Pollen Variations among some Cultivated Citrus Species and its Related Genera in Egypt

Abstract
The present investigation aims to study the pollen morphology and ultrastructure of pollen grain characteristics for nine Citrus species and three related genera cultivated in Egypt. The pollen grains were photographed by using both Light Microscope (LM) and Scanning Electron Microscope (SEM). Twelve qualitative and quantitative pollen morphological characters were used to differentiate among the studied taxa. Statistical analysis of palynological data indicated that the pollen size, shape, colpi length, apertures number and type, ora size, amb shape, mesocolpium diameter, and the exine ornamentation were the most distinguished characters in the circumscription of the studied taxa and were of taxonomic value. On the contrary, the other studied pollen characters including the ratio between Polar length/Equatorial diameter (P/E), ora shape and exine thickness were not found to be of taxonomic value in the differentiation among the closely related taxa of Citrus, Fortunella margarita, Limequat hybrid and Poncirus trifoliata in the present study.

Keywords: Citrus; Fortunella margarita; Limequat hybrid; Pollen morphology; Poncirus trifoliata; Rutaceae

Abbreviations: LM: Light Microscope; SEM: Scanning Electron Microscope; LSD: Least Significant Difference; SAS: Statistical Analysis System

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Introduction
Cultivated Citrus are derived from various Citrus species found in the wild. Some are only selections of the original wild types, while others are hybrids between two or more ancestors. Many Citrus types were identified and named by individual taxonomists, resulting in a large number of identified species: 870 by a 1969 count [1].

Citrus plants belong to family Rutaceae, they are characterized by having different life forms as trees and shrubs. Citrus species may frequently contain aromatic compounds with pellucid glands on the stems, leaves and fruits. The leaves are usually oppositely, compound and without stipules, sometimes with thorns [2]. According to Engler [3], Rutaceae is divided into seven subfamilies, he defined these subfamilies primarily by gynoecium characters especially the fruit type. Citrus species and its related genera are closely related and all belong to subtribe Citrinæ, tribe Citreae, of the orange subfamily Aurantioideae. The Citrus fruit is of berry or hesperidium type. Species within the genus Citrus are highly economic and medicinal plants distributed all over the world [4]. Several taxonomists have classified various kinds of Citrus species into groups and given them valid names [5-9]. Nicolosi [10] mentioned that there are two currently outstanding systems of classification for Citrus. They are those of Swingle [11] and Swingle and Reece [4] of the USA, and Tanaka [12] of Japan. From the standpoint of the grower, most horticulturists and other plant scientists, Swingle’s system appears to be the most useable.

According to Swingle’s system the Citrinæ subtribe is subdivided into three groups, the ‘primitive Citrus’ distant relatives, the closer ‘near Citrus’ including Citrus-related genera like Atalantia, and the ‘true Citrus’, which included Poncirus, Citrus, Fortunella, Eremocitrus, Microcitrus, and Clymenia, all but the first now viewed to fall within Citrus. He subdivided Citrus into two subgenera: the first subgenus Eucitrus (later called simply subgenus Citrus) includes citrons, pomeles, mandarins, oranges, grapefruits, and lemons, while the hardy but slow-growing trees with relatively unpalatable fruit he placed in subgenus Papeda.
Distinguishing of *Citrus* species and related genera according to morphological and geographical distribution are very difficult because *Citrus* contains an enormous degree of genetic variation, with abundant natural hybridization [13]. The classification of the genus *Citrus* are complex and the precise number of natural species is unclear, as many of the named species are hybrids clonally propagated through seeds (by apomixes) and there is genetic evidence that even some wild, true-breeding species are of hybrid origin [4,14]. As a result of hybridizations between *Citrus* species, there is confusion around correct botanical names of commonly known *Citrus* [10,15]. In Egypt, there are no wild *Citrus* species [16]. All the present species are introduced and cultivated. Now, Egypt is considered as one of the most leading countries in cultivating and exporting orange, ranking as the sixth-largest producer and the second-largest exporter in the world [17].

The use of pollen morphological characters is important in plant taxonomy, as Davis and Heywood [18] indicated. The use of pollen morphology in solving taxonomic problems has been used for a long time ago [19-26]. This work is considered as a step in finding way in differentiating between nine *Citrus* species and its related genera.

This study aims to investigate and assess the relationships among nine *Citrus* species as well as three related genera cultivated in Egypt using pollen morphological characters.

**Materials and Methods**

The present investigation was carried out on mature trees of nine *Citrus* species and three related genera; *Citrus aurontifolia* (Christm.) Swing., *Citrus aurantium* (L.), *Citrus grandis* (L.) Osbeck, *Citrus latifolia* Tanaka, *Citrus limetta* Risso. *Citrus paradisi* Macf., *Citrus reshni* Hort. ex Tanaka, *Citrus reticulate* Blanco, *Citrus sinensis* (L.) Osbeck, *Furtunella margarita* (Lour.) Swing. and *Limquat* hybrid, which is a cross hybrid between the *Citrus aurontifolia* (Christm.) Swing. and *Furtunella japonica* (Thunb.) Swing., and *Poncirus trifoliate* (L.) Raf. grown in a private orchard. This orchard located 120 Km away from Alexandria on Alexandria-Cairo desert road. These species are identified by the aid of the faculty of Agriculture, Alexandria University, as well as vouchers of the studied taxa are allocated there.

The trees were grown in sandy soil and received the same cultural practices as usually done in each orchard. Four uniform trees were selected from each *Citrus* species and related genera, from which mature anthers were taken from the uppermost flowers of the branches to obtain the mature pollen grains used in this investigation.

Pollen grain samples of all studied taxa were acetolized according to Erdtman’s technique [19]. The acetolyzed samples were used for both Light and Scanning Electron Microscopy. Slides were prepared from acetolized portion of pollen grains for Light Microscope examination by mounting in glycerin jelly, examined and measured using Zeiss Light Microscope with a pre-calibrated eye-piece micrometer. Measurements given are the means of forty acetolized well-developed pollen grains from each taxon.

Pollen grains of the acetolized portion were dehydrated in ethanol series placed onto coverslips, left for ethanol evaporation then attached to copper stubs by double-sided tape, coated with 30 nm gold using fine coat ion sputter JEOL JFC 1100E, examined and photographed at 30 KV using JEOL JSM-3500 Scanning Electron Microscope present in the Faculty of Science, Alexandria University. The terminology used here is those of Faegri [27] and Erdtman [19].

**Statistical analysis**

The mean values of the pollen characters, of all the taxa under investigation, were separated and calculated. They were then compared using the Least Significant Difference (LSD) test at 0.05 level of probability, according to Snedecor and Cochran [28]. The statistical analysis was performed using Statistical Analysis System (SAS) version 9.13 [29].

**Results**

**Pollen grain morphology**

**Shape:** The results obtained from the twelve studied taxa are summarized in Table 1 and were illustrated in plates 1-12. The pollen grains of all taxa were monads, radially symmetric, isopolar and were different in size. The pollen shape varied from prolate spheroidal to sub-prolate except in *F. margarita* (Figure 1), where it was oblate spheroidal. The mean polar axis length varied from a minimum of 26 μm in both *F. margarita* and Limquat to a maximum of 34.48 μm in *C. grandis*. Moreover, the mean equatorial diameter ranged from a minimum of 26 μm in both *F. margarita* and Limquat to a maximum of 33.44 μm in *C. grandis*. While the minimum ratio of the mean length of Polar Axis/ Mean Equatorial Diameter (P/E) was 1 in both *F. margarita* and Limquat, while the maximum mean ratio was 1.2 in *C. latifolia*.

**Aperture:** The types of apertures were either colpate or colporate ranged from three to five in number. The variations in the type and number of apertures were found to be within the same taxa and within the same anther as well. Five groups of aperture types were found; the first group included three taxa characterized by tri-tetra-colporate aperture types, *C. limetta* (Figure 2a and 2b), *C. reshni* (Figure 3a, 3b and 3d) and Limquat (Figure 4a, 4b and 4d). The second group included also three taxa characterized by tetra-penta-colporate aperture types in *C. aurontifolia* (Figure 5a, 5b and 5d), *C. reticulate* (Figure 6a, 6b and 6d) and *P. trifoliate* (Figure 7a-7c). The third group has only *F. margarita* which included the tetra-penta-colporate types, in addition to the tri-colporate ones (Figure 1a, 1b, 1d and 1e). The taxa in the fourth group characterized by tri-tetra-colpate and tri-tetra-colporate aperture types in *C. grandis* (Figure 8a-8c and 8e) and *C. sinensis* (Figure 9a and 9b). Finally, group five comprised *C. aurantium*, *C. latifolia* and *C. paradisi* with pollen grains that have tetra-penta-colpate and tetra-penta-colporate types of apertures (Figures 10a-10d, 11a-11c, 11e and 12a, 12c), respectively.

The ecto-aperture colpi, in all the studied taxa were long, wide, with rounded or pointed ends, equally spaced around the equator. They were characterized by uneven margins and covered with granular membranes. The mean colpi length varied within the studied taxa from a minimum of 20 μm in both *F. margarita* and...
Table 1. Pollen morphological characters of the studied Citrus species and its related taxa.

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<tbody>
<tr>
<td>1</td>
<td>C. aurantifolia (Christm.) Swing.</td>
<td>Mexican lime</td>
<td>31.3</td>
<td>27.5</td>
<td>1.14</td>
<td>3</td>
<td>2</td>
<td>27</td>
<td>9.7</td>
<td>2.08</td>
<td>7.71</td>
<td>2</td>
<td>2.4</td>
<td>4</td>
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<td>2</td>
<td>C. aurantium L.</td>
<td>Sour orange</td>
<td>30.7</td>
<td>28.3</td>
<td>1.09</td>
<td>2</td>
<td>5</td>
<td>25.4</td>
<td>9.36</td>
<td>3.2</td>
<td>8.3</td>
<td>2</td>
<td>2.7</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>C. grandis (L.) Osbeck</td>
<td>Pummelo</td>
<td>34.5</td>
<td>33.4</td>
<td>1.03</td>
<td>2</td>
<td>4</td>
<td>29.1</td>
<td>11.22</td>
<td>3.06</td>
<td>7.9</td>
<td>1</td>
<td>2.4</td>
<td>1</td>
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<td>4</td>
<td>C. latifolia Tanaka</td>
<td>Tahiti lime</td>
<td>31.6</td>
<td>26.4</td>
<td>1.2</td>
<td>3</td>
<td>5</td>
<td>26.4</td>
<td>9.36</td>
<td>2.97</td>
<td>7.69</td>
<td>2</td>
<td>2.4</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>C. limetta Riso</td>
<td>Sweet lime</td>
<td>33.7</td>
<td>31.8</td>
<td>1.07</td>
<td>2</td>
<td>1</td>
<td>28.4</td>
<td>12.72</td>
<td>3.2</td>
<td>7.3</td>
<td>1</td>
<td>2.4</td>
<td>4</td>
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<tr>
<td>6</td>
<td>C. paradise Macf.</td>
<td>Marsh grapefruit</td>
<td>33.1</td>
<td>30.6</td>
<td>1.09</td>
<td>2</td>
<td>5</td>
<td>27.9</td>
<td>10.54</td>
<td>3.3</td>
<td>6.9</td>
<td>2</td>
<td>2.4</td>
<td>1</td>
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<tr>
<td>7</td>
<td>C. reshni Hort. ex Tanaka</td>
<td>Cleopatra mandarin</td>
<td>29.8</td>
<td>27.5</td>
<td>1.09</td>
<td>2</td>
<td>1</td>
<td>24.5</td>
<td>9.78</td>
<td>4.2</td>
<td>8.1</td>
<td>1</td>
<td>2.4</td>
<td>2</td>
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<tr>
<td>8</td>
<td>C. reticulata Blanco</td>
<td>Clementine tangerine</td>
<td>30.5</td>
<td>28.1</td>
<td>1.09</td>
<td>2</td>
<td>2</td>
<td>24.8</td>
<td>10.3</td>
<td>2.9</td>
<td>6.8</td>
<td>2</td>
<td>2.4</td>
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<tr>
<td>9</td>
<td>C. sinensis (L.) Osbeck</td>
<td>Succari orange</td>
<td>32.2</td>
<td>29.6</td>
<td>1.1</td>
<td>2</td>
<td>4</td>
<td>27.6</td>
<td>10.4</td>
<td>3.6</td>
<td>7.2</td>
<td>1</td>
<td>2.4</td>
<td>1</td>
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<td>10</td>
<td>Fortunella margarita (lour.) Swing.</td>
<td>Oval Kumquat</td>
<td>26</td>
<td>26.2</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>21</td>
<td>9.82</td>
<td>3.5</td>
<td>6.08</td>
<td>3</td>
<td>2.4</td>
<td>3</td>
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<tr>
<td>11</td>
<td>Limequat (Hybrid) [C. aurifolia (Christm.) Swing. × F. japonica (Thunb.) Swing.]</td>
<td>Limequat (hybrid)</td>
<td>26.6</td>
<td>26.1</td>
<td>1.02</td>
<td>2</td>
<td>1</td>
<td>21</td>
<td>9.82</td>
<td>3</td>
<td>6.4</td>
<td>1</td>
<td>2.4</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>Poncirus trifoliata (L.) Raf.</td>
<td>Trifoliata orange</td>
<td>32.3</td>
<td>31.2</td>
<td>1.04</td>
<td>2</td>
<td>2</td>
<td>26.4</td>
<td>10.72</td>
<td>3.2</td>
<td>6.4</td>
<td>2</td>
<td>2.4</td>
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LSD_{0.05} 0.93 0.89 0.03 0.94 0.71 0.44 0.8 0.04


Limequat to a maximum of 29.08 μm in C. grandis. Moreover, the mean mesocolpium diameter varied from a minimum of 9.36 μm in C. aurantium and C. latifolia to 12.72 μm in C. limetta. The endoapertures pori were lalongate in all the studied taxa, where the ora width ranged from 6.08-6.4 μm in F. margarita, Limequat and P. trifolia; slightly wider from 6.8 to 7.3 μm in C. reticulate, C. paradise, C. sinensis and C. limetta and more than 7.3 μm in the rest of the taxa. The amb shapes are mostly rounded triangular or square and sometimes both shapes are found. The pollen amb was rounded triangular and square in C. limetta (Figure 2a and 2b), C. reshni (Figure 3b and 3d), Limequat (Figure 4b and 4d), C. grandis (Figure 8c and 8e) and C. sinensis (Figure 9a and 9b). While it was square and rounded in C. aurantifolia (Figure 5b and 5d), C. reticulata (Figure 6b and 6d) and P. trifoliata (Figure 7b and 7d), C. aurantium (Figure 10b), C. latifolia (Figure 11c and 11e), C. paradisi (Figure 12a and 12c). In addition, the amb was rounded triangular, square and rounded F. margarita (Figure 1b, 1d and 1e).

Exine: The exine is considerably thin; it was 2.4 μm thick in all the studied taxa, except in C. aurantium it was 2.7 μm. The exine ornamentation of the pollen grains of the studied taxa; as observed by the scanning electron microscope appeared in four different types. The first type was tectate perforate with smooth tectum, which is provided by more or less rounded pores in C. grandis (Figure 8d), C. sinensis (Figure 9c) and C. paradisi (Figure 12b). The second type was tectate perforate to microreticulate with latimurate reticulum, which is characterized by more or less straight and smooth muri and rounded to oval small-sized lumina in C. reshni (Figure 3c), and C. reticulata (Figure 6c). The third type was foveolate with latimurate reticulum, which is characterized by more or less straight and smooth muri and nearly rounded large-sized lumina in F. margarita (Figure 1c), Limequat (Figure 4c) and P. trifoliata (Figure 7c) and C. aurantium (Figure 10c and 10d). The fourth type was reticulate with angustimurate reticulum, which is characterized by straight and rough muri and the lumina were different in size and shape in C. limetta (Figure 2c), C. aurantifolia (Figure 5c) and C. latifolia (Figure 11d).

Pollen grains classification

Accordingly, the studied taxa can be classified into three different...
groups according to their pollen characters: The first group included five taxa; *C. grandis, C. limetta, C. paradise, C. sinensis*
and *P. trifoliata*. These five taxa were characterized by the biggest pollen size; where the polar axis length was more than 32.20 µm, with prolate spheroidal pollen shape, colpi length more than 26.44 µm and mesocolpium diameter exceed 10.30 µm.

The second group included five taxa as well; *C. aurantifolia*, *C. aurantium*, *C. latifolia*, *C. reshni* and *C. reticulate*. These taxa...
Pollen morphology of Rutaceae has been examined by several workers; they found their taxonomic significance in different taxonomic levels, depending on the plant groups [19,25,33,34]. However, there are no reports on pollen morphology of Citrus species in Egypt. Inyama et al. [25] found that palynological characters were useful in delimiting six studied Citrus species and thus could be exploited in conjunction with other evidence in species identification and characterization, while they were insignificant in the reclassification of the investigated taxa. In the present study, palynological investigations indicated that variations in pollen morphological characters were of taxonomic significance.

In particular, the twelve studied taxa were significantly different from each other in six quantitative pollen characters: polar length, equatorial diameter, colpi length, ora length, ora diameter, mesocolpi diameter. These results were in agreement with those reported by Breis et al. [35] and Mohammad et al. [36]. The pollen shape varies from oblate-spheroidal, prolate-spheroidal to sub-prolate in all the studied taxa. This finding agrees with that found by Ye et al. [37] and Mohammad et al. [36]. The variations of pollen size were suggested by Kozaki and Hirai [38] and Mohammad et al. [36] where they reported that pollen grain of C. grandis and Poncirus trifoliate had larger pollen than C. latifolia, C. limetta and F. margarita, while those of C. aurantium, C. sinensis and C. reshni were intermediate in size. These suggestions were in agreement with the results of the present study where the studied taxa classified into three different groups according to their pollen size. The first group included C. grandis, C. limetta, C. paradise, C. sinensis and P. trifoliate, which have the largest pollen grains. The second group included C. aurantifolia, C. aurantium, C. latifolia, C. reshni and C. reticulate have medium size pollen grains. While the third one included two taxa F. margarita and Limequat with the smallest pollen grains. On the contrary, these groups did not coordinate with Al-Anbariet al. [34], which recognized four groups in the Iraqi pollen grains based on pollen size and exine ornamentation only.

Meanwhile, the most variable characters found in the present investigation were within the number and type of apertures, exine ornamentations, ora width as well as mesocolpium diameters. Ye et al. [37] and Mohammad et al. [36] used both ora width and mesocolpi diameters as valuable characters in the identification of Citrus species; these results agree with the results of this work.

Grant et al. [39] found considerable variation in pollen morphology of subfamily Aurantioideae, which divided the studied taxa into five pollen types. The differences include aperture number, ektocolpus shape and size, exine ornamentation and wall structure. When designating pollen types for the subfamily Aurantioideae, the principal characters used were the aperture number and exine ornamentation. These characters were in concomitant with the obtained results and as a conclusion, the aperture type and ora size were the most distinguished characters in the circumscription of the studied taxa. According to the type and number of aperture five types were observed in the studied taxa. Type (1) Tri-tetra-colporate was found in C. limetta, C. reshni and Limequat. Type (2) Tetra-penta-colporate was found in C. aurantifolia, C. reticulata and P. trifoliate. Type (3) Penta-

### Statistical analysis

In the present study, palynological investigations indicated that variations in pollen morphological characters were of taxonomic significance. In particular, the twelve studied taxa were found to be significantly different from each other in six quantitative pollen characters out of eight; this includes polar length, equatorial diameter, colpi length, ora length, ora diameter, mesocolpi diameter. While the ratio between the Polar length and Equatorial diameter (P/E) and the exine thickness were insignificantly different from each other (Table 1).

### Discussion

Pollen morphology has been used since a long time ago in solving taxonomic problems at different levels; families, genera and species [19,23,27,30] Genus Citrus and its related two genera: Fortunella and Poncirus are considered one of the important economic and medicinal fruits in the world, they are rich plants in vitamin C and volatile oils. The problem within these taxa is the frequent hybridizations between their species, which made their taxonomy very confusing.

The classification and species delimitation of the genus has long been a controversial issue by a number of authors as Swingle [11], who included only 16 species in Citrus, while Tanaka [9] described 162 species, but Scora [15] and Baret and Rodes [31] defined only three true species within the genus Citrus, which are Pummelo (C. grandis L. Osbeck), Citron (C. medica L.) and Mandarin (C. reticulata Blanco.). They indicated that all other Citrus species resulting from hybridization between these basic species. Later, Scora [32] added another true species C. halimii Stone.
colporate was found in *F. margarita*. Type (4) included both "tri-tetracolpate and tri-tetra-colporate" were found in *C. grandis* and *C. sinensis*. Finally, type (5) included both "tetra-penta-colpate and tetra-penta-colporate" were found in *C. aurantium*, *C. latifolia* and *C. paradisi*. These multi types of pollen apertures were found in the studied species from the same anther which may be due to chromosomal abnormalities as mentioned by Stace et al. [40].

In this study, the exine thickness was an insignificant character, while the exine ornamentations showed great variations in the sculpturing types and have taxonomic value in the classification of the studied taxa. According to the exine ornamentations four different types were observed. Type (1) Tectate perforate in *C. grandis*, *C. paradisi* and *C. sinensis*. Type (2) Tectate perforate to micro reticulate in *C. reshni*, *C. reticulata*. While Type (3) Foveolate with latimurate reticulum in *C. aurantium*, *F. margarita*, Limequat and *P. trifoliata*. Type (4) Reticulate with angustimurate reticulum, in *C. aurantifolia*, *C. latifolia* and *C. limetta*. These findings agree with those found by Ye et al. [37] and Mohammad et al. [36]. While disagree with the results of Kozaki and Hirai [38], who stated that the exine patterns was sub-reticulate in the species of *Citrus*, *Poncirus* and *Fortunella*.

**Conclusion**

In the present investigation, the pollen size, pollen shape, colpi length, the apertures number and type, ora size, amb shape, mesocolpium diameter, and exine ornamentation were the most distinguished characters in the circumscription of the studied taxa. All the studied pollen grain characters except ora shape and exine thickness could be considered of taxonomic value in the differentiation among the closely related taxa of *Citrus*, *Fortunella*, Limequat hybrid and *Poncirus* in the present study.
References


