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Molecular Taxonomy Matches Pollen Grains Taxonomy in Differentiation Between Some Caesalpinioideae Taxa

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ABSTRACT

Phylogeny investigation relying on chloroplast genome sequence is an established approach in differentiation between plants derived from related ancestors. In this study, we compared the phylogenetic tree resulted from chloroplast genome sequences to phylogenetic tree generated from pollen grains features of ten taxa belonging to the Caesalpinioideae sub-family, grown in Egypt. Chloroplast genome sequences of taxa under investigation, retrieved from the National Center for Biotechnology Information (NCBI), were used to generate a phylogenetic tree using RStudio® software. Pollen grain features were investigated using the Scanning Electron Microscope (SEM) and data were combined in a numerical cluster to compose a phylogenetic tree. Both phylogenetic trees, originating from chloroplast genome sequences and pollen grains features in our study indicated high similarity in relatedness between taxa. Our results suggest the utilization of both pollen grains features and molecular characteristics in discrimination between plants stemming from common ancestors. The novelty of this work lies in comparing similarities and differences of the investigated species through combining their pollen and molecular characteristics altogether. The identification key of the investigated species, reported in this manuscript, is hoped to be a reliable approach and can be utilized by taxonomists for taxa identification in the future.

***Keywords*:** Phylogeny, pollen grains taxonomy, Molecular taxonomy, Caesalpinioideae.

INTRODUCTION

Taxonomic significance of the sub-family Caesalpinioideae has long been recognized (Hubbard and Hutchinson, 1948; Klitard, 2010). The name of this sub-family is derived from its genus *Caesalpinia*. The study of pollen grains' features is one of the most important techniques in plant taxonomy, especially in the continuous development in electron microscope applications. Pollen grains' features was, and still, a reliable source in the field of taxonomy (Erdtman, 1954). In addition, genetic approaches have been utilized by modern systematic community (Wen et al., 2017).

Botanical features of Mimosaceae were compared to those of Caesalpinioideae using pollen grains characteristics (Kattab et al., 2007). Ullah et al. (2021) concluded that pollen traits are a powerful tool in determining boundaries between related species at various taxonomic levels; therefore, strengthening the taxonomy of the Caesalpinioideae sub-family. Similarly, ElKholy et al. (2023) differentiated between members of Mimosoideae and Caesalpinioideae on the basis of pollen features. Abdalla and El Ghazali (2016) demonstrated that pollen characteristics diagnose and characterize plants of the genus *Cassia*.

Numerous molecular markers obtained from intragenic and intergenic regions of chloroplast genes have been used as plant DNA barcodes (Kress et al., 2005; Awad et al., 2017). MatK chloroplast gene has also been used for plant identification and classification (Hollingsworth et al., 2009). In this manuscript, we used chloroplast genome sequences and pollen characteristics to distinguish between ten species from Caesalpinioideae subfamily grown in Egypt.

MATERIALS AND METHODS

Taxa collection and identification:

Ten specimens, representing subfamily Caesalpinioideae and belonging to five genera (*Bauhinia*, *Cassia*, *Ceratonia*, *Delonix* and *Parkinsonia*), namely *Bauhinia madagascariensis*, *Bauhinia monandra*, *Bauhinia tomentosa*, *Bauhinia veriegata*, *Cassia bicapsularis*, *Cassia fistula*, *Cassia nodosa*, *Ceratonia siliqua*, *Delonix regia* and *Parkinsonia aculeate* were collected from the herbarium of the Department of Agricultural-botany, Faculty of Agriculture, Al-Azhar university. Collected specimens were identified through comparing morphological, flowering, and fruiting characteristics with previously identified samples. Specimen were also compared to references, such as Tackholm, 1974; and Boulos, 2002. The source of descriptive terminology used in this manuscript is from Erdtman (1952 and 1954); Faergri et al., 1989; Punt, 2007; Stephen, 2014.

Pollen samples preparation for scanning electron microscopy (SEM):

To ensure dryness of all samples prior to starting this investigation, mature anthers from each sample were separated and kept overnight in drying oven at 70 °C. Specimens were prepared for SEM examination by mashing three anthers on a slide to separate pollen from the anthers. Pollen grains were further mounted on clean SEM stubs, covered with double-sided sellotape, and coated with gold-palladium. SEM , model JEOL JSM-6510LV (operated by the Electron Microscope Unit, Mansoura University, Egypt), was used to observe the samples at 30 KV.

Bioinformatics and data analysis

Data retrieved from pollen grains' characters were analyzed utilizing Multi-Variate Statistical Package Program

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(MVSP; version 3.1), (Sneath, 1973). A total of ten chloroplast genome sequences, representing taxa under investigation, were retrieved from the National Center for Biotechnology Information (NCBI) nucleotide database, and used for phylogeny investigation. RStudio (R Core Team, 2022) software (version 4.2.0) was used to generate the phylogenetic tree, utilizing the required packages for alignment and visualization.

RESULTS AND DISCUSSION

Pollen grains in all examined samples of this study were monads. Pollen samples were classified in three distinct groups; according to their sizes. The first group contained genus *Bauhinia* preserved large-sized pollen grains, compared to the other groups investigated. The second group, genus *Delonix*, *Ceratonia* and *Cassia*, preserved medium-sized pollen grains. The third group, genus *Parkinsonia*, preserved the smallest pollen grains. It is worth mentioning that pollen grains from *Cassia bicapsularis*, which belongs to genus *Cassia* in the second group, preserved smaller pollen grains; similar to taxa grouped in the third group. These results are in line with those reported by Aftab and Perveen (2006) and Abo-Elnaga et.al. (2022).

Two types of aperture were found in pollen samples of this study. The majority of taxa preserved colporate aperture, such as *Bauhinia veriegata* (Fig. 1-D). These results agree with finding reported by Taia et.al. (2022) and Abdalla and El Ghazali (2014). In contrast, colpate aperture was only found in four taxa; *Bauhinia madagascariensis*, *Bauhinia monandra*, *Bauhinia tomentosa*, *and Cassia bicapsularis* (Fig. 1- E); similar to findings mentioned by Arogundade et al. (2019) and Banks et al. (2013).

We noticed a difference in the number of aperture. In most taxa, Tricolporate, such as *Delonix regia* was observed (Fig. 1-I), while Tetracolporate was observed only in *Ceratonia siliqua* and pentacolpate was only observed in *Bauhinia tomentosa* (Fig 1- H and 1-C, respectively). Such observations are in accordance with findings mentioned by Abdalla and El Ghazali, 2014; Graham et.al., 1980; and Banks et al., 2014, respectively.

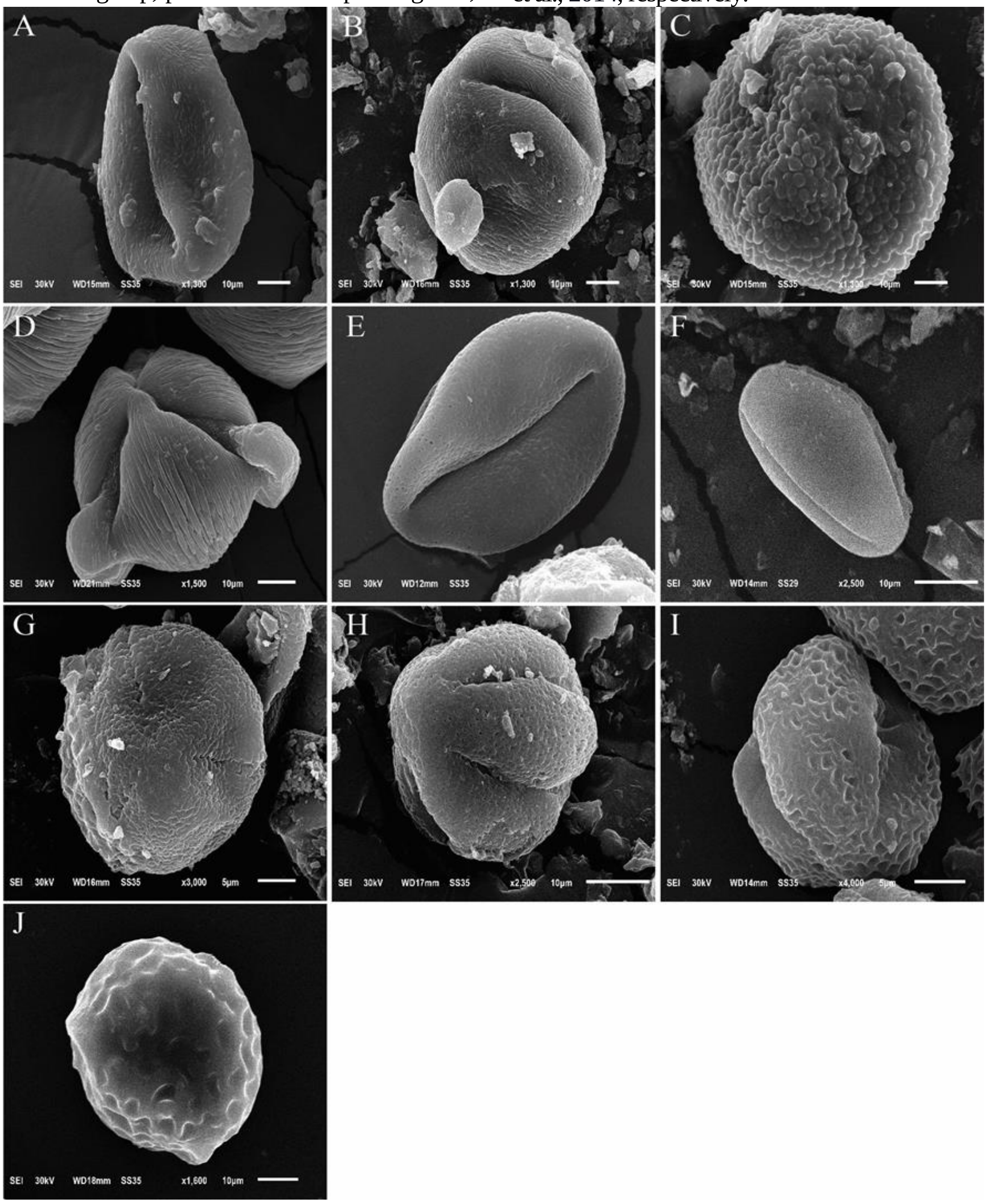

Fig. 1. Scanning electron micrographs of pollen grains collected from 10 taxa of the sub-family Caesalpinioideae; A) *Bauhinia madagascariensis;* B) *Bauhinia monandra;* C) *Bauhinia tomentosa*; D) *Bauhinia veriegata*; E) *Cassia bicapsularis;* F) *Cassia fistula*; G) *Cassia nodosa*; H) *Ceratonia siliqua*; I) *Delonix regia*; J) *Parkinsonia aculeate*.

Exine sculpture patterns of the taxa investigated have shown unique distinction. Seven distinguished patterns were observed; 1) Reticulate in three taxa *Bauhinia monandra*, *Delonix regia* and *Parkinsonia aculeate* (Fig. 2-B, I and J), in agreement with Ullah et al. (2022) and Banks and Rudall, 2016; 2) Psilate in two taxa, *Cassia fistula* and *Ceratonia siliqua* (Fig. 2-F and H), in agreement with Santos et al., 2012; 3) Microreticulate in *Bauhinia madagascariensis* (Fig 2-A), in agreement with Santos et al., 2012 and Sarwar et al., 2015; 4) Clavate in *Bauhinia tomentosa* (Fig. 2-C), in agreement with Banks et al., 2014; 5) Striate in *Bauhinia veriegata* (Fig. 2-D), in line with the findings reported by Ullah et al., 2022 and Taia et al, 2022; 6) Perforate in *Cassia bicapsularis* (Fig. 2-E), in agreement with Sarwar et al., 2015; 7) Coarsely regulate in *Cassia nodosa* (Fig. 2-G), in conformity with the findings of Banks et al., 2013 and Sarwar et al., 2015.

In the current study, shape of pollen grains, as determined by the ratio between polar axis and equatorial axis was also recorded and used as a diagnostic feature to distinguish between studied taxa (Tab. 1 and Fig. 3). The majority of the examined taxa exhibited prolate or spherical shapes, such as *Bauhinia tomentosa* (Fig. 1-C); while *Bauhinia madagascariensis* and *Cassia fistula* showed prolate shapes (Fig. 1-A and F). Oblate-spheroidal shape was observed in *Bauhinia monandra* and *Bauhinia veriegata* (Fig. 1, B and D). Polar axis varied from 23.48 µm to 70.15 µm and equatorial axis ranged from 20.80 µm to 61.90 µm; while diameter in polar view varied from 17.99 µm to 78.99 µm (Tab. 1). Colpus length ranges from 17.99 µm to 52.41 µm and width was 3.93 µm to 13.47 µm; while the ratio between colpus length and colpus width varied from 3.15 µm to 7.28 µm (Tab. 1 and Fig. 4). It is worth mentioning that apocolpium index ranged from 3.01 (in *P. aculeate*) to 6.67 (in *B. madagascariensis*) (Tab.1 and Fig. 5). These results agree with Aftab and Perveen (2006) and Gharnit et al. (2004).

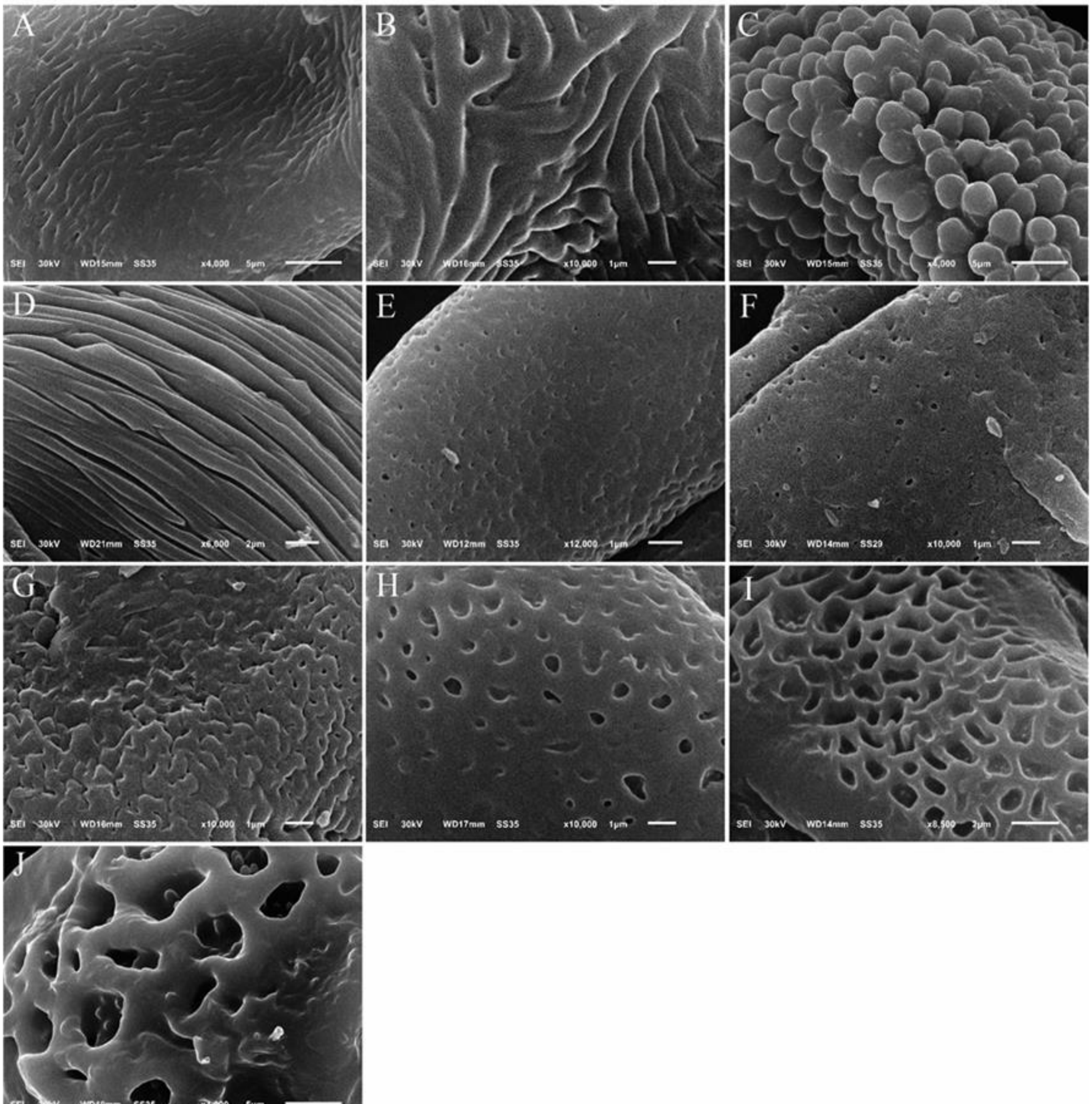

Fig. 2. Exine sculpture patterns of pollen grains collected from the 10 taxa of the sub-family Caesalpinioideae; A) *Bauhinia madagascariensis;* B) *Bauhinia monandra;* C) *Bauhinia tomentosa*; D) *Bauhinia veriegata*; E) *Cassia bicapsularis;* F) *Cassia fistula*; G) *Cassia nodosa*; H) *Ceratonia siliqua*; I) *Delonix regia*; J) *Parkinsonia aculeate*.

Table 1: Data matrix of the observed characters of taxa

Taxa	Accession Number	Polar axis µm	Equatorial axis µm	P/e µm	Shape in Equatorial view	diameter in polar view µm	Aperture type	Number of aperture	colpus length µm	Colpus width µm	colpus length/ Colpus width µm	distance between the apices of two ectocolpi µm	Apocolpium index	Exine sculpture pattern	Size type
Bauhinia madagascariensis	JN881376	66.66	47.62	1.39	Prolate	57.25-52.02	Colpate	Tricolpate	51.95	7.05	7.28	7.14	6.67	Microreticulate	MA
Bauhinia monandra	JN881378	58.56	61.74	0.95	Oblate spheroidal	62.22-59.04	Colpate	Tricolpate	52.41	9.52	5.00	10.47	5.90	Reticulate	MA
Bauhinia tomentosa	JN881403.1	70.15	61.90	1.13	Prolate spherìdal	70.47-78.88	Colpate	Pentacolpate	42.85	13.47	3.20	12.04	5.15	Clavate	MA
Bauhinia veriegata	MT176420.1	51.69	53.07	0.97	Oblate spheroidal	50.51-50.99	Colporate	Tricolporate	42.13	7.24	5.89	9.65	5.50	Striate	MA
Cassia bicapsularis	MT559309.1	25.70	22.49	1.14	Prolate spheridal	21.12-19.99	Colpate	Tricolpate	19.88	3.93	5.05	3.75	6.00	Perforate	MI
Cassia fistula	ON099431.1	32.61	22.69	1.43	Prolate	32.61-18.69	Colporate	Tricolporate	28.78	5.04	5.71	4.31	5.26	Psilate	ME
Cassia nodosa	EU361910.1	30.82	28.53	1.08	Prolate spheridal	28.05-27.91	Colporate	Tricolporate	25.75	5.00	5.15	8.00	3.56	Coarsely rugulate	ME
Ceratonia siliqua	KJ468096.1	33.60	30.77	1.09	Prolate spheridal	33.24-30.77	Colporate	Tetracolporate	26.59	5.60	4.75	8.78	3.50	Psilate	ME
Delonix r egia	MN893243.1	41.50	40.53	1.03	Prolate spheridal	41.52-40.53	Colporate	Tricolporate	33.30	7.57	4.40	9.09	4.46	Reticulate	ME
Parkinsonia aculeate	MW628953.1	23.48	20.80	1.12	Prolate spheridal	20.40-17.99	Colporate	Tricolporate	17.99	5.71	3.15	6.92	3.01	Reticulate	MI

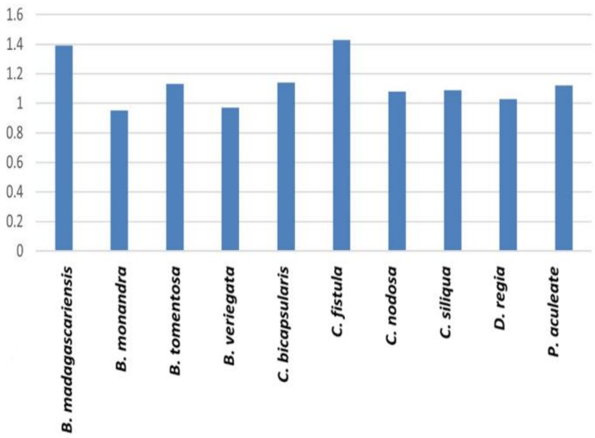

Fig. 3: The ration of polar axis to equatorial axis.

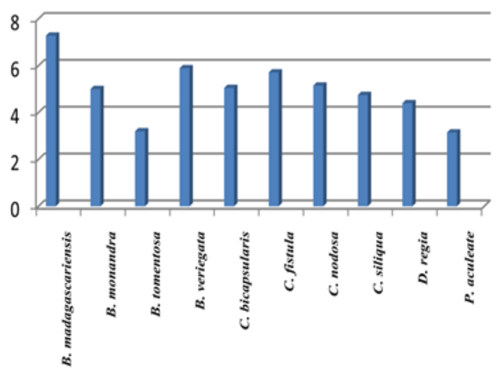

Fig. 4: The ratio of colpus length to colpus width.

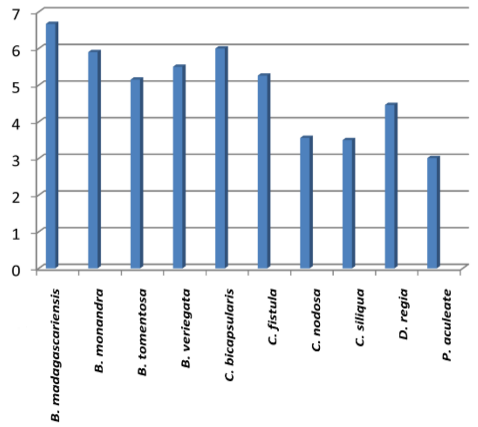

Fig. 5: Apocolpium index.

Cluster analysis

Numerical analysis of the pollen characteristics of ten taxa in this study (data matrix; Tab. 1) resulted in a phylogenetic tree (Fig. 6) that divided the taxa into two groups; the 1st group included genus *Bauhinia* as well as two sub-groups; *Bauhinia veriegata* and the rest of *Bauhinia* (due to its difference a number of characteristics such as exine sculpture striate and aperture type colporate); the 2nd group was also divided into two sub-groups; the 1st sub-group contained genus *Cassia* (it was also noted that *Cassia bicapsularis* differed from the rest of the genus within this group due to its unique characteristics such as exine sculpture, which was perforate); the 2nd sub-group contained three genera (*Ceratonia*, *Delonix* and *Parkinsonia*) and the last two were closer to each other, compared to *Ceratonia*. Our numerical cluster analysis results is in harmony with El Kholy et al. (2023).

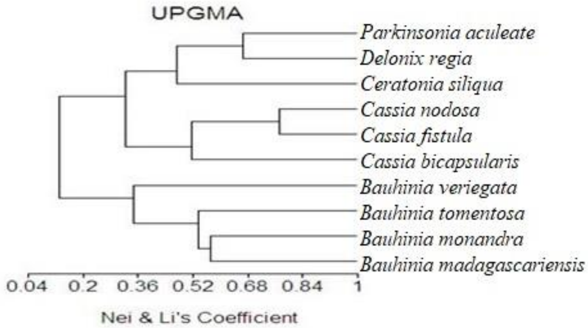

Fig. 6. Phylogenetic tree resulting from pollen characteristics of investigated taxa.

Phylogeny analysis

The history of descent of taxa under investigation, from their common ancestors, revealed that taxa are grouped in three clusters (Fig. 7). The 1st cluster contained *D. regia* and *P. aculeata*. The 2nd cluster included *C. fistula*, *C. nodosa* and *C. Bicapsularis*. However, *C. fistula* and *C. nodosa* are more closely related to each other than *C.* bicapsularis. The third cluster included *B. monandra*, *B. madagascariensis*, *B. tomentosa*, *B. variegata* and *C. siliqua*, with *B. monandra* and *B. madagascariensis* were more closely related (sharing the most recent common ancestor) than other taxa in this group. *C. siliqua* was the least related taxon in this cluster though it shares the same ancestor with the remaining four taxa. Considering the aforementioned relationships as revealed from cluster and phylogeny analyses, it is evident that chloroplast genome sequences phylogeny results (Fig. 7) agree with numerical pollen characteristics results (Fig. 6).

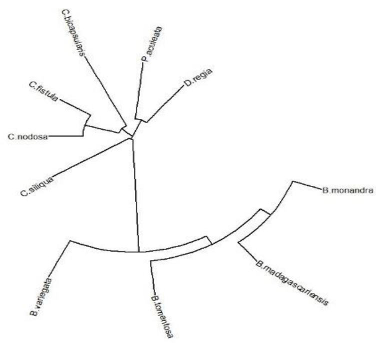

Fig. 7. Phylogenetic tree resulted from chloroplast genome sequences. RStudio® software was used to visualize the tree.

Identification key of ten taxa belonging to sub-family Caesalpinioideae, based on observed pollen grains' features:

1- Pollen grains, Colpate:

A- Pollen grains, Pentacolpate……… *Bauhinia tomentosa*

B- Pollen grains, Tricolpate

B1- Prolate, Microreticulate……… *Bauhinia madagascariensis*

B2- Oblate spheroidal, Reticulate……… *Bauhinia monandra*

B3- Prolate spheroidal, Perforate……… *Cassia bicapsularis*

2- Pollen grains, Colporate:

a- Pollen grains, Tricolporate

a1- Large spores (50 to 100 µm): Striate oblate spheroidal……… *Bauhinia veriegata*

a2- Medium spores (25 to 50 µm):

a2a- Psilate, Prolate……… *Cassia fistula L.*

a2b- Coarsely regulate Prolate spheroidal……… *Cassia nodosa*

a2c- Reticulate Prolate spheroidal……… *Delonix regia*

a3- Small spores (10 to 15 µm): Reticulate Prolat spheroidal……… *Parkinsonia aculeate* .

b- Pollen grains, Tetracolporate……… *Ceratonia siliqua*

CONCLUSION

In this manuscript, we compared related ten taxa from Caesalpinioideae using molecular and pollen characteristics. Sequences from the NCBI were used to generate phylogenetic tree of the investigated taxa using RStudio software. Pollen grains' features of the investigated taxa have also been recorded using SEM imaging, and data have been used to generate an identification key as well as a phylogenetic tree. Phylogenetic tree from molecular and pollen characteristics were compared to each other and they were similar. Reliable taxonomic approaches should be based on numerous characteristics (such as pollen, morphological and molecular characteristics, etc.) to weed out superficial affinity and/or differences between investigated taxa.

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"تشابه التقسيم الجزيئي مع تقسيم حبوب اللقاح فى التفرقة بين بعض انواع تحت الفصيلة البقمية"

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الملخص

يعتبر التصنيف التطورى الذي يعتمد على تسلسل جينوم البلاستيدات الخضراء نهجًا راسخًا في التمييز بين النباتات الناتجة من أسلاف ذات قرابة. في هذه الدراسة، قمنا بمقارنة شجرة القرابة الناتجة من تسلسل جينوم البلاستيدات الخضراء مع شجرة القرابة الناتجة من بعض خصائص حبوب اللقاح لعشرة أصناف تنتمي إلى تحت فصيلة Caesalpinioideae المنزرعة في مصر. تم استخدام تسلسل جينوم البلاستيدات الخضراء للأصناف قيد الدراسة، والتي تم إستنباطها من المركز الوطني لمعلومات التكنولوجيا الحيوية (NCBI)، لإنشاء شجرة التطور وذلك باستخدام برنامج RStudio®. فى المقابل، تم فحص بعض خصائص حبوب اللقاح باستخدام الميكروسكوب الإلكتروني الماسح (SEM) وتم دمج البيانات الناتجة في مجموعة عددية لتوليد شجرة قرابة. أظهرت نتائج كلا من شجرتى القرابة (الناتجتين من تسلسل جينوم البلاستيدات الخضراء المستنبطه من NCBI وكذلك بعض خصائص حبوب اللقاح) في هذه الدراسة تشابهًا كبيرًا في الترابط بين الوحدات التصنيفية محل الدراسة . تشير النتائج إلى اهمية إستخدام خصائص حبوب اللقاح جنبا الى جنب مع الخصائص الجزيئية في التمييز بين النباتات التي لها أسلاف مشتركة. تكمن حداثة هذا العمل في مقارنة أوجه التشابه والاختلاف بين الأنواع التي تم فحصها من خلال الجمع بين خصائص حبوب اللقاح والخصائص الجزيئية معًا. ويعد مفتاح التعريف الذي تم إنشاؤه من هذه الدراسة للأنواع التي تم فحصها مصدرًا موثوقًا يمكن استخدامه في الدراسات التصنيفية المستقبلية.