

## Comparative Study on Pollen Morphology of *Epimedium sagittatum* and *Epimedium myrianthum* from Tianpingshan: Postprint

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### Abstract

*Epimedium sagittatum* and *Epimedium tiangpingshanense* are closely related, with many macro-morphological characteristics, even key taxonomic traits, showing transitions. To investigate the taxonomic relationship between these two groups, scanning electron microscopy (SEM) was employed to observe and compare pollen morphology and exine ornamentation across 13 populations of *Epimedium sagittatum* (including one population of *Epimedium sagittatum* var. *glabrum*) and 7 populations of *Epimedium tiangpingshanense*. The results indicate that pollen grains of both groups are subspheroidal and tricolpate, with similar sizes. Pollen size of *Epimedium sagittatum* ranges from (18.21~21.66)  $\mu\text{m} \times$  (19.55~23.63)  $\mu\text{m}$ , while that of *Epimedium tiangpingshanense* ranges from (18.32~21.43)  $\mu\text{m} \times$  (19.73~23.48)  $\mu\text{m}$ . Exine ornamentation is an important trait in pollen morphology. Considerable variation exists in pollen exine ornamentation among different populations, which can be primarily classified into three main types: reticulate ornamentation, striate-reticulate ornamentation, and granulate ornamentation, and further subdivided into six subtypes based on muri type and lumina density. Specifically, pollen morphology of *Epimedium sagittatum* encompasses all three main types and six subtypes, whereas *Epimedium tiangpingshanense* includes three main types and four subtypes. The pollen characteristics of *Epimedium tiangpingshanense* represent a subset of the morphological variation observed in *Epimedium sagittatum*. Overall, exine ornamentation types exhibit a transition from reticulate to striate-reticulate patterns with increasing latitude and altitude. The main morphological pollen traits are significantly correlated with altitude. In terms of pollen morphology and exine ornamentation characteristics, *Epimedium tiangpingshanense* falls within the range of variation of *Epimedium sagittatum*, consistent with variation patterns observed in other macro-morphological traits. Pollen morphological traits cannot effectively distinguish the two groups and fail

to provide strong support for *Epimedium tiangpingshanense* as an independent species. This suggests that pollen morphological evidence has relatively limited significance in clarifying phylogenetic relationships among closely related taxa, though whether it can provide robust evidence for delimiting major clades within the genus requires further investigation.

## Full Text

### Comparative Study on Pollen Morphology of *Epimedium sagittatum* and Its Related Species *E. myrianthum*

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## Abstract

*Epimedium sagittatum* and *E. myrianthum* are closely related taxa with transitional states in many macro-morphological characters and even key taxonomic features. To explore their taxonomic relationship, we conducted comparative observations of pollen morphology and exine ornamentation using scanning electron microscopy (SEM) on 13 populations of *E. sagittatum* (including one population of *E. sagittatum* var. *glabratum*) and 7 populations of *E. myrianthum*. The results showed that pollen grains of both taxa were subspheroidal with tricolpate apertures and similar sizes. Pollen size of *E. sagittatum* was (18.21–21.66)  $\mu\text{m} \times$  (19.55–23.63)  $\mu\text{m}$ , while that of *E. myrianthum* was (18.32–21.43)  $\mu\text{m} \times$  (19.73–23.48)  $\mu\text{m}$ . Exine ornamentation represents a crucial character in pollen morphology. Significant variation in exine ornamentation existed among populations, which could be classified into three main categories: reticulate, striate-reticulate, and granular ornamentation, further subdivided into six subcategories based on muri type and lumina density. *Epimedium sagittatum* exhibited all three categories and six subcategories, whereas *E. myrianthum* displayed three categories and four subcategories. The pollen characteristics of *E. myrianthum* thus represent a subset of the morphological variation observed in *E. sagittatum*. Overall, exine ornamentation types showed a transition from reticulate to striate-reticulate patterns with increasing latitude and altitude. The main morphological pollen characters were closely correlated with elevation. Regarding pollen morphology and exine ornamentation features, *E. myrianthum* fell within the variation range of *E. sagittatum*, consistent with variation patterns in other macro-morphological characters. Pollen morphological characters could not effectively distinguish the two taxa and failed to provide strong support for *E. myrianthum* as an independent species. This suggests that pollen morphological evidence has relatively limited significance in clarifying phylogenetic relationships among closely related taxa, though whether it can provide robust evidence for defining major clades in the genus phylogeny requires further

investigation.

**Keywords:** population, *Epimedium sagittatum*, *Epimedium myrianthum*, pollen morphology, morphological variation

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*Epimedium* is a genus of perennial herbs in the family Berberidaceae and represents an important traditional and endemic medicinal plant in China. It possesses effects of tonifying kidney-yang, strengthening bones and muscles, and dispelling wind-dampness (National Pharmacopoeia Commission, 2015), and also demonstrates outstanding efficacy in improving cardiovascular diseases, enhancing immunity, preventing and treating cancer, and alleviating depression (Zhao et al., 2018; Choi et al., 2019). It serves as a crucial raw material for products such as Hui ren Shen bao tablets, Xian ling Gubao capsules, and China Jingjiu liquor. Additionally, *Epimedium* species exhibit excellent ornamental value due to their peculiar floral shapes and diverse colors, making them popular horticultural plants in the United Kingdom, United States, Japan, and Belgium (Ward, 2004; Ren et al., 2008; Avent, 2010; Probst, 2017).

The genus *Epimedium* L. comprises approximately 68 species worldwide, with 58 species originally described from China, revised to 46 species, representing about 85% of the taxa endemic to China (Xu et al., 2019; Xu et al., 2020). China is the modern distribution center and diversity center of *Epimedium*, likely also the origin and differentiation center, encompassing a continuous evolutionary process from primitive to advanced forms that remains actively diverging (Ying, 2002; Stearn, 2002). *Epimedium* is a taxonomically special and complex genus, with over half of the species described by foreign researchers based on few cultivated individuals. Between 1990 and 1999, the number of new species increased rapidly, with 26 species described, all from China.

Due to this rapid increase in species number within a short period, insufficient morphological investigation, and fertile interspecific hybridization leading to complex relationships among species, the genus has formed numerous species complexes or aggregates (Xu et al., 2014a). Among these, the *E. sagittatum* species complex represents one of the most complicated concerns in *Epimedium*, with at least 10 taxa closely related to *E. sagittatum* (Xu et al., 2014a). Xu et al. previously conducted a preliminary review of the classification of the *E. sagittatum* complex, treating *E. coactum*, *E. coactum* var. *longtouhum*, *E. lobophyllum*, *E. multiflorum*, and *E. jingzhouense* as synonyms of *E. myrianthum*, and *E. sagittatum* var. *guizhouense* and *E. myrianthum* var. *jianaheense* as synonyms of *E. sagittatum*, while downgrading *E. pudingense* to *E. sagittatum* var. *glabratum*. The classification of the *E. sagittatum* complex was ultimately traced back to the relationship between *E. sagittatum* and *E. myrianthum*.

*Epimedium sagittatum* is distributed across Hubei, Hunan, Jiangxi, Anhui, Zhejiang, Guangdong, and Fujian provinces. It is a widely distributed species in the genus with the greatest morphological variation and longest medicinal history. Its taxonomic issues are significant for phylogenetic reconstruction and taxonomic research of *Epimedium* (Stearn, 2002; Guo and Xiao, 2003; Xu, 2008; Xu et al., 2013; Xu et al., 2014b). Furthermore, *E. sagittatum* has been included in all editions of the *Pharmacopoeia of the People's Republic of China* (National Pharmacopoeia Commission, 2015), whereas *E. myrianthum* is a non-pharmacopoeial species. Clarifying their taxonomic relationship is crucial for accurate species identification, medicinal resource utilization, and ensuring 药材 quality and efficacy. Different scholars have conducted taxonomic studies on *E. sagittatum* from perspectives including plant morphology, genetic diversity, leaf abaxial non-glandular trichome characters, chemical active components, and chemotypes, all revealing rich variation among and within populations (Liang et al., 2013; Xu et al., 2013; Xu et al., 2015a; Xu et al., 2015b).

From macro-morphological perspectives, the two taxa show overlapping variation ranges in characters such as leaf number on flowering stems, young leaf color, outer sepal color, petal color, and petal shape, with sympatric distribution (Xu et al., 2014a). The primary distinction lies in inflorescence axis type—whether a narrow, straight panicle (*E. sagittatum*) or a diffuse panicle (*E. myrianthum*)—and the resulting differences in flower number and size (Xu et al., 2014a). Therefore, whether *E. myrianthum* should be treated as an independent species or merged with *E. sagittatum* requires additional evidence (Xu et al., 2014b).

Pollen of higher plants possesses stable geometric morphology, large production, easy preservation, high temperature resistance, and extreme resistance to oxidation (Wang and Wang, 1983; Tang et al., 2018; Zhang et al., 2019). As one of the stably inherited characters in plants, pollen morphology holds important value for species delimitation and even phylogenetic reconstruction (Zhang et al., 2019; Xiong et al., 2019; Xu et al., 2019). Since the 1990s, scholars have successively studied pollen morphology of *Epimedium* species (Liang and Yan, 1991; Yu and Liu, 1991; He and Guo, 1997; Guo et al., 1998; Wang et al., 2015). Wang et al. (2015) studied pollen morphology of 31 *Epimedium* species, demonstrating that pollen size and shape, particularly exine ornamentation (reticulate, striate-reticulate, and striate), could serve as important bases for plant classification and medicinal material identification. However, different scholars have described *E. sagittatum* pollen morphology quite differently. Yu and Liu (1991) described relatively large pollen grains of  $(29.7-32.6) \mu\text{m} \times (32.4-37.5) \mu\text{m}$  with reticulate exine sculpture. Liang and Yan (1991) described pollen size as  $(25-30) \mu\text{m} \times (28.7-31.9) \mu\text{m}$  with striate-reticulate ornamentation and irregular lumina. Guo (1998) and He (1997) described pollen size as  $20.5 \mu\text{m} \times (22.1-25.2) \mu\text{m}$  with reticulate ornamentation. Pollen morphology differed significantly among the three localities, ranging from prolate to subspheroidal shapes, with variations in muri morphology and lumina density. Wang et al. (2015) described *E. sagittatum* pollen as  $(18.2-21.8) \mu\text{m} \times (39.1-42.7) \mu\text{m}$  with reticulate ornamentation

and circular lumina.

Previous studies often used small samples to represent entire species, neglecting the “population” concept and failing to capture intraspecific and inter-individual variation. Sampling limitations led to incomplete understanding of interspecific and intraspecific variation ranges and patterns, making correct assessment of taxonomic value difficult and causing bias in determining “exclusivity” (Xu et al., 2013; Xu et al., 2014b; Xu et al., 2019), resulting in inconsistent findings among researchers. Wang et al. (2015) explicitly stated that pollen characteristics of *E. sagittatum* required investigation of more populations. Moreover, previous studies used dry pollen from herbarium specimens, which may have inconsistent developmental stages and risk of cross-contamination from pollination among sympatric species.

The “population” concept is crucial in taxonomy and must be emphasized to correctly understand character variation and taxonomic value (Ge and Hong, 1995; Liu et al., 2016; Liu et al., 2017; Xu et al., 2019). This study, using “populations” as units, conducted systematic pollen morphology research on *E. sagittatum* and its related species *E. myrianthum*, providing palynological reference for their taxonomic relationship.

## 1 Materials and Methods

### 1.1 Reagents

Ethanol (Tianjin Damao Chemical Reagent Factory, Tianjin), acetic acid (Tianjin Damao Chemical Reagent Factory, Tianjin; Xilong Scientific Co., Ltd., Guangdong), methanol (Tianjin Damao Chemical Reagent Factory, Tianjin; Xilong Scientific Co., Ltd., Guangdong), and glycerol (Xilong Scientific Co., Ltd., Guangdong) were all analytical grade.

### 1.2 Materials

Research materials were collected strictly following population sampling during the flowering period, with 19–30 individuals sampled per population. Experimental materials were identified by Professor Xu Yanqin from Jiangxi University of Traditional Chinese Medicine and cultivated in a common garden at the university. A total of 20 populations were collected, including 13 populations of *E. sagittatum* (including one population of *E. sagittatum* var. *glabratum*) and 7 populations of *E. myrianthum*. The populations covered the main distribution areas of the species, including seven provinces: Hunan, Hubei, Guizhou, Jiangxi, Anhui, Guangdong, and Guangxi. Information on collection locations, population codes, longitude, latitude, and elevation is provided in Table 1. Pollen observations were conducted during the 2019 flowering period (March–April) using plants cultivated in the common garden. For each population, 5–6 individuals were randomly selected, and 10–20 flowers with mature stamens were collected and immersed in FAA solution (5 mL of 38% formaldehyde, 5 mL of glacial acetic acid, 90 mL of 70% ethanol) for storage in the dark.

### 1.3 Instruments and Methods

A small amount of pollen was washed using an ethanol gradient (50-90% concentration), dried, and then a small quantity was picked up with a toothpick and evenly adhered to conductive adhesive paper on a sample stage. Observations were performed using an FEI Quanta 250 field emission environmental scanning electron microscope (SEM) at an operating voltage of 5 kV. Clear and representative fields of view were selected at different magnifications: pollen overall appearance was observed at 800 $\times$  magnification with 20 pollen grains randomly selected for measurement of polar axis length and equatorial axis length; polar and equatorial views were examined at 10,000 $\times$  magnification; exine ornamentation features were observed at 24,000 $\times$  magnification, with mesh diameter (lumina) and mesh ridge width (muri) measured. All fields of view were photographed for documentation.

### 1.4 Data Analysis

The ratio of polar axis length to equatorial axis length (P/E, Polar axis/equator axis) and the ratio of mesh diameter to mesh ridge width (D/W, Diameter/width) were calculated. Data were analyzed using SPSS 21.0 software for one-way ANOVA to determine means and standard deviations of pollen morphological characters for each population. The three exine ornamentation types (reticulate, striate-reticulate, and granular) were assigned values of 1, 2, and 3, respectively, for correlation analysis between exine ornamentation and main geographical-ecological factors (longitude, latitude, and elevation). MVSP 3.0 software was used for data standardization and cluster analysis of exine types and quantitative morphological characters. DPS 9.01 software was employed for grey correlation analysis between ecological factors and pollen grain morphological features, with pollen morphological characters as mother factors and ecological factors as son factors. Images were edited using Photoshop software. To ensure systematic and comparable research, terminology and classification of pollen morphological characters in this study followed previous studies on *Epimedium* pollen morphology (Liang and Yan, 1991; Guo et al., 1998; Wang et al., 2015).

## 2 Results

### 2.1.1 Pollen Size

Pollen grains of both *E. sagittatum* and *E. myrianthum* were single, subspheroidal, and tricolpate. The two species showed minimal difference in pollen size. *Epimedium sagittatum* pollen measured approximately (18.21-21.66) m  $\times$  (19.55-23.63) m with a P/E ratio of 1.06-1.08, while *E. myrianthum* pollen measured approximately (18.32-21.43) m  $\times$  (19.73-23.48) m with a P/E ratio of 1.07-1.09.

### 2.1.2 Pollen Exine Ornamentation

Based on exine ornamentation, three main categories were identified: reticulate ornamentation (I), striate-reticulate ornamentation (II), and granular ornamentation (III).

#### Category I: Reticulate Ornamentation

This category was further divided into three subcategories based on muri characteristics and lumina density.

##### I-1: Reticulate ornamentation with smooth muri and sparse or absent lumina

This type included *E. sagittatum* populations JXNF and HNJY, and *E. myrianthum* populations GXLZ, GZSS, and GZLC.

##### I-2: Reticulate ornamentation with verrucate muri and abundant lumina

This type included *E. sagittatum* population HNJS and *E. myrianthum* population HNSZ.

##### I-3: Reticulate ornamentation with smooth muri and abundant lumina

This type included *E. sagittatum* populations HNJJ, HNLY, and GDRY. Notably, populations GDRY and HNLY exhibited localized irregular striate-reticulate ornamentation within the reticulate pattern.

#### Category II: Striate-Reticulate Ornamentation

This category was subdivided into two subcategories based on ornamentation orientation and lumina density.

##### II-1: Irregular striate-reticulate ornamentation with sparse or absent lumina

This type included *E. sagittatum* populations AHHS and HNZZ, *E. sagittatum* var. *glabratum* population JXSL, and *E. myrianthum* populations GZZY and GZKY.

##### II-2: Regular striate-reticulate ornamentation with abundant lumina

This type included *E. sagittatum* populations HBLT, JXWN, and JXWY. In population JXWN, one-third of pollen grains displayed striate-reticulate ornamentation, while two-thirds showed striate-reticulate ornamentation with localized reticulate patterns. Population JXWY exhibited striate-reticulate ornamentation with localized reticulate patterns.

#### Category III: Granular Ornamentation

Pollen exine with granular protrusions and no lumina. This included *E. sagittatum* population HBHF and *E. myrianthum* population HBTP.

These results demonstrate that both *E. sagittatum* and *E. myrianthum* exhibit rich variation in pollen morphology, encompassing all three ornamentation categories. However, *E. sagittatum* displayed all six subcategories, while *E. myri-*

*anthum* contained only four subcategories. Therefore, the pollen morphological characters of *E. myrianthum* represent a subset of the variation observed in *E. sagittatum*.

Table 2 presents the pollen morphological characteristics of different populations of *E. sagittatum* and *E. myrianthum*, including measurements of polar axis length, equatorial axis length, P/E ratio, mesh ridge width, mesh diameter, D/W ratio, shape, and exine ornamentation descriptions for each population.

### **Geographic Distribution and Ornamentation:**

The figure shows the distribution and ornamentation characteristics. Triangles represent *E. sagittatum* (including var. *glabratum*), squares represent *E. myrianthum*. Green indicates reticulate ornamentation; red indicates striate-reticulate; black indicates granular; red with green (JXWN, JXWY) indicates predominantly striate-reticulate with localized reticulate; green with red (GDRY, HNLY) indicates predominantly reticulate with localized striate-reticulate. Solid symbols indicate few or no lumina; hollow symbols indicate abundant lumina.

[Figure 1: see original paper]

## **2.2.1 Reticulate Ornamentation (Category I)**

### **2.2.1.1 Reticulate Ornamentation—Smooth Muri, Absent or Sparse Lumina (I-1)**

**JXNF:** Pollen grain size (18.51–19.32)  $\mu\text{m} \times$  (19.82–20.72)  $\mu\text{m}$ , subspheroidal or suboblate, with flat poles, trilobate polar view appearing triangular-round, and circular equatorial view. Colpus membrane with sparse granular protrusions with blunt apices, relatively wide colpi. Exine with reticulate ornamentation, raised and smooth muri, no lumina, with blurred reticulation near poles and colpi margins.

**HNJY:** Pollen grain size (20.50–21.64)  $\mu\text{m} \times$  (21.88–23.50)  $\mu\text{m}$ , subspheroidal, with flat poles, trilobate polar view circular, equatorial view circular. Colpus membrane with verrucate protrusions distributed uniformly. Exine with reticulate ornamentation, relatively blurred reticulation, no lumina, muri surface without protrusions.

### **2.2.1.2 Reticulate Ornamentation—Verrucate Muri, Abundant Lumina (I-2)**

**HNJS:** Pollen grain size (20.00–20.54)  $\mu\text{m} \times$  (20.97–21.72)  $\mu\text{m}$ , subspheroidal, circular polar view with tricolpi, relatively wide in the middle. Colpus membrane wrinkled with granular protrusions. Exine with clear reticulate ornamentation, uneven muri surface with verrucate protrusions, dense circular lumina distributed uniformly, smaller lumina near poles, muri width similar to lumina diameter.

### **2.2.1.3 Reticulate Ornamentation—Smooth Muri, Abundant Lumina (I-3)**

**HNJH:** Pollen grain size (21.69–22.64)  $\mu\text{m} \times$  (23.09–24.07)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view, relatively large polar view, elliptical equatorial view. Relatively wide colpi, colpus membrane with uniformly distributed granular protrusions with blunt apices. Exine with reticulate ornamentation, lumina of various sizes near equatorial axis, but no lumina near poles and colpi margins, with some blurred areas lacking lumina.

**HNLV:** Pollen grain size (19.97–20.66)  $\mu\text{m} \times$  (20.73–21.47)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view, flat poles. Colpus membrane with uniformly distributed granular protrusions with blunt apices. Exine with reticulate ornamentation locally appearing striate-reticulate, clear reticulation, relatively large lumina near equatorial axis, irregularly and uniformly distributed, occasionally showing columellae through lumina. Smaller lumina near poles and colpi margins.

**GDRY:** Pollen grain size (18.79–19.98)  $\mu\text{m} \times$  (20.83–22.23)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view, colpus membrane with relatively large granular protrusions. Exine with reticulate ornamentation locally appearing irregular striate-reticulate, with lumina of various sizes and irregular shapes.

It should be noted that populations GDRY and HNLV exhibited variation in pollen ornamentation, predominantly reticulate but with localized irregular striate-reticulate patterns. Based on overall exine ornamentation, they were classified under reticulate ornamentation.

## 2.2.2 Striate-Reticulate Ornamentation (Category II)

### 2.2.2.1 Striate-Reticulate Ornamentation—Sparse or Absent Lumina, Irregular Pattern (II-1)

**AHHS:** Pollen grain size (20.67–21.49)  $\mu\text{m} \times$  (21.53–22.64)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view, elliptical equatorial view. Colpus membrane densely covered with uniformly distributed granular protrusions with blunt apices. Exine with striate-reticulate ornamentation, irregular orientation, clear pattern, raised striate muri, blurred striate-reticulate pattern near poles and colpi margins, with extremely few uneven small lumina occasionally visible.

**HNZJ:** Pollen grain size (20.52–21.66)  $\mu\text{m} \times$  (22.33–23.76)  $\mu\text{m}$ , trilobate circular polar view, colpus membrane with dense verrucate protrusions. Exine with striate-reticulate ornamentation, irregular orientation, no lumina or extremely sparse lumina near equatorial axis.

**JXSL:** Pollen grain size (20.39–21.26)  $\mu\text{m} \times$  (21.92–23.04)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view, poles gradually narrowing in equatorial view. Relatively narrow colpi, colpus membrane with uniformly distributed granular protrusions with blunt apices. Exine with striate-reticulate ornamentation, clear pattern, smooth muri, sparse lumina near equatorial axis, unevenly distributed, no lumina near poles and colpi margins.

### 2.2.2.2 Striate-Reticulate Ornamentation—Abundant Lumina (II-2)

**HBLT:** Pollen grain size (18.22–19.27)  $\mu\text{m} \times$  (19.55–20.56)  $\mu\text{m}$ , subspheroidal or prolate, circular polar view, equatorial view gradually narrowing at both ends, wider in the middle. Densely packed granules on colpus membrane, uniformly distributed with blunt apices. Exine with striate-reticulate ornamentation, extending along polar axis direction, smooth and dense muri. Relatively small, irregular lumina, muri width greater than lumina diameter, abundant lumina near equatorial axis, smaller and fewer lumina near colpi and poles.

**JXWN:** Pollen grain size (18.69–19.93)  $\mu\text{m} \times$  (20.01–21.10)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view, flat poles. Relatively narrow colpi, colpus membrane with granular protrusions with blunt apices. Exine with striate-reticulate or reticulate ornamentation, clear pattern, irregular orientation, dense lumina, uniformly distributed, smaller lumina near poles or colpi margins. Some pollen grains lack lumina near equatorial axis, with sparse lumina near colpi.

**JXWY:** Pollen grain size (20.51–21.14)  $\mu\text{m} \times$  (22.27–22.90)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view, flat poles. Relatively narrow colpi, colpus membrane with granular protrusions with blunt apices. Exine with striate-reticulate ornamentation, locally reticulate, clear pattern, lumina of various sizes and irregular shapes near equatorial axis, no lumina near poles or colpi margins.

It should be noted that populations JXWN and JXWY exhibited variation in pollen ornamentation. In JXWN, one-third of pollen grains showed striate-reticulate ornamentation, while two-thirds displayed striate-reticulate ornamentation with localized reticulate patterns. Population JXWY showed striate-reticulate ornamentation with localized reticulate patterns.

### 2.2.3 Granular Protrusions (Category III)

**HBHF:** Pollen grain size (19.77–20.81)  $\mu\text{m} \times$  (21.14–22.28)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view, circular equatorial view. Colpus membrane with irregular granular protrusions. Exine uneven, densely covered with verrucate small protrusions, extremely sparse lumina at poles, a few pollen grains with very few lumina near equatorial axis, extremely small lumina, smooth muri.

### 2.3.1 Reticulate Ornamentation (Category I)

#### 2.3.1.1 Reticulate Ornamentation—Sparse or Absent Lumina (I-1)

**GXLZ:** Pollen grain size (19.99–20.46)  $\mu\text{m} \times$  (22.30–23.34)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view, poles gradually narrowing in equatorial view. Relatively narrow colpi, gradually pointed or sharply pointed near poles, colpus membrane with dense irregular granular protrusions with blunt apices, uniformly distributed. Exine with reticulate ornamentation, clear pattern, extremely sparse small lumina.

**GZSS:** Pollen grain size (18.32–18.98)  $\mu\text{m} \times$  (19.73–20.27)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view, poles gradually narrowing in equatorial view. Relatively narrow colpi, gradually pointed or sharply pointed near poles, colpus

membrane with dense irregular granular protrusions. Exine with reticulate ornamentation, locally with sparse lumina of various sizes and irregular shapes. Some pollen grains have blurred reticulation without lumina.

**GZLC:** Pollen grain size (19.08–19.97)  $\mu\text{m} \times$  (20.80–21.63)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view. Relatively wide colpi, colpus membrane with granular protrusions. Exine with reticulate ornamentation, some pollen grains with blurred reticulation, no lumina or sparse irregular lumina near equatorial axis.

### **2.3.1.2 Reticulate Ornamentation—Abundant Lumina, Verrucate Muri (I-2)**

**HNSZ:** Pollen grain size (19.10–20.60)  $\mu\text{m} \times$  (20.90–21.64)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view. Colpus membrane with granular protrusions. Exine with clear reticulate ornamentation, dense verrucate protrusions on muri, lumina of various sizes and subcircular shapes near equatorial axis.

## **2.3.2 Striate-Reticulate Ornamentation (Category II)**

### **2.3.2.1 Striate-Reticulate Ornamentation—Sparse or Absent Lumina (II-1)**

**GZZY:** Pollen grain size (19.46–20.90)  $\mu\text{m} \times$  (21.10–22.09)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view, poles gradually narrowing in equatorial view. Colpus membrane with granular protrusions. Exine with irregular striate-reticulate ornamentation, clear pattern, extremely sparse and uneven small lumina near equatorial axis.

**GZKY:** Pollen grain size (20.35–21.43)  $\mu\text{m} \times$  (22.41–23.48)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view, poles gradually narrowing in equatorial view. Relatively narrow colpi, gradually pointed or sharply pointed near poles, colpus membrane with dense irregular granular protrusions. Exine with irregular striate-reticulate ornamentation, blurred pattern near poles and colpi margins, occasionally with extremely sparse small lumina.

### **2.3.3 Granular Protrusions (Category III)**

**HBTP:** Pollen grain size (20.27–21.06)  $\mu\text{m} \times$  (21.10–21.81)  $\mu\text{m}$ , subspheroidal, trilobate circular polar view. Relatively narrow colpi, gradually pointed near poles, colpus membrane with dense irregular granular protrusions. Exine with dense granular protrusions, extremely sparse and uneven small lumina near equatorial axis.

[Figure 2: see original paper], [Figure 3: see original paper], [Figure 4: see original paper], [Figure 5: see original paper]

## **2.4.1 Correlation Analysis Between Exine Ornamentation and Ecological Factors**

Based on geographic distribution and exine ornamentation characteristics, certain patterns emerged (Fig. 1 [Figure 1: see original paper]). Reticulate

ornamentation (Type I) was primarily distributed in northern Guangdong and Guangxi, and southern Guizhou, Jiangxi, and Hunan (populations GZLZ, HNJY, HNJH, GZLC, GZSS, and JXNF). Striate-reticulate ornamentation (Type II) was mainly found in central Guizhou, Hunan, and Jiangxi, as well as southern Anhui and adjacent Hubei regions (populations GZKY, GZZY, HNZJ, JXSL, AHHS, and HBLT). Granular ornamentation (Type III) was observed in populations HBHF and HBTP from western Hubei. Overall, exine ornamentation showed a gradual transition pattern with latitude. Correlation analysis revealed significant relationships between ornamentation type and latitude ( $P = 0.006$ ), with ornamentation transitioning from Type I (reticulate) to Type II (striate-reticulate) and Type III (granular) as latitude increased. Ornamentation type also showed significant correlation with elevation ( $P = 0.046$ ) but not with longitude ( $P = 0.829$ ).

#### 2.4.2 Grey Correlation Analysis Between Pollen Morphology and Ecological Factors

Grey correlation analysis between pollen morphological characters and ecological factors across different populations showed varying impacts of different ecological factors on pollen morphology (Table 3). For equatorial axis length, mesh diameter, mesh diameter/mesh ridge width ratio, and exine ornamentation, the influence order was elevation > latitude > longitude. For polar axis length and P/E ratio, the order was elevation > longitude > latitude. For mesh ridge width, the order was longitude > elevation > latitude.

Overall, grey correlation coefficients ( $r_i$ ) between *Epimedium* pollen morphological indices and ecological factors ranged from 0.5896 to 0.7346, indicating relatively close relationships. Elevation showed the highest correlation with polar axis length, equatorial axis length, P/E ratio, mesh diameter, mesh diameter/mesh ridge width ratio, and ornamentation type ( $r_i = 0.6650-0.7346$ ), suggesting elevation is the dominant factor affecting main pollen morphological characters. Longitude was the dominant factor affecting mesh ridge width ( $r_i = 0.6388$ ). Notably, latitude was also an important factor influencing pollen size characters and exine ornamentation ( $r_i = 0.5896-0.7120$ ).

Based on these correlation analyses, pollen morphological characters showed close relationships with ecological factors. Regarding the relationship between exine ornamentation and ecological factors, different software produced slightly different results. SPSS 21.0 analysis showed both latitude and elevation were significantly correlated with exine ornamentation, but latitude showed stronger correlation. DPS 9.01 analysis indicated both elevation and latitude were closely related to exine ornamentation, but elevation showed higher correlation. Overall, both elevation and latitude had prominent effects on pollen morphology.

## 2.5 Pollen Morphology Clustering

Cluster analysis based on pollen morphology data divided all populations into five groups at a genetic distance of 3.2 (Fig. 6 [Figure 6: see original paper]). Population HNZJ, with relatively large pollen grains, formed a separate group. The second group included *E. sagittatum* HBHF and *E. myrianthum* HBTP, both with granular ornamentation. The third group comprised four *E. sagittatum* populations (HNJH, JXSL, HNJY, JXWY) and two *E. myrianthum* populations (GXLZ, GZKY), containing both reticulate and striate-reticulate types. The fourth group consisted solely of *E. sagittatum* HBLT with striate-reticulate ornamentation. The fifth group included six *E. sagittatum* populations (JXNF, GDRY, HNJS, JXWN, HNLV, AHHS) and four *E. myrianthum* populations (HNSZ, GZSS, GZLC, GZZY), encompassing both reticulate and striate-reticulate ornamentation types.

These results indicate no clear distinction or discontinuity between *E. sagittatum* and *E. myrianthum* based on pollen morphological characters, with populations of the two taxa intermingling in the dendrogram.

## 3 Discussion

### 3.1 Pollen Morphological Characteristics of *E. sagittatum* and Taxonomic Implications

*Epimedium sagittatum* exhibits the greatest intraspecific variation range within the genus, showing rich diversity in plant morphology, genetic variation, and chemical profiles, suggesting it may be a polymorphic species (Xu, 2008; Liang et al., 2013; Xu et al., 2013; Xu et al., 2015a). This implies that sample selection for *E. sagittatum* studies requires extreme caution (Xu et al., 2013). Samples must be as comprehensive as possible, as insufficient sampling can make continuous variation patterns appear discontinuous. Establishing species and infraspecific taxa should be based on natural populations—a fundamental principle of modern taxonomy (Tang and Xiang, 1989). Since variation exists among and within populations of the same species, population-based systematic research is essential for comprehensively understanding variation ranges and patterns and evaluating their taxonomic significance (Wang et al., 2015; Liu et al., 2016; Liu et al., 2017; Xu et al., 2019). However, previous palynological studies on *Epimedium* often neglected the “population” concept, using small samples from single localities to represent entire species, leading to significant discrepancies in pollen morphology descriptions among researchers and the taxonomic pitfall of oversplitting.

This study systematically investigated pollen morphology of 12 *E. sagittatum* populations and one variety population, revealing extensive variation in pollen characters similar to macro-morphological variation, with significant differences among populations in key features. Three main categories of exine ornamentation were observed—reticulate, striate-reticulate, and granular—further subdivided into six subcategories based on muri and lumina characteristics. Re-

garding exine ornamentation, this study encompassed all variations reported in previous research, including the striate-reticulate ornamentation observed by Liang and Yan (1991), the reticulate pattern reported by Yu and Liu (1991), and the reticulate ornamentation described by He and Guo (1997) and Wang et al. (2015). Additionally, this study observed striate-reticulate and granular ornamentation not previously documented, reaffirming the importance and necessity of comprehensive sampling in *E. sagittatum* research.

Wang et al. (2015), based on pollen morphology of 31 *Epimedium* species, proposed that exine ornamentation could serve as a basis for plant classification. Their study supported *E. pudingense* as an independent species based on its striate-reticulate ornamentation, contrasting with the reticulate ornamentation of *E. sagittatum*. However, our results demonstrate that multiple *E. sagittatum* populations exhibit striate-reticulate ornamentation (e.g., AHHS, HNZJ, HBLT, JXWN, JXWY), proving this character is not exclusive to *E. pudingense*. Therefore, pollen characteristics cannot support *E. pudingense* as a separate species. Xu et al. (2014b) previously downgraded *E. pudingense* to *E. sagittatum* var. *glabratum* based on macro-morphological characters. The striate-reticulate ornamentation described for *E. pudingense* by Wang et al. (2015) matches that of the *E. sagittatum* var. *glabratum* JXSL population in our study, providing further pollen morphological support for this taxonomic treatment.

### 3.2 Taxonomic Relationship Between *E. sagittatum* and *E. myrianthum*

The classification issues surrounding the *E. sagittatum* species complex ultimately trace back to the relationship between *E. sagittatum* and *E. myrianthum* (Xu et al., 2014b). Based on extensive field morphological observations, our research group found overlapping key characters between the two taxa. For instance, when Stearn (1998) described *E. myrianthum* as a new species, he noted that *E. sagittatum* had wavy leaf margins and green young leaves, while *E. myrianthum* had flat leaf margins and young leaves with distinct red spots. However, most populations of both taxa actually exhibit young leaves with varying numbers and intensities of red spots or patches. Additionally, characters such as leaf number on flowering stems, outer sepal color, petal color, and petal shape all show transitions and intersections between the two groups (Xu et al., 2014b). The primary distinction lies in inflorescence type—whether a narrow, straight panicle (*E. sagittatum*) or a diffuse panicle (*E. myrianthum*)—and the resulting differences in flower number and size (Xu et al., 2014b). Xu et al. (2015a, 2015b, 2016) conducted quality evaluations based on icariin content in 16 populations of *E. sagittatum* and 8 populations of *E. myrianthum*, revealing significant variation among populations of both taxa and showing that the three chemical types of *E. myrianthum* fell within the chemical variation range of *E. sagittatum*.

Pollen morphology data and cluster analysis results (Table 2, Fig. 5) demonstrate no clear distinction or discontinuity between *E. myrianthum* and *E. sagittatum*, with exine ornamentation variation of the former falling entirely within

the variation range of the latter, consistent with macro-morphological variation trends. Therefore, based on palynological evidence, no clear discontinuity exists between *E. myrianthum* and *E. sagittatum*, failing to provide strong support for *E. myrianthum* as an independent species. However, we cannot reject the species status of *E. myrianthum* simply because its pollen morphological variation falls within that of *E. sagittatum*. Palynological studies of different *Epimedium* species (Liang and Yan, 1991; Yu and Liu, 1991; He and Guo, 1997; Guo et al., 1998; Wang et al., 2015) show that the exine ornamentation variation observed in *E. sagittatum* in this study encompasses variations found in most species of the genus. Therefore, the taxonomic significance of palynological evidence requires systematic investigation of pollen morphological characters across the entire genus to reach objective conclusions.

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