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Full Paper

Pollen morphology of 16 species of *Fritillaria* L. and its taxonomic implications

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Abstract: *Fritillaria* spp. have an abundance of germplasm resources, but there is still controversy over the delimitation of some species. The morphological characteristics of pollen are important for classification and evolutionary research. In this study, we assessed the pollen morphology of 16 samples from 11 species covering four subgenera. The pollen is subprolate, prolate or perprolate, mostly with bilateral symmetry, monosulcate almost up to the poles, oblong-ellipsoidal in equatorial view and suborbicular in polar view. A total of ten exine sculpturing types (striate-reticulate, rugulate-reticulate, perforate, reticulate-perforate, psilate-perforate, suprareticulate, reticulate, microreticulate, macroreticulate and granular), and eight sulcus membrane ornamentation types (psilate, rugulate, granulate, granulate-striate, verrucate, verrucate-granulate, plicate-granular and macrogranular) were observed across these species. The apex of the sulcus was sharp or round. The murus consisted of single- or multiple-line particles and a more or less verrucate-granulate mesh was observed in some species. Combined with molecular evidence, pollen sculpture patterns appear to reflect phylogenetic relationships and are useful for delimiting species or subsections. Using the characteristic pollen morphology of all the examined species, we also analysed evolutionary trends and taxonomic implications while discussing the application of pollen morphology in the classification of cultivars. These data provide palynological evidence for understanding similarities and relationships within the *Fritillaria* genus.

Keywords: classification, evolution trend, fritillaries, pollen morphological characteristics, scanning electron microscopy

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INTRODUCTION

Fritillaria spp. form a genus of perennial bulbous plants belonging to the Liliaceae family with important medicinal and ornamental values [1]. The genus *Fritillaria* L., one of the largest genera in the Liliaceae, is comprised of about 130 species that have been divided into five sections [2]. However, in Rix's opinion, *Fritillaria* is represented worldwide by eight subgenera (*Fritillaria*, *Rhinopetalum*, *Petillium*, *Theresia*, *Liliorhiza*, *Korolkowia*, *Japonica* and *Davidii*), two sections (*Fritillaria* and *Olostylea*), and 165 taxa (139 species, 17 subspecies and 9 varieties) [3]. Regarding the number of *Fritillaria* species, at the end of December 2010, the Plant List, which is curated by the Royal Botanical Garden and Missouri Botanical Garden, showed that there were 171 taxa, containing 30 subspecies and varieties [4]. They are widely distributed in temperate regions of Europe, Asia and North America, and are especially distributed in the Mediterranean region, primarily in Iran and Turkey, which have the greatest abundance of species [5]. In China there are 24 *Fritillaria* species and two varieties, 15 species being endemic [6, 7]. Therefore, China is considered to be one of the major distribution centres of diversity in fritillaries.

In China, except for the last decade and over a span of 30 years preceding 2004, researchers discovered and named many new species, as many as 50, in the *Fritillaria* genus [5]. The emergence of this phenomenon may be due to the high variability of morphological characters or probable interspecific hybridisation among fritillaries [8]. In addition, the newly discovered species almost all belong to the subgenus *Fritillaria*. The classification of these species is complicated and is still in a state of flux in China. Although there have been some studies on fritillary classification, most have focused on plant morphological characters. Luo and Chen [9, 10] revised the species of *Fritillaria* L. in the Hengduan Mountains and adjacent regions in China, mainly based on their geographic distribution and some morphological characteristics such as the number of bulb scales, the living state of leaves, the colour of perianth segments, and others. Through a field investigation, and referring to specimens and the literature, Zhang and Cheng considered that the names of six *Fritillaria* species, four varieties and one cultivar should be reduced to synonyms, and recognised six species and one variety in Gansu province, mainly distinguished by plant morphology [8]. In their research pollen morphology was also used to distinguish individual species. For example, they claimed that *F. taipaiensis* var. *zhouquensis* should be considered as a variety of *F. taipaiensis* or as a separate species based on evidence from an esterase isozyme zymogram, chromosome karyotype and pollen morphology, in addition to plant morphological traits [8]. However, few studies have used palynological, chromosomal, or molecular methods to determine the status of a plant for most species, and these methods may be more precise in modifying current classification and systematic relationships.

In recent years some research showed pollen morphology to be useful for clarifying discrepancies in systematic classifications [11, 12]. In Iran pollen grains of five taxa (*F. poluninii*, *F. avramanica*, *F. assyriaca*, *F. fleischeriana* and *F.* sp.) from the genus *Fritillaria* L. were studied by scanning electron microscopy (SEM). Results showed that sculpturing of the exine, pollen membrane ornamentation, and lumina shape provided valuable characteristics for separating species, and based on those characteristics, three main pollen types were determined with three different forms of exine sculpturing: reticulate, reticulate-perforate and suprareticulate [13]. In addition, pollen grains of 19 *Fritillaria* (Liliaceae) taxa and two hybrids from Greece were investigated by SEM. Seven types of exine sculpturing were observed: regulate-reticulate, regulate-perforate, psilate-perforate, perforate-reticulate, reticulate, reticulate-heterobrochate and psilate-reticulate [14].

These studies indicate that morphological characteristics of pollen may positively contribute to the classification of *Fritillaria* taxa at the species level.

The structure of angiosperm pollen is strongly conservative and is weakly affected by environmental factors [15]. Palynological evidence that takes into consideration pollen shape, size, surface ornamentation, and aperture type and location has important value in the classification of higher plants [16, 17]. Pollen morphology can define the taxonomic status of a genus or species and confirm whether it is newly discovered, as well as reflect its state of evolution [18]. Differences in pollen morphology have supported the classification of *Caatinganthus*, *Stilpnopappus*, *Strophopappus* and *Xiphochaeta* as separate genera, reinforcing the currently accepted taxonomic classification [19]. In taxonomic revisions of the Vernonieae, pollen morphology served as an important source of information to re-establish or to fragment genera [20]. Pollen morphology was useful in deciphering the systematics of eight wild *Lilium* species belonging to three sections [21]. Pollen morphology was used to classify *Lilium amoenum* as a subsection under sect. *Lophophorum* [22]. Based on a synthetic analysis of the pollen morphology of *Lilium* L., an evolutionary trend in pollen type was proposed (Martagon → Callose → Concolor → Formosanum), although the authors indicated that the evolution trends in exine sculpture were not definitively correlated with pollen size and shape [18]. To date, a few studies have employed light microscopy, SEM or transmission electron microscopy (TEM) to evaluate the pollen morphology of *Fritillaria* species [23-27]. As a result, over 50 species covering seven subgenera (except for subgenus *Japonica*) have been reported. Six types of ornamentation in pollen morphology (reticulate, reticulate-perforate, suprareticulate, rugulate-reticulate, psilate-perforate and perforate) were determined in 39 *Fritillaria* species or subspecies from Turkey [25]. Ozler and Pehlivan reported the pollen morphological parameters of 12 species or subspecies from Turkey [28], Kosenko examined nine species of *Fritillaria* L. [29], and Li et al. researched 27 species or varieties from China [30]. However, a comprehensive analysis of pollen morphology in the genus *Fritillaria* or between different subgenera is lacking and the systematic significance of such an assessment is also unclear.

High-resolution electron microscopy allows for an in-depth examination of differences in pollen morphological characteristics between species, cultivars or individuals [31]. In the present study we observe the pollen characteristics of 16 samples using SEM. These 16 samples include 4 different subgenera, 11 different species and 7 varieties. The selection of these samples enables us to analyse the pollen morphology among different subgenera, species and varieties of *Fritillaria*, providing references for the investigation of their systematic evolutionary relationships from a palynological point of view. Our aim is to present detailed quantitative and qualitative data on the pollen morphology of these species, as well as to investigate whether these pollen traits support the identification of species or varieties in the genus *Fritillaria* and provide palynological evidence for the disputed species. In addition, complementing the findings of previous studies, and combined with their pollen morphological (sculpture and size) and molecular evidence, phylogenetic relationships and evolutionary trends of seven subgenera (except for subgenus *Japonica*) of *Fritillaria* L. are comprehensively discussed.

MATERIALS AND METHODS

Plant Materials

Table 1 shows 16 *Fritillaria* species in seven subgenera reported in the literature and used in our pollen morphology research. Fresh pollen grains of these taxa cultivated at the experimental farm of Beijing Forestry University or the Beijing Botanical Garden were collected from mature stamens in March and April of 2015. These species, varieties or cultivars were introduced from the Netherlands, the U.S., and Xinjiang and Anhui provinces of China, and were cultivated in the Beijing area. Fresh pollen grains were dried naturally at room temperature (about 25°C) for two days or over silica gel. Dry pollen was used for SEM observations.

Dry pollen grains were directly mounted onto the surface of polished aluminum stubs with double-sided adhesive tape. After each stub was sputter-coated with a gold layer, samples were taped to the object stage. Observation of pollen morphological characteristics was carried out with a scanning electron microscope (Model S-3400N, Hitachi, Japan) at the Biotechnology Centre of Beijing Forestry University. Photographs of pollen grains were taken at 200, 2500 and 10,000 × magnifications.

Table 1. Code, flower colour, blooming date (2015), section and source of 16 fritillaries used in this pollen morphology research

Code	Plant material	Flower colour	Blooming date	Subgenus name	Source
A	*F. imperialis* 'Maxima Lutea'	Bright yellow	13 April	*Petillium*	Netherlands
B	*F. imperialis* 'Maxima Rubra'	Orange-red	13 April	*Petillium*	Netherlands
C	*F. imperialis* 'Aurora'	Orange with red stripes	17 April	*Petillium*	Netherlands
D	*F. imperialis* 'The Premier'	Orange- yellow	20 April	*Petillium*	Netherlands
E	*F. persica* 'Adiyaman'	Dark-purple	17 April	*Theresia*	Netherlands
F	*F. persica* 'Ivory Bell'	Yellow- green	15 April	*Theresia*	Netherlands
G	*F. meleagris*	Deep red with lighter tessellation	2 April	*Fritillaria*	Netherlands
H	*F. uva-vulpis*	Golden-brown	28 April	*Fritillaria*	U.S.
I	*F. pallidiflora*	Pale yellow with stripes	20 April	*Fritillaria*	Xinjiang, China
J	*F. tortifolia*	Lavender with red speckles	13 April	*Fritillaria*	Xinjiang, China
K	*F. verticillata*	White with purple speckles	16 April	*Fritillaria*	Xinjiang, China
L	*F. verticillata* var. *albiflora*	White with purple- red speckles	14 April	*Fritillaria*	Xinjiang, China
M	*F. albidoflora*	White	15 April	*Fritillaria*	Xinjiang, China
N	*F. yuminensis*	Lavender or pink	10 April	*Fritillaria*	Xinjiang, China
O	*F. karelinii*	Pink with dark speckles	30 March	*Rhinopetalum*	Xinjiang, China
P	*F. anhuiensis*	Purple-red with tessellation	12 April	*Fritillaria*	Anhui, China

Methods of Measurement and Analysis

Twenty pollen grains of each species, variety or cultivars were measured to assess their size and shape, whereas other measurements were based on five pollen grains. All the pollen grains were randomly chosen and measured by Adobe Photoshop CS3® for Windows. Measurements consisted of four quantitative features: length of the polar axis (P), length of the equatorial axis (E), pollen perforation diameter (D) and ridge width (W), and two ratios: P/E and D/W. Pollen characteristics, viz. pollen shape, type of perforations and sexine ornamentation, were observed in equatorial and polar front views, all based on SEM images. The description of pollen shape was based on the P/E ratio according to the following classification: peroblate (<0.50); spheroidicity (0.50-0.88);

subsphaeroidal (0.88-1.14); subprolate (1.14-2.00); or perprolate (>2.00). Palynological terminology followed that suggested by Wang and Wang [32] and Punt et al. [33].

Raw data were entered into Microsoft Excel 2007 and analysis of variance and significant differences among means at $P < 0.05$ following Duncan's multiple range test were performed in SPSS (version 20.0; IBM, USA). In addition, Q-cluster analysis was conducted in SPSS with 11 pollen morphological characteristics, i.e. six quantitative traits (P, E, P/E, D, W and D/W) and five qualitative features (pollen shape, pollen exine sculpture type, murus features, mesh features and other features). If there were no values for D, W and D/W, then these were assigned a zero value. The codes of qualitative features used for cluster analysis were: shape (1: subprolate, 2: perprolate), exine sculpture type (0: granular, 1: striate-reticulate, 2: rugulate-reticulate, 3: perforate, 4: reticulate-perforate, 5: reticulate, 6: suprareticulate), murus features (0: no murus, 1: multi-line granule, 2: single-line granule), mesh features (0: no mesh, 1: no significant differences in size, 2: significant differences in size), other features (0: no surface perforation, 1: surface perforation, 2: no verrucate-granulate in mesh, 3: verrucate-granulate in mesh). The values of these 11 measured or encoded indicators were first normalised by descriptive statistics and then clustered by system clustering. The Euclidean coefficient distance factor was adopted as the genetic distance of clustering units [34]. All these analyses were conducted in SPSS.

RESULTS

Pollen Morphological Characteristics

The pollen of 16 *Fritillaria* species, variety or cultivars that were investigated had different morphological characteristics (Figure 1). Tables 2, 3 and 4 show the measurements of pollen characteristics and a description of pollen morphology. A detailed description follows next.

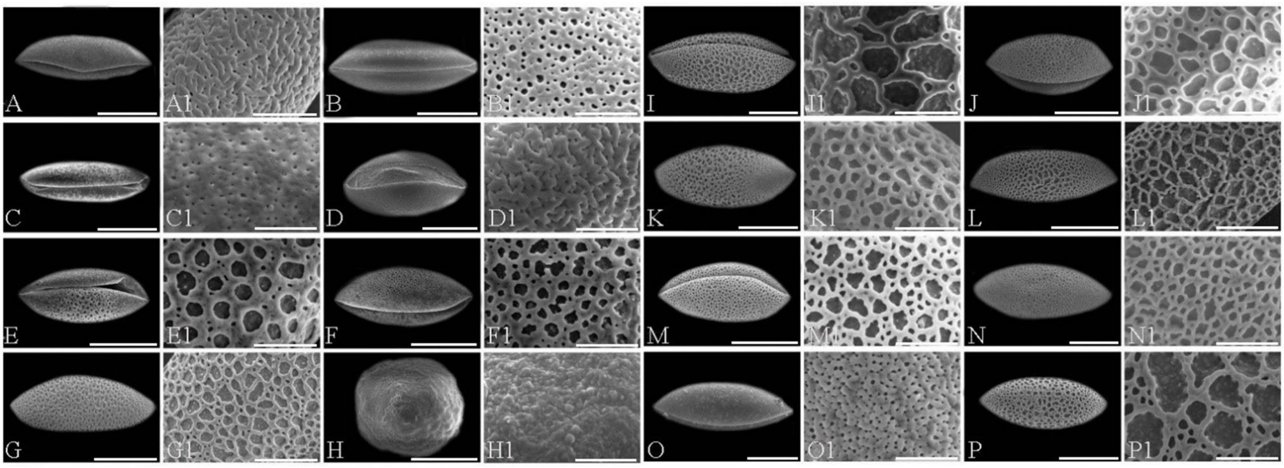

Figure 1. Observation of pollen morphology of 16 species or varieties of *Fritillaria*: *F. imperialis* 'Maxima Lutea' (A, A1), *F. imperialis* 'Maxima Rubra' (B, B1), *F. imperialis* 'Aurora' (C, C1), *F. imperialis* 'The Premier' (D, D1), *F. persica* 'Adiyaman' (E, E1), *F. persica* 'Ivory Bell' (F, F1), *F. meleagris* (G, G1), *F. uva-vulpis* (H, H1), *F. pallidiflora* (I, I1), *F. tortifolia* (J, J1), *F. verticillata* (K, K1), *F. verticillata* var. *albiflora* (L, L1), *F. albidoflora* (M, M1), *F. yuminensis* (N, N1), *F. karelinii* (O, O1) and *F. anhuiensis* (P, P1). A–P: shape of a single pollen grain; A1–P1: surface ornamentation. Scale bars: 40 µm (A–C), 30 µm (D-H, J-M and P), 20 µm (I, N and O), 10 µm (G1 and L1), 5 µm (A1-F1, H1-K1 and M1-P1).

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Table 2. Mean and standard deviation (n=20) of six indices related to pollen morphology characteristics of 16 fritillaries

Code	Plant material	P[1] (mean ± SD (μm)	E[2] (mean ± SD) (μm)	P/E (mean ± SD)	D[3] (mean ± SD) (μm)	W[4] (mean ± SD) (μm)	D/W (mean ± SD)
A	*F. imperialis* 'Maxima Lutea'	84.35 ± 6.60 j	30.05 ± 1.78 e	2.82 ± 0.33 g	—*	—	—
B	*F. imperialis* 'Maxima Rubra'	112.77 ± 7.18 l	37.45 ± 3.47 f	3.04 ± 0.35 h	—	—	—
C	*F. imperialis* 'Aurora'	94.07 ± 3.91 k	30.02 ± 1.95 e	3.15 ± 0.25 h	—	—	—
D	*F. imperialis* 'The Premier'	62.75 ± 2.52 e	36.62 ± 1.34 f	1.72 ± 0.10 b	—	—	—
E	*F. persica* 'Adiyaman'	60.35 ± 2.23 cd	23.33 ± 2.55 a	2.61 ± 0.27 e	1.46 ± 0.31 de	0.89 ± 0.20 e	1.77 ± 0.75 b
F	*F. persica* 'Ivory Bell'	63.45 ± 3.45 ef	24.88 ± 1.54 b	2.56 ± 0.18 de	0.92 ± 0.25 b	0.56 ± 0.11 d	1.71 ± 0.61 b
G	*F. meleagris*	66.43 ± 3.58 gh	28.15 ± 2.11 d	2.37 ± 0.18 c	1.10 ± 0.21 bc	0.30 ± 0.07 b	3.78 ± 1.03 d
H	*F. uva-vulpis*	49.25 ± 6.47 a	37.25 ± 4.56 f	1.32 ± 0.10 a	—	—	—
I	*F. pallidiflora*	74.28 ± 3.11 i	26.92 ± 1.37 c	2.76 ± 0.15 fg	2.92 ± 0.57 g	0.54 ± 0.11 d	5.57 ± 1.35 e
J	*F. tortifolia*	64.17 ± 3.99 efg	27.56 ± 1.16 cd	2.33 ± 0.18 c	1.54 ± 0.30 e	0.46 ± 0.04 c	3.41 ± 0.79 cd
K	*F. verticillata*	66.73 ± 3.67 h	25.52 ± 1.62 b	2.62 ± 0.20 e	1.15 ± 0.32 bcd	0.44 ± 0.06 c	2.64 ± 0.76 c
L	*F. verticillata* var. *albiflora*	65.34 ± 4.99 fgh	26.79 ± 1.52 c	2.44 ± 0.18 cd	1.26 ± 0.38 cde	0.25 ± 0.03 a	5.23 ± 1.97 e
M	*F. albidoflora*	63.45 ± 2.74 ef	26.92 ± 1.72 c	2.37 ± 0.19 c	1.24 ± 0.37 bcde	0.44 ± 0.05 c	2.86 ± 0.98 c
N	*F. yuminensis*	58.03 ± 2.63 b	22.88 ± 1.31 a	2.55 ± 0.18 de	0.93 ± 0.29 bc	0.33 ± 0.04 b	2.88 ± 0.89 c
O	*F. karelinii*	59.08 ± 2.24 bc	22.33 ± 1.45 a	2.66 ± 0.20 ef	0.30 ± 0.06 a	0.45 ± 0.06 c	0.69 ± 0.18 a
P	*F. anhuiensis*	62.52 ± 2.80 de	25.73 ± 1.33 b	2.44 ± 0.20 cd	2.48 ± 1.17 f	0.46 ± 0.08 c	5.49 ± 2.62 e

Note: Different letters within a column indicate significant differences at $P \leq 0.05$ according to Duncan's multiple range test.
[1] Polar axis length; [2] Equatorial axis length; [3] Perforation diameter; [4] Ridge width; * no perforation

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Table 3. Comparison of pollen shape and exine sculpture type among 16 fritillaries (n = 20)

Code	Plant material	P × E (μm)*	Pollen shape	Exine sculpture type
A	*F. imperialis* 'Maxima Lutea'	(72.00-96.00) × (25.50-33.50)	Perprolate	Striate-reticulate
B	*F. imperialis* 'Maxima Rubra'	(102.50-124.50) × (32.00-43.50)	Perprolate	Rugulate-reticulate
C	*F. imperialis* 'Aurora'	(84.50-99.00) × (26.00-33.00)	Perprolate	Perforate
D	*F. imperialis* 'The Premier'	(58.00-68.50) × (33.50-39.00)	Subprolate	Striate-reticulate
E	*F. persica* 'Adiyaman'	(56.50-64.50) × (19.50-29.50)	Perprolate	Reticulate-perforate
F	*F. persica* 'Ivory Bell'	(57.50-70.00) × (22.00-28.50)	Perprolate	Reticulate-perforate
G	*F. meleagris*	(60.00-73.50) × (24.00-32.00)	Perprolate	Suprareticulate
H	*F. uva-vulpis*	(40.00-56.50) × (30.50-44.50)	Subprolate	Granular
I	*F. pallidiflora*	(68.00-81.00) × (24.00-29.85)	Perprolate	Suprareticulate
J	*F. tortifolia*	(57.20-72.50) × (25.83-29.93)	Perprolate	Suprareticulate
K	*F. verticillata*	(58.02-70.84) × (22.5-29.4)	Perprolate	Reticulate
L	*F. verticillata* var. *albiflora*	(58.29-73.50) × (24.23-29.50)	Perprolate	Suprareticulate
M	*F. albidoflora*	(58.50-68.00) × (24.00-29.50)	Perprolate	Reticulate
N	*F. yuminensis*	(53.50-64.50) × (20.50-26.00)	Perprolate	Reticulate
O	*F. karelinii*	(55.50-63.00) × (19.50-25.00)	Perprolate	Striate-reticulate
P	*F. anhuiensis*	(56.00-68.00) × (22.5-28.00)	Perprolate	Suprareticulate

* P = polar axis length; E = equatorial axis length

Table 4. Comparison of pollen morphological characteristics among four subgenera

Subgenus name	Range of polar axis length (μm)	Range of equatorial axis length (μm)	Range of P/E	Range of mesh diameter (μm)	Range of ridge width (μm)	Range of D/W	Pollen shape	Exine sculpture type	Ridge features	Mesh features	Notes
Petillium	62.75-112.77	30.02-37.45	1.72-3.15	—*	—	—	Perprolate subprolate	Striate-reticulate, rugulate-reticulate perforate	—	—	Unevenly distributed perforations
Theresia	60.35-63.45	23.33-24.88	2.56-2.61	0.92-1.46	0.56-0.89	1.71-1.77	Perprolate	Reticulate-perforate	Multi-line of particles	No significant differences in size with ridge	No verrucate-granulate
Fritillaria	58.03-74.28 (one case = 49.25)	22.88-28.15 (one case = 37.25)	2.33-2.76 (one case = 1.32)	0.93-2.92	0.25-0.54	2.64-5.57	Perprolate (subprolate)	Reticulate, suprareticulate, granular	A single-line of particles	Mesh is bigger than ridge	Verrucate-granulate
Rhinopetalum	59.08	22.33	2.66	0.30	0.45	0.69	Perprolate	Striate-reticulate	—	—	Perforations

* No mesh

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Pollen Shape and Size

The shapes of the studied pollen grains of all 16 *Fritillaria* L. species, varieties or cultivars are similar (Table 3). They are almost all perprolate (Figure 2C) except for *F. imperialis* 'The Premier' and *F. uva-vulpis*, which are subprolate (Figure 2D). They are oblong-ellipsoidal in equatorial view and suborbicular in polar view. The pollen grains show a nearly bilateral symmetry with a single colporate, extending almost up to the poles. However, there is no germinal aperture in the pollen grains of *F. uva-vulpis* (Figure 1H). The size of the pollen grains vary. The length of the polar axis ranges from 49.25 to 112.77 µm and the length of the equatorial axis ranges from 22.33 to 37.45 µm. Of note, *F. imperialis* 'Maxima Rubra' (Figure 1B) has the longest polar and equatorial axes, whereas *F. uva-vulpis* (Figure 1H) and *F. karelinii* (Figure 1O) have the shortest axes. *F. imperialis* 'Maxima Rubra' (Figure 1B) has the largest pollen (P×E = 112.77 × 37.45 µm), whereas *F. karelinii* (Figure 1O) has the smallest pollen (P×E = 59.08 × 22.33 µm). The range of P/E is 1.32 to 3.15, with *F. imperialis* 'Aurora' (Figure 1C) displaying the maximum value (3.15) and *F. uva-vulpis* (Figure 1H) the minimum value (1.32). In addition, the numerical values for pollen grain size of the four sections are summarised in Table 4. Subgenus *Petillium* has the largest pollen grains; subgenus *Rhinopetalum* has the smallest ones, while pollen grains of the other two sections display an intermediate size.

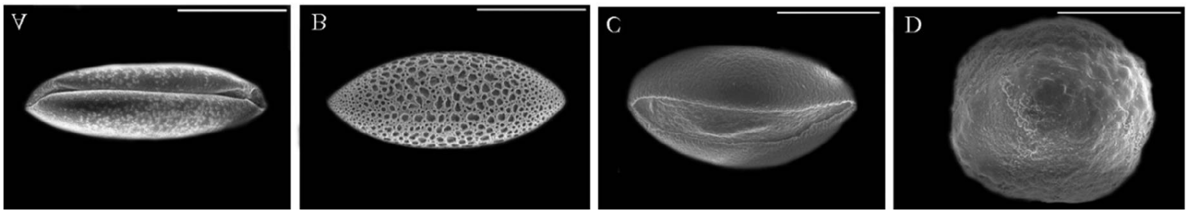

Figure 2. Two types of pollen shape: perprolate (A and B) and subprolate (C and D). Scale bars: 40 µm (A), 30 µm (B, C and D).

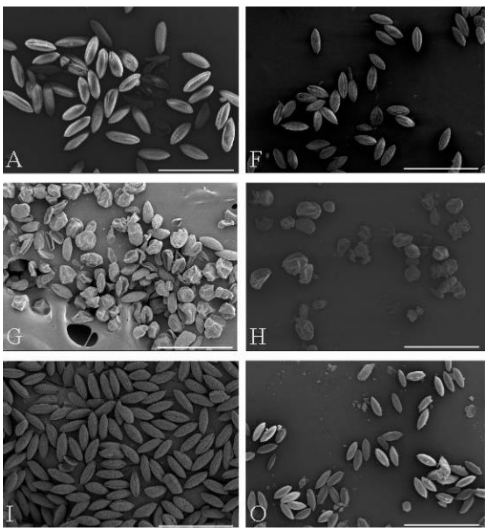

Figure 3. Comparison of uniformity of pollen morphology in *Fritillaria* species of different subgenera. *F. imperialis* 'Maxima Lutea' (A) belongs to subgenus *Petillium*; *F. persica* 'Ivory Bell' (F) belongs to subgenus *Theresia*; *F. meleagris* (G), *F. uva-vulpis* (H) and *F. pallidiflora* (I) belong to subgenus *Fritillaria*; *F. karelinii* (O) belongs to subgenus *Rhinopetalum*. Scale bars: 200 µm.

The uniformity of pollen morphology in different sections is shown in Figure 3. From the photographs, we found that most pollen grains have a regular shape and uniform size, except for *F. meleagris* (Figure 3G) and *F. uva-vulpis* (Figure 3H), which belong to subgenus *Fritillaria*. The majority of their pollen grains are empty flat shells or have heteromorphic pollen attached.

Types of Pollen Sexine Ornamentation

On the basis of the exine sculpture, six ornamentation types (Figure 4) are recognised in the investigated pollen grains. The ornamentation in *Fritillaria* is usually reticulate (Figure 4H), rarely striate-reticulate (Figure 4A, B), reticulate-perforate (Figure 4E), perforate (Figure 4F), suprareticulate (Figure 4G), or granular (Figure 4I). In subgenus *Petillium*, there are two types of exine sculpture. The *F. imperialis* varieties 'Maxima Lutea' and 'The Premier' have a striate-reticulate exine sculpture whereas the other two varieties of *F. imperialis* have a perforate exine sculpture. Reticulate-perforate, striate-reticulate and granular sculpturing patterns are observed in *F. persica* (subgenus *Theresia*), *F. karelinii* (subgenus *Rhinopetalum*) and *F. uva-vulpis* (subgenus *Fritillaria*) respectively. The other species in subgenus *Fritillaria* have a reticulate exine sculpture.

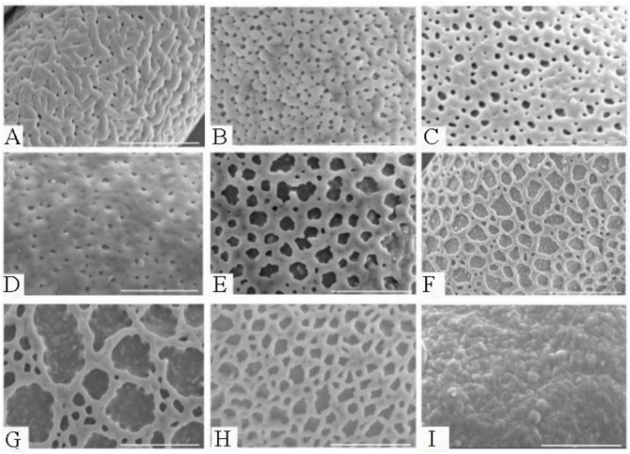

Figure 4. Types of pollen exine sculpture. A: Striate-reticulate (*F. imperialis* 'Maxima Lutea'); B: striate-reticulate (*F. karelinii*); C: regulate-reticulate (*F. imperialis* 'Maxima Rubra'); D: regulate-reticulate (*F. imperialis* 'Aurora'); E: reticulate-perforate (*F. persica* 'Ivory Bell'); F: perforate (*F. meleagris*); G: suprareticulate (*F. anhuiensis*); H: reticulate (*F. yuminensis*); I: granular (*F. uva-vulpis*). Scale bars: 5 μm.

Characteristics of Ridge and Mesh

Mesh diameter, ridge width and their features were recorded (Tables 2 and 4). There is no obvious mesh and ridge in the pollen of *F. imperialis*, but there are many small perforations on the surface. These perforations have different sizes and show an uneven distribution, which are the characteristics of subgenus *Petillium* pollen. The same features are observed for the pollen of *F. uva-vulpis*, but there are three distinct differences: a rugged surface, verrucous granules and no perforations. *F. uva-vulpis* is a special species in subgenus *Fritillaria*, which has an obvious mesh and ridge on the pollen surface. In subgenus *Rhinopetalum*, *F. karelinii* shows a similar brain-striated exine sculpture, although the surface of its pollen grains has obvious overlapping curves and perforations. The brain-striated pattern consists of single-line particles and the width of each brain stria (0.45 μm) is greater than the aperture (0.30 μm).

The other *Fritillaria* species have an obvious mesh and ridge, but their size varies (Table 2). However, a common feature is that the mesh is widest in the middle but tapers and becomes smaller towards the apices. Mesh diameter ranges from 0.92 to 2.92 μm and ridge width ranges between 0.25 and 0.89 μm, and the ratio of mesh diameter and ridge width (D/W) ranges from 1.71 to 5.57. *F. persica* 'Ivory Bell' has the smallest aperture and *F. pallidiflora* has the largest, and they also display the minimum and maximum value of D/W respectively.

Pollen morphological characteristics of the four *Fritillaria* sections are summarised in Table 4. Most ridges consist of multi-line particles (i.e. with more than a single line; Figures 5c, 5d), and a ridge width varies in subgenus *Theresia*. Mesh size differs but approaches ridge width ($0.5 < D/W < 2.0$), and meshes are not verrucate-granulate. In subgenus *Fritillaria*, except for *F. uva-vulpis*, most ridges consist of single-line particles (Figures 5a, 5b) and mesh diameter is significantly greater than ridge width ($D/W > 2.0$) (Table 4). The junction of adjacent meshes usually expands, and verrucate-granulate meshes are visible, or not.

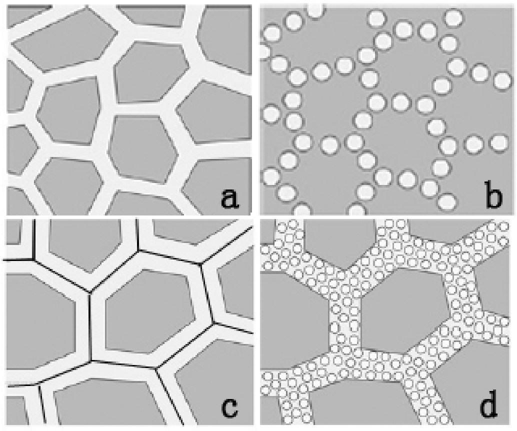

Figure 5. Schematic diagram of ridge features: single line of particles (a and b); multi-line of particles (c and d) [33] (copyright permission granted by Elsevier).

Cluster Analysis

Pollen morphological characteristics including six quantitative traits (P, E, P/E, D, W and D/W) and five qualitative features were analysed by Q-cluster analysis in a bid to assess the genetic relationships among the 16 fritillaries studied. Clustering results are shown in Figure 6; the 16 species, varieties or cultivars can be divided into four groups with a distance of L = 8.0. Group I includes seven species and one variety, all belonging to subgenus *Fritillaria*. Among them, *F. pallidiflora* and *F. anhuiensis* are in the same cluster, whereas the other six species or varieties are separate, but clustered together. The pollen grains of this group are all perprolate and have a reticulate exine sculpture. The mesh is significantly larger than the width of the ridge, which consists of single-line particles, and there is a visible verrucate-granulate pattern. Group II includes one species and two cultivars: two cultivars of *F. persica* belonging to subgenus *Theresia* are clustered together and *F. karelinii*, which belongs to subgenus *Rhinopetalum*, forms a separate cluster. Group III includes one species and one cultivar, *F. imperialis* 'The Premier' and *F. uva-vulpis*. The shape of their pollen grains is subprolate. Group IV includes three cultivars of *F. imperialis* belonging to subgenus *Petillium* but *F. imperialis* 'Maxima Lutea' and *F. imperialis* 'Aurora' are clustered together as a small cluster.

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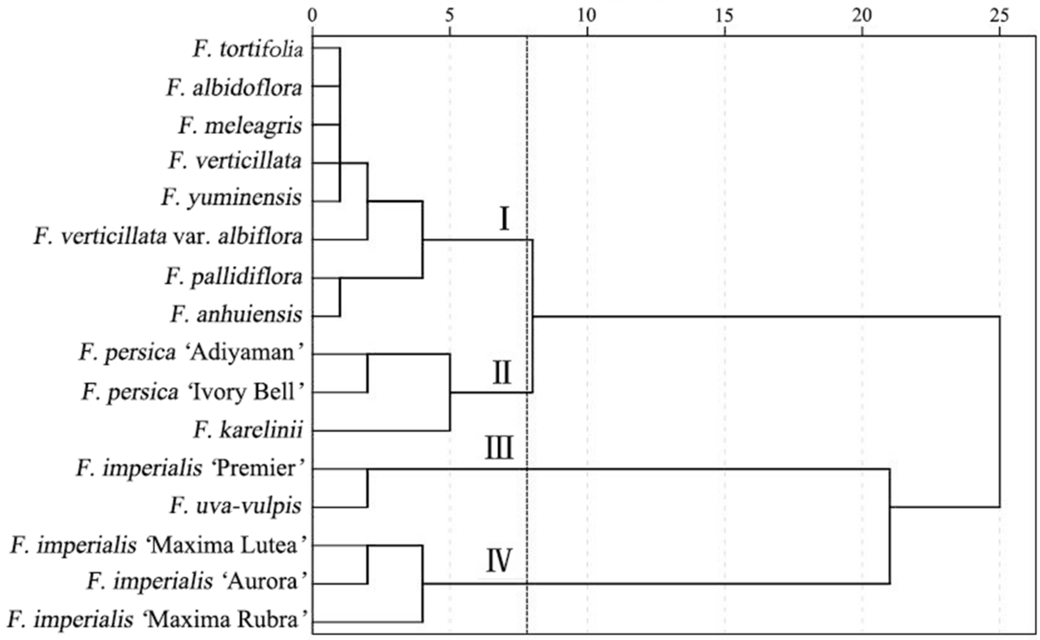

Figure 6. Clustering results of 16 *Fritillaria* species based on pollen characteristics and Q-cluster analysis.

DISCUSSION

In this palynological study on 16 *Fritillaria* species, varieties and cultivars, the focus is on pollen shape, size and exine ornamentation. The pollen grains investigated in this study are subprolate or perprolate, have nearly bilateral symmetry and are single-colporate, almost up to the poles, or oblong-ellipsoidal in equatorial view and suborbicular in polar view. Six exine sculpturing types are observed, and most of the ridges consist of single- or multi-line particles. There are significant differences in mesh size and meshes show obvious or no verrucous protuberances.

Pollen morphological characteristics can reflect common characteristics of a family or genus and can be used to identify taxa [35-37]. They can also be used to classify some species or varieties, and plant classification was formerly mainly based on phenotypic characteristics [38]. However, in recent years pollen morphological characteristics have been used to provide palynological evidence for taxonomy due to their stability. Using electron microscopy, especially SEM, to explore pollen ultrastructure is an important method to identify plant species or cultivars.

In our study we found that most *Fritillaria* species that have similar pollen morphology are clustered into one group, and the clustered species almost belong to one section. For example, different cultivars of *F. imperialis* or *F. persica*, which are considered affinity species of *F. albidoflora* and *F. yuminensis* [30], or as synonym species of *F. albidoflora* and *F. verticillata* [39], are clustered together (Figure 6). In the Plant List [4] *F. albidoflora* is considered a synonym of *F. agrestis*. However, whether *F. albidoflora* should be a separate species or treated as a synonym also needs additional evidence to support this change in classification. A more interesting question is the status of *F. verticillata* and *F. verticillata* var. *albiflora*. According to the Plant List [4] *F. verticillata* var. *albiflora* is treated as a synonym of *F. verticillata*. However, based on the results of our cluster analysis, the distance between them is relatively greater than that between *F. verticillata* and *F. yuminensis*, indicating that there are some differences between their pollen morphological characteristics, and that *F. verticillata* var. *albiflora* might be considered as a separate species.

Understandably, a single method such as SEM or a single technique such as pollen observation is not sufficient to correctly determine the classification status of a species or other taxon. A comprehensive analysis would ideally need to combine various other methods and techniques such as plant phenotype, molecular markers, cytology and genetics [40].

In this study we discover significant differences in pollen morphology among the four *Fritillaria* subgenera. Moreover, there are some differences in ridge width, mesh size and distribution, and ridge features among members of the same subgenus. This suggests that pollen morphological characteristics may serve as important parameters in the classification, sorting and identification of *Fritillaria* species.

The degree of similarity in pollen morphology can reflect the genetic relationships of species [41]. A cluster analysis was used to assess the evolutionary relationships of lily (*Lilium* L.) germplasm based on pollen characteristics and it was found that species and cultivars could be divided into two sections, *Lilium* and *Sinomartagon* [42]. Thus, in the case of wild lily species, the classification based on pollen morphological features was consistent with the plant's phenotypic classification [5]. In our study a similar result is achieved: species or cultivars within the same subgenus have close genetic distance and similar pollen morphology while species or cultivars in different subgenera have a relatively large genetic distance and different pollen morphology. Based on this clustering result, we found that subgenera *Petillium* and *Fritillaria* are genetically most distant.

Pollen morphology can be used to effectively predict plant evolutionary relationships and evolutionary trends in pollen surface ornamentation, and the corresponding evolutionary order (primitive to evolutionary) is psilate, reticulate, striate, verruciform, and spinous [43]. Based on this theory, we speculate that subgenus *Fritillaria* might be the most primitive and subgenera *Petillium* and *Rhinopetalum* are more evolved, while *F. uva-vulpis* is the most evolved. Pollen exine ornamentation is another important aspect for investigating the evolution of pollen morphology, and a greater D/W ratio reflects a higher degree of evolution [26]. If this theory is followed, then the species belonging to subgenus *Fritillaria* should be the most evolved among the 16 examined *Fritillaria* species and cultivars, but this contradicts the view of Liang [5], who indicated that subgenus *Fritillaria* was the most primitive taxon in the *Fritillaria* L. genera.

Notably, amongst all pollen grains examined, the pollen morphology of *F. uva-vulpis* lacks attributes consistent with those defining subgenus *Fritillaria* and is significantly different from other species. Its pollen grains are almost entirely empty flat shells, and exine sculpturing is granular. The reason for this irregular shape and the lack of a germinal aperture may be that this species is a triploid or aneuploid variety [44]. Moreover, the description of morphological features is not consistent with the findings of Teksen et al. [25], who stated that the pollen grains of *F. uva-vulpis* are regular and single-colporate with suprareticulate exine sculpturing. It is possible that the species we studied is a cultivar of *F. uva-vulpis*.

We also discover that there are two types of exine sculpturing (brain-striate and perforate) in *F. imperialis*. Based on different pollen exine sculpturing, the four *F. imperialis* varieties can be distinguished (Figure 1: A1, B1, C1, D1). We also find significant differences in the size of *F. imperialis* pollen grains (62.75-112.77 μm) compared to the findings of Teksen et al. (57.9 μm) [25], but no significant differences in the case of *F. persica*. Compared to the study of Li et al. [30], who investigated the pollen morphology of 27 *Fritillaria* species or varieties in China, which belong to three sections (*Fritillaria*, *Rhinopetalum* and *Liliorhiza*), we found no obvious differences in pollen features, but only subtle differences in pollen size.

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CONCLUSIONS

This study has analysed the pollen morphology of 16 *Fritillaria* species, varieties or cultivars, and a cluster analysis based on six quantitative traits (P, E, P/E, D, W and D/W) and five qualitative features divided these taxa into four groups. Significant differences in the pollen morphology of the four groups are observed, and different members of the same group also display differences in ridge width, mesh size and distribution, and ridge features. The difference in pollen size within the same species of *Fritillaria* may be due to environmental influences. This study provides a robust theoretical basis for the palynological study of these 16 species, varieties or cultivars of *Fritillaria*, and can be used as a reference for the classification and phylogeny of *Fritillaria*.

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