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### ORIGINAL ARTICLE

## PALYNOLOGICAL STUDIES FOR SOME CULTIVATED SPECIES OF *PINUS* L., 1753 (PINALES, PINACEAE) IN EGYPT



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### ABSTRACT

Pollen grains of the five cultivated species of *Pinus* L., 1753 from Subsect. *Pinaster* (Order Pinales, Family Pinaceae) were collected from the Orman Botanic Gardens at Giza in addition to the herbarium specimens. They were examined by light and scanning electron microscopy to detect the taxonomic value of their pollen characteristics. The pollen grains were bisaccate. An artificial key constructed according to the morphology of pollen grains recognizes the five species that belong to *Pinus*: *P. pinea* Linnaeus, 1753; *P. canariensis* Smith, 1828; *P. halepensis* Miller, 1768; *P. roxburghii* Sargent, 1897; and *P. brutia* Tenore, 1811. The differential items included the presence or absence of apertures, e.g. the monosulcate colpus that presents in *P. pinea* and *P. brutia*; pollen shape without sacchi that could be perprolate as in *P. pinea* or prolate as in the remaining species; pollen shape (outlined with sacchi) in polar view that could be haploxylo-noid as in *P. pinea* and *P. roxburghii* or diploxylo-noid as in the remaining species; in addition to cappa and sacchi exine sculpture. A dendrogram from the Community Analysis Package statistical program for data analysis supported the separation of five species of *Pinus* in Egypt and showed that *P. canariensis* and *P. halepensis* were closely related, as well as *P. brutia* and *P. roxburghii*. The cluster separated *P. pinea* into a separate group, but it was more closely related to *P. canariensis* and *P. halepensis*. The cluster tree was illustrated, visualized, and confirmed by a heat map based on the R programming language for effective manipulation of the data.

Keywords: Cultivated trees, Data analysis, Egypt, *Pinus*, Pollen morphology.

### INTRODUCTION

The family Pinaceae Spreng. ex F. Rudolphi (1830) belongs to the order Pinales Gorozh (1904), which included 200 species grouped under 11 genera and distributed in the Northern Hemisphere (Farjon, 2001). *Pinus* species are distributed over North Africa, Asia, Europe, and North America (Shu, 1999). *Pinus* trees dominate Northern Hemisphere forests, including boreal, subalpine, temperate, tropical, and arid woodlands (Richardson and Rundel, 1998). They are sources of economically important wood, paper, resins, and charcoal, and their seeds are used as food as well as for ornamentation (Le Maitre, 1998).

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Large winter buds with numerous scales. Leaves are needle-like, straight or twisted, with persistent or deciduous membranous sheaths at the base (Shu, 1999).

Genus *Pinus* has two subgenera, [Subgenus *Pinus* L. (1753) and Subgenus *Strobus* (D. Don) Lemmon (1895)] as classified by Gernandt *et al.* (2005). In turn, the subgenus *Pinus*, 'hard pines' has two sections [sect. *Pinus* L. (1753) and sect. *Trifoliae* Duhamel (1755)]. The section *Pinus* is comprised of two subsections: Subsect. *Pinus* L. (1753), and subsect. *Pinaster* Loudon (1838); the latter subsection includes the five studied *Pinus* species: *P. brutia*, *P. canariensis*, *P. halepensis*, *P. pinea*, and *P. roxburghii*. The section *Trifoliae* comprises three subsections [subsect. *Contortae* Little and Critchfield (1969), subsect. *Australes* Loudon (1838), and subsect. *Ponderosae* Loudon (1838)]. The subgenus *Strobus*, 'soft pines' has two sections: [Sect. *Quinquefoliae* Duhamel (1755), including three subsections: Subsect. *Strobui* Loudon (1838), Subsect. *Krempfianae* Little and Critchfield (1969), and subsect. *Gerardianae* Loudon (1838), and sect. *Parrya* Mayr (1890), with three subsections: namely, *Balfourianae* Engelm. (1880), *Cembroides* Engelm. (1880), and *Nelsonianae* Burgh (1973).

Farjon (2001) recognized 109 species in the genus *Pinus*, while Price *et al.* (1998) proposed 111 species in two subgenera, four sections, and 17 subsections based on cross-ability, secondary metabolites, protein, DNA, morphological, anatomical, and cytological studies. Pines are trees or rarely shrubs, evergreen, with regularly whorled branches; branchlets are strongly dimorphic: long branchlets bear scale-like leaves; short branchlets are persistent or deciduous and bear acicular leaves in fascicles of 2-5. Male cones are axillary, upright, densely clustered at the base of short shoots, sessile, small, soft, cylindrical, oblong, or ovoid, up to 15 mm long; female cones are terminal or sub-terminal, woody, and collapse as a whole; scales are cuneate, thickened at the apex, and frequently extended; seeds are winged or not (Shu, 1999).

Pollen morphology such as size, shape, exine thickness, and sculpture pattern are very important characteristics and can be used for identification and differentiation between related species (Goda and Gabr, 2018). Pollen identification is widely used in the reconstruction of vegetation, past climates, and plant biodiversity. Studies concerning pollen structure, size, and form are key issues in basic sciences such as plant taxonomy and evolution (Dafni *et al.*, 2000). According to Okwulehie and Okoli (1999), pollen morphology differences can not only reclassify the investigated taxa, but also their morphological similarities can indicate interspecific relationships.

In general, gymnosperms have great diversity in pollen morphology, which is significant in the classification of their taxa. Pollens of the order Pinales are distinguished by their two sacci and a proximal part called the cap (Khan *et al.*, 2018). Pinaceae is one of the conifer families that is well-known for its saccate pollen as a modified form of pollen grain to facilitate pollination (Tomlinson, 1994). *Pinus* male cones in clusters have numerous microsporophylls that are considered stamens in angiosperms, within which two microsporangia contain millions of bisaccate microspores, or pollen grains, that are carried

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by the wind during pollination. The recognition of pollen grain characteristics can be used to trace the history of taxa; these have significant taxonomic value (Moore and Webb, 1978).

According to Khan *et al.* (2017), both light microscopy (LM) and scanning electron microscopy (SEM) have significant value in the investigation and identification of pollen grains from various plant taxa. Using SEM, in addition to pollen examination and description by LM, greatly increases resolution and confirms the exine surface morphology, which can be more significant at the species level (Bagnell, 1975; Weir and Thurston, 1977). SEM can be used to identify pollen grains based on external exine features (Jones and Bryant, 2007; Faria *et al.*, 2021), and the internal structure of *Pinus* pollen bladders (Sivak and Caratini, 1973).

Ting (1965) used pollen morphological characteristics to identify 20 taxa within *Pinus* and other Pinaceae taxa in California. Several authors investigated the pollen morphology of *Pinus*: Erdtman (1969); Ueno (1978); Iwanami *et al.* (1988); Song *et al.* (2012); and Nikolić *et al.* (2019). Also, the evolutionary study of Liu and Basinger (2000) was carried out on some fossil taxa of Pinaceae (including *Pinus*) in Canada. Khan *et al.* (2018) assessed the pollen flora of gymnosperms in Pakistan, and Chropeňová *et al.* (2016) estimated the *Pinus* pollen's sensitivity to air pollution in some sites in the Slovak Mountains.

In Egypt, the genus *Pinus* contributes to 4.1% of the total pollen production of the taxa in the Rosetta district as a result of the work of Taia *et al.* (2019). As a result, the study of its pollen should be of great interest in Egypt. Palyno-morphological studies of *Pinus* in Egypt need more elaborated investigation, accordingly, the present study will focus on the pollen grain qualitative and quantitative morphological characters of five *Pinus* species cultivated in gardens. To provide palynological data to support the taxonomy of the studied *Pinus* species cultivated in Egypt.

#### MATERIALS AND METHODS

**Materials data:** Herbarium specimens of *Pinus* from CAI and CAIM were studied, and the freshly collected specimens were compared with authentic specimens by R. Hamdy at the Herbarium of Cairo University. The new collections of the five species from the Orman Botanic Garden east of Cairo University at Giza, Egypt, were done to provide materials for pollen studies. A total of 30 pollen grains were taken from the third pollen sacs from the apex of male strobili for each species and were examined by both light and scanning electron microscopy. The pollen specimens have been preserved in the herbarium of Fayoum University (proposed abbreviation "CAIF"), Fayoum Province, Egypt. An artificial key of pollen morphological characters (observed by both LM and SEM) was constructed for the studied species.

**The microscopic study by Light microscopy (LM):** The collected pollen grains were acetolyzed with glycerin according to a modified protocol of pollen extraction by Moore *et al.* (1991). By using a light microscope, the Carl Zeiss M305973 at x400, the parameters measured are described in Table (1). Examination of the pollen grains was done in polar and

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equatorial views. Light microscopy photos were captured using an Axiocam 208 colour microscope camera, and the investigation was performed by a PrimoStar Carl Zeiss microscope.

**Table (1):** Pollen morphological data of the examined *Pinus* species [PL1 (corpus length), PL2 (pollen length with sacci), PW1 (corpus width), PW2 (pollen width with sacci), AL (saccus length), AW (saccus width), ET (exine thickness), FL (furrow length), PPS (Pollen shape in polar view), PS= PL1/PW1, CS (cappa sculpture), and SS (succus sculpture)].

Species	PL1 (µm)	PL2 (µm)	PW1(µm)	PW2 (µm)	AL (µm)	AW (µm)	ET (µm)	FL (µm)
<i>P. pinea</i>	29-33 (31)	34-39 (36.5)	12-18 (15)	17-20 (18.5)	15-18 (16.5)	10-14 (12)	1	1-10 (5.5)
<i>P. canariensis</i>	30-37 (33.5)	33-42 (37.5)	14-20 (17)	20-25 (22.5)	11-20 (15.5)	15-19 (17)	1	1-8 (4.5)
<i>P. halepensis</i>	27-32 (29.5)	34-43 (38.5)	16-26 (21)	22-28 (25)	17-26 (21.5)	15-25 (20)	1-2 (1.5)	5-9 (7)
<i>P. roxburghii</i>	23-30 (26.5)	32-39 (35.5)	16-18 (17)	20-24 (22)	14-18 (16)	13-18 (15.5)	1	5-7 (6)
<i>P. brutia</i>	16-25 (20.5)	30-36 (33)	9-20 (14.5)	17-24 (20.5)	12-17 (14.5)	12-18 (15)	1	2-10 (6)

**Table (1, cont.)**

Species	Size	PS		Aperture	Colpus length	PPS	Sculpture	
		Shape	Ratio				CS	SS
<i>P. pinea</i>	Medium-sized grains	Perprolate	2.06	Monosulcate	15-17 (16)	Haploxylo-noid	Scabrate	Perforate
<i>P. canariensis</i>		Prolate	1.97	Absent	—	Diploxylo-noid	Granulate	Rugulate
<i>P. halepensis</i>			1.4		—		Verrucate	Scabrate
<i>P. roxburghii</i>			1.55		—			

<i>P. brutia</i>	Small grains		1.41	Monosulcate	10-21 (15.5)	Diploxytonoid		
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**The microscopic study by scanning electron microscopy (SEM):** Pollen grains were directly dusted onto carbon stubs provided with double-sided tape, then coated with a 150 Å (15 nm) gold layer. The coating step was made by using the JFC-1100E ion sputter device. The sculpturing pattern of pollen grains per taxon was investigated by using the JSM-5400LV scanning microscope at (x 500-x 10.000).

**Pollen terminology used:** Pollen grains of the studied species were described according to Pragłowski and Punt (1973).

**Pollen data analysis:** Pollen morphological data were based on 14 morphological characters and 30 character states that described pollen dimensions, shape, and exine sculpture as qualitative binary characters and quantitative multi-state characters, respectively, and both were used as operational taxonomic units (OTUs) (Tab. 2). Using the Community Analysis Package (CAP) statistical program, an average linkage agglomerative clustering method was used to obtain a classification dendrogram, and a reciprocal averaging (RA) ordination method was used to obtain an ordination plot, as described by Schütze and Silverstein (1997). The "gg heat map" package version 2.1 (Luo, 2021) was used to create a heat map for clustering visualization.

**Table (2):** Pollen morphological characters, character states, and data matrix applied to the numerical analysis of the examined *Pinus* species.

Characters	Character states	<i>P. pinea</i>	<i>P. canariensis</i>	<i>P. halepensis</i>	<i>P. roxburghii</i>	<i>P. brutia</i>
Pollen outline in polar view	Diploxytonoid	0	1	1	0	1
	Haploxytonoid	1	0	0	1	0
Corpus length (µm)	>28.2	1	1	1	0	0
	<28.2	0	0	0	1	1
Pollen length with sacchi (µm)	>36.2	1	1	1	0	0
	<36.2	0	0	0	1	1
Corpus width (µm)	>16.9	0	1	1	1	0
	<16.9	1	0	0	0	1

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Pollen Width with sacci (µm)	>21.7	0	1	1	1	0
	<21.7	1	0	0	0	1
Saccus length (µm)	>16.8	0	0	1	0	0
	<16.8	1	1	0	1	1
Saccus width (µm)	>15.9	0	1	1	0	0
	<15.9	1	0	0	1	1
Exine thickness (µm)	=1	1	1	0	1	1
	1-2	0	0	1	0	0
Furrow length (µm)	>5.8	0	0	1	1	1
	<5.8	1	1	0	0	0
Equatorial pollen shape	Perprolate	1	0	0	0	0
	Prolate	0	1	1	1	1
Ratio between the polar axis and the equatorial diameter	>1.67	1	1	0	0	0
	<1.67	0	0	1	1	1
Cappa sculpture	Scabrate	1	0	0	0	0
	Granulate	0	1	0	0	0
	Verrucate	0	0	1	1	1
Succus sculpture	Psilate	1	0	0	0	0
	Rugulate	0	1	0	0	0
	Scabrate	0	0	1	1	1
Monosulcate aperture	Present	1	0	0	0	1
	Absent	0	1	1	1	0

**RESULTS**

The pollen grains of the studied *Pinus* species were heteropolar, bilaterally symmetric, and bisaccate monads; Table (2) summarized the pollen morphological data of the studied species.

**Pollen's morphological description of each species (Pl. 1-3)**

*Pinus pinea* L., 1753: Tree with a rounded crown; reddish-grey bark with deep longitudinally fissured; yellowish shoots; stiff gray-green leaves arranged in bundles of two,

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10–20 cm long; male cones broad ovoid; female cones up to 15 cm long, sessile. Corpus lengths of 29–33  $\mu\text{m}$ , pollen lengths with sacci of 34–39  $\mu\text{m}$ , corpus widths of 12–18  $\mu\text{m}$ , pollen widths with sacci of 17–20  $\mu\text{m}$ , saccus lengths of 15–18  $\mu\text{m}$ , saccus widths of 10–14  $\mu\text{m}$ , exine thickness of one  $\mu\text{m}$ , and furrow lengths (leptoma) of 1–10  $\mu\text{m}$ . Equatorial pollen with perprolate shape (PL1/PW1 ratio of 2.06). Monosulcate aperture between pollen sacci with a length of 15–17  $\mu\text{m}$ . Pollen haploxytonoid in polar view. Exine coarse and cappa sculpture scabrate. Sacci sculptures perforate.

*Pinus canariensis* C. Sm. ex DC., 1825: Tree; reddish bark deeply fissured; leaves arranged in bundles of 3, consisting of very long, string-like pendant needles, glossy blue-green, up to 30 cm long, drooping; sheath about 2 cm long; male cones conical-elongated, orange-yellow; female cones cylindrical-ovoid, 20 cm long, arranged in clusters of 2–3, conical-elongated, purplish. Pollen grains with a corpus length of 30–37  $\mu\text{m}$ , pollen length with sacci 33–42  $\mu\text{m}$ , corpus width of 14–20  $\mu\text{m}$ , pollen width with sacci of 20–25  $\mu\text{m}$ , saccus length 11–20  $\mu\text{m}$ , saccus width 15–19  $\mu\text{m}$ , exine thickness of one  $\mu\text{m}$  and furrow length 1–8  $\mu\text{m}$ . Equatorial pollen with prolate shape (PL1/PW1 ratio of 1.97). Pollen diploxytonoid in polar view. Exine coarse and cappa sculptures granulate. Sacci sculptures regulate.

*Pinus halepensis* Mill., 1768: Tree, reddish-brown bark glabrous, fissured; pale green shoots; yellowish-brown branches; leaves needle-like, flexible, up to 10 cm long, in clusters of two on dwarf shoots within a sheath; male cones in terminal clusters appear in spring, lanceolate; female cones solitary, pendulous on short axillary branches, ovoid to conical, reddish, and up to 12 cm long. Pollen grains with corpus length of 27–32  $\mu\text{m}$ , pollen length with sacci of 34–43  $\mu\text{m}$ , corpus width of 16–26  $\mu\text{m}$ , pollen width with sacci of 22–28  $\mu\text{m}$ , saccus length 17–26  $\mu\text{m}$ , saccus width 15–25  $\mu\text{m}$ , exine thickness 1–2  $\mu\text{m}$  and furrow length 5–9  $\mu\text{m}$ . Equatorial pollen with prolate shape (PL1/PW1 ratio of 1.40). Pollen diploxytonoid in polar view. Exine coarse and cappa sculpture verrucate. Sacci sculpture scabrate.

*Pinus roxburghii* Sargent, 1897: Evergreen tree, up to 20 m high; branches symmetrically whorled; brownish bark thick, deeply fissured into large plates; leaves needle-like, in groups of threes on dwarf shoots. 20–35 cm long (hence the name *longifolia*), sheath persistent, 1.2–2.5 cm long; male cones ovoid, about 1.5 cm long, yellowish in dense terminal clusters; female cones solitary, or 2–3 on the short recurved peduncle, ovoid, 10–20 cm long. Pollen grains had corpus lengths of 23–30  $\mu\text{m}$ , pollen lengths of 32–39  $\mu\text{m}$ , corpus widths of 16–18  $\mu\text{m}$ , pollen widths of 20–24  $\mu\text{m}$ , saccus lengths of 14–18  $\mu\text{m}$ , saccus widths of 13–18  $\mu\text{m}$ , exine thickness of one  $\mu\text{m}$ , and furrow lengths of 5–7  $\mu\text{m}$ . Equatorial pollen with prolate shape (PL1/PW1 ratio of 1.55). Pollen haploxytonoid in polar view. Exine coarse and cappa sculpture verrucate. Sacci sculpture scabrate.

*Pinus brutia* Ten., 1811: Tree; orange-red bark deeply fissured; shoots straight, slender; leaves arranged in pairs, bright to yellowish green, 10–18 cm long, slender, about 1 mm thick, with a persistent 1–1.5 cm sheath; female cones sessile to subsessile. Pollen grains had corpus lengths of 16–25  $\mu\text{m}$ , pollen lengths of 30–36  $\mu\text{m}$ , corpus widths of 9–20  $\mu\text{m}$ , pollen widths of 17–24  $\mu\text{m}$ , saccus lengths of 12–17  $\mu\text{m}$ , saccus widths of 12–18  $\mu\text{m}$ , exine

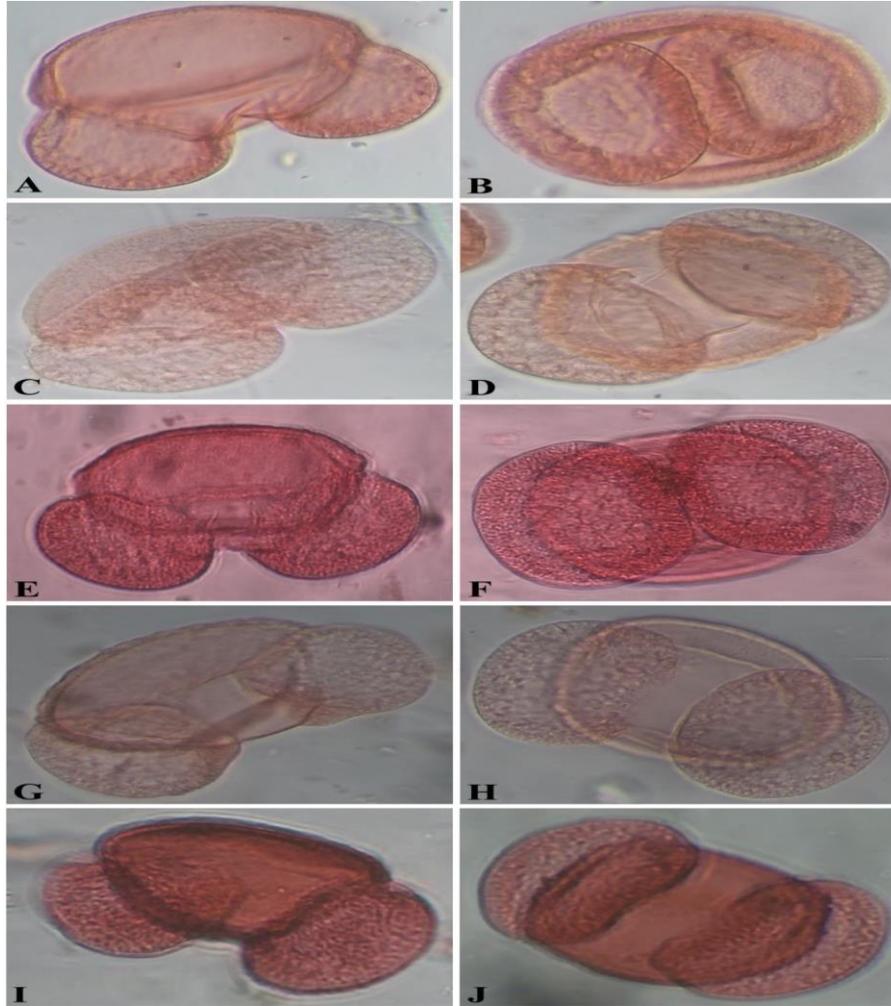
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thickness of one  $\mu\text{m}$ , and furrow lengths of 2–10  $\mu\text{m}$ . Equatorial pollen with prolate shape (PL1/PW1 ratio of 1.41). Monosulcate aperture between pollen sacchi with a length of 10–21  $\mu\text{m}$ . Pollen diploxytonoid in polar view. Exine coarse and cappa sculpture verrucate. Sacchi sculpture scabrate. A key to species was made for the studied specimens to differentiate and correlate among them, according to data based on LM and SEM, resulting in the differentiation of five species as follows:

**Artificial key to *Pinus* studied species:**

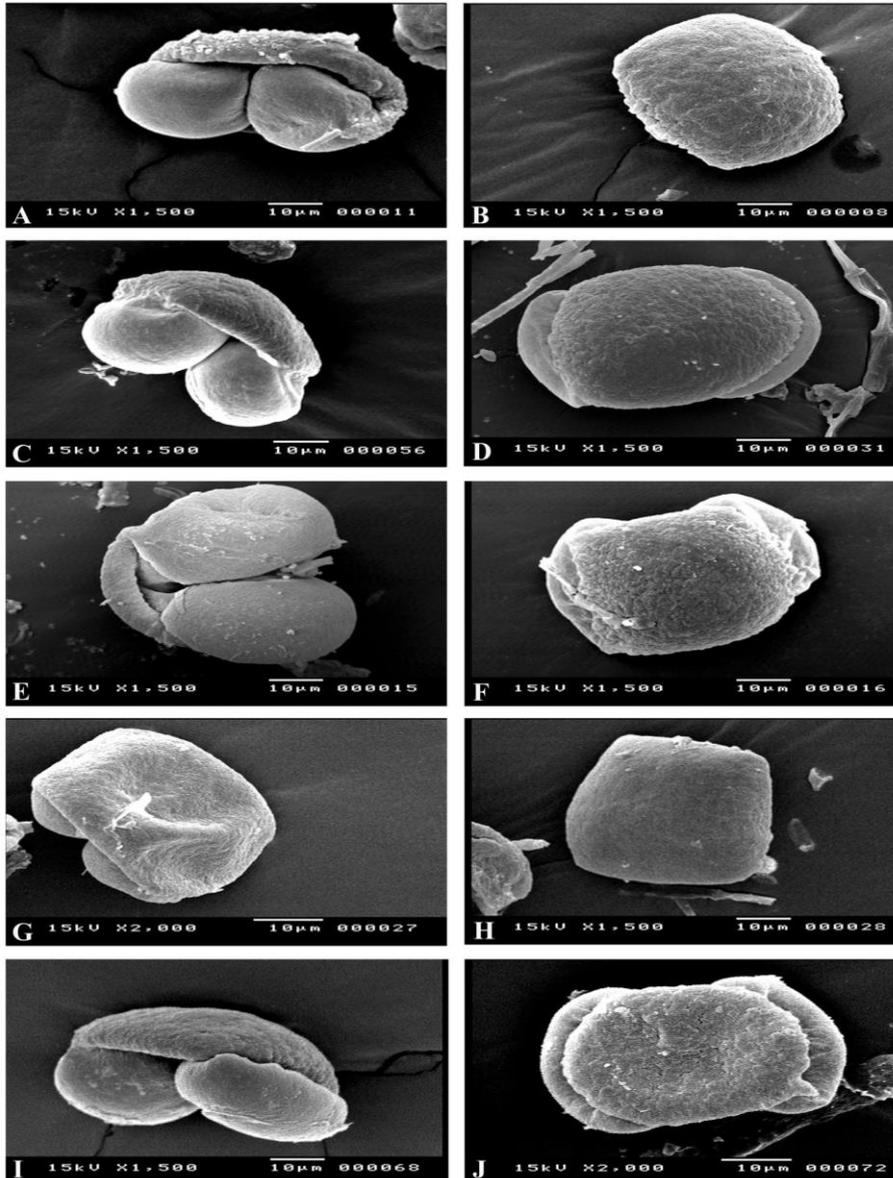
- 1. Aperture monosulcate ..... 2
- 1. Aperture absent..... 3
- 2. Corpus (without sacchi) perprolate..... *P. pinea*
- 2. Corpus (without sacchi) prolate ..... *P. brutia*
- 3. Haploxytonoid in polar view ..... *P. roxburghii*
- 3. Diploxytonoid in polar view ..... 4
- 4. Cappa sculpture granulate, while sacchi regulate ..... *P. canariensis*
- 4. Cappa sculpture verrucate, while sacchi scabrate ..... *P. halepensis*

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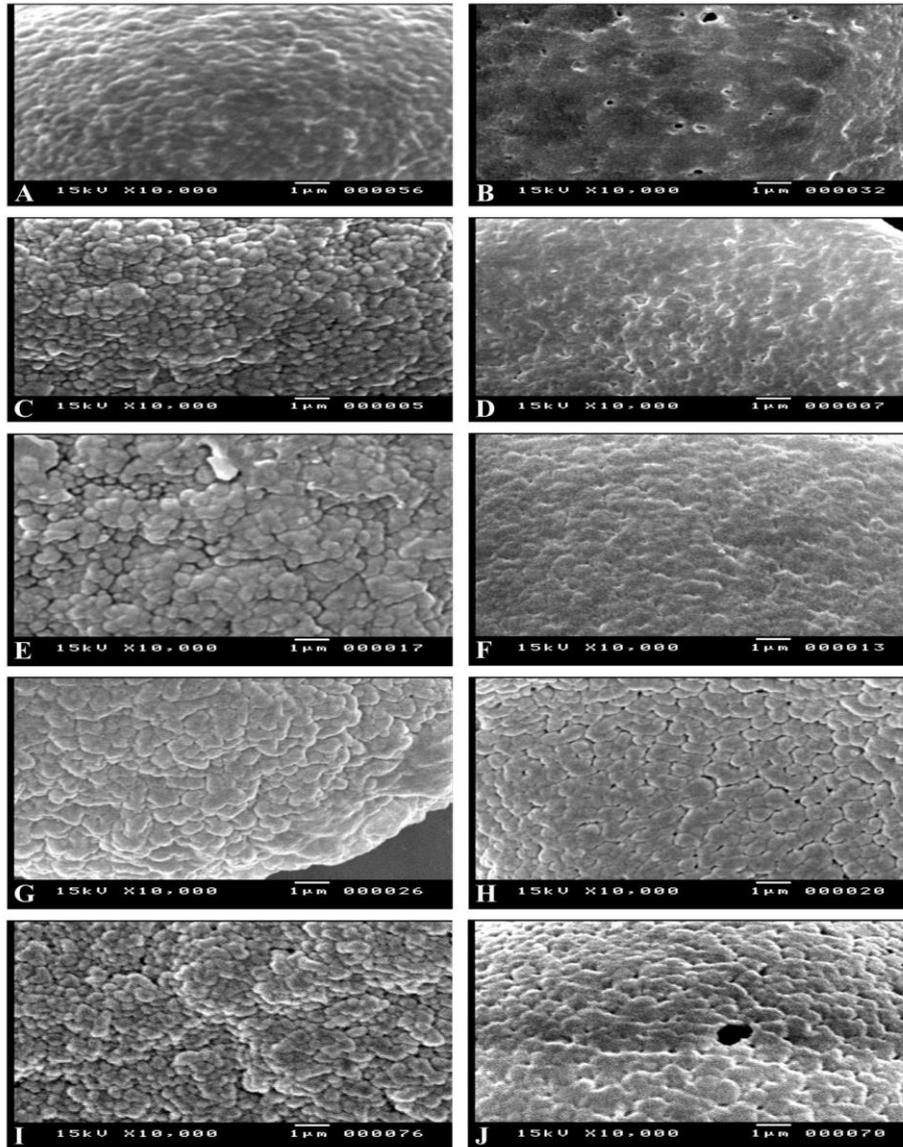


**Plate (1):** Light microscopy images, show equatorial view (A, C, E, G, I), and polar view (B, D, F, H, J) of the studied *Pinus* species; A, B: *P. pinea*; C, D: *P. canarensis*, E, F: *P. halepensis*, (G, H) *P. roxburghii*, (I, J) *P. brutia*.

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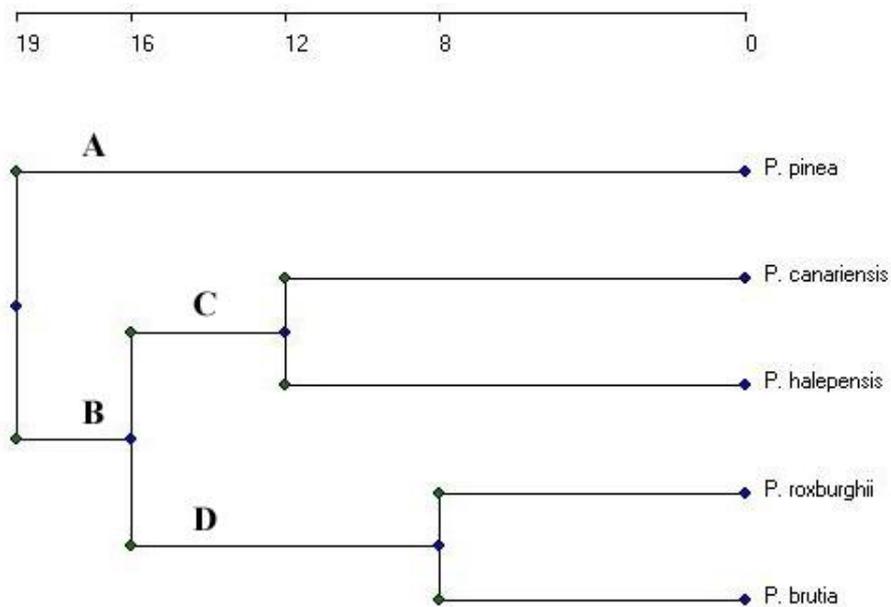
**Plate (2):** Scanning electron microscopy images, show equatorial view (EV) and polar view (PV) of the studied *Pinus* species: *P. pinea*: A. EV, B. PV; *P. canarensis*: C. EV, D. PV; *P. halepensis*: E. EV, F. PV; *P. roxburghii*: G. EV, H. PV; *P. brutia*: I. EV, J. V.



**Plate(3):** Scanning electron microscopy images, show cappa exine sculpture (A, C, E, G and I) and sacci exine sculpture (B, D, F, H, J) of the studied *Pinus* species: A, B: *P. pinea*; C, D: *P. canarensis*; E, F: *P. halepensis*; G, H: *P. roxburghii* and I, J: *P. brutia*.

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The dendrogram based on the pollen morphological data matrix (Diag. 1) was forked into two clusters; the first one represented *P. pinea* separately, while the second one combined all the remaining species and was branched into two sub-clusters. The first sub-cluster shared both *P. canariensis* and *P. halepensis* then *P. roxburghii* and *P. brutia* came together in the second sub-cluster according to some mutual characteristics. A heatmap was obtained for cluster confirmation by utilizing the R program. It depicts the relationships between the studied species (Diag. 2). The reciprocal averaging ordination plot (Diag. 3) also confirmed the dendrogram results.



**Diagram (1):** Dendrogram based on pollen morphological data of the studied *Pinus* species.

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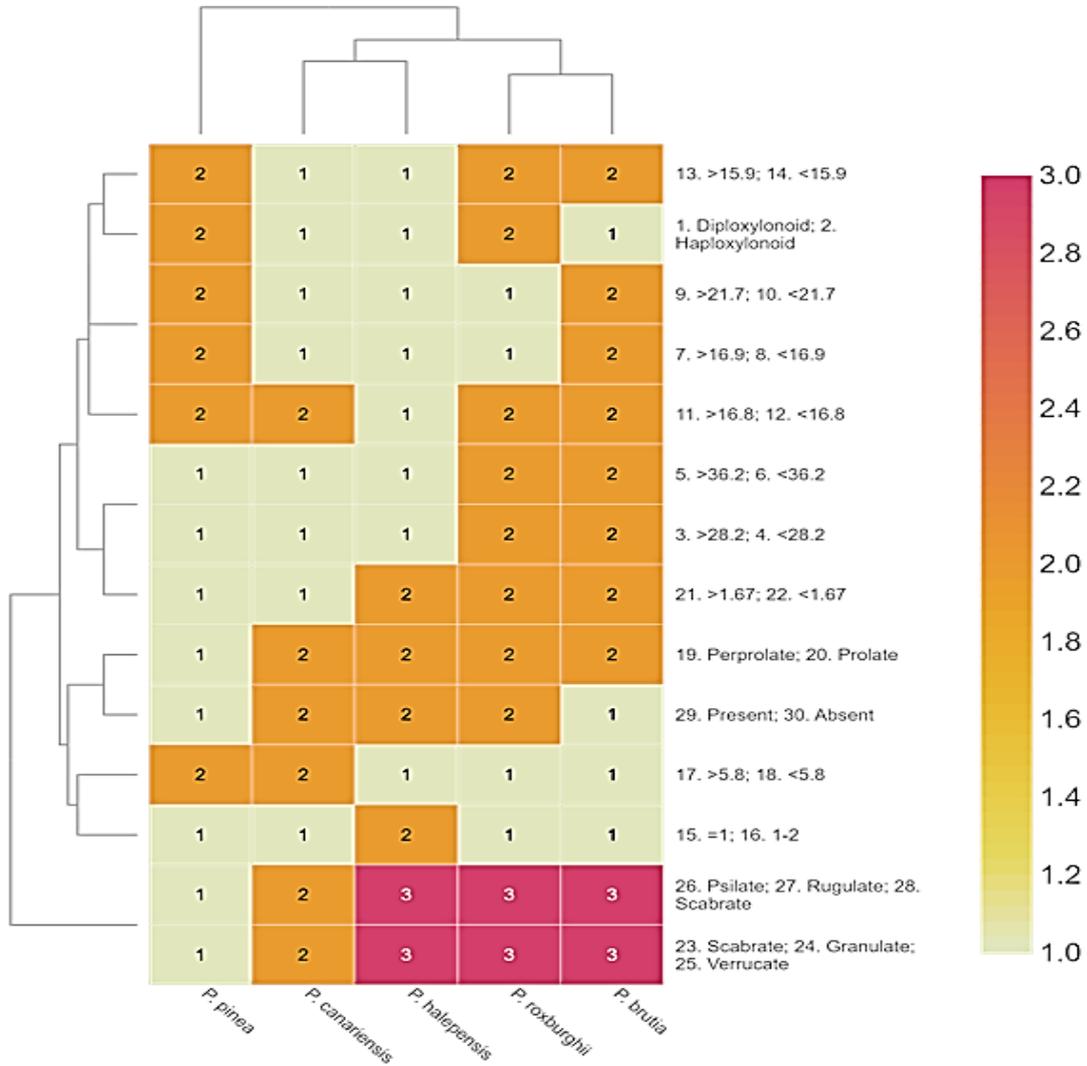
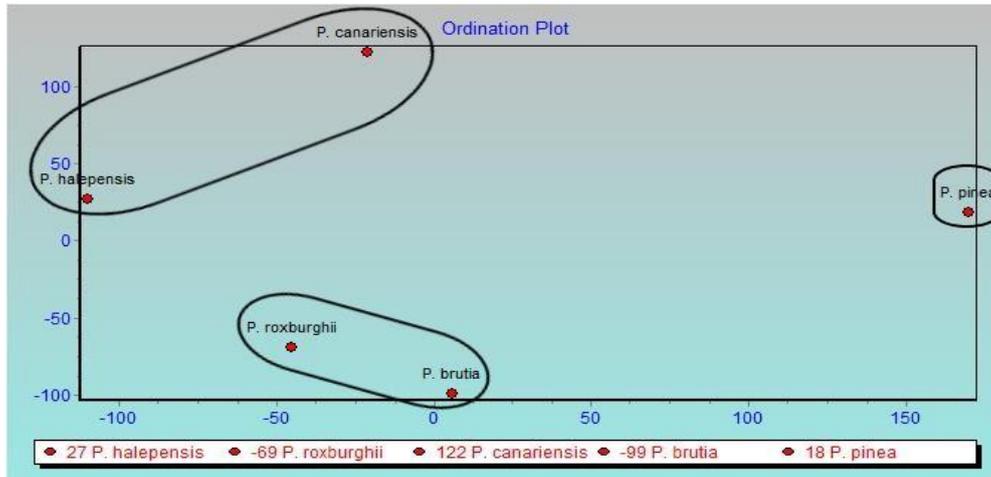


Diagram (2): Heat map visualize the cluster tree based on pollen morphological data of the studied *Pinus* species.

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**Diagram (3):** Reciprocal Averaging (RA) ordination plot based on pollen morphological data of the studied *Pinus* species.

## DISCUSSION

By applying numerical analyses to the scored data, the agglomerative clustering dendrogram based on pollen morphological data (Diag. 1) revealed the separation of two main groups. *Pinus pinea* comes in a separate group in 19 due to the perprolate shape of its pollen, scabrate cappa sculpture, and perforate succus sculpture, which are diagnostic characteristics in identification and separation; this matches with the study of Heigl (2020a).

The second group made up of 4 species, is divided into two subgroups at 16; the first subgroup consists of *P. canariensis* and *P. halepensis*, which appear in a separate group at 12, though each of these two species has distinct characteristics from the other, *P. canariensis* has granulate cappa sculpture and rugulate succus sculpture, while *P. halepensis* has average succus length  $>16.8 \mu\text{m}$  and exine thickness  $1-2 \mu\text{m}$ , they belong to the same subgroup due to diploxylonoid pollen shape, average corpus length  $> 28.2 \mu\text{m}$ , average pollen length with sacci  $> 36.2 \mu\text{m}$  [this agrees with that of Heigl (2020b) study where pollen length  $41-50 \mu\text{m}$ ], corpus width  $> 16.9 \mu\text{m}$ , average pollen width with sacci  $> 21.7 \mu\text{m}$  [this is smaller than pollen width of Heigl (2020b) study  $51-100 \mu\text{m}$ ], average saccus width  $> 15.9 \mu\text{m}$  and prolate pollen shape in equatorial pollen view, in this study the pollen shape of *P. halepensis* disagrees with the oblate shape of Heigl (2020b) study, it may be due to the differences in pollen width.

The second subgroup in the second group combines *P. roxburghii* and *P. brutia* at 8. They are closely related and sharing some pollen morphological characteristics such as average corpus length  $< 28.2 \mu\text{m}$ , average pollen length with sacci  $< 36.2 \mu\text{m}$  [that is smaller than those of Heigl (2021) study  $41-50 \mu\text{m}$ ], succus length  $< 16.8 \mu\text{m}$ , average saccus width  $< 15.9 \mu\text{m}$ , exine thickness of  $1 \mu\text{m}$ , average furrow length  $> 5.8 \mu\text{m}$ , prolate pollen shape whereas pollen of Heigl (2021) study have oblate shape due to the differences in pollen

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length. In addition to the verrucate cappa and scabrate succus sculptures that match Heigl (2021), the ratio between the polar axis and the equatorial diameter is 1.67. As seen in pollen data dendrogram, the cluster of *P. canariensis* and *P. halepensis* is more related to that of *P. roxburghii* and *P. brutia* because they assemble more characters than those with *P. pinea*. They are common in prolate pollen shapes; at the same time, *P. canariensis*, *P. halepensis*, and *P. roxburghii* are joined in average corpus width  $> 16.9 \mu\text{m}$ , average pollen width with sacci  $> 21.7 \mu\text{m}$ , and their grains without apertures. Also, *P. canariensis*, *P. halepensis*, and *P. brutia* join in their diploxylonoid pollen. *P. canariensis*, *P. roxburghii*, and *P. brutia* share a saccus length of  $< 16.8 \mu\text{m}$  and an exine thickness of  $1 \mu\text{m}$ . In addition, *P. halepensis*, *P. roxburghii*, and *P. brutia* join in an average furrow length of  $> 5.8 \mu\text{m}$ , a ratio between the polar axis and the equatorial diameter of  $< 1.67$ , a verrucate cappa sculpture, and a scabrate succus sculpture.

It is noticeable that the subgroup of *P. canariensis* and *P. halepensis* is more adjacent to the cluster of *P. pinea* because they share common characteristics such as an average corpus length of  $> 28.2 \mu\text{m}$ , which is dissimilar to that of *P. pinea*  $31\text{-}35 \mu\text{m}$  and *P. halepensis*  $41\text{-}50 \mu\text{m}$  in the Heigl (2020 a, b) study, and an average pollen length with sacci  $> 36.2 \mu\text{m}$ . *P. pinea* has a furrow length of  $< 5.8 \mu\text{m}$  on average and a ratio of the polar axis to the equatorial diameter of more than 1.67. However, *P. pinea* shows a connection with the subgroup of *P. roxburghii* and *P. brutia* but is less adjacent; that is, all have an average saccus width of  $< 15.9 \mu\text{m}$ ; both *P. pinea* and *P. roxburghii* have haploxylonoid pollens in polar view; in addition, *P. pinea* joints with *P. brutia* in average corpus width  $< 16.9 \mu\text{m}$ , average pollen width with sacci  $< 21.7 \mu\text{m}$  and monosulcate apertures are present on the pollen distal part of both species.

A heatmap was created using the R-program to confirm the clustering of the studied species and to visualise the relationships between the studied species using a colour gradient, where the darker the shade, the higher the quantitative value and the most common qualitative character (Diag. 2).

The resultant five species are separated clearly on a reciprocal averaging ordination plot (Diag. 3) according to the presence or absence of the recorded quantitative and qualitative pollen character states and support the outcomes of the clustering dendrogram. *P. pinea* looks like a separate entity at 18; *P. canariensis* is separated at 122 but in the same direction as *P. halepensis* axis, which is separated at 27. *P. roxburghii* is separated at -69 and is adjacent to *P. brutia*, which is separated at -99. The region of separation of *P. canariensis* and *P. halepensis* is near the region of separation of *P. roxburghii* and *P. brutia*.

This is owed to the accuracy of the statistical data analysis in using more diagnostic characteristics in the statistical classification and finding an accurate classification of the studied entities.

Among all the studied species, the quantitative and qualitative characters are varied and significant in the characterization of the cultivated *Pinus* species in Egypt by the

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construction of an artificial key. The exine thickness has significance in the recognition of the species 1-2  $\mu\text{m}$  (1.5  $\mu\text{m}$  on average). This differs from that of the studied *P. halepensis*, *P. brutia*, and *P. roxburghii* in Pakistan (0.2-0.5  $\mu\text{m}$ ) by Khan *et al.* (2018).

For the same species, the cluster results of the current study can be compared with the results of the plant morphological description cluster of seven *Pinus* species by Mohamed (2018), the taxonomic relationships among *Pinus* taxa according to 21 morphological characters were investigated by using the Average Linkage of the SPSS program; two major clusters resulted; *P. halepensis* comes in one of these major clusters, while *P. pinea*, *P. canariensis*, *P. roxburghii*, and *P. brutia* form the other one that is similar to Farjon's (1998) classification of *Pinus*. The two studies are combatable in the grouping of *P. canariensis*, *P. roxburghii*, and *P. brutia* in one major cluster, but disagree on the combination of *P. pinea* or *P. halepensis* with them.

A recent study revealed that the bisaccate pollen size (PL2 x PW2) is 30-43 x 17-28  $\mu\text{m}$ , the corpus size (PL1 x PW1) is 16-37 x 9-26  $\mu\text{m}$ , the saccus size (AL x AW) is 11-26 x 10-25  $\mu\text{m}$ , and the corpus distal part that is called "furrow" has a length of 1-10  $\mu\text{m}$ . The sacci size is smaller than the corpus size. The largest size of bisaccate grains is of *P. halepensis* (34-43 x 22-28  $\mu\text{m}$ ), while the smallest ones are of *P. brutia* (30-36 x 17-24  $\mu\text{m}$ ). These are bigger than those in Pakistan (Khan *et al.* 2018), 22-27 x 14-17  $\mu\text{m}$  for pollen of *P. halepensis* and 24-33 x 17-21  $\mu\text{m}$  for pollen of *P. brutia*. Also, as it is noticeable, the pollen of Pakistani *P. brutia* is bigger than that of *P. halepensis*. In the same debate, the Egyptian pollens of *P. roxburghii* (32-39 x 20-24  $\mu\text{m}$ ) are bigger than the Pakistani ones (25-28 x 15-18  $\mu\text{m}$ ) in the same study.

On one hand, in the equatorial view, only *P. pinea* is with perprolate corpus, where the ratio between the polar axis and the equatorial diameter of the investigated pollens exceeds two (2.06), whereas all remaining species are prolate where the ratio is in the range of 1.33-2 (1.40 for *P. halepensis* and 1.9 for *P. canariensis*). Also, the pollen of *P. pinea* and *P. brutia* has an elongated monosulcate aperture (a single germinal colpus) on the distal pole between the two sacci (Pl. 1, 2). Subjected to the current argument that the pollens of the Egyptian *P. halepensis*, *P. roxburghii*, and *P. brutia* are bigger than the Pakistani ones of the same species, this may be due to the habitual differences that affect pollen size. It is expected that the pollen shape should be different and the pollens appear as oblate in shape and the ratio in a range between 0.5-0.75, where the ratio is 0.63, 0.68, and 0.64 for the pollens of the previously mentioned three species, respectively. These proposed ranges of the ratio between the corpus length and the corpus width are according to Pragłowski and Punt (1973).

On the other hand, the polar pollen shape is haploxytonoid for *P. pinea* and *P. roxburghii*. This is due to the more or less continuous outline of the sacci with the outline of the corpus of the examined pollen then the pollen grains appear more or less smooth ellipsoidal form. However, a diploxytonoid outline is observed for *P. canariensis*, *P. halepensis*, and *P. brutia* because of the discontinuity of the outline of the sacci with the outline of the corpus (Pl. 1, 2), thus the pollen grains appear as three distinct more or less oval parts according to

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Pragłowski and Punt (1973). Although the light microscopy investigation data provided valuable characteristics that enabled the construction of the artificial key, these data alone are not sufficient for the differentiation and classification of the species, so SEM investigations are helpful in the present study in the pollen identification of the different species.

SEM investigations show that the corpus exine sculpture is varied for the studied species. In *P. pinea*, exine sculpture is scabrate of irregular shapes less than one  $\mu\text{m}$  in all directions, while in *P. canariensis*, granulate with more or less rounded granules. The sculpturing pattern of the sacci is perforated with a smooth surface and rugulate with irregular elements more than one  $\mu\text{m}$  long in both species. While, *P. roxburghii*, *P. halepensis*, and *P. brutia* have the same corpus sculpture, viz., verrucate with wart-like elements of a width of more than one  $\mu\text{m}$  and there are no constrictions at the base, and the same sacci sculpture (scabrate) as shown in Pl.3. These disagree with the observation of Khan *et al.* (2018), where their described pollen exine of *P. halepensis*, *P. brutia*, and *P. roxburghii* in Pakistan showed only one pattern (rugulate). In the case of LM examinations, the sculpture patterns appear reticulate for all studied species, which indicates that LM observations are inaccurate in sculpture descriptions and limited in a taxonomy based on palynological data. So, in addition to LM and SEM, 3D imaging microscopy can be important to understand the structures of the studied pollens, as discussed by Shen *et al.* (2020).

Using advanced microscopy techniques might be more helpful for the investigation and identification of pollen morphology such as Fourier Transform Infrared (FTIR) microspectroscopy as used by Zimmermann (2018) in his study on two Norwegian species, *P. mugo* and *P. sylvestris*. Also, the investigation of other parameters than the morphological ones, such as the sequencing of DNA products using whole-genome amplification (WGA) can be considered a precious tool for pollen identification at the species level, as in the work of Nakazawa *et al.* (2018) on some Russian *Pinus* species.

The dissimilarities between the current results and those of the different studies on the same taxa but from different localities or countries may be due to variations in ecological or geographical conditions or both or may be due to the normal variation among the taxa of the same rank.

#### CONCLUSIONS

It is possible to conclude that five species were identified, and the closely related species including *P. canariensis* and *P. halepensis*, also *P. roxburghii* and *P. brutia* are closely related species; depending on that *P. pinea* and *P. brutia* could be distinguished by their monosulcate colpus, pollen of *P. pinea* was easily identified from the other species by its perprolate shape, *P. roxburghii* was discriminated by prolate haploxylo-noid pollen. The SEM examination distinguished accurately among all the examined species, resulting in the identification of the remaining two species as *P. canariensis* by the granulate cappa sculpture and rugulate saccus sculpture of its pollen grains, while the pollen grains of *P. halepensis* were of verrucate cappa sculpture and scabrate saccus sculpture. Finally, based on

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the certainty of the statistical analysis results, two clusters were formed to represent the considered species, *P. pinea* was easily identified and separated from the other species, *P. canariensis* and *P. halepensis* were separated into one group, while both *P. roxburghii* and *P. brutia* were closely related in another group, but the first group was more related to *P. pinea* than the second one. This is required to be confirmed by additional taxonomic tools in the future, such as anatomical and molecular studies.

CONFLICT OF INTEREST STATEMENT

"The authors declare no conflict of interest".

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دراسة حبوب اللقاح لبعض أنواع جنس الصنوبر *Pinus* L., 1753  
في مصر (Pinales, Pinaceae)

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### الخلاصة

تضمنت هذه الدراسة خمس وحدات تصنيفية من جنس الصنوبر *Pinus* L., 1753، تم تجميع حبوب اللقاح الخاصة بها من حديقة الأورمان النباتية بالجيزة، بالإضافة إلى فحص بعض العينات المعشبية لتحديد القيمة التصنيفية لصفات العينات قيد الدراسة. بدراسة الشكل الظاهري لحبوب اللقاح من خلال الميكروسكوب الضوئي والميكروسكوب الماسح الإلكتروني، تبين أنها أحادية ذات كيسين جانبيين. تحددت خمسة أنواع لجنس الصنوبر بواسطة مفتاح تعريفي اصطناعي لتعريف الوحدات التصنيفية قيد الدراسة: *P. pinea* Linnaeus, 1753؛ *P. canariensis* Smith, 1828؛ *P. brutia* Tenore, و *P. roxburghii* Sargent, 1897؛ *P. halepensis* Miller, 1768 (1811)، بناءً على دراسة الصفات الشكلية لحبوب اللقاح، والتي تتضمن وجود أو غياب شق أحادي كما في *P. pinea* و *P. brutia*، جسم حبة اللقاح بدون الأكياس الجانبية يمكن أن يكون فوق بيضاوي كما في *P. pinea* أو بيضاوي كما في باقي الأنواع، يظهر محيط حبة اللقاح في المنظر القطبي كوحدة واحدة كما في *P. pinea* و *P. roxburghii* أو كوحدين متراكبتين كما في باقي الأنواع، بالإضافة إلى شكل نحت الطبقة الخارجية لحبوب اللقاح. من خلال هذه الدراسة تبين أن الصفات الشكلية لحبوب اللقاح نجحت في تعريف، فصل، والتقييم التصنيفي للوحدات التصنيفية قيد الدراسة. كما دعمت الشجرة التصنيفية الناتجة من برنامج التحليل الاحصائي

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Community Analysis Package فصل خمسة أنواع من جنس الصنوبر في مصر، فأوضحت الشجرة التصنيفية وجود علاقة قوية بين نوعي *P. canariensis* و *P. halepensis*، و أيضاً وجود علاقة قوية بين نوعي *P. brutia* و *P. roxburghii*. جاء النوع *P. pinea* في مجموعة تصنيفية منفصلة عن باقي المجموعات، ولكنه في نفس الوقت ذو ارتباط بالنوعين *P. canariensis* و *P. halepensis*. من أجل معالجة أفضل للبيانات تم ايضاح وتأكيد الشجرة التصنيفية عن طريق عمل خريطة حرارية بالاعتماد على لغة برمجة R للتحليل الاحصائي.