Pollen Morphology of 31 Taxa in the Genus Iris and Its Taxonomic Significance: Postprint

Authors: Zhu Ying, Song Hua, Li Kai, Lei Zhang, Wang Baibing

Date: 2025-03-19T00:00:00+00:00

Abstract

To investigate the pollen morphology of Iris taxa and its potential taxonomic significance, pollen from 31 taxa across three subgenera of Iris was used as experimental material, prepared using the critical point drying method, and subjected to scanning electron microscopy observation and hierarchical cluster analysis. The results showed that: (1) The pollen of all 31 taxa was heteropolar monad pollen with a distal colpus, medium to large in size, and oblate spheroidal, suboblate, or spheroidal in shape; the distal colpus membrane was smooth or ornamented; the exine was semitectate or intectate, with exine ornamentation typically being various types of heterobrochate reticulate patterns, and a few being baculiform-clavate. (2) Pollen grains with annulate distal colpi were found in Iris bungei, Iris pumila, and Iris ventricosa; bipolar colpi were observed on pollen grains of Iris hoo, which is reported for the first time in Iris. (3) Hierarchical cluster analysis revealed that at a squared Euclidean distance of 10, the pollen of the 31 Iris taxa clustered into six types: Siberian Iris type, German Iris type, short-standard Iris type, purple-bract Iris type, swallow-flower Iris type, and lute Iris type, with the tested taxa generally clustering according to the relationships of subgenera, sections, and series in morphological taxonomy. Among the six types, the German Iris type had the largest pollen grains, while the purple-bract Iris type had the smallest. The short-standard Iris type pollen was intectate, with baculiform-clavate exine ornamentation. The lute Iris type pollen was suboblate, with an ornamented colpus membrane on the distal colpus. (4) The possible phylogenetic trends in pollen morphology of the three subgenera suggest that subgenus Limniris is more primitive than subgenus Iris, series Ruthenicae is the most primitive group among the studied taxa, and subgenus Pardanthopsis and section Pseudoregelia may be intermediate types in the transition from subgenus Limniris to subgenus Iris. (5) The critical point drying method and the 2,2-dimethoxypropane (DMP) direct method are suitable methods for preparing Iris pollen materials. These results reflect that the pollen morphology of the 31 Iris taxa exhibits both consistency and variation, and palynological characteristics can serve as an auxiliary means for dividing subgenera, sections, and series within the genus, providing reliable palynological evidence.

Full Text

Preamble

DOI: 10.11931/guihaia.gxzw202409031

Pollen Morphology of 31 Taxa of the Genus Iris L. (Iridaceae) and Its Taxonomic Implications

ZHU Ying*, SONG Hua, LI Kai, ZHANG Lei, WANG Baibing

(Beijing Botanical Garden, Key Laboratory of National Forestry and Grassland Administration on Plant Ex Situ Conservation, Beijing Floriculture Engineering Technology Research Centre, Beijing 100093, China)

Abstract: To investigate the pollen morphology and its potential taxonomic implications for the genus Iris L. (Iridaceae), pollen grains from 31 taxa across three subgenera were examined using scanning electron microscopy (SEM) and subjected to hierarchical cluster analysis. Pollen grains were prepared using the critical-point drying method. The results revealed: (1) All pollen grains from the 31 taxa were monad, heteropolar, and sulcate, with medium to large size and oblate, suboblate, or spheroidal shapes. The sulcus membrane was either smooth or ornamented. The exine was semitectate or atectate, with different types of heterobrochate reticulation (rarely gemmate-clavate) ornamentation. (2) Zonasulcate pollen grains were found in *Iris bungei*, *Iris kobayashii*, and *Iris ventricosa*. For the first time, disulcate pollen grains were observed in *Iris hookeri* within the genus Iris. (3) Cluster analysis showed that at a squared Euclidean distance of 10, the 31 taxa could be grouped into six types: Sibirica type, Germanica type, Pumila type, Ruthenica type, Laevigata type, and Spuria type. The clustering pattern largely corresponded to the subgenera, sections, and series recognized in morphological taxonomy. Among the six types, the Germanica type exhibited the largest pollen grains, while the Ruthenica type had the smallest. The Pumila type was characterized by atectate pollen with gemmate-clavate exine ornamentation, and the Spuria type by suboblate pollen with an ornamented sulcus membrane. (4) The possible evolutionary trends in pollen morphology across the three subgenera suggest that subgenus Limniris is more primitive than subgenus Iris, with series Ruthenicae representing the most primitive group among the studied taxa. Subgenus Pardanthopsis and section Lophiris may represent intermediate types in the transition from subgenus Limniris to subgenus Iris. (5) Both the critical-point drying method and the 2,2-dimethoxypropane (DMP) direct method are suitable for preparing Iris pollen samples. These findings demonstrate that the pollen morphology of the 31 Iris taxa reflects both consistency and variation, and that palynological characteristics can serve as supplementary criteria for delimiting subgenera, sections, and series, providing reliable palynological evidence for classification.

Keywords: Iris, critical-point drying method, pollen morphology, taxonomic implications, SEM

The genus *Iris* L. comprises approximately 260 species of world-renowned ornamental plants distributed across temperate regions of the Northern Hemisphere, primarily in Eurasia and North America, with about four species in North Africa (Wilson, 2011). China represents one of the distribution centers of the genus, with approximately 58 native species, of which 21 are endemic (Zhao et al., 2000). Current infrageneric classification of *Iris* is primarily based on morphology. However, the great diversity of species, coupled with rich phenotypic variation and genetic diversity, has created numerous taxonomic challenges, leading to the establishment of multiple classification systems. The most influential among these are the systems proposed by Dykes (1913), Lawrence (1953), Rodionenko (1987), Zhao Yutang (1985), and Mathew (1989) (Cheng et al., 2021). These systems emphasize different characteristics and show inconsistencies in their treatment of crested irises, spuria irises, *Pardanthopsis* irises, and bulbous irises. In recent years, molecular studies have prompted adjustments and revisions to infrageneric classification systems (Wang and Zhuo, 2006; Wilson, 2009; Mu et al., 2011; Kang et al., 2020). Building on previous work, Wilson (2011) proposed a revised classification system integrating multiple lines of evidence, though this system has not yet gained widespread acceptance, with Mathew’ s (1989) system remaining the most internationally recognized.

Pollen, as a highly reduced male gametophyte, exhibits morphological characteristics controlled by plant genotype and largely unaffected by environmental conditions, demonstrating strong genetic conservatism. Its size, shape, exine ornamentation, and apertures serve as important palynological indicators that are more stable than other traits (Wu et al., 2012). At lower taxonomic levels (genus and species), unique combinations of pollen morphological and structural characteristics often point to specific taxa, making even subtle traits systematically valuable (Halbritter et al., 2021). Particularly in taxon delimitation, when results from multigene phylogenetic studies conflict, pollen data combined with other morphological evidence have become important criteria for evaluating the most representative gene trees (Ulrich et al., 2013). Palynology was applied to phylogenetic studies of *Iris* as early as the 1990s, with scholars conducting detailed investigations of specific subgenera or regional taxa and identifying evolutionary trends and taxonomic value of infrageneric pollen characteristics (Colasantem et al., 1989; Goldblatt and Thomas, 1992; Pinar and Dönmez, 2000; Dönmez and Pinar, 2001). Domestic researchers have examined the pollen morphology of 34 Chinese *Iris* species, providing preliminary palynological data for systematic classification and evolution of the genus (Qi and Zhao, 1987; Yang, 2002; Yu et al., 2010; Ma et al., 2017). However, these studies employed natural air-drying or acetolysis methods, which caused severe pollen shrinkage and deformation, preventing the acquisition of clear images for accurate morphological description.

Therefore, based on the *Iris* germplasm resource nursery at the National Botanical Garden (North Garden), this study employed the critical-point drying method for pollen preparation. Through SEM observation and cluster analysis of pollen from 31 *Iris* taxa native to or cultivated in China, we aimed to address the following questions: (1) What are the similarities and differences in pollen morphology among subgenera, sections, series, and species across the 31 taxa? (2) What is the taxonomic significance of palynological evidence and the evolutionary trends of pollen morphology in *Iris*? (3) What are the suitable methods for preparing *Iris* pollen samples?

1.1 Experimental Materials

The experimental materials comprised 31 taxa of *Iris* (28 species, 2 varieties, and 1 forma) belonging to 3 subgenera, 5 sections, and 7 series . The classification of subgenus *Pardanthopsis* followed Wilson (2011), while other subgenera followed Mathew (1989). All materials were sourced from the resource nursery at the National Botanical Garden (North Garden). *Iris confusa* and *I. hookeri* were⋯

1.2.1 Pollen Collection and Processing

Freshly opening flower buds were selected and bagged with paper bags. When flowers opened and pollen was released, anthers were collected and placed in 2 mL centrifuge tubes, immediately fixed with 2.5% glutaraldehyde fixative, and stored at 4°C. The fixation time was at least 10 hours but not exceeding 10 days.

1.2.2 SEM Sample Preparation

Pollen was removed from the fixative and dehydrated through an ethanol gradient of 30%-50%-70%-100%-100%, with 15 minutes at each concentration. After dehydration, samples were removed from 100% ethanol and subjected to critical-point drying in a critical-point dryer (Tousimis Autosamdri-815, Series A) for 1 hour. The dried pollen was mounted on stubs with conductive adhesive tape, sputter-coated with gold (HIYACHI MC1000), and observed under scanning electron microscopy (HIYACHI SU8020).

Observations included pollen populations (150-200×), equatorial view (1,000-2,000×), polar view (1,000-2,000×), exine ornamentation (5,000×, 15,000×), and germination regions (15,000×). For stored pollen of *I. confusa* and *I. hookeri*, two methods were employed: (1) natural air-drying, where dry pollen was directly sputter-coated and observed (hereinafter referred to as dry pollen); and (2) fixation with 2.5% glutaraldehyde followed by the same procedure as for fresh pollen.

1.2.3 Analysis Methods

For each taxon, 20 normal pollen grains were selected, and polar axis length (P) and equatorial axis length (E) were measured using Nano Measurer 1.2 software, with P/E values calculated. Pollen morphology terminology followed *Pollen Flora of China Woody Plants by SEM* and *Illustrated Pollen Terminology* (Li et al., 2011; Halbritter et al., 2021). Eight indicators were selected for hierarchical cluster analysis using SPSS software with squared Euclidean distance: polar axis length, equatorial axis length, sulcus length, pollen grain shape, polar view outline, sulcus membrane, ornamentation type, and reticulum size level.

2.1.1 Morphological Characteristics of Dry Pollen

The dry pollen of *I. confusa* and *I. hookeri* exhibited a boat-shaped morphology with invaginated germination regions, making the sulcus membrane barely visible and exine ornamentation unclear [Figure 1: see original paper]. Unless otherwise specified, the pollen morphology described below refers to pollen prepared by critical-point drying.

2.1.2 Pollen Shape and Size

All 31 taxa produced heteropolar monad pollen grains (Table 1, Figures 2-7) of medium to large size. Three species—*I. ruthenica*, *I. uniflora*, and *I. kobayashii* —were medium-sized (maximum diameter 26-50 m), while all other taxa were large (maximum diameter 51-100 m). Polar and equatorial axis lengths differed significantly among species (Table 1). The three species in section *Iris* (subgenus *Iris*) had larger pollen grains, with both polar and equatorial axes exceeding 70 m. *Iris tectorum* from section *Lophiris* also had large pollen grains, with polar and equatorial axes around 80 m, similar to *I. pallida*. The two species in series *Ruthenicae* had the smallest pollen grains, with polar and equatorial axes around 40 m—only half the size of *I. tectorum* and *I. pallida*.

The shape of most pollen grains (based on P/E ratio) was spheroidal (Figures 2-7), while eight species and one variety—including *I. spuria*, *I. halophila*, and *I. kobayashii*—were suboblate, and *I. ensata* and its variety *I. ensata* var. *spontanea* were oblate. Spheroidal pollen grains showed a circular polar outline, whereas oblate and suboblate grains showed an elliptical polar outline.

2.1.3 Sulcus Characteristics

All 31 taxa possessed a single distal sulcus (hereinafter referred to as sulcus) (Figures 2-7). Only *I. hookeri* exhibited a small number of pollen grains with disulci [FIGURE:8:1]. Sulcus length and width varied considerably among taxa. In most species, sulcus length was approximately half the pollen grain circumference, but in some taxa the sulcus extended toward the proximal surface, forming an extended sulcus or even a zonasulcate condition. For example, *I. mandshurica* had an extended sulcus [FIGURE:2:4] reaching two-thirds of the pollen circumference. *Iris bungei*, *I. ventricosa*, and *I. kobayashii* also had extended sulci, with some pollen grains developing zonasulcate patterns [FIGURE:8:2-4]. Sulcus width also varied substantially. *Iris pallida* [FIGURE:2:1], *I. scariosa* [FIGURE:2:2], the three species in section *Lophiris* [FIGURE:3:6-8], and *I. tenuifolia* [FIGURE:4:17] had relatively wide sulci with length-to-width ratios approaching 1:1. The three species in series *Spuriae* [FIGURE:3:9-11], *I. laevigata*, *I. ensata*, and its variety had relatively narrow, elongated sulci with length-to-width ratios of about 2:1. Taxa with extended sulci typically had narrow, ribbon-like sulci.

The sulcus membrane was smooth in most taxa, but seven species and one variety—including *I. tigridia*, *I. spuria*, *I. kobayashii*, and *I. versicolor*—showed varying degrees of ornamented membranes [FIGURE:2:5; FIGURE:3:9-11; FIGURE:4:14-16; FIGURE:5:21].

2.1.4 Exine Tectum and Ornamentation

Among the studied taxa, only *I. chamaeiris* [FIGURE:2:3] and *I. tigridia* [FIGURE:2:5] lacked a tectum, exhibiting gemmate-clavate ornamentation. All other taxa were semitectate with various types of heterobrochate reticulation; some possessed free-standing columellae. Reticulum size varied considerably. Generally, subgenus *Iris* (excluding atectate species), subgenus *Pardanthopsis*, and section *Lophiris* had larger reticula, while subgenus *Limniris* had relatively smaller reticula, particularly in series *Ruthenicae*, where reticula reached extremely small sizes (diameter <0.5 m). *Iris japonica* and *I. confusa* showed specialized reticulation: the former exhibited reticulum cristatum with free-standing columellae [FIGURE:3:7], while the latter showed gemmate-reticulate patterns [FIGURE:3:8]. *Iris pseudacorus* had disrupted muri forming incomplete reticula [FIGURE:5:22].

2.2 Cluster Analysis of Pollen Morphology

Hierarchical cluster analysis [Figure 9: see original paper] revealed that at a squared Euclidean distance of 10, the 31 *Iris* taxa grouped into six types: Sibirica type, Germanica type, Pumila type, Ruthenica type, Laevigata type, and Spuria type.

The **Sibirica type** was characterized by spheroidal pollen grains with smooth sulcus membranes and sulcus length approximately half the pollen circumference (except in *I. mandshurica*). Reticulum dimensions were mostly grade B (1-5 m diameter), occasionally grade C (5.1-10 m). This type included the most taxa: 12 species and 1 forma across 3 subgenera, 3 sections, and 4 series. All species from series *Sibericae* and *Tripetalae* of subgenus *Limniris* and the entire subgenus *Pardanthopsis* clustered here, along with *I. tenuifolia* from series *Tenuifoliae*, *I. japonica* and *I. confusa* from section *Lophiris*, *I. pseudacorus* from series *Laevigatae*, and *I. mandshurica* from section *Psammiris*.

The **Germanica type** had the largest pollen grains and largest reticula among all types, with spheroidal shape, smooth sulcus membrane, relatively wide sulcus (length-to-width ratio ~1:1), and reticulum dimensions of grade C or even grade D (>10 m). This type included only three species: *I. pallida* and *I. scariosa* from section *Iris* of subgenus *Iris*, and *I. tectorum* from section *Lophiris* of subgenus *Limniris*.

The **Pumila type** was characterized by atectate exine with gemmate-clavate ornamentation and spheroidal pollen shape. This type included only two species from subgenus *Iris*: *I. chamaeiris* from section *Iris* and *I. tigridia* from section *Pseudoregelia*.

The **Ruthenica type** had the smallest pollen grains and smallest reticula among all types, comprising only two species from series *Ruthenicae* of subgenus *Limniris*: *I. ruthenica* and *I. uniflora*.

The **Laevigata type** was distinguished by oblate or suboblate pollen grains with elliptical polar outlines and relatively small reticula (grade A or B). This type included four species: *I. laevigata*, *I. ensata*, its variety *I. ensata* var. *spontanea* from series *Laevigatae*, and *I. lactea* from series *Ensatae*.

The **Spuria type** was characterized by suboblate pollen grains with elliptical polar outlines, ornamented sulcus membranes, sulcus length approximately half the pollen circumference or extending to zonasulcate patterns, and relatively small reticula (grade A or B). This type included seven species: *I. spuria*, *I. halophila*, its variety *I. halophila* var. *sogdiana* from series *Spuriae*; *I. kobayashii*, *I. ventricosa*, and *I. bungei* from series *Tenuifoliae*; and *I. versicolor* from series *Laevigatae*.

Different colored arrows in [Figure 9: see original paper] indicate taxa that did not cluster according to morphological taxonomy but were embedded in other clades.

Overall, the 31 taxa largely clustered according to morphological subgenera, sections, and series, with only a few species embedded in other groups (as indicated by arrows in [Figure 9: see original paper]). This demonstrates that *Iris* pollen morphology exhibits both consistency and variation, with palynological characteristics serving as supplementary criteria for delimiting subgenera, sections, and series.

3.1.1 Pollen Morphology and Classification of Subgenus Iris

In subgenus *Iris*, section *Iris* shows considerable variation in plant height, ranging from dwarf to tall types, while sections *Psammiris*, *Pseudoregelia*, and *Hexapogon* are exclusively dwarf. Our study included five species from subgenus *Iris* representing three sections: *Iris*, *Psammiris*, and *Pseudoregelia*. Cluster analysis placed these five species into three major clades: *I. mandshurica* in the Sibirica type, *I. chamaeiris* and *I. tigridia* in the Pumila type, and *I. pallida* and *I. scariosa* in the Germanica type.

The Germanica and Pumila types correspond to Mitić' s types I and II, respectively (Mitić et al., 2013). Mitić et al. (2013) studied 11 species from section *Iris* and proposed that type I (Germanica type) generally matches tall bearded irises, while type II (Pumila type) corresponds to dwarf bearded irises. Our results, along with previous palynological studies of sections *Psammiris* and *Hexapogon* (Qi and Zhao, 1987; Colasantem et al., 1989; Halbritter and Buchne, 2016), support this trend and extend the range of dwarf bearded irises to include some species from sections *Hexapogon* and *Psammiris*. However, exceptions exist: *I. scariosa* is a dwarf species but exhibits the Germanica type, while *I. marsica* is tall but shows the Pumila type (Colasantem et al., 1989). The clustering of dwarf *I. scariosa* and tall *I. pallida* in the Germanica type indicates their close phylogenetic relationship, consistent with molecular studies grouping *I. scariosa* with tall bearded irises (Zhong, 2010; Feng, 2016).

The taxonomic position of *I. mandshurica* has long been controversial. Wang and Zhuo (2006) suggested that despite belonging to subgenus *Iris*, *I. mandshurica* shares many characteristics, including seedling morphology and pollen morphology, with subgenus *Limniris*, making it a transitional type between the two subgenera. Qin et al. (2000) found that based on chemical constituents, cytology, and distribution, *I. mandshurica* is relatively distant from other members of subgenus *Iris*. In our study, *I. mandshurica* possessed a semitectate exine, differing from the Pumila type of its section-mates *I. potaninii* and *I. curvifolia* (Qi and Zhao, 1987), and more closely resembling the Germanica type of tall bearded irises. However, its extended sulcus resembles those of three species from series *Tenuifoliae* of subgenus *Limniris*. Molecular studies also failed to cluster *I. mandshurica* with *I. potaninii* (Feng, 2016). Therefore, the placement of *I. mandshurica* in section *Psammiris* requires further investigation.

3.1.2 Pollen Morphology and Classification of Subgenus Pardanthopsis

The two species in subgenus *Pardanthopsis*—*I. dichotoma* and *I. domestica*—have been subject to considerable taxonomic debate due to their dichotomously branched stems, a feature distinct from other *Iris* species. *Iris dichotoma* possesses petaloid style branches typical of *Iris*, leading Dykes (1913), Lawrence (1953), Rodionenko (1987), and Zhao Yutang (1985) to treat it as a subgenus of *Iris*. *Iris domestica* (formerly *Belamcanda chinensis*), however, differs significantly from typical *Iris* flowers, with similar inner and outer perianth segments and only shallowly lobed, non-petaloid stigmas, leading to its establishment as a separate genus *Belamcanda*. Mathew (1989) treated both as monotypic genera. Molecular studies have revealed that *I. domestica* is nested within *Iris*, forming a clade with *I. dichotoma* that is sister to subgenus *Iris* (Wilson, 2004; Goldblatt and Mabberley, 2005; Kang et al., 2020). Our study found that both species have spheroidal pollen with distal sulci, typical of *Iris* pollen morphology, and both cluster within the Sibirica type. Combined with their similar vegetative morphology and ability to hybridize and produce fertile offspring, these findings support molecular evidence for including *I. domestica* within *Iris* and establishing both species in subgenus *Pardanthopsis* (Wilson, 2011).

3.1.3 Pollen Morphology and Classification of Subgenus Limniris

Our study included 21 species, 2 varieties, and 1 forma from subgenus *Limniris*, representing 2 sections and 7 series. Except for the Pumila type, all other five types contained taxa from subgenus *Limniris*. The Ruthenica, Laevigata, and Spuria types consisted exclusively of subgenus *Limniris* taxa.

Section *Lophiris* represents a particularly controversial group. Our study revealed that the three species in this section differed significantly from other subgenus *Limniris* types but showed similarities to *I. pallida*. *Iris japonica* and *I. confusa* possessed specialized exine ornamentation: the former exhibited reticulum cristatum with prominent secondary sculpture on the muri, while the latter showed gemmate-reticulate patterns, with the gemmate elements approaching the characteristics of the Pumila type in subgenus *Iris*. Cluster analysis placed *I. tectorum* with *I. pallida* and *I. scariosa* from section *Iris* of subgenus *Iris*, indicating a closer relationship with bearded irises. *Iris japonica* and *I. confusa* formed a separate subclade within the Sibirica type, sister to other Sibirica type taxa. Molecular studies have shown that *I. japonica* and *I. confusa* are closely related, clustering together (Japonica clade), while *I. tectorum* is more distantly related, forming a separate clade (Tectorum clade) (Guo and Wilson, 2013). The palynological characteristics observed in our study support these molecular findings and the elevation of this group to subgenus *Lophiris* (Wilson, 2011).

The two species in series *Ruthenicae* formed a distinct clade, representing the type with the smallest pollen grains and smallest reticula within subgenus *Limniris*. These clear palynological distinctions from other series, combined with the presence of arils on seeds of *I. ruthenica* and *I. uniflora*, support Zhao Yutang' s (1985) elevation of this series to sectional status.

The three species and one variety in series *Spuriae* showed consistent pollen morphology, forming a subclade within the Spuria type, sister to the three species from series *Tenuifoliae*.

Pollen morphology varied among the four species in series *Tenuifoliae*. *Iris kobayashii*, *I. ventricosa*, and *I. bungei* showed similar pollen morphology, clustering together with typical characteristics of suboblate shape and extended sulci, including zonasulcate patterns. *Iris tenuifolia*, however, had spheroidal pollen with larger reticula, more similar to *I. pallida* in shape, but its smaller pollen size resulted in clustering within the Sibirica type. This suggests that *I. tenuifolia* is phylogenetically distant from the other three species. Additionally, clear differences exist in leaf width and scape length between *I. tenuifolia* and the other three species, raising questions about whether these four species should be classified within the same series.

The five species and one variety in series *Laevigatae* showed considerable variation in pollen morphology. *Iris laevigata*, *I. ensata*, and its variety exhibited consistent pollen morphology, forming a clade sister to the sole species of series *Ensatae* (*I. lactea*), together constituting the Laevigata type. *Iris versicolor*, with its ornamented sulcus membrane, clustered within the Spuria type, possibly due to natural hybridization during long-term cultivation—a question requiring further investigation. *Iris pseudacorus*, with spheroidal pollen, clustered with the four species of series *Sibericae*, consistent with Mitić et al. (2013). Molecular studies have shown that *I. pseudacorus* clusters with *I. setosa*, forming a clade sister to *I. sibirica* (Wilson, 2009), which aligns with our palynological clustering results and suggests a closer relationship between *I. pseudacorus* and both *I. setosa* and *I. sibirica*. This raises questions about the correct placement of *I. pseudacorus* within series *Laevigatae*. Our study found *I. pseudacorus* exine ornamentation to be incomplete reticulate, differing from the complete reticulate pattern reported by Halbritter et al. (2020). This discrepancy may result from variation due to long-term cultivation or could indicate polymorphism in *I. pseudacorus* pollen exine structure, requiring further investigation.

The pollen morphology of series *Sibericae* and *Tripetalae* was relatively consistent, with both clustering within the Sibirica type as sister groups. In cultivation practice, *I. sanguinea* and *I. sibirica* are difficult to distinguish, and the leaf width of *I. typhifolia* seedlings shows continuous variation, preventing accurate differentiation from *I. sanguinea*. Combined with their similar palynological characteristics, these findings support Boltenkov et al.' s (2020) conclusion that *I. sanguinea*, *I. typhifolia*, and *I. sibirica* are conspecific.

In summary, the 31 taxa largely clustered according to morphological subgenera, sections, and series, with only a few species embedded in other groups. This demonstrates that *Iris* pollen morphology exhibits both consistency and variation, with palynological characteristics serving as valuable supplementary criteria for infrageneric classification.

3.2 Systematic Significance of Pollen Morphology in Iris

Evolutionary trends in pollen morphology tend toward larger grain size and heterobrochate reticulate exines (Pinar and Dönmez, 2000), with larger reticulate exines being derived (Goldblatt and Thomas, 1992). Our results indicate that subgenus *Limniris* (excluding section *Lophiris*) has smaller pollen grains and reticula, representing a more primitive condition than subgenus *Iris*, consistent with the conclusion that rhizomatous growth and lack of appendages are ancestral characteristics in *Iris* (Wilson, 2006). Series *Ruthenicae*, with the smallest pollen grains and reticula, represents the most primitive group among the studied taxa. The pollen morphology of section *Lophiris* and subgenus *Pardanthopsis* trends toward that of subgenus *Iris*, suggesting they may represent intermediate types in the transition from subgenus *Limniris* to subgenus *Iris*.

The Germanica type possesses semitectate exines, while the Pumila type lacks a tectum. According to the theory of exine structural evolution in angiosperms from semitectate to atectate (Wang and Wang, 1983), the Pumila type would be derived from the Germanica type. However, the Germanica type has larger pollen grains than the Pumila type, contradicting the trend that larger pollen grains are more evolved. Therefore, Mitić et al. (2013) and Colasantem et al. (1989) favor the derivation of the Germanica type from the Pumila type. Our study found that *I. confusa* exhibits gemmate-reticulate ornamentation, where dense gemmate elements connect to form a reticulum, possibly representing a transitional type from atectate to semitectate. This supports the theory that semitectate exines are more evolved, i.e., that the Germanica type is derived from the Pumila type.

Monosulcate pollen grains are considered the most primitive in seed plants (Pinar and Dönmez, 2000), with extended sulci potentially providing an evolutionary pathway to the more advanced zonasulcate condition (Goldblatt and Thomas, 1992). Zonasulcate germination regions represent an optimal mechanism for directional regulation, adapting to pollen tube formation through contraction or expansion—a phenomenon demonstrating both adaptive and systematic value, particularly in monocot pollen (Halbritter et al., 2021). Our study confirmed that extended sulci in *I. kobayashii*, *I. ventricosa*, and *I. bungei* can develop into zonasulcate patterns in some grains, supporting Goldblatt and Thomas' s (1992) theory. In germination experiments with *I. kobayashii*, we observed some pollen grains producing two or three pollen tubes simultaneously [Figure 10: see original paper], with rare double-tube pollen also observed in *I. ventricosa* and *I. bungei.* This may represent an advantageous adaptation for species growing in arid environments. Extended and zonasulcate sulci are rare in *Iris*, previously reported only in *I. graminea* of the spuria group (Mitić et al., 2013) and in seven species of subgenus *Hermodactyloides* (Goldblatt and Thomas, 1992; Pinar and Dönmez, 2000).

We report for the first time in *Iris* the occurrence of disulcate abnormal pollen grains in *I. hookeri* [FIGURE:8:1]. Within Iridaceae, Rudall and Wheeler (1988) clearly observed disulci in three genera—*Alophia*, *Cipura*, and *Tigridia*—using SEM. Some scholars suggest that increased numbers of germination regions provide more potential germination sites, offering selective advantages by ensuring at least one aperture contacts the stigma surface, potentially increasing fertilization rates (Dajoz et al., 1991; Furness and Rudall, 2004). Abnormal pollen grains arise from genetic (polyploidization), chemical, or environmental induction and frequently occur in cultivated, ornamental, crop, annual, vegetatively propagated, and hybrid plants (Halbritter et al., 2021). The *I. hookeri* used in our study is a cultivated plant that may have undergone beneficial mutations under long-term cultivation. In conclusion, the occurrence of disulci and zonasulcate germination regions in *Iris* likely has systematic and functional significance, though the underlying causes remain unclear and require further investigation.

3.3 Sample Preparation Methods for SEM Observation of Iris Pollen

Proper sample preservation and preparation to obtain complete, clear images is a primary concern in palynological SEM studies. Common methods for *Iris* pollen preparation include natural air-drying, critical-point drying, and 2,2-dimethoxypropane (DMP) direct methods. Natural air-drying involves direct sputter-coating of dry pollen, offering short processing time and low cost but suitable only for pollen with rigid exines; softer exines suffer severe shrinkage and deformation (Zhang et al., 2016). Most palynological studies of *Iris* have employed this method (Yang, 2002; Yu et al., 2010; Ma et al., 2017; Georgescu and Dobrin, 2023), but failed to obtain ideal images. Our study found that *Iris* pollen has relatively thin exines; pollen of *I. confusa* and *I. hookeri* prepared by natural air-drying showed severe shrinkage and deformation, invaginated germination regions, boat-shaped morphology, and unclear exine ornamentation.

The critical-point drying method involves chemical fixation, dehydration, and critical-point drying, which may cause exine shrinkage but generally preserves pollen shape, size, and germination regions well (Halbritter, 1998). Goldblatt and Thomas (1992) and Choi (2022) used critical-point drying for *Iris* pollen preparation with satisfactory results. Our study also employed this method; although some species showed exine shrinkage, important details including pollen shape, size, germination regions, and exine ornamentation were well preserved. Stored dry pollen of *I. confusa* and *I. hookeri* that was first fixed with glutaraldehyde and then critical-point dried could maintain a basically spheroidal shape. While most grains showed exine wrinkling, germination regions were not invaginated, and some pollen maintained normal morphology with clear exine ornamentation, meeting research requirements. This provides a new method for preparing stored dry pollen of *Iris*.

The DMP direct method is similar to critical-point drying but involves direct immersion of fresh samples in acidified DMP solution to accomplish fixation and dehydration simultaneously, followed by acetone treatment and critical-point drying. This method preserves important details of hydrated pollen grains well, avoiding shrinkage, deformation, or dissolution, and yields clean, clear images (Halbritter, 1998). The DMP method is widely used internationally; Mitić et al. (2013) employed it for palynological studies of pollen from Croatia and surrounding regions, obtaining clear, clean images. Based on previous studies and our results, we conclude that natural air-drying is unsuitable for *Iris* pollen preparation, while either critical-point drying or the DMP direct method is appropriate.

In conclusion, the pollen morphology of the 31 *Iris* taxa studied exhibits both consistency and variation, with palynological characteristics serving as supplementary criteria for delimiting subgenera, sections, and series, and providing palynological evidence for investigating phylogenetic relationships and systematic evolution among subgenera, sections, series, and species. Critical-point drying or the DMP direct method is suitable for preparing *Iris* pollen samples.

References

Boltenkov E, Artyukova E, Kozyrenko M, et al., 2020. *Iris sanguinea* is conspecific with *I. sibirica* (Iridaceae) according to morphology and plastid DNA sequence data [J]. *PeerJ*, 8: e10088.

Cheng L, Feng SC, Xiao YE, et al., 2021. Advances in molecular phylogeny of Iris [J]. *Guihaia*, 41(1): 31-39.

Choi B, Ryu J, Jang TS, 2022. Can pollen exine ornamentation contribute to species delimitation in Korean *Iris* L. taxa (Iridaceae)? [J]. *Palynology*, 46(4): 1-9.

Colasantem M, Difford M, Vosa CG, 1989. Scanning electron microscopy of some critical bearded Irises pollen: preliminary observations [J]. *Webbia*, 43(2): 339-350.

Dajoz I, Till-Bottraud I, Gouyon PH, 1991. Evolution of pollen morphology [J]. *Science*, 253(5015): 66-68.

Dönmez EO, Pinar M, 2001. The clypeate pollen grains of Turkish *Iris* L. (Iridaceae): subgenus *Scorpiris* [J]. *Turkish Journal of Botany*, 25(2): 57-62.

Dykes WR, 1913. *The Genus Iris* [M]. New York: Dover Publications: 124-178.

Feng YM, 2016. Molecular phylogenetics of some *Iris* species in China based on ITS and *matK* markers [D]. Sichuan: Sichuan Agricultural University: 44.

Furness CA, Rudall PJ, 2004. Pollen aperture evolution—a crucial factor for eudicot success? [J]. *Trends in Plant Science*, 9(3): 154-158.

Georgescu MI, Dobrin A, 2023. The pollen morphology of different *Iris* L. species from Romania [J]. *Scientific Papers. Series B. Horticulture*, 67(1): 376-381.

Goldblatt P, Mabberley DJ, 2005. *Belamcanda* included in *Iris*, and the new combination *I. domestica* (Iridaceae: Irideae) [J]. *Novon*, 15(1): 128-132.

Goldblatt P, Thomas AL, 1992. Pollen apertures, exine sculpturing and phylogeny in Iridaceae subfamily Iridoideae [J]. *Review of Palaeobotany and Palynology*, 75(3-4): 301-315.

Guo JY, Wilson CA, 2013. Molecular phylogeny of crested Iris based on five plastid markers (Iridaceae) [J]. *Systematic Botany*, 38(4): 987-995.

Halbritter H, 1998. Preparing living pollen material for scanning electron microscopy using 2,2-dimethoxypropane (DMP) and critical-point drying [J]. *Biotechnic & Histochemistry*, 73(3): 137-143.

Halbritter H, Buchner R, 2016. *Iris hookeriana.* In: PalDat—A palynological database [EB/OL]. https://www.paldat.org/pub/Iris_{hookeriana}/302857; accessed 2024-07-22.

Halbritter H, Auer W, Svojtka M, 2020. *Iris pseudacorus.* In: PalDat—A palynological database [EB/OL]. https://www.paldat.org/pub/Iris_{pseudacorus}/304784; accessed 2024-07-25.

Halbritter H, Silvia U, Friogeir G, et al., 2021. *Illustrated Pollen Terminology* [M]. 2nd ed. Yao YF, Li JF, Xie G, et al., trans. Wuhan: Hubei Science and Technology Press: 8-426.

Kang YJ, Kim S, Lee J, et al., 2020. Identification of plastid genomic regions inferring species identity from de novo plastid genome assembly of 14 Korean-native *Iris* species (Iridaceae) [J]. *PLoS ONE*, 15(10): e0241178.

Lawrence GHM, 1953. A reclassification of the genus *Iris* [J]. *Gentes Herb*, 8: 346-371.

Li TQ, Cao HJ, Kang MS, et al., 2011. *Pollen Flora of China Woody Plants by SEM* [M]. Beijing: Science Press: 4-11.

Mathew B, 1989. *The Iris* [M]. London: Batsford: 89-156.

Ma YM, Zhang Y, Qin JY, et al., 2017. Pollen morphology, viability and stigma receptivity of *Iris scariosa* Willd [J]. *Xinjiang Agricultural Sciences*, 54(1): 110-116.

Mitić B, Halbritter H, Šostarić R, et al., 2013. Pollen morphology of the genus *Iris* L. (Iridaceae) from Croatia and surrounding area: taxonomic and phylogenetic implications [J]. *Plant Systematics and Evolution*, 299(1): 271-288.

Mu SH, Peng ZH, Qie GF, et al., 2011. Genetic diversity of *Iris* determined by AFLP markers [J]. *Journal of Northeast Forestry University*, 39(1): 124-126.

Pinar NM, Dönmez EO, 2000. Pollen morphology of Turkish *Iris* L. (Iridaceae) with reference to evolutionary trends at the infrageneric level [J]. *Israel Journal of Plant Sciences*, 48(2): 129–141.

Qi YB, Zhao YT, 1987. Studies on the pollen morphology of the genus *Iris* in China [J]. *Acta Phytotaxonomica Sinica*, 25(6): 430-436.

Qin MJ, Xu LS, Toshihiro T, et al., 2000. A preliminary study on the distribution pattern of isoflavones in rhizomes of *Iris* from China and its systematic significance [J]. *Acta Phytotaxonomica Sinica*, 38(4): 343-349.

Rodionenko GI, 1987. *The Genus Iris* (English translation) [M]. London: The British Iris Society: 132-143.

Rudall P, Wheeler A, 1988. Pollen morphology in Tigridieae (Iridaceae) [J]. *Kew Bulletin*, 43(4): 693-701.

Ulrich S, Hesse M, Bröderbauer D, et al., 2013. *Calla palustris* (Araceae): New palynological insights with special regard to its controversial systematic position and to closely related genera [J]. *Taxon*, 62(4): 701-712.

Wang KF, Wang XZ, 1983. *Palynology Introduction* [M]. Beijing: Peking University Press: 30-34.

Wang L, Zhuo LH, 2006. Systematic classification of some species of *Iris* based on ITS sequences [J]. *Journal of Northeast Forestry University*, 34(4): 54-56+75.

Wilson CA, 2004. Phylogeny of *Iris* based on chloroplast *matK* gene and *trnK* intron sequence data [J]. *Molecular Phylogenetics and Evolution*, 33(2): 402-412.

Wilson CA, 2006. Patterns of evolution in characters that define *Iris* subgenera and sections [J]. *Aliso*, 22(1): 425-433.

Wilson CA, 2009. Phylogenetic relationships among the recognized series in *Iris* section *Limniris* [J]. *Systematic Botany*, 34(2): 277-284.

Wilson CA, 2011. Subgeneric classification in *Iris* re-examined using chloroplast sequence data [J]. *Taxon*, 60(1): 27-35.

Wu HX, Liu LT, Liao BY, et al., 2012. Pollen morphology and resulting taxonomic implications of 20 *Eucalyptus* species and progenies [J]. *Journal of Central South University of Forestry Technology*, 32(3): 29-36.

Yang RL, 2002. Examination on pollen morphology of *Belamcanda chinensis* (L.) DC. and *Iris lactea* var. *chinensis* Koidz [J]. *Guihaia*, 22(3): 237-238+290.

Yu XF, Zhang HQ, He XM, et al., 2010. Pollen morphology of 12 species of *Iris* L. and its systematic significations [J]. *Acta Horticulturae Sinica*, 37(7): 1175-1182.

Zhang GY, Zhang WT, Yao JN, et al., 2016. Characterization of several *Pinus* species pollens via different treatments by SEM [J]. *Journal of Chinese Electron Microscopy Society*, 35(1): 49-52.

Zhao YT, 1985. *Flora Reipublicae Popularis Sinicae*, Vol. 16(1) [M]. Beijing: Science Press: 133-197.

Zhao YT, Noltie HJ, Mathew B, 2000. Iridaceae [M]// Wu ZY, Raven PH. *Flora of China*. Beijing: Science Press; St. Louis: Missouri Botanical Garden Press: 24: 297-313.

Zhong Y, 2010. The system classification research based on multi-genome of some *Iris* species [D]. Heilongjiang: Northeast Forestry University: 27.

Note: Figure translations are in progress. See original paper for figures.