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ORIGINAL ARTICLE

MORPHOLOGICAL, PALYNOLOGICAL, AND MOLECULAR STUDIES ON *PSEUDERUCARIA CLAVATA* (BOISS. & REUT.) O. E. SCHULZ (1916) (BRASSICALES, BRASSICACEAE) IN EGYPT

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ABSTRACT

No detailed morphological, palynological, or molecular studies had previously been conducted on *Pseuderucaria clavata* (Boiss. & Reut.) O. E. Schulz (1916) (Brassicales, Brassicaceae). This species exhibits two distinct forms with different flower colors: violet and white. A taxonomic revision of these color forms identified two subspecies *Pseuderucaria clavata* (Boiss. & Reut.) O. E. Schulz subsp. *clavata* (1916), and *P. clavata* subsp. *tourneuxii* (Cross.) Maire (1933). Molecular study using the chloroplast barcoding markers rbcL and matK to elucidate the taxonomic relationships approved, and supported that the two color forms represent two different subspecies. The sequences of the two confirmed subspecies have been documented and deposited in the global database under the accession numbers OR573855, OR573856, OR573857, OR573858, OR573859, OR573860, OR636847, OR636848, OR636849, OR636850, OR636851, and OR636852. Additionally, the analysis of pollen grains characteristics in both subspecies confirmed the high taxonomic value of pollen features.

Keywords: Barcoding, Egypt, Pollen, *Pseuderucaria clavata*, Subspecies.

INTRODUCTION

Brassicaceae (Cruciferae) or mustard family is one of the largest Angiosperm families, comprising 52 tribes, 351 genera, and 3977 species (Kiefer *et al.*, 2014), mainly distributed in temperate areas with highest diversity in Mediterranean, Irano-Turanian and west north American regions (Tai-yien *et al.*, 1987). This family is one of the ten most economically important plant families (Rich, 1991), as it contains a considerable diversity of cultivated food crops. Many of these crops are used to produce oils and animal fodder as well (Warwick, 2010). There is also a wide range of condiments and ornamental plants. *Pseuderucaria* O.E. Schulz (1916) is one of the genera of family Brassicaceae in Egypt belonging to tribe Brassiceae de Candolle (1821) (German *et al*., 2023). Genus *Pseuderucaria* O.E. Schulz,

1916 is represented by two species *P. teretifolia* (Desf.) O.E. Schulz which is native to Egypt, Libya, Tunisia, and Algeria (Dobignard and Chatelain, 2011), and *Pseuderucaria clavata* (Boiss. and Reut.) O.E. Schulz. which tolerates high temperature and is found in sandy and stony deserts and plains. It is native to Algeria, Tunisia, Libya, Egypt, Palestine, and Jordan. There are two accepted subspecies representing *Pseuderucaria clavata* which are *P. clavata* subsp. *clavata* (Boiss. & Reut.) O.E. Schulz, and *Pseuderucaria clavata* subsp. *tourneuxii* (Coss.) Maire (Roggero *et al*., 2002). *Pseuderucaria clavata* subsp. *clavata* which is native to Egypt and Palestine (Dobignard and Chatelain, 2011).

Pollen morphology of family Brassicaceae is more closely related to Tamaricaceae where both families have tricolpate pollen with reticulate tectum (Perveen et al., 2004). According to the number and position of apertures, the pollen grains belong to the trizonocolpate aperture class and have reticulate or finely reticulate exine ornamentation (Erdtman, 1972; Moore and Webb, 1978; Reile, 1992; El Ghazali, 1993; Abdel Khalik *et al.*, 2002).

Bicolor in flowering plants can be caused by unstable flora gene alleles, infections, or genetically inherited patterns (Lokko and Josephine, 2010). Recently, DNA barcoding has gained popularity as a credible method of species identification. It is employed globally to support taxonomy, biodiversity assessment, and genetic resource conservation, as well as to get beyond the limitations of morphology-based taxonomy (Ho *et al*., 2021). rbcL and matK coding genes were used to carry out a phylogenetic analysis of 16 Egyptian species of Brassicaceae by Abdelhady *et al*. (2021). RbcL and matK, as potential DNA barcodes were used to distinguish Brassicaceae economic species by Sun *et al*. (2015).

The aim of this study is investigate the variation range of the examined *P. clavata* populations, to identify the taxonomic status of these color forms and document these taxa using DNA markers. Additionally, the study seeks to clarify the molecular relationships among these color forms, and determine the pollen grain characters of these two color forms using SEM.

MATERIALS AND METHODS

Plant material: The herbarium specimens housed at Cairo University herbarium, along with other authentic specimens housed in virtual herbaria available online (the JSTOR Global Plants database) were examined, Index Herbariorum is followed for acronyms, and authors' names are abbreviated using IPNI (Brummit and Powell, 1992). Fieldwork conducted between 2020 and 2023; fresh representative specimens of *Pseuderucaria clavata* belonging to 50 populations were collected from the Eastern Desert (10 populations from five different localities with coordinates: 29°53'33" & 31°52'20", 29°58'14" & 31°20'04", 30°01'34" & 31°38'29", 30°04'18" & 31°19'50", and 30°06'39"& 31°37'15"). 20 individuals / population were investigated to determine the morphological diversity in all localities. Different stem, leaf, flower, and fruit morphological criteria were examined. Voucher specimens were placed in (CAI).

Sample Preparation for Scanning Electron Microscope (SEM): For the pollen study, Wadi Digla was selected as the study site, as it represents both colour forms of *P. clavata*. Where the two color forms of *P. clavata* were represented. Fresh anthers were collected from the floral buds of the studied color forms. The Investigated anthers were placed through an ETOH dehydration series, and dried using the liquid CO2 (carbon dioxide)-based CPD drying technique with a Denton DCP-1 Critical Point Drying device. Using this method, surface tension didn't influence the removal of liquid from tissues. Pollen samples were prepared and scanned at 20 kv using a JEOL 1200 EX SEM (JEOL, Japan). Size measurements were recorded from 25 randomly selected grains. The pollen terminology followed the guideline of Punt *et al*., (2007).

Molecular study: The genomic DNA was extracted from one gram of juvenile leaves (3 samples/color form) that had been powdered in liquid nitrogen using the CTAB (Cetyl-trimethyl ammonium bromide) extraction buffer approach, as described by Doyle and Doyle (1990) and modified by Allen *et al*. (2006).

matK and rbcL barcoding analysis:

PCR Reactions: PCR amplification reaction mixture for rbcL and matK included 40 ng DNA, 1x buffer (Promega), 0.2 mM dNTPs, 15 mM MgCl2, 1µ of Taq DNA polymerase (GoTaq, Promega), 20 pmol of each primer, and ultra-pure water to a final volume of 50 µL (Wattoo *et al*., 2016). The primer sequences were as follows: matk-F: 5'-CGATCTATTCATTCAATATTTC-3' and matk-R: 5'-TCTAGCACACGAAAGTCGAAGT-3'; with 900bp/each and rbcL-F: 5'-ATGTCACCACAAA CAGAGA CTAAAGC-3' and rbcL-R: 5'-TCGCAT GTACC TGCAGTAGC-3'; with 600bp/each.

PCR amplification and product detection: The Perkin Elmer/ GeneAmp® PCR System 9700 (PE Applied Biosystems) was used to carry out the PCR amplification, which was designed to complete 40 cycles following the initial denaturation cycle, which would last for 5 min at 94°C. Each cycle was made up of three steps: a denaturation step at 94°C for 30 seconds, an annealing step at 50°C for 30 seconds, and an elongation stage at 72°C for one minute. The primer extension phase was prolonged in the last cycle to 7 min at 72°C. By electrophoresis on a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TBE buffer, run at 95 volts, the PCR products were detected and separated (El-Sayed, 2022). A 100 bp DNA ladder was used as a molecular size standard. UV light was used to view PCR products, and they were photographed using (BIO-RAD 2000) Gel Documentation System (Abdo *et al.*, 2023).

PCR Products Purification: PCR reaction mixture was transferred to a 1.5 ml microfuge tube, and then three volumes of binding buffer 1 were added. The mixture solution was centrifuged after standing in the EZ-10 spin column for 2 minutes at room temperature. The column was then centrifuged for two minutes at 10.000 rpm after adding 750 µl of wash solution. The column was washed again and spun at 10.000 rpm for another minute to eliminate any leftover wash solution. The column was transferred to a clean 1.5 ml microfuge tube, then 50 µl elution buffer was added, and the mixture was kept for 2 minutes at room temperature. Pure DNA was then stored at -20 °C (Elian *et al*., 2021).

matk and rbcL sequencing analysis: The PCR product was sequenced in an automated sequencer ABI PRISM 3730XL Analyzer using Big Dye TM Terminator Cycle Sequencing Kits and the manufacturer's suggested methods (Mohdly *et al.*, 2023).

Computational analysis: For *P. clavata* color forms, the obtained sequences were aligned using the BLAST (Basic Local Alignment Search Tool), which is available online at http://www.ncbi.nlm.nih.gov/BLAST. MEGA5 was used to create a phylogenetic tree, and determine pairwise distances using the sequences that were deposited on the gene bank with the following accessions OR573855, OR573856, OR573857, OR573858, OR573859, OR573860, OR636847, OR636848, OR636849, OR636850, OR636851, and OR636852.

RESULTS

Pseuderucaria clavata (Boiss. and Reut.) O.E.Schulz (1916)
Pseuderucaria clavata (Boiss. and Reut.) O.E.Schulz, Beibl. Bot. Jahrb. Syst. 119: 54 (1916).
Basionym: *Moricandia clavata* Boiss. & Reut.

P. clavata is an annual, glabrous, glaucous herb; tap root length 8-11 cm; stem terete, ascending, branching from the base, by 10-40 cm; leaves are petiolate, fleshy, 1-9 cm long, pinnatisect, with linear, terete, obtuse lobes; racemes loose, and few flowered; flowers are conspicuous, ebracteate, pedicellate, sepals violet, or violet with green tip. The two outer sepals 7.5-10 × 2.5-5 mm, and the two inner sepals 7-9 × 1-1.5 mm. The length of petals with long clawed, is 1.3-2.3, and limb width 0.5-0.7 cm. Filaments of long stamens 7-10 x 0.5-2 mm; anthers yellow, 2-4 x 0.5-1 mm, filaments of short stamens 6 x 0.15 mm; anthers 2.5 x 0.8 mm. Ovary, 5.5-9 x 0.9-1 mm; style short, 0.5-1 x 0.2-1 mm, stigma 0.5 x 0.4-1 mm; pedicel 2-8 x 0.5-1mm, accrescent in fruit. Fruit linear siliqua, 4-6.5 x 0.1-0.15 cm. Seeds are small, brownish, 100-120 per fruit, in 2 rows, ellipsoid, and compressed.

P. clavata exhibits two color forms that are identical in all morphological features except the color of petals. The first form has flowers with violet petals, while the flowers of the second form having white to pale violet petals (Plate 1). The geographical distribution of the two color forms of *Pseuderucaria clavata* in Egypt co-existing along the mediterranean coast, in Nile Delta, and Sinai. The studied populations of *Pseuderucaria clavata* were collected from the sandy and stony deserts, as well as plains in Wadi Digla, and along the Cairo-El Qattamia Suez Road (Galala Desert).

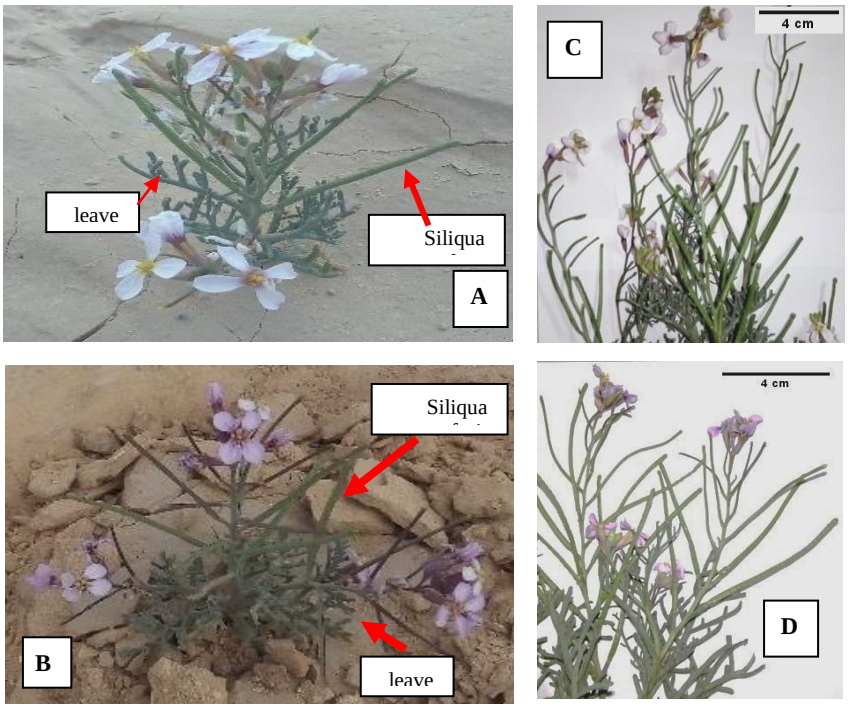

Plate (1): Phenotypic features of the *Pseuderucaria clavata* subspecies, A & C subsp. *tourneuxii*; and B & D subsp. *clavata*.

Genetic correlation between the two color forms of *P.* clavata: According to the phylogenetic dendrogram created using matk sequences, two clusters were identified (Diag. 1). All accessions of the first form with violet flowers (M1, M2, and M3) generated the first cluster, with very low genetic distance ranging from 0.003 between M2 and M3 to 0.008 between M1 and M3. The second clusters included all accessions of the second form characterized by white to pale violet flowers. The genetic distances between the accessions were also very low ranging from 0.001 between M4 and M5 to 0.016 between M4 and M6.

Similarly, the phylogenetic dendrogram based on rbcl sequences also split into two main clusters (Diag. 2). The first cluster included all accessions of the first form (R1, R2, and R3) with very low genetic distance ranging from 0.001 between R1 and R3 to 0.002 between R2 and R3. The second form accessions (R3, R4, and R5) constructed the second cluster with a genetic distance of 0.001.

The molecular data indicated that genetic factors were responsible for the phenotypic variability between the two identified color forms, leading to their classification as two subspecies: *P. clavata* subsp. *clavata* represent the first form (violet) and *P. clavata* subsp. *tourneuxii* represent the second color form (white). The confirmed sequences of the two subspecies were the first to be added to the global database. Our nucleotide sequences of both

matK and rbcL genes were deposited with the following accession codes OR573855, OR573856, OR573857, OR573858, OR573859, OR573860, OR636847, OR636848, OR636849, OR636850, OR636851 and OR636852.

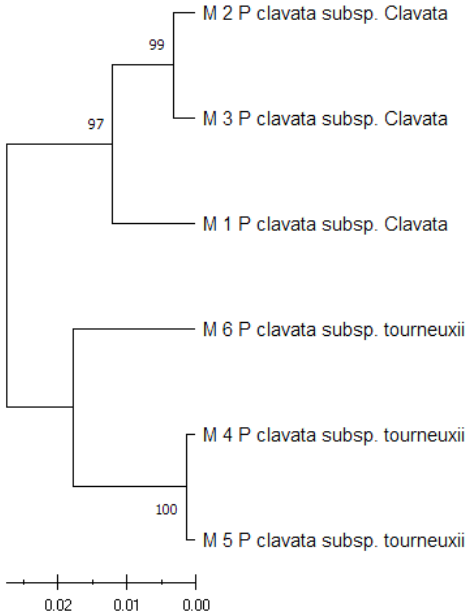

Diagram (1): Phylogenetic dendrogram based on matK sequences, constructed by UPGMA based on values of genetic distance between the studied populations of *P. clavata* subsp. *clavata* (M1, M2, & M3) and *P. clavata* subsp. *tourneuxii* (M4, M5, & M6).

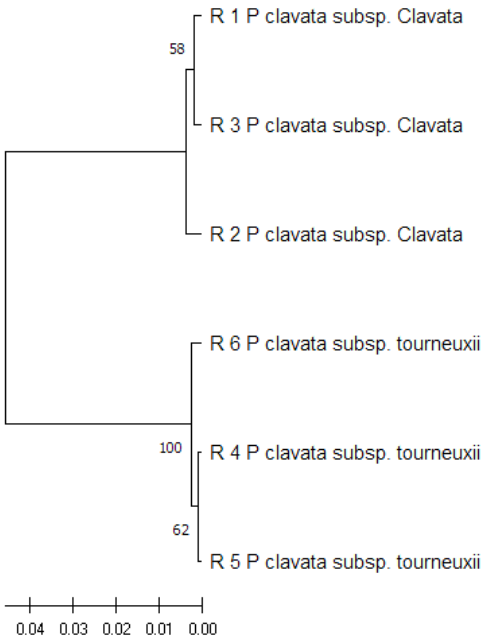

Diagram (2): Phylogenetic dendrogram based on rbcl sequences, constructed by UPGMA based on values of genetic distance between the studied populations of *P. clavata* subsp. *clavata* (R1, R2, & R3) and *P. clavata* subsp. *tourneuxii* (R4, R5, & R6).

Pollen features using scanning electron microscope (SEM): Pollen grains of the two identified *Pseuderucaria* subspecies are isopolar, radially symmetrical, tricolpate, with microreticulate to reticulate exine ornamentation (Pl. 2).

Size: Pollen grains are small in the two subspecies; the pollen grains of *P. clavata* subsp. *tourneuxii* have a polar axis length of 13.63 µm, and an equatorial diameter of 15.02 µm. The pollen grains of *P. clavata* subsp. *clavata* have a polar axis length of 15 µm, and an equatorial diameter of 15.7 µm (Tab. 1).

Shape: Based on the P/E ratio (Tab. 1), the shape of pollen grains is oblate-spheroidal in both subspecies; P/E ratio is 0.91 in subsp. *tourneuxii*. While subsp. *clavata* has P/E ratio of 0.96.

Apertures: Both subspecies have tricolpate pollen grains. The colpi are wider at the equator and gradually narrow as they approach the poles. Also, both have different colpi dimensions (Tab. 1). subsp. *clavata* possesses longer 8.28 µm and wider colpi 3.62 µm. While subsp. *tourneuxii* has colpi with a length of 5.01 µm and a width of 2.77 µm.

Exine structure and ornamentation: The exine ornamentation in both subspecies is microreticulate (lumina less than 1 µm in diameter) to reticulate (1 µm < lumina diameter < 2

µm). The variable lumina size and shape (ranging from polygonal to circular or indeterminate shape) makes the exine appear heterobrochate. The luminal diameter is greatest near the equator and gradually shrank towards the poles. In the two subspecies, there are no interluminal tissues, and the reticulum appears open. The muri walls have a warty surface in both subspecies (Pl. 2).

Table (1): Pollen morphological characters of the two identified subspecies of *P. clavata* in Egypt.

Pollen character	*P. clavata* subsp. *tourneuxii*	*P. clavata* subsp. *clavata*
Polar axis (P, µm)	12.78- (13.63) -14.48	14.48- (15) -15.53
Equatorial axis (E, µm)	14.91- (15.02) -15.12	15.53- (15.7) -15.87
P/E (µm)	0.91	0.96
Pollen shape	oblate spheroidal	oblate spheroidal
Colpus length (L, µm)	4.69- (5.01) -5.33	7.94- (8.28) -8.63
Colpus width (W, µm)	2.34- (2.77) -3.20	3.45- (3.62) -3.80
L/W	1.81	2.29
No. of lumina/µm	1.16- (1.74) -2.33	1.55- (1.94) -2.33
Lumina diameter (µm)	0.26- (0.73) -1.20	0.34- (0.82) -1.29
Muri width (µm)	0.17- (1.29) -2.41	0.17- (1.59) -3.01
Muri wall surface	warty	warty

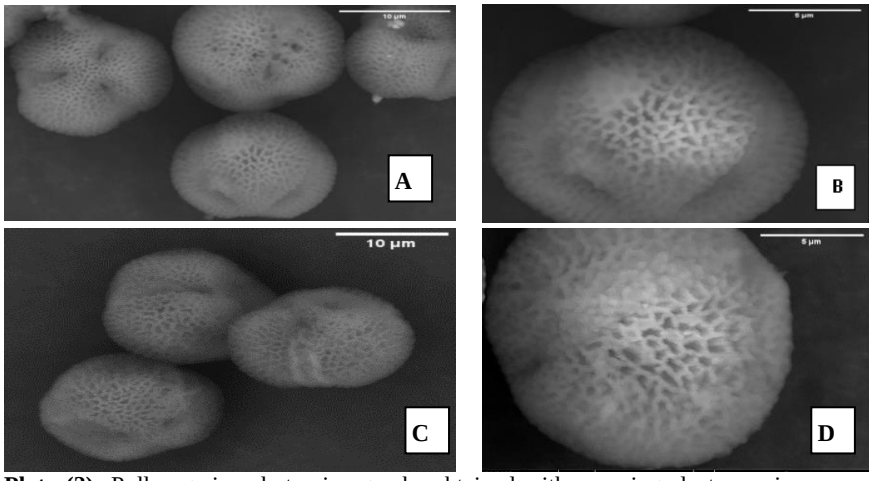

Plate (2): Pollen grains photomicrographs obtained with scanning electron microscopy (SEM) of *P. clavata* subsp. *tourneuxii* (A & B), and *P. clavata* subsp. *clavata* (C & D) showing pollen shape and exine pattern.

DISCUSSION

The current taxonomic investigation on the Egyptian *Pseuderucaria clavata*, based on morphological characters, revealed the presence of two Petal colour forms (violet and white, Pl. 1). The investigation of *P. clavata* genetic diversity had received little attention for this species in the earlier researches. The study aims to explain how genetic variation affects the phenotypic diversity of Egyptian color forms.

It is possible to determine the genetic diversity of individuals or populations using morphological and molecular markers, given that morphological traits are constrained by the environment and the stage of plant development (El-Domyati *et al*., 2011). Therefore, this work used molecular markers to examine the interspecific similarities between the identified morphological forms of *P. clavata*. The molecular markers have been applied in the fields of taxonomy, genetics, physiology, and embryology.

The created matK dendrogram (Diag. 1) clustered all accessions of the first form into a single cluster with very low genetic distance ranging from 0.003 to 0.008. Similarly, when rbcL was used, the genetic distance ranges from 0.001 to 0.002 between the first form accessions (Diag. 2). The second form accessions gave the second cluster in both matK and rbcL dendrograms with a genetic distance of 0.001 for rbcL and a range of 0.001 to 0.016 for matK.

The retrieved molecular results confirmed that barcoding technique using matK and rbcL sequences can differentiate between the two color forms. These findings revealed that the morphological diversity between the two identified forms is genetically controlled consequently, these colour forms were treated as two distinct subspecies. *P. clavata* subsp. *clavata* represent the first color form (violet) and this agrees with Boulos (1999), who recorded that subsp. *clavata* is characterized by violet to pink petals, and *P. clavata* subsp. *tourneuxii* represent the second color form (white to pale violet). According to Maire (1933), the type of subsp. *tourneuxii* was collected from Egypt, in addition this subsp. *tourneuxii* is distributed in neighboring countries of Egypt including Libya, Tunisia, and Algeria (Dobignard and Chatelain, 2011), as all of these countries are located within the same phytogeographic region known as Saharo-Sindian region. According to BrassiBase, *Pseuderucaria clavata* is represented by two subspecies *P. clavata* subsp. *clavata* (Boiss. & Reut.) O.E. Schulz and *P. clavata* subsp. *tourneuxii* (Coss.) Maire.

The morphological diversity of *P. clavata* pollen has never been thoroughly investigated using SEM. The SEM study of pollen grains showed differences in pollen size and apertures (colpus length and width) between the two identified subspecies. The Palynological results supported the molecular results achieved by RbcL and matK genes sequencing. Our findings highlight the potential of pollen features for taxonomic classification, particularly in describing Egyptian subspecies. These findings align with those of Amer and Abdo's (2014) who demonstrated that pollen characteristics have a high taxonomic significance at the infra-specific level.

Morphological, palynological, and molecular studies

Research studies by Rollins and Banerjee (1979), Anchev and Deneva (1997), and Arora and Modi (2011) on pollen grains from Brassicaceae confirm our results on pollen morphology. Pollen grains of *P. clavata* were small in the two identified subspecies, with a polar axis 12.78-15.53 µm. Erdtman (1969) divided pollen grains into six sizes based on the polar axis: very small grains < 10 µm, small grains 10–25 µm, medium grains 25–50 µm, large grains 50–100 µm, very large grains 100–200 µm, gigantic grains > 200 µm.

The pollen grains of the two identified subspecies are you oblate-spheroidal in shape. This agrees with Perveen *et al*., (2004) who recorded that pollen grains in Brassicaceae are generally prolate to sub-prolate or prolate–spheroidal , but rarely oblate–spheroidal. In subsp. *tourneuxii*, the P/E ratio was 0.91, while the P/E ratio in subsp. *clavata* was 0.96, oblate spheroidal shape having 0.88 ≤ P/E ratio ≤ 0.99, according to Erdtman (1986). Abdel Khalik *et al*., (2002) confirmed minor differences in the size, shape and apertures in pollen of the Brassicaceae, but significant variation in exine ornamentation, which varies among genera within tribes and among species within the same genus.

Both identified subspecies have tricolpate pollen grains with microreticulate (lumina less than 1 µm in diameter) to reticulate (1 µm < lumina diameter < 2 µm). The tricolpate pollen grains of the Brassicaceae were also observed by Abdel-Khalik *et al*., (2002), who additionally identified three forms of exine ornamentation (coarsely reticulate, reticulate, and microreticulate) based on lumina size. Lahham and El-Eisawi (1987) studied the pollen grains of 87 species of Brassicaceae and found that the majority were tricolpate. Apple and Al-Shehbaz (2002) also reported tricolpate reticulate pollen in the family Brassicaceae. Rollins and Banerjee (1979) also noted that the surface of crucifer pollen is reticulate reporting a strong reticular pattern of the muri. Anchev and Deneva (1997) indicated two types: the first type is reticulate with lumina equal to or exceeding the size of the muri, this type is found in all tribes; the second type has aperforated reticulate ornamentation with lumina equal in size or smaller than the muri, this type found in *Erysimum*, *Alyssum*, and *Brassica*.

CONCLUSIONS

Two subspecies of *P. clavata* (Boiss. & Reut.) O.E. Schulz were identified in Egypt as a result of the taxonomic revision of this species which are *P. clavata* (Boiss. & Reut.) O.E. Schulz subsp. *clavata* and *P. clavata* subsp. *tourneuxii* (Cross.) Maire, This identification was confirmed and validated through the molecular investigation using the chloroplast barcoding markers matK and rbcL. It was confirmed by examining the characteristics of pollen grains in both subspecies that these features had a high taxonomic value.

CONFLICT OF INTEREST STATEMENT

"The authors declare no conflicts of interest".

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دراسة مورفولوجية ،بالينولوجية و جزيئية لنبات *Pseuderucaria Clavata* (Boiss. & Reut.) O.E. Schulz في مصر

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الخلاصة

لم يتم إجراء أي دراسات مورفولوجية أو بالينولوجية أو جزيئية تفصيلية سابقة لنبات *Pseuderucaria clavata* (Brassicales, Brassicaceae). يُظهر هذا النوع شكلين بألوان زهور مختلفة (البنفسجي والأبيض). ولقد كانت الدراسة الجزيئية باستخدام rbcL وmatK لتوضيح العلاقات التصنيفية مفيدة للغاية، ودعمت أن الشكلين يمثلان نوعين فرعيين مختلفين هما *P. clavata* subsp. *clavate*، و *P. clavata* subsp. *turneuxii*. تم إيداع تسلسلات النوعين الفرعيين في قاعدة البيانات العالمية بأرقام الانضمام التالية: OR573855، OR573856، OR573857، OR573858، OR573859، OR573860، OR636847، OR636848، OR636849، OR636850، OR636851 و OR636852 وكذلك أكدت دراسة صفات حبوب اللقاح في كلا النوعين على القيمة التصنيفية العالية لخصائص حبوب اللقاح.