi Khorreit 40 km east of Kom Ombo	ED	24°28'38"	33°10'45"	4.3.1961	V. Täckholm *et al.* s.n. (CAI)
33	Kom Ombo desert	ED	24°28'10"	32°55'45"	20.1.1927	Gunnar Täckholm s.n. (CAI)
34	Kom Ombo desert, Wadi Natash	ED	24°26'39"	34°03'55"	5.2.1964	V. Täckholm *et al*. s.n. (CAI)
35	Wadi Seyal, Res Sea	ED	23°39'54"	32°26'26"	9.2.1956	N. El-Hadidi s.n. (CAI)
36	Wadi Seyal, N. Galala	ED	23°39'23"	32°26'43"	9.2.1958	Amal Amin and Sami Kenawi s.n. (CAI)
37	Wadi Hodein, Gebel Elba	ED	23°14'34"	35°06'44"	11.2.1967	J. Osborn and Ibrahim Helmy s.n. (CAI)
38	A wadi across Gebel El-Shaieb, Res Sea	ED	22°03'44"	35°44'26"	11.2.1960	V. Täckholm *et al*. s.n. (CAI)

** localities of freshly collected specimens which were subjected to pollen, cytogenetic and molecular studies, GR: phytogeographical region, M: Mediterranean coastal strip, ED: Eastern Desert.*

Table (2). Characteristic morphological features of the studied *Z. 146pinose* morphotypes.

No.	**Character**	
1-	life cycle	perennial shrub
2-	Plant nature	Erect to ascending
3-	Stem branching	Densely dichotomously branched
4-	Stem length (cm)	50 to 150
Basal leaves		
5-	Leaf structure	simple
6-	Leaf length	2-10 cm
7-	Leaf width	2-5 cm
8-	Leaf shape	Spathulate
9-	Petiole length	2-3 cm
10-	Petiole width	2-4 mm
11-	Leaf margin	Entire, dentate or pennatifid
12-	Leaf apex	acute to obtuse
13-	Leaf base	cuneate
14-	Leaf color	green
15-	Stipules	absent
16-	Leaf surface	glabrous
Cauline leaves		
17-	Leaf structure	simple
18-	Leaf length (cm)	1.5-3
19-	Leaf width (cm)	(0.2) 0.25-0.4

Table (2) : Cont’		
20-	Leaf shape	Lanceolate to oblong
21-	Leaf margin	entire
22-	Inflorescence type	Racemes (few flowered)
23-	Flower color	Pink to violet or white
24-	Flower sexuality	hermaphrodite
25-	Flower pedicel length (mm)	1-2.5
26-	Flower pedicel width (mm)	1
27-	Epicalyx	absent
28-	Number of sepals	Four (two wide, two narrow)
29-	Sepal length (mm)	4.5-8
30-	Wide sepal width (mm)	2.5
31-	Narrow sepal width (mm)	1-1.5
32-	Sepal color	green
	Sepal shape	oblong-ovate
33-	Number of petals	four
34-	Petal length (cm)	0.7-1.8
35-	Petal width (cm)	0.2-3.5
36-	Petal shape	oblong-spathulate
37-	Number of stamens	6
38-	Length of long stamen filament (cm)	0.5-0.7
39-	Length of long stamen anther (cm)	0.4

Table (2) : Cont’		
40-	Length of short stamen filament (cm)	0.4-0.6
41-	Length of short stamen anther (cm)	0.4
42-	Width of filament and anther (mm)	1
43-	Ovary length (mm)	1-2
44-	Ovary width (mm)	1-2
45-	Style length (mm)	1.5-4
46-	Style width (mm)	0.5-1
47-	Stigma length (mm)	0.5-1
48-	Stigma width (mm)	0.5-1
49-	Fruit type	Indehiscent silicula
50-	Fruit shape	ovoid-globose (2-celled), tapering into conical beak
51-	Seed shape	ellipsoid
53-	Number of seeds per fruit	two (1 per locule)
54-	Seed color	black
55-	Seed diameter (mm)	1

Table (3). The list of primer, Total Number of Bands (TB), Monomorphic Bands (MB), Polymorphic Bands (PB), Percentage of Polymorphism (%P), Frequency (F) and Polymorphism Information Content (PIC) as revealed by SCoT analysis of 5 samples representing the two morphotypes.

PRIMER	TB	MB	PB	% P	F	PIC
SCOT-01	9	6	**3**	33	0.87	0.20
SCOT-02	11	5	6	55	0.66	0.35
SCOT-03	**17**	6	**11**	65	0.66	0.35
SCOT-04	13	7	6	46	0.69	0.31
SCOT-05	7	4	3	43	0.77	0.29
SCOT-07	13	3	10	77	0.55	0.37
SCOT-09	15	4	**11**	73	0.68	0.34
SCOT-11	13	4	9	69	0.63	0.36
SCOT-12	15	8	7	47	0.71	0.33
TOTAL	113	47	66	-	-	-
AVERAGE	12.5	5.2	7.3	56	0.69	0.32

Table (4). Similarity matrix among five samples according to Dice coefficient as revealed by SCoT markers.

	1S	2S	3B	4B	5S
1S	1.00				
2S	0.84	1.00			
3B	0.76	0.83	1.00		
4B	0.73	0.72	0.83	1.00	
5S	**0.85**	0.82	0.78	**0.70**	1.00

Table (5). Pollen morphological characters of the two subspecies of Egyptian *Z. spinose.*

Pollen character	subsp. *biparmata*	subsp. *spinosa*
Polar axis (P, µm)	31.85- (33.63) -35.4	32.9- (33.25) -33.6
Equatorial axis (E, µm)	16.8- (18.1) -19.3	16.45- (16.8) -17.15
P/E	1.86	1.98
Pollen shape	Prolate	Prolate
Colpus length (L, µm)	26.25- (28.53) -30.8	28- (29.24) -30.45
Colpus width (W, µm)	0.35- (0.44) -0.53	0.35- (0.44) -0.53
L/W	64.83	66.45
No. of lumina/µm	0.6- (0.75) -0.9	0.6- (0.75) -0.9
Lumina diameter (µm)	0.7- (1.84) -2.98	0.7- (1.75) -2.8
Muri width (µm)	0.53- (0.65) -0.77	0.35- (0.42) -0.49
Muri wall surface	Smooth	Smooth

Table 6. Karyotyping data for *Z. spinosa* subsp. *spinosa*, and subsp. *Biparmata*.

Chro. No.	*Mean Relative Length (MRL)*		*Mean Centromeric index (MCI)*			
	Z. spinosa subsp. *spinosa*	*Z. spinosa* subsp. *biparmata*	*Z. spinosa* subsp. *spinosa*		*Z. spinosa* subsp. *biparmata*	
1	7.95	9.16	48.76	m	49.53	m
2	7.68	7.96	47.73	m	46.24	m
3	7.53	7.1	47.45	m	48.19	m
4	7.45	7.02	46.88	m	48.78	m
5	6.89	6.93	47.3	m	48.15	m
6	6.87	6.8	45.76	m	47.8	m
7	6.46	6.76	48.11	m	48.1	m
8	6.24	6.76	46.64	m	49.37	m
9	5.64	6.08	43.3	sm	43.66	sm
10	5.53	5.91	44.21	sm	44.93	sm
11	5.53	5.82	44.21	sm	44.12	sm
12	5.47	5.22	44.68	sm	44.26	sm
13	5.18	5.13	44.94	sm+st	43.33	sm
14	5.18	4.96	44.94	sm	41.38	sm
15	5.18	4.28	44.94	sm+st	40	sm+st
16	5.18	4.11	44.94	sm	41.67	sm

m: Metacentric, sm: Submetacentric, st: satellite chromosome, L: large, M: medium, S: small.

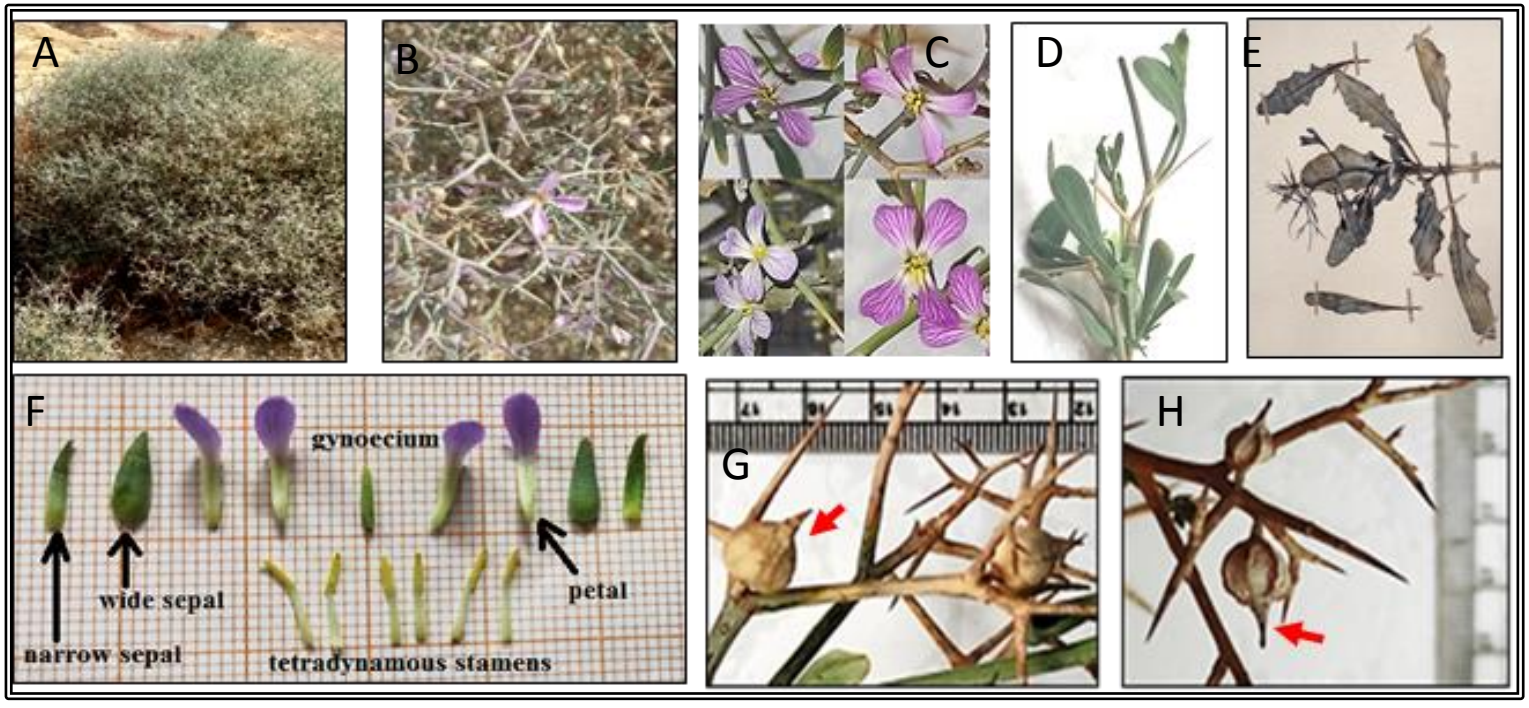

Fig. (1). A, B: Field pictures of *Z. spinosa* morphotypes; C: Morphological diversity in the flower of both morphotypes; D, E: Morphological diversity in the basal leaves within both morphotypes; F: Parts of the flower in both morphotypes; G: Fruit in first morphotype (silicula is smooth without outgrowths); H: Fruit in second morphotype (silicule is ribbed and irregularly wrinkled).

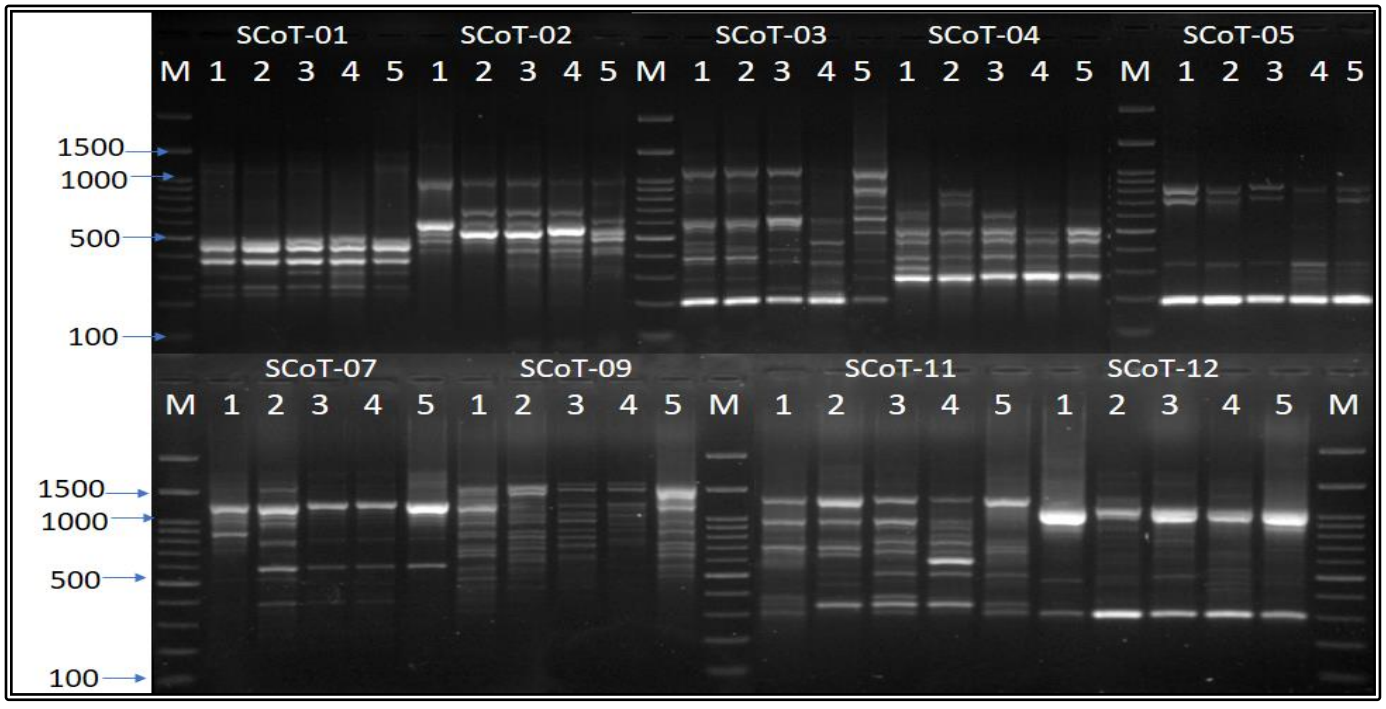

Fig. (2). SCoT profiles, the PCR patterns of the five samples using the nine SCoT Primers; (SCoT-01, SCoT-02, SCoT-03, SCoT-04, SCoT-05, SCoT-07, SCoT-09, SCoT-11 and SCoT-12). M: 100bp DNA ladder (Fermentas, Germany). Lanes 1 to 5 represent: 1S, 2S, 3b, 4b and 5S specimens, respectively (1S, 2S and 5S representing the first morphotype; 3b and 4b representing the second morphotype).

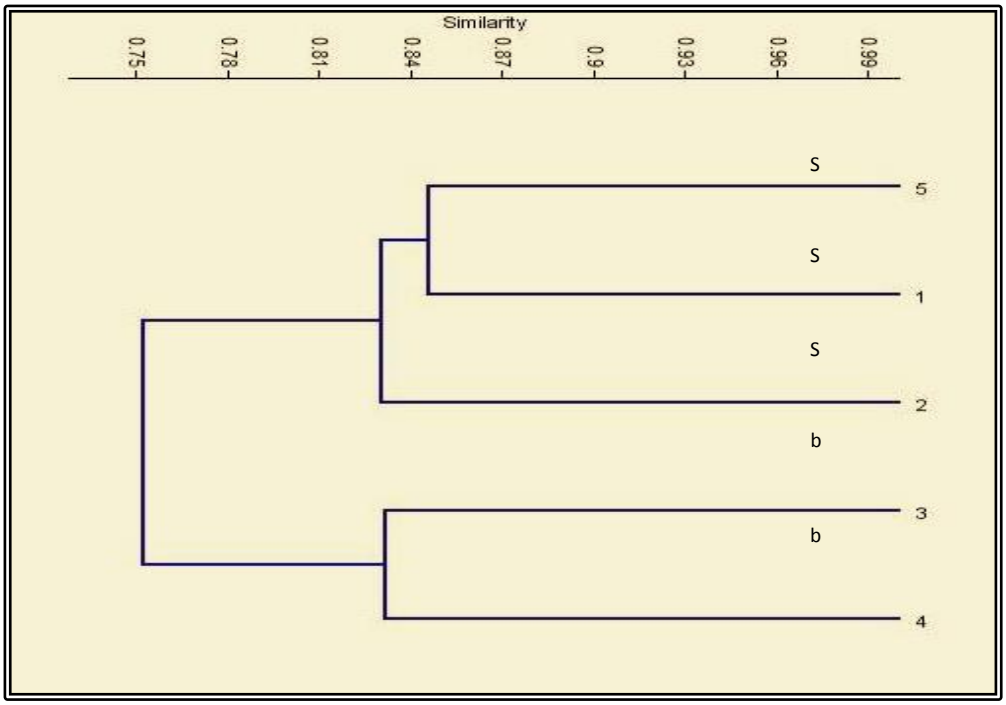

Fig. (3). Dendrogram for the five samples constructed from SCoT data using UPGMA and similarity matrix computed according to Dice coefficient.

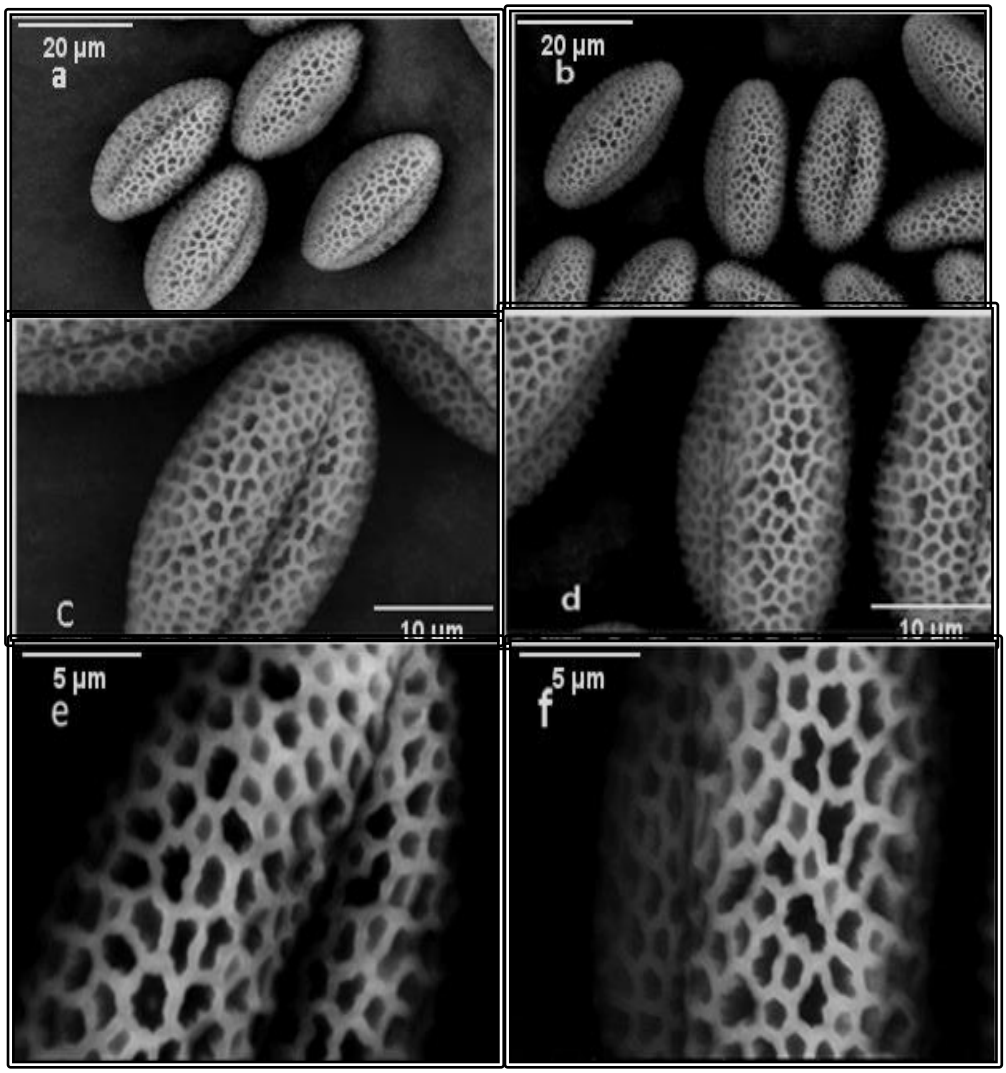

Fig. (4). Photomicrographs of pollen grains obtained with scanning electron microscopy (SEM) of *Z. spinosa* subsp. *biparmata* (a, c, e), and *Z. spinosa* subsp. *spinosa* (b, d, f) showing pollen shape and exine pattern.

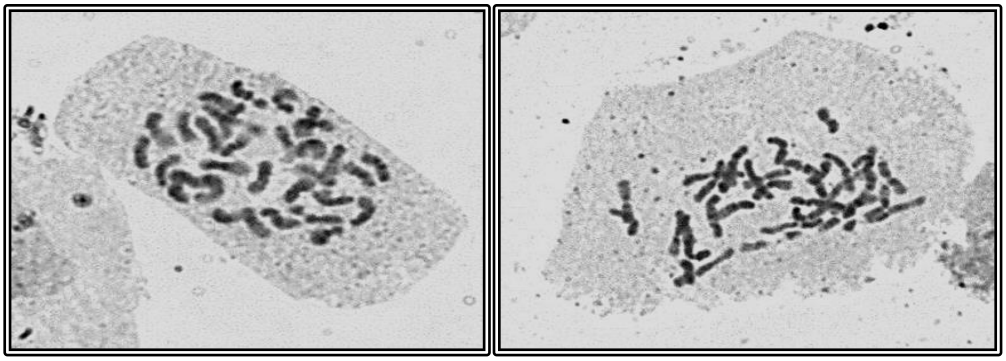

Fig.. (5). Photomicrographs of well spread mitotic metaphase in the two subspecies of *Z. spinosa*, a: subsp. *spinosa*; b: subsp. *Biparmata.*

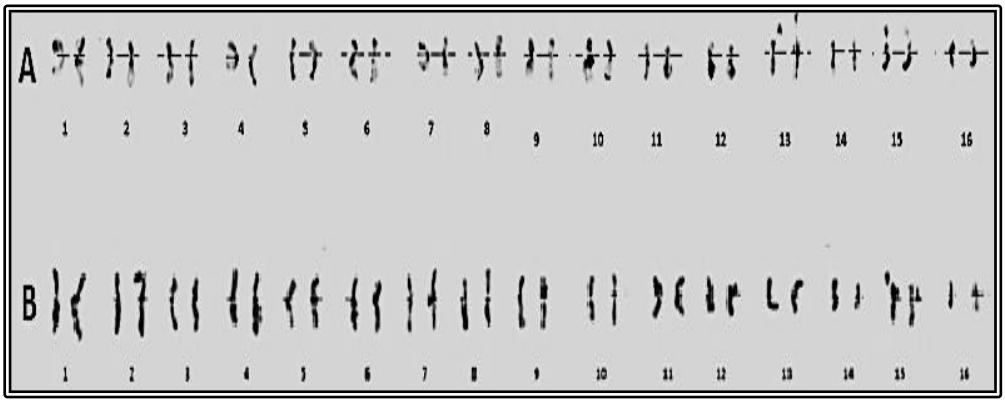

Fig. (6). A: Karyotype of *Z. spinosa* subsp. *spinosa* (2n=2x=32), B: Karyotype of *Z. spinosa* subsp *biparmata*. (2n=2x=32).

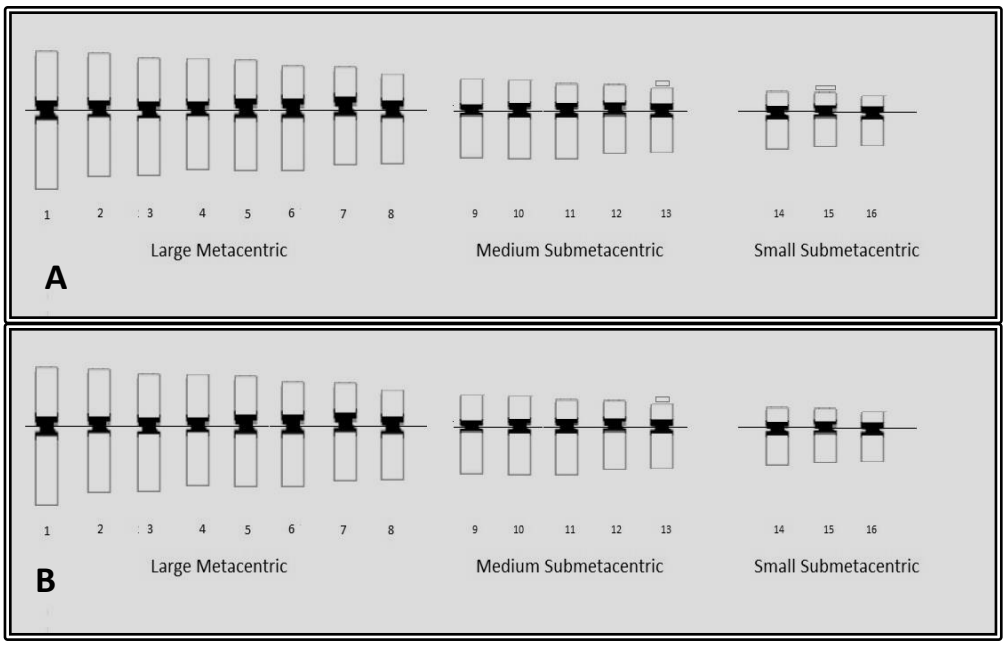

Fig. (7). A: Idiogram of *Z. spinosa* subsp. *spinosa*, B: Karyotype of *Z. spinosa* subsp *biparmata*. (2n=2x=32).

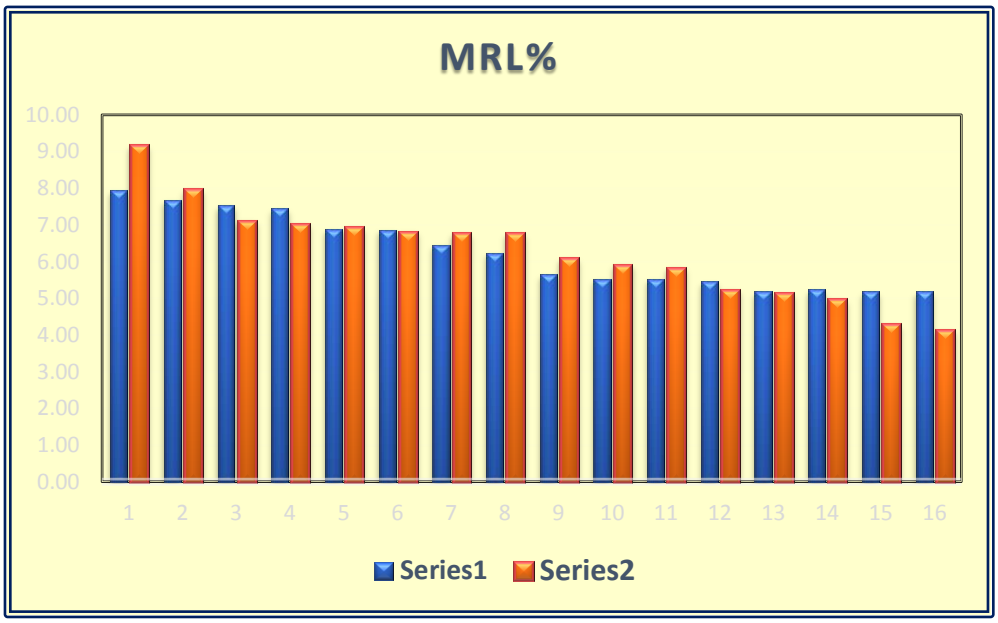

Fig. (8). Mean relative length (MRL) of each chromosome pair for *Z. spinosa* subsp. *spinosa* (series 1), and *Z. spinosa* subsp. *biparmata* (series 2).