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POLLEN GRAINS TAXONOMY MATCHES DNA-BARCODING TAXONOMY IN DISCRIMINATION BETWEEN SOME *CONVOLVULACEAE* TAXA

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Abstract

Palynological investigation relays on morphology and seed coat characters. It's a well-known approach to be used in discrimination between related plants. While the DNS barcoding used specific regions in the plant genome to identify the plant species/sub-species. Here, we compared pollen grain taxonomy and DNA barcoding taxonomy of 12 taxa belonging to the *Convolvulaceae* family growing in Egypt. The pollen grains were examined by the scanning electron microscope (SEM) then their features were recorded in a data matrix to compare their characters and create a phylogenetic tree. The gene sequences of the MatK gene, that is wildly used in the DNA barcoding, of the same 12 plants were compared to build another phylogenetic tree based on the sequence similarity between the gene sequences. Our results show high similarity between the two phylogenetic trees indicating the high efficiency of the pollen grain taxonomy and a promising integration of micro-morphological approaches and molecular approaches in discrimination between plants.

Keywords**:** Pollen grains taxonomy, DNA barcoding, *Argyria, Convolvulus, Ipomoea, Merremia.*

Introduction

A palynological investigation is a popular tool in plant taxonomy, especially with the invention of the high-resolution power microscopes as the scanning electron microscopes (SEM). The basis of this branch is Erdmann's 1952 handbook of palynology and its second edition by Nilson and Praglowski (Erdtman, 1952). On the other hand, the modern systematics communities depend on genetic bases (Wen *et al*., 2017). DNA barcode is the most popular DNA-based method for species identification and taxonomy clarification (Hollingsworth *et al*., 2011). Several chloroplast genes, intragenic and intergenic regions are used as a plant DNA barcodes such as trnfM-trnT, trnH-psbA, trnL(p6) and ycf1 (Awad *et al*., 2017; Choi *et al*., 2015; Kress *et al*., 2005) The consortium for the barcode of life adopted MatK and rbcL chloroplast genes as core genes for plant taxonomy, identification and systematics (Hollingsworth *et al*., 2009).

The taxonomic significance of pollen morphology in *Convolvulaceae* has long been recognized. Hallier (1893) was the first to divide this family based on pollen features into two groups, *Echinoceniae* and *Psiloconiae* based on their echinate or psilate exine, respectively (Hallier, 1893). The genus *Convolvulus* was put in *Psiloconiae* in which the pollen has either a psilate or a granulated surface. In the division of Gamble 1923, the family was divided into two groups based on echinate and non-echinate pollen grains with the genus *Convolvulus* included in the latter one (Gamble, 1923). Erdtman 1952 separated the *Convolvulacious* pollen grains into two groups. Namely *Ipomoea* type and other types, in which he includes genus *Convolvulus* (Erdtman, 1952). The *Ipomoea* type possesses pollen grains which are polyporate with a thick exine and are echinate, whereas the *Convolvulus* type is distinctly perforate. O. Donell 1955 separated genus *Convolvulus* and *Calystegia*on the basis of Halliers aperture descriptions, but he reported that in *Convolvulus* the pollen is 3-colporate (Ṓ Donell, 1955).

Sengupta 1972 carried out a comprehensive study in the family and divided it into four main pollen types based on the number and distribution of apertures (Sengupta, 1972). Pollen characters provide taxonomically valuable information that has long been used in the classification of the *Convolvulaceae*. Indeed, many keys require knowledge of the pollen surface features and apertures to arrive at a genus name (Austin *et al*., 1998). According to Boulos 2000, 12 Convolvulaceae species are growing in Egypt (Boulos, 1999). In this article are using pollen characters and the sequence of MatK gene for the 12 species which grow in Egypt to discrimination these species and compare the pollen characters and the DNA barcoding results.

Materials and Methods

Taxa acquisition

Twelve taxa of family *Convolvulaceae*, some species of genus *Convolvulus* collected from Marsa Matruh, a resort town on Egypt's Mediterranean coast, and the other taxa were collected from the Herbarium of Orman garden (Giza governorate, Egypt) through 2014 and 2016 (Table.1). The identification of the collected specimens was achieved by comparing their morphological characters with the characters of the previously identified plants as published by (Boulos, 1999).

Pollen grains imaging

Pollen grains were separated from anthers, for each taxon three specimens were used, and from each specimen at least five anthers were examined. For Scanning Electron Microscope (SEM) examination, pollen grains were coated by a gold sputter coater (Sp1-Module), then examined by SEM (JEOL JSM-5500LV) JEOL Ltd., Japan, by using low vacuum mode at a magnification of 1200-1600x at the Regional Center of Mycology and Biotechnology, Cairo, Egypt. We used the terminology of Punt *et al*. 2007 and

Sugandha to describe the morphological characters (Punt *et al*., 2007).

Bioinformatics and data analysis

Multi-Variate Statistical Package Programme (MVSP) was used to analyze the data of pollen grains characters (Sneath, 1973). Twelve MatK sequences were retrieved from NCBI for the 12 species (Table 1). TTCATTACTCKAAAGAGGTC and AADATTTCTGYA TATACG manually designed and used as a forward and reverse primers respectively, for *In-silico* PCR. A sequence length of 675 bp from the MatK gene was used for multiple sequence alignment and maximum likelihood (ML) phylogenetic tree construction using CLC v.7 (CLC bio, Aarhus, Denmark) with 100 bootstrap replicates. We used iTol v.5 for tree visualization (Letunic and Bork, 2019). The trees were redrawn for clarity and the original trees are included in the supplementary files for references (Figure S1).

Results and Discussion

Palynological investigation

The pollen taxa exhibited a wide range of size variation. All investigated taxa of three genera *Argyria*, *Ipomoea* and *Merremia* are large (50-100um) while genus *Convolvulus* is medium (25-50um). All taxa are isopolar except *C. arvensis* (Figure 1-c) and *C. oleifolius* (Figure 1-f) are apolar. All taxa show radically symmetrical as in *Argyria*, *Ipomoea* and two species belonging to *Convolvulus* genus (*C. dorycnium* and *C. pilosellifolius*) (Figure 1-d and g) while the other species are bilateral such as (*C. althaeoides*, *C. arvensis*, *C. lineatus* and *C. oleifolius* (Figure 1-b, c and f). The examined taxa were separated into two groups, a group with colpate aperture (tricolpate) included genus *Convolvulus* and *Merremia*, (Figure 1-b, c, d, e, f, g and i) and the other group with porate (polyporate) included *Argyria* and *Ipomoea* (Figure 1-a, h, i, j and k).

According to the ratio between polar axis and equatorial diameter in the equatorial view determined by the shape of pollen grains, most of the examined taxa are spheroidal shapes such as *Argyria nervosa*, *Ipomoea stolonfera* and *Merremia dissecta* (Figure 1-a, k, and l), two taxa only have prolate shape, *Convolvulus arvensis* and *C. oleifolius* (Figure 1-c and f) while *C. althaeoides* only has prolate-spheroidal (Figure 1-b) and *Ipomoea cairica* has oblate shape (Figure 1-h). Sculpturing is granulate in all species of *Convolvulus* and *Merremia dissecta*, (Figure. 1-b, c, d, e, f, g and i) while it is echinate in *Argyria nervosa* and species of *Ipomoea* (Figure. 1-a, h, i, j and k). Polar axis length varies from 35µm. to 91µm. Equatorial diameter varies from 20µm. to 91µm. while aperture length varies from 5µm. to 35µm. All observed palynological characters (both quantitative and qualitative) of 12 investigated taxa belonging to family *Convolvulaceae* are listed in Table 1 and the SEM images are shown in Figure 1.

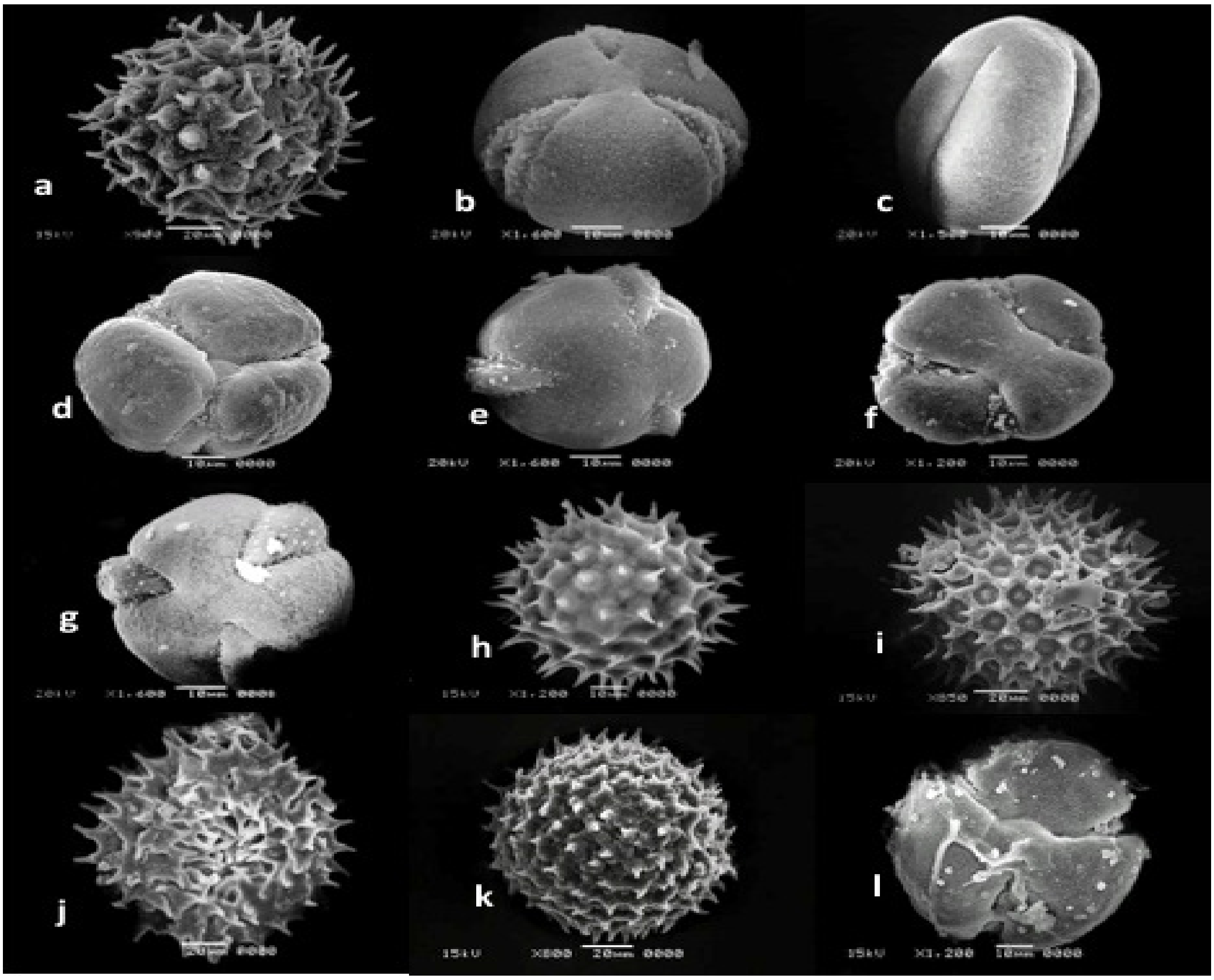

Fig. 1 : SEM images of pollen grains of the 12 taxa of the family *Convolvulaceae.* A) *Argyria nervosa, B) Convolvulus althaeoides,* C) *C. arvensis,* D) *C. dorycnium,* E) *C. lineatus,* F) *C. oleifolius,* G) *C. pilosellifolius H) Ipomoea cairica,* I) *I. eriocarpa*, J) *I. hederacea* K) *I. stolonfera* and L) *Merremia dissecta*

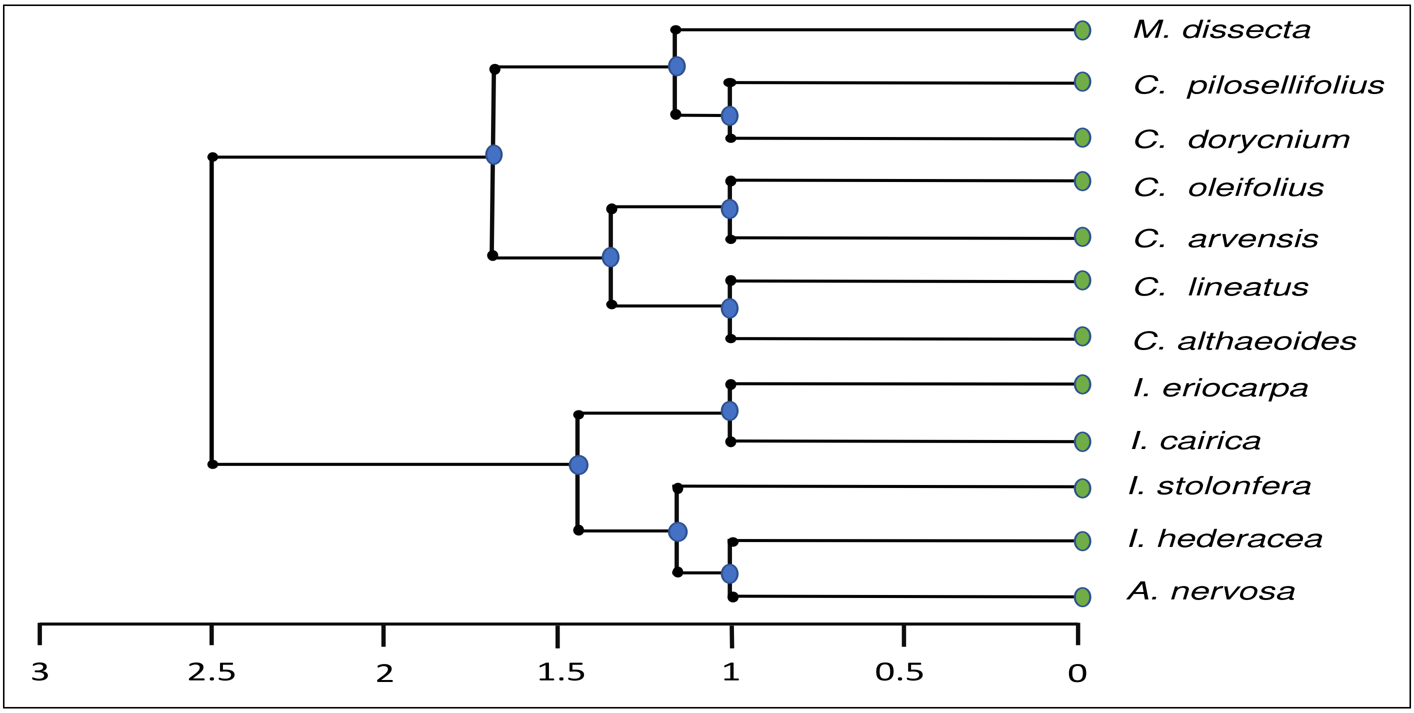

Fig. 2-A

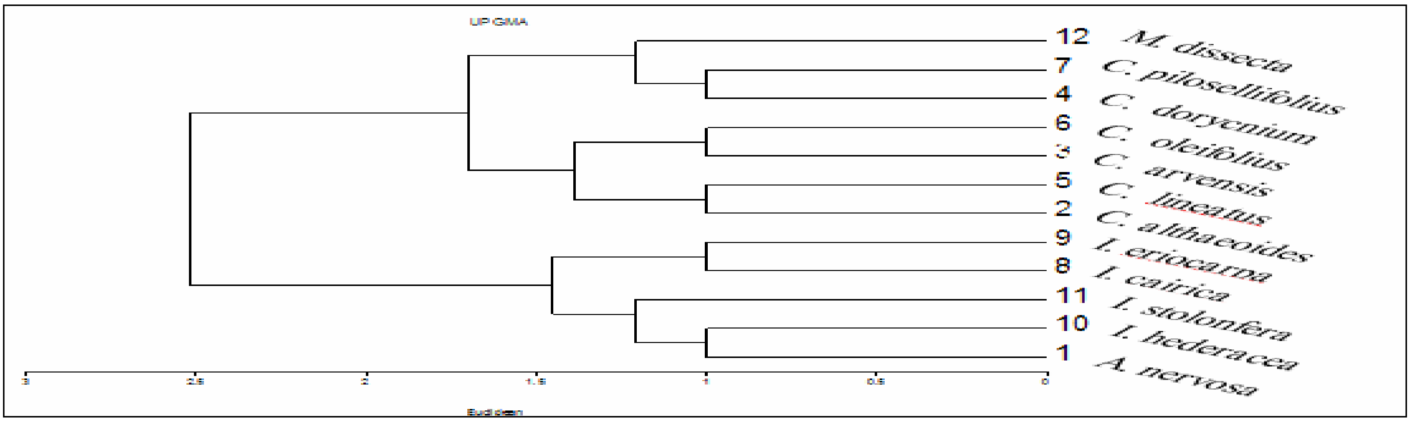

Fig. 2-B

Fig. 2 : The phylogenetic trees resulted from **A)** the Palynological investigation and **B)** the DNA-barcoding using MatK gene sequence. iTol v.5 was used for tree visualization and the trees were redrawn for clarity.

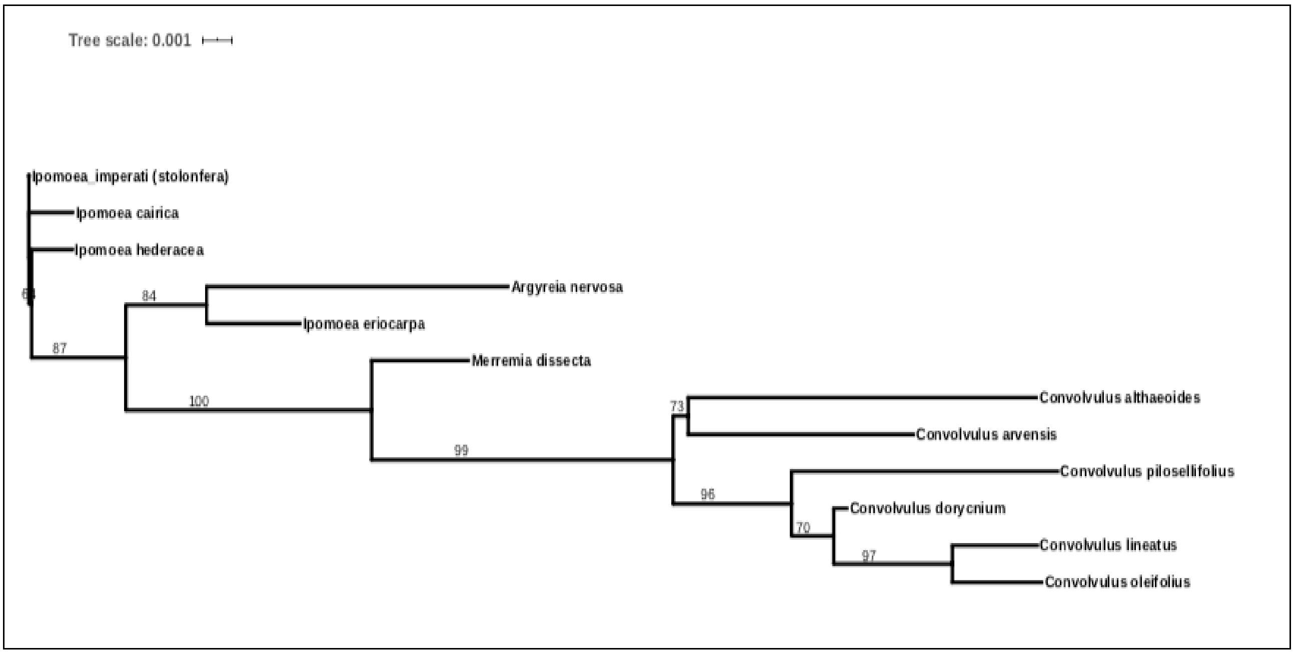

Fig. S1 : Original phylogenetic trees. A) Dendrogram is constructed on the basis of 12 pollen grains features. B) The phylogenetic tree resulted from the MatK gene sequence analysis.

These results are in agreement with the known features of pollen grains of *Convolvulus arvensis* (medium size, prolate shape, and tricolpate) (Ashfaq *et al*., 2018). Also, it is in agreement with what was reported about *Ipomoea cairica* in size and sculpturing but contrary to wat reported about its shape, which was reported as subspheroidal (V *et al*., 2011). The four species belonging to genus *Ipomoea* are in agreement with the present work in the type of sculpturing and the present work in features of *Argyreia nervosa* in symmetry, polarity, aperture type, shape and sculpturing (Saensouk, S. and Saensouk, 2018).

Cluster analysis

Cluster analysis produces a phylogenetic tree of 12 taxa, based on the data matrix (Table 1). The resultant phylogenetic tree is shown in Figure 2A which shows that the studied taxa are divided into two groups. The first group includes all species belonging to genus *Convolvulus* plus *Merremia dissecta* which showed clear similarity with *Convolvulus* except in the pollen grain size. Species of *Convolvulus* differed in polarity, similarity, the shape of pollen grains in addition to polar axis and equatorial axis as shown in Table 1. The second group includes both *Argyria nervosa* and *Ipomoea* species and we depend on statics characters to discriminate between *Ipomoea* species and *Argyria.* Both were similar in polarity, symmetry, apertures type, number of aperture and exine sculpturing. The present numerical analysis results were in harmony with those obtained before on some species of fabaceous plants (Khattab and El–Kobisy, 2007).

DNA-barcoding investigation

To investigate the agreement between palynological taxonomy and genomic taxonomy in *Convolvulaceae* members discrimination we compared the MatK gene sequence in the 12 taxa. MatK is a chloroplast gene that is widely used for taxonomical and species identification purposes. A representative MatK sequence was retrieved from NCBI for each species. Then, we designed a forward and a reverse degenerative primers (see methods) to unify the sequence borders to avoid the sequence bias coming from unaligned nucleotides in flanks. In total, 675 pb used for ML gene tree construction. MatK phylogenetic tree showed a harmonious taxonomical topology with those palynological taxonomy approaches (Figure 2B).

Convolvulus species distinguished in a separate clade with a sister lineage *Merremia dissecta*, both shared the same ancestral node. This clade agreed with *colpate operture* clade in palynological dendrogram (Figure 2B). On the other hand, *Ipomoea* species showed a gradual lineage development from *I. imperati* (stolonfera), *I. cairica* and *I. hederacea*, respectively, then *I. eriocarpa* and *Argyria nervosa* as a sister lineages from the same internal node (Figure 2B). The overall figure agreed with porate clade in palynological dendrogram by grouping *Argyria nervosa* with *Ipomoea* species. Moreover, this agreed with the modern taxonomy (NCBI:txid139741). The only difference is that the Palynological dendogram shows that *Argyria nervosa* has an internal node with I. *hederacea*, while the MatK phylogenetic tree and Pollen grain identification key showed *I. eriocarpa* is closer to *Argyria nervosa* than *I. hederacea*. This also agreed with the whole chloroplast phylogenetic tree (Eserman *et al*., 2014).

Identification key of the 12 *Convolvulaceae* family taxa based on the observed pollen grains features

1- Pollen grains: tricolpate, granulate
  a-pollen grains, large size (50-100) µm, spheroidal…... *Merremia dissecta*
  b-pollen grains, medium size (25- 50)µm
    bb- pollen grains, bilateral
      bb1- pollen grains, prolate-spheroidal……. *C. althaeoides*
      bb2- pollen grains, spheroidal……………….. *C. lineatus*

  c- pollen grains, radially symmetrical, spheroidal shape
      c1- polar axis 40um…………………..…… *C. dorycnium*
      c2- polar axis 35um. ………………… *C. pilosellifolius*

  d- pollen grains, isopolar, prolate shape
      d1- polar axis 45um……………………….. *C. arvensis*
      d2- polar axis 30um. ……………………… *C. oleifolius*

2- Pollen grains polyporate, echinate
  A. pollen grains, spheroidal shape
    A1- polar axis 90um., aperture length 7.5um. … *Argyria nervosa*
    A2- polar axis 84um., aperture length 6um. …… *I. eriocarpa*
    A3- polar axis 89um., aperture length …… *I. hederacea*
    A4- polar axis 91um., aperture length 7.7um. .. *I. stolonfera*

  B. pollen grain, oblate shape, polar axis 40um. aperture length 5um. ….. *I. cairica*

Conclusion

We investigated the taxonomy of all the members of the *Convolvulaceae* family that grow in Egypt, 12 taxa in total. We used palynological taxonomy based on pollen grain characters observed through SEM imaging and genomic taxonomy through the analysis of the MatK chloroplast gene sequence. We built two phylogenetic trees based on the two analyses and compare them to investigated the agreement between the two approaches and we created an identification key for the discrimination between the 12 taxa based on the palynological analysis. Our results show that the 12 taxa are divided into two groups, a group includes all species belonging to genus *Convolvulus* plus *Merremia dissecta* and another group includes both *Argyria nervosa* and *Ipomoea* species. Furthermore, our results show a harmony between the palynological and genomic approaches as both of them gave similar results.

Table 1 : Data matrix and accession numbers of the observed characters in the different taxa. The data matrix based on 11 characters of 12 taxa.

	Taxa	**Accession Number**	**Polar axis µm**	**Equatorial axis µm**	**P/e µm**	**Shape of pollen grain***	**Polarity Apolar+/isopolar-**	**Symmetry Radially symmetrical+/bilaterial-**	**Size Large+/medum-**	**Number of aperture Tri+/poly-**	**Apertural type Colpate+/porate-**	**aperture length µm**	**Exine sculpturing Granulate+/echinate-**
1	*Argyria nervosa*	KR024898	90	90	90/90	S	+	+	+	-	-	7.5	-
2	*Convolvulus althaeoides*	KC529015	45	42	45/42	P-S	+	-	-	+	+	18	+
3	*C. arvensis*	KC529020	45	28	45/28	P	-	-	-	+	+	35	+
4	*C. dorycnium*	KC529040	40	40	40/40	S	+	+	-	+	+	20	+
5	*C. lineatus*	KC529081	40	37	40/37	S	+	-	-	+	+	12	+
6	*C. oleifolius*	KC529089	30	20	30/20	P	-	-	-	+	+	12	+
7	*C. pilosellifolius*	KC529016	35	35	35/35	S	+	+	-	+	+	12	+
8	*Ipomoea cairica*	FJ795793	40	60	40/60	O	+	+	+	-	-	5	-
9	*I. eriocarpa*	KF242483	84	84	84/84	S	+	+	+	-	-	6	-
10	*I. hederacea*	MG973747	89	89	89/89	S	+	+	+	-	-	7	-
11	*I. stolonfera*	MF064347	91	91	91/91	S	+	+	+	-	-	7.7	-
12	*Merremia dissecta*	KR024956	68	68	68/68	S	+	+	+	+	+	5.5	+

* S: spheroidal, P-S: prolate-spheroidal, P: prolate, O: oblate)

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