Pollen morphology of 19 species of Aristolochiaceae and its taxonomic significance

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Abstract

The delimitation of genera in Aristolochiaceae has long been a core issue in the taxonomic study of this family, as macromorphological characters are readily affected by convergent evolution, making it essential to incorporate stable micromorphological traits. To clarify the taxonomic value of pollen morphology, this study examined 11 species and 1 subspecies of Isotrema and 7 species of Aristolochia in Aristolochiaceae. Using an improved DMP dehydration preparation method, high-resolution scanning electron microscopy, and multivariate statistical analyses, key characters including pollen size, morphological outline, and exine ornamentation were systematically quantified, and their taxonomic significance was revealed through principal component analysis (PCA) and cluster analysis (UPGMA). The results showed that: (1) pollen grains of the studied taxa were all monads and subspheroidal (P/E=1.02~1.13), exhibiting clear family-level morphological conservatism. (2) Pollen morphology differed significantly between the two genera. Isotrema mostly had small to medium-sized pollen, with polar axis length of 23.21–28.17 μm and exine ornamentation dominated by fine reticulate patterns; Aristolochia mainly had medium to large-sized pollen, with polar axis length of 25.44–46.14 μm and mostly coarse reticulate or cerebriform ornamentation. Pollen size and exine ornamentation type can serve as important diagnostic criteria for distinguishing the two genera. (3) Quantitative parameters such as lumina diameter of 0.17–0.40 μm, muri width of 0.48–2.89 μm, and the polar-equatorial ratio can effectively distinguish closely related species; among them, the specialized germinal furrow structure of Aristolochia bracteolata and the unique cerebriform ornamentation of Aristolochia gigantea are distinctive diagnostic characters at the species level. (4) The results of principal component and cluster analyses were consistent, supporting the independent differentiation of Isotrema and Aristolochia. The established three-level quantitative discrimination system based on pollen size, shape, and ornamentation enables precise delimitation of problematic closely related species. This study improves and supplements the basic palynological data for Aristolochiaceae, optimizes an electron microscopy preparation method suitable for thin-walled and delicate pollen, and provides reliable palynological evidence for taxonomic revision, species delimitation, and conservation of medicinal wild resources in this family. It also provides data supporting the shift of angiosperm palynological research from traditional qualitative description toward quantitative identification.

Full Text

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Pollen morphology of 19 species of Aristolochiaceae and its taxonomic significance

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Abstract: Persistent disputes regarding the circumscription of genera within Aristolochiaceae have long constituted a core problem impeding taxonomic research on this family. Macroscopic morphological traits are susceptible to interference from convergent evolution, and stable micromorphological characters urgently need to be supplemented. To clarify the taxonomic value of pollen morphology, this study selected 11 species and 1 subspecies of the related genera and 7 species of *Aristolochia* as study objects. Using an improved DMP dehydration preparation method, high-precision scanning electron microscopy, and multivariate statistical analysis, we systematically quantified key traits such as pollen size, morphological outline, and exine ornamentation, and revealed their taxonomic significance through principal component analysis (PCA) and cluster analysis (UPGMA). The results showed that: (1) The pollen of the study taxa was single-grained and subspheroidal (P/E=1.02–1.13), showing a distinct family-level morphological conservation. (2) The pollen morphology of the two genera differed significantly: the related genera mostly had medium- to small-sized pollen, with polar-axis length 23.21–28.17 μm and an exine dominated by fine reticulate ornamentation; *Aristolochia* mainly had medium- to large-sized pollen, with polar-axis length 25.44–46.14 μm, mostly with coarse reticulate or cerebriform ornamentation. Pollen volume and exine ornamentation type can serve as important diagnostic evidence for distinguishing the two genera. (3) Quantitative parameters such as lumina diameter 0.17–0.40 μm, muri width 0.48–2.89 μm, and polar-equatorial ratio can effectively distinguish closely related species. Among them, the specialized budding-groove structure of *A. petelotii* and the unique cerebriform ornamentation of *A. gigantea* are both species-specific diagnostic traits. (4) The results of principal component analysis were consistent with those of cluster analysis, supporting the independent separation of the related genera and *Aristolochia*. The constructed three-level quantitative identification system of pollen size–shape–ornamentation can achieve precise delimitation of difficult closely related species. This study improves and supplements the basic palynological data of Aristolochiaceae, optimizes and adapts an electron-microscopy preparation method for thin-walled and delicate pollen, provides reliable palynological evidence for systematic revision, species delimi-

tation, and conservation breeding of medicinal wild resources in this family, and also provides data for the study of angiosperm pollen from traditional qualitative description toward quantitative identification.

Keywords: Aristolochiaceae; pollen morphology; taxonomic identification; DMP preparation; systematic evolution

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Abstract: Persistent disputes regarding generic circumscriptions within Aristolochiaceae have long hindered taxonomic research of this family, as macroscopic morphological traits are prone to convergent evolution. This highlights an urgent need for stable micromorphological diagnostic

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evidence. To clarify the taxonomic significance of pollen morphology, we sampled 11 species and one subspecies of *Isotrema*, alongside seven species of *Aristolochia* for this study. We employed a modified DMP dehydration preparation protocol coupled with high-resolution scanning electron microscopy (SEM) and multivariate statistical analysis to quantitatively characterize key pollen traits, including pollen size, outline shape and exine ornamentation. We further performed principal component analysis (PCA) and unweighted pair-group

method with arithmetic averaging (UPGMA) clustering to reveal the taxonomic implications of pollen micromorphology. The results were as follows: (1) All pollen grains from the investigated taxa are monads and subspherical, with polar/equatorial axis ratios (P/E) of 1.02–1.13, exhibiting pronounced family-level morphological conservatism. (2) Pollen morphology differed distinctly between the two genera. *Isotrema* generally produces small to medium-sized pollen grains with polar axis of 23.21–28.17 μm, whose exine is predominantly finely reticulate. In contrast, *Aristolochia* bears medium to large pollen grains with polar axis length of 25.44–46.14 μm, characterized by coarsely reticulate or cerebroid exine ornamentation. Pollen size and exine ornamentation type constitute critical diagnostic traits for distinguishing these two genera. (3) Quantitative metrics such as lumina diameter (0.17–0.40 μm), muri width (0.48–2.89 μm) and P/E ratio enable reliable discrimination of closely related congeneric species. The distinctive germinal aperture structure of *Aristolochia bracteolata* and the unique cerebroid exine ornamentation of *A. gigantea* serve as exclusive species-specific diagnostic traits. (4) PCA and cluster analyses yielded congruent results, supporting the independent evolutionary divergence of *Isotrema* and *Aristolochia*. The established three-tier quantitative discrimination framework based on pollen size, shape and exine ornamentation enables precise delimitation of taxonomically ambiguous closely related taxa. This study enriches the fundamental palynological dataset for Aristolochiaceae, and optimizes SEM preparation protocols tailored to thin-walled, fragile pollen grains. It provides reliable palynological evidence for taxonomic revision, species boundary delimitation and conservation management of wild medicinal Aristolochiaceae resources. Moreover, this research provides baseline data to advance angiosperm palynology from traditional qualitative morphological documentation toward quantitative taxonomic identification.

Key words: Aristolochiaceae, pollen morphology, taxonomic delimitation, DMP dehydration preparation, phylogenetic evolution

Aristolochiaceae is a group of angiosperms with important medicinal and ecological value. It comprises more than 770 species worldwide (The Royal Botanical Gardens, Kew, 2026), distributed mainly in tropical and subtropical regions. In the traditional classification system of angiosperms, this family has been placed within the paleoherb group, and its systematic position and intergeneric delimitation have long been focal issues in taxonomic research (Hutchinson, 1959). China is one of the modern centers of distribution for this family (González & Stevenson, 2002), with five genera and more than 150 species (http://sp2000.org.cn/). However, its taxonomic study has long been hindered by disputes over generic delimitation, especially the highly controversial separation between *Aristolochia* and *Isotrema* (Nakonechnaya & Kalachev, 2018; Zhu Xinxin et al., 2019; Zhu et al., 2019; The Royal Botanical Gardens, Kew, 2026). Macromorphological classification, such as that based on perianth morphology and floral structure, is susceptible to convergent evolution and intraspecific variation, resulting in unstable inference of phylogenetic relationships; thus, there is an urgent need to introduce genetically more stable micromorphologi-

cal characters as supplements (Endress & Matthews, 2012; Zhang Shuo et al., 2014). Pollen morphology, as a highly genetically stable micromorphological character, can provide key clues when macromorphology is ambiguous (Walker & Skvarla, 1975). In recent years, some progress has been made in studies of pollen morphology in Aristolochiaceae. For example, Polevova (2019) observed the development of the pollen outer wall in *Aristolochia* and revealed a potential association between developmental timing and ornamentation formation. However, existing studies have mostly focused on developmental descriptions of a small number of species and lack systematic quantitative analyses of interspecific variation, making it difficult for them to directly serve taxonomic practice. Existing studies have shown that pollen grains of Aristolochiaceae generally

They are oblate-spheroidal, nearly spherical, and lack germination pores; the exine ornamentation (e.g., granulate, reticulate) shows marked differentiation at both the genus and species levels (Mi Qiushuang and Yang Chunshu, 1991; Perveen & Qaiser, 2008). Further studies have found that the spheroidal morphology of pollen in this family is also stable and is highly consistent with exine-ornamentation characteristics, and thus can serve as supplementary taxonomic evidence (Oak et al., 2022). Scanning electron microscopy (SEM), with its high-resolution advantages, can directly reveal the fine ornamentation structure of the pollen exine and has been widely applied in taxonomic studies of groups such as *Isotrema*, *Hordeum*, and *Acmella* (Gu Yanan et al., 2025; Hu Meng et al., 2025; Zhu Ying et al., 2025), indicating that pollen morphological characteristics are important evidence for interpreting close and distant phylogenetic relationships and reconstructing the history of systematic evolution (Zhang Yuxia and Chen Qingfu, 2002; Zhao Yixuan et al., 2021; Hayat et al., 2023; Wan Xianqin et al., 2025). In addition, at the level of morphological development, Li Jun and Ren Yi (2008) observed the floral morphogenetic process of *Aristolochia contorta* Bunge by SEM, revealing the origin of the synstemonous lobes and the mechanism of formation of the central axial placenta, thereby providing microscopic evidence for understanding the evolution of floral structure in Aristolochiaceae.

Although palynological methods are widely applied in plant systematics, palynological studies of Aristolochiaceae still have three core shortcomings: first, sampling coverage is insufficient. Existing reports mostly focus on widely distributed species or groups in particular geographical regions, and systematic coverage of the diversity of domestic Aristolochiaceae species is inadequate (Li Jun, 2008); second, preparation methods need optimization. Traditional methods (such as natural air-drying and ethanol fixation) readily cause high-water-content, thin-walled pollen to shrink and deform, resulting in distortion of exine ornamentation (Nakonechnaya & Kalachev, 2018; Ermolaev et al., 2024). Although the 2,2-dimethoxypropane (DMP) chemical dehydration method has shown advantages in preparing delicate pollen of families such as Orchidaceae and Fabaceae (Halbritter, 1998; Zhao Ying, 2021; Subramanyan et al., 2023), a standardized application procedure has not yet been developed for Aristolochiaceae; third, analytical methods are rather uniform. Most studies remain at the

stage of qualitative description and lack the integration of quantitative indices with multivariate statistical analyses, making it difficult to objectively reveal patterns of morphological differentiation and phylogenetic relationships among groups.

In view of the above research gaps, this study selected 19 species of Aristolochiaceae (11 species and 1 subspecies of *Isotrema*, and 7 species of *Aristolochia*) as the study subjects, and constructed an integrated research framework of "optimized DMP preparation–high-precision SEM observation–multivariate statistical analysis." By systematically quantifying core traits such as pollen size, shape, exine ornamentation, and germination pores, and combining principal component analysis (PCA) with hierarchical clustering analysis (UPGMA), this study clarifies the taxonomic value and systematic evolutionary patterns of pollen morphological characteristics, and verifies their effectiveness in delimiting genera and distinguishing species in Aristolochiaceae (Do, 2016). The study aims to provide key palynological evidence for revision of the classification system, species delimitation, and reconstruction of phylogenetic relationships in this family, to supplement the basic palynological data of domestic Aristolochiaceae, and to improve the global palynological database.

1 Materials and Methods

1.1 Experimental materials

The test materials were collected from introduced cultivated or wild populations in the liana garden of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (101°16′E, 21°55′N, elevation 570 m), and from regions such as Baoshan, Yunnan. Mature pollen at full flowering was collected from February 2022 to March 2025 (Table 1). For each species, 3–5 healthy, pest- and disease-free plants were selected; newly opened flowers were collected and immediately brought back to the laboratory for preparation. The taxonomic names of the study materials follow the taxonomic treatments of Zhu et al. (2019) and Zhu Xinxin et al. (2019).

Table 1 List of Aristolochiaceae materials studied

No.	Chinese name	Scientific name	Accession number	Collection number	Collection date
1	Diannan guanmutong	*Isotrema austroyunnanense*	00,2000,0677	zr21	2022-09-23
2	Cangyuan guanmutong	*I. cangyuanense*	00,2021,0067	zr50	2024-10-16

No.	Chinese name	Scientific name	Accession number	Collection number	Collection date
3	Qiaojing guanmutong	*I. caulialatum*	00,2009,0263	zr23	2022-02-26
4	Huangmao guanmutong	*I. fulvicomum*	00,2019,0665	zr48	2024-08-28
5	Hainan guanmutong	*I. hainanense*	10,2008,0028	zr25	2022-02-26

No.	Chinese name	Scientific name	Accession/collection No.	Code	Date
6	Yingjiang pipevine	*I. hainanense* subsp. *yingjiangense*	00,2019,1220	zr55	2025-03-03
7	Nanyue pipevine	*I. howii*	00,2013,0428	zr46	2024-08-13
8	Pei pipevine	*I. petelotii*	00,2019,1069	zr49	2024-10-08
9	Sanya pipevine	*I. sanyaense*	00,2019,0679	zr17	2024-10-30
10	Tongbiguan pipevine	*I. tongbiguanense*	00,2017,0592	zr41	2024-08-05
11	Variegated pipevine	*I. versicolor*	00,2020,0049	zr22	2022-02-26
12	Yang' s pipevine	*I. yangii*	No	zr20	2022-09-23
13	Prismatic Aristolochia	*Aristolochia anguicida*	00,2020,0637	zr15	2024-10-22
14	Bracteate Aristolochia	*A. chlamydophylla*	00,2019,0696	zr24	2022-02-26
15	Flowing-sutured Aristolochia	*A. galeata*	29,2018,0032	zr42	2024-08-05

No.	Chinese name	Scientific name	Accession/collection No.	Code	Date
16	Gilbert' s Aristolochia	*A. gilbertii*	00,2018,1856	zr45	2024-08-05
17	Giant Aristolochia	*A. gigantea*	52,2010,0008	zr47	2024-08-13
18	Beautiful Aristolochia	*A. littoralis*	2020030013	zr44	2024-08-05
19	Pelican flower	*A. ringens*	04,2014,0016	zr32	2022-03-20

Note: Except for *Isotrema yangii*, which was collected from the wild population in Baoshan, Yunnan, all other materials were collected from cultivated individuals introduced and grown at XTBG.

1.2 Experimental Methods

1.2.1 Preparation of Pollen Samples

Pollen was prepared using an optimized DMP dehydration–critical-point drying method. The specific procedure was as follows. Pollen collection: under a dissecting microscope, mature anthers were cut open with a sterile dissecting needle, and pollen was collected into 2 mL centrifuge tubes; chemical dehydration: 1 mL of freshly prepared acidified DMP working solution [10 mL DMP (Macklin, purity ( )99%, water content <0.1%) + 50 μL concentrated hydrochloric acid] was added, and the samples were soaked at room temperature for 4 h, during which they were inverted and mixed 3 times to ensure that the pollen fully contacted the dehydration solution; rinsing and purification: 1 mL anhydrous ethanol was added for rinsing twice, 20 min each time, to remove residual reaction products and acid catalyst; critical-point drying: the pollen samples were wrapped in filter paper and transferred to a Leica EM CPD 300 critical-point dryer, with liquid CO_2 as the transition medium. The parameters were set as follows: CO_2 injection rate fast, delay 400 s, exchange rate 5, 18 cycles, exhaust heating slow, speed slow, to avoid damage to pollen structure. Compared with traditional methods, this optimized procedure can significantly reduce the shrinkage and deformation rate of pollen with high water content and effectively preserve the original fine structure of the outer-wall ornamentation.

1.2.2 Scanning Electron Microscopy Observation and Morphological Measurement

(1) Sample mounting: dried pollen was evenly distributed on an aluminum specimen stub affixed with 8 mm × 8 mm double-sided conductive carbon tape; (2) conductive coating: multi-angle oblique sputter coating was performed using a Q150R S ion sputter coater, with parameters set to a sputtering current of 15 mA, sputtering time of 300 s, and coating thickness controlled at 8–10 nm; (3) imaging optimization: observations were carried out with a Zeiss EVO LS10 scanning electron microscope, with accelerating voltage set to 8 kV and working distance 12–15 mm. Images of the polar view, equatorial view, and ornamentation details of each sample were systematically taken, with magnification ranging from 500× to 10 000×; (4) morphological measurement: for each species, 10 intact pollen grains were randomly selected to measure indices including polar-axis length (P), equatorial-axis length (E), lumina diameter, and murus width; the polar/equatorial ratio (P/E) and pollen volume (P×E) were calculated, and results were expressed as "mean ± standard deviation."

1.2.3 Criteria for Defining Morphological Characters

Pollen shape followed the standards of Wang Fuxiong et al. (1995): P/E values of 0.88–1.14 were defined as subspheroidal; pollen size classes were based on polar-axis length: 10–25 μm as small and 25–50 μm as medium (Erdtman, 1978); exine ornamentation types were classified, in combination with SEM observations and palynological terminology (Wang Kaifa and Wang Xian, 1983), into four types: granulate, finely reticulate, coarsely reticulate, and rugulate; the surface morphology of the muri was divided into smooth and undulate types.

1.3 Data Analysis

Microsoft Excel 2010 was used for statistical analysis of pollen morphological data, Adobe Photoshop 2024 for image processing, and SPSS 27.0 software for significance-difference analysis and principal component analysis of indicators such as pollen polar-axis length, equatorial-axis length, and muri width. Using Euclidean distance as the measure, systematic cluster analysis was performed with the between-groups linkage method.

2 Results and Analysis

2.1 Pollen Morphological Characteristics

2.1.1 Pollen Size and Shape The pollen of the 19 species of Meliaceae plants all occurs as single grains, and no aggregated pollen was observed; this is consistent with the typical characteristics of pollen in Meliaceae (Nakonechnaya & Kalachev, 2018). The polar/equatorial ratio (P/E) of the pollen of all species ranged from 1.02 to 1.13. According to the pollen-shape classification standard

of Wang Fuxiong et al. (1995), all were classified as subspheroidal. Both polar view and equatorial view were nearly circular, indicating a high degree of evolutionary conservatism in pollen shape in this family (Table 2). In terms of intergeneric differences, *Chukrasia* and *Aglaia* showed clear differentiation in pollen size, and interspecific differences were significant ($P<0.05$): species of *Chukrasia* were generally dominated by small- to medium-sized pollen, with polar-axis length 23.21–28.17 μm, equatorial-axis length 21.96–26.94 μm, and pollen size (P × E) 509.63–759.54 μm^2; overall, pollen volume was relatively small and the values were concentrated within a narrow range. Species of *Aglaia* were mainly characterized by medium-sized to relatively large pollen, with polar-axis length 25.44–46.14 μm, equatorial-axis length 24.44–43.76 μm, and pollen size (P × E) 622.83–2 026.67 μm^2; the numerical span was much greater than that of *Chukrasia*, and the pollen grains were significantly larger overall. Among them, the pollen size of *Aglaia odorata* reached 2 026.67 μm^2, making it the species with the largest pollen among all groups and forming a diagnostic feature.

The pollen shape of both *Chukrasia* and *Aglaia* was nearly spheroidal, but the P/E value showed a certain degree of differentiation both between and within genera. In *Chukrasia*, *Chukrasia tabularis* had the highest P/E value (1.13 ± 0.05), being slightly prolate-spheroidal and the group with the most elongated shape among all species; the remaining *Chukrasia* species had P/E values of 1.03–1.08 and were overall close to spheroidal. In *Aglaia*, *Aglaia spectabilis* (1.02 ± 0.03) and *Aglaia lawii* (1.03 ± 0.02) had the lowest P/E values and were closest to spheroidal; the remaining *Aglaia* species had P/E values of 1.04–1.06 and were likewise generally subspheroidal. In terms of the degree of variation, P/E within *Chukrasia* was more stable: *Chukrasia tabularis* had the largest coefficient of variation in P/E (5.66%), whereas most groups such as *Chukrasia velutina* and *Chukrasia tabularis* var. *velutina* had coefficients of variation <2%, showing a significantly high consistency in pollen shape. Although *Aglaia* as a whole was also nearly spheroidal, the P/E range among different species was wider (1.02–1.06), and differentiation was more evident, reflecting greater diversity in the evolution of pollen shape than in *Chukrasia*. Correlation analysis showed that polar-axis length and equatorial-axis length were extremely significantly positively correlated ($r=0.986$, $P<0.001$), indicating that the polar and equatorial axes of pollen in the two genera elongate synchronously during development and that their growth patterns are highly coordinated. In summary, the family-level conservatism of pollen shape and the genus-level differentiation of size form a clear contrast; pollen size can serve as a core indicator for distinguishing the two genera.

2.1.2 Ornamentation of the Pollen Exine Through high-resolution SEM observation, the exine ornamentation of the pollen of the 19 plant species was divided into four types (granulate, microreticulate, coarsely reticulate, and cerebriform), and the ornamentation types showed clear intergeneric differentiation patterns (Table 3, Fig. 1–Fig. 3). Microreticulate ornamentation was the dominant ornamentation type in *Chukrasia* (11 species), with lumina diameter 0.17–

0.40 μm and muri width 0.48–1.72 μm. The lumina were nearly circular and arranged uniformly and densely, constituting the typical diagnostic feature of the pollen exine in *Chukrasia*. Only *Aglaia odorata* was an exceptional case within *Aglaia* with microreticulate ornamentation, forming clear differentiation from other species of the same genus. Within *Chukrasia*, the micromorphology of the ornamentation was clearly differentiated: *Chukrasia tabularis* had the largest lumina diameter, at (0.40 ± 0.05) μm, while *Chukrasia velutina* had the smallest lumina diameter, at (0.17 ± 0.03) μm; the muri width of *Chukrasia assamica* reached (1.72 ± 0.15) μm, the largest muri value in the genus. These fine-scale features can serve as evidence for identifying closely related species within the genus. Coarsely reticulate and cerebriform ornamentation were mainly concentrated in *Aglaia*: coarsely reticulate ornamentation (4 species) had muri width of 1.64–2.89 μm, significantly coarser than the microreticulate type in *Chukrasia*, among which the muri width of *Aglaia lawii* was (2.89 ± 0.69) μm, the largest among all species. Cerebriform ornamentation was unique to *Aglaia grandis*, and this ornamentation type is reported here for the first time within the genus. Its features are irregular lumina and cerebriform wrinkles, with wrinkle width of (1.23 ± 0.29) μm; the ornamentation paths are mutually connected, resembling cortical sulci, making it a unique pollen-classification feature of *Aglaia*. Granulate ornamentation was sporadically distributed in both genera and occurred only in two species, *Aglaia elaeagnoidea* and *Chukrasia tabularis*; the exine was dominated by granular protrusions and had no distinct lumina, representing a relatively specialized ornamentation type in the two genera.

The overall intergeneric ornamentation differences can be summarized as follows: *Isotrema* is dominated by a uniformly stable fine reticulate ornamentation, with medium-sized lumina and uniformly small muri; *Aristolochia* has more diverse ornamentation types, a higher proportion of coarse reticulate and cerebriform patterns, larger muri spans (0.48–2.89) μm, and a more complex ornamentation arrangement. Analysis of 16 reticulate-ornamented species showed that lumen diameter was not significantly correlated with murus width ($r = 0.234$, $P > 0.05$), indicating that pollen lumen size and murus width in the two genera are independently genetically regulated. In addition, the surface morphology of the muri also showed intergeneric differentiation: *Isotrema* mostly had smooth or finely undulate muri, whereas the coarse reticulate group of *Aristolochia* mostly had broadly undulate muri, further strengthening the differentiation characteristics of the micro-morphology of the outer wall between the genera.

2.1.3 Aperture characteristics

Aperture characteristics likewise exhibited intergeneric differentiation: clear apertures were observed only in *Aristolochia debilis* of the genus *Aristolochia*, located at the poles, with an aperture length of (28.6 ± 3.2) μm and an aperture width of (4.2 ± 0.5) μm, and with a distinct aperture margin; the remaining 18 species, including all *Isotrema* species and most *Aristolochia* species, had no clearly identifiable apertures. Only a few *Aristolochia* species, such as *Aris-*

tolochia elegans and *A. manshuriensis*, had weak thin-walled zones. This result indicates that aperture traits are highly conservative in *Isotrema*, whereas specialized types occur in *Aristolochia*, reflecting the diversity of evolutionary patterns in the pollen structure of the latter. This is consistent with the overall characteristic in Aristolochiaceae that most pollen grains have no apertures or inconspicuous apertures (Nakonechnaya & Kalachev, 2018), while also supplementing detailed differences in aperture traits between the two genera.

Table 2 Pollen size and shape of 19 Aristolochiaceae species

Table 2 Pollen size and shape of 19 Aristolochiaceae species

No.	Plant material	P (μm) Polar axis	E (μm) Equatorial axis	P/E	Pollen shape	Pollen size	P × E (μm²)
1	Diannan *Isotrema* *Isotrema austroyunnanense*	25.40± 2.16def (22.99~28.22)	23.93± 1.33efgh (22.86~26.17)	1.06 ± 0.05abc	Subspheroidal	Medium	609.66± 86.54efg (525.55~740.35)
2	Cangyuan *Isotrema* *I. cangyuanense*	26.15± 1.33def (24.64~28.23)	25.09± 1.52cde (23.47~26.95)	1.04 ± 0.04bc	Subspheroidal	Medium	657.59± 67.21efg (593.58~760.26)
3	Caudate *Isotrema* *I. caudilatum*	28.08± 1.63bcd (26.04~29.48)	26.80± 1.07bc (25.52~27.67)	1.05 ± 0.02bc	Subspheroidal	Medium	753.78± 72.44cde (670.53~814.53)
4	Yellow-haired *Isotrema* *I. fulvicomum*	25.96± 0.79def (24.83~26.72)	24.74± 0.63defg (24.17~25.78)	1.05 ± 0.04bc	Subspheroidal	Medium	642.14± 23.29efg (607.09~670.02)
5	Hainan *Isotrema* *I. hainanense*	27.15± 0.41cde (26.75~27.85)	26.43± 0.89bcd (25.27~27.16)	1.03 ± 0.03c	Subspheroidal	Medium	717.79± 32.89def (675.97~755.05)

No.	Plant material	P (μm) Polar axis	E (μm) Equatorial axis	P/E	Pollen shape	Pollen size	P × E (μm^2)
6	Yingjiang *Isotrema* *I. hainanense* subsp. *yingjiangense*	27.01 ± 1.07cde (25.61~28.44)	25.82 ± 1.20cd (24.18~27.17)	1.05 ± 0.02bc	Subspheroidal	Medium	698.44 ± 62.52def (619.25~771.63)
7	Nanyue *Isotrema* *I. howii*	26.61 ± 0.79def (25.30~27.32)	24.86 ± 0.69def (23.19~25.83)	1.07 ± 0.04abc	Subspheroidal	Medium	661.66 ± 30.76efg (620.86~705.68)
8	Petelot's *Isotrema* *I. petelotii*	23.82 ± 0.82fg (22.78~24.91)	22.53 ± 0.69h (21.72~23.28)	1.06 ± 0.03abc	Subspheroidal	Small	537.07 ± 30.39g (494.78~564.31)
9	Sanya *Isotrema* *I. sanyaense*	23.21 ± 0.87fg (21.68~23.75)	21.96 ± 1.04h (20.85~23.14)	1.06 ± 0.06abc	Subspheroidal	Small	509.63 ± 32.28g (465.04~546.01)
10	Tongbiguan *Isotrema* *I. tongbiguanense*	28.16 ± 1.13bcd (26.35~29.29)	26.94 ± 1.23bc (24.99~28.00)	1.05 ± 0.02bc	Subspheroidal	Medium	759.54 ± 61.60cde (658.49~810.75)
11	Variegated *Isotrema* *I. versicolor*	28.17 ± 1.33bcd (27.30~30.46)	24.86 ± 0.75def (23.05~25.62)	1.13 ± 0.05a	Subspheroidal	Medium	700.62 ± 47.18def (658.25~780.39)

No.	Plant material						
12	Yang's pipevine *I. yangii*	24.73 ± 1.17efg (23.43~26.20)	22.79 ± 0.93gh (21.68~23.76)	1.08 ± 0.05ab	Subspheroidal	Small	563.82 ± 40.56fg (507.96~615.96)

No.	Plant material						
13	Snake Aristolochia *Aristolochia anguicida*	25.44± 1.20def (24.37~26.78)	24.44± 1.19efg (23.50~26.45)	1.04 ± 0.03bc	Subspheroid	Medium	622.83± 57.22efg (574.11~705.69)
14	Bracteate Aristolochia *A. chlamydophylla*	46.14± 2.92a (43.94~49.39)	43.76± 3.63a (39.85~48.59)	1.05 ± 0.04bc	Subspheroid	Medium	2026.67± 267.49a (1751.01~2371.19)
15	Sickle Aristolochia *A. galeata*	34.95± 1.28b (33.45~36.30)	33.86± 1.59b (31.57~35.50)	1.03 ± 0.02c	Subspheroid	Medium	1185.05± 95.75b (1056.02~1288.65)
16	Gibert's Aristolochia *A. gibertii*	39.04± 2.81b (34.90~42.20)	37.68± 3.41b (32.31~41.77)	1.04 ± 0.03bc	Subspheroid	Medium	1478.42± 231.61b (1127.62~1762.69)
17	Giant Aristolochia *A. gigantea*	36.88± 1.11b (35.80~38.13)	34.95± 0.45b (34.21~35.40)	1.06 ± 0.03abc	Subspheroid	Medium	1289.14± 48.70b (1233.27~1349.80)
18	Littoral Aristolochia *A. littoralis*	37.58± 3.90b (33.03~41.22)	36.77± 3.89b (32.04~40.83)	1.02 ± 0.03c	Subspheroid	Medium	1393.30± 285.01b (1093.29~1683.01)
19	Pipevine flower *A. ringens*	31.23± 0.81b (29.90~32.09)	29.61± 1.10b (28.43~31.10)	1.06 ± 0.04abc	Subspheroid	Medium	924.97± 48.61bcd (874.28~978.72)

Note: Different lowercase letters within the same column indicate significant differences ($P<0.05$). Values in parentheses are minimum to maximum.

Table 3 Pollen morphological characteristics of 19 Aristolochiaceae species

No.	Plant material	Colpi	Lumen	Lumen shape	Lumen size	Murus	Murus width (μm)	Lumen diameter (μm)	Lumen width (μm)	Exine ornamentation
1	Diannan pipevine *Isotrema austroyunnanense*	Not observed	–	–	–	Smooth, wide	0.84± 0.15gh (0.68~1.04)	–	–	Granulate
2	Cangyuan pipevine *I. cangyuanense*	Not observed	Present	Subround	Smaller	Wavy, wide	0.84± 0.19gh (0.62~1.08)	0.35± 0.07ab (0.25~0.43)	0.22± 0.05bc (0.14~0.28)	Microreticulate
3	Wingstem pipevine *I. caulialatum*	Not observed	Present	Subround	Smaller	Wavy, narrow	0.90± 0.33fgh (0.40~1.52)	0.30± 0.04bc (0.27~0.39)	0.20± 0.04cd (0.15~0.24)	Microreticulate
4	Yellow-hairy pipevine *I. fulvicomum*	Not observed	Present	Subround	Smaller	Wavy, narrow	0.83± 0.18gh (0.62~1.06)	0.40± 0.05a (0.32~0.44)	0.37± 0.03a (0.32~0.41)	Microreticulate

No.	Taxon	Colpi	Annulus	Shape	Size	Aperture margin	Value 1	Value 2	Value 3	Ornamentation
5	Hainan Aristolochia *I. hainanense*	Not observed	Present	Subround	Smaller	Wavy, narrow	0.72 ± 0.15hi (0.64~0.99)	0.27 ± 0.02cde (0.23~0.28)	0.21 ± 0.01bcd (0.20~0.23)	Microreticulate
6	Yingjiang Aristolochia *I. hainanense* subsp. *yingjiangense*	Not observed	Present	Subround	Smaller	Smooth, narrow	0.69 ± 0.12hi (0.55~0.88)	0.17 ± 0.03e (0.13~0.20)	0.14 ± 0.02e (0.13~0.18)	Microreticulate
7	Nanyue Aristolochia *I. howii*	Not observed	Present	Subround	Smaller	Wavy, narrow	1.72 ± 0.15c (1.51~1.92)	0.19 ± 0.02e (0.18~0.23)	0.18 ± 0.03de (0.16~0.23)	Microreticulate
8	Pei's Aristolochia *I. petelotii*	Not observed	Present	Subround	Smaller	Smooth, narrow	0.88 ± 0.16fgh (0.68~1.04)	0.32 ± 0.09b (0.24~0.48)	0.21 ± 0.03bcd (0.18~0.26)	Microreticulate
9	Sanya Aristolochia *I. sanyaense*	Not observed	Present	Subround	Smaller	Wavy, narrow	0.59 ± 0.07i (0.51~0.69)	0.27 ± 0.07cde (0.20~0.37)	0.19 ± 0.03cde (0.14~0.21)	Microreticulate
10	Tongbiguan Aristolochia *I. tongbiguanense*	Not observed	Present	Subround	Smaller	Wavy, narrow	0.74 ± 0.19hi (0.53~1.04)	0.24 ± 0.07cde (0.16~0.31)	0.18 ± 0.04de (0.14~0.24)	Microreticulate

No.	Taxon	Colpi	Annulus	Shape	Size	Aperture margin	Value 1	Value 2	Value 3	Ornamentation
11	Variegated Aristolochia *I. versicolor*	Not observed	Present	Subround	Smaller	Wavy, narrow	0.73 ± 0.20hi (0.43~0.91)	0.23 ± 0.04de (0.18~0.26)	0.19 ± 0.04cde (0.14~0.24)	Microreticulate
12	Yang's Aristolochia *I. yangii*	Not observed	Present	Subround	Smaller	Smooth, narrow	0.66 ± 0.11hi (0.56~0.85)	0.19 ± 0.04e (0.16~0.25)	0.14 ± 0.01e (0.12~0.15)	Microreticulate
13	Aristolochia anguicida *Aristolochia anguicida*	Not observed	–	–	–	Smooth, wide	0.92 ± 0.14fgh (0.69~1.05)	–	–	Granulate
14	Aristolochia chlamydophylla *A. chlamydophylla*	Colpus, visible	Present	Subround	Smaller	Smooth, narrow	0.48 ± 0.05i (0.43~0.56)	0.24 ± 0.03cde (0.19~0.28)	0.18 ± 0.02de (0.16~0.22)	Microreticulate

No.	Taxon	Observations	Pores	Shape	Size	Wall	Measurement 1	Measurement 2	Measurement 3	Sculpture
15	Galeate Dutchman's pipe *A. galeata*	Not observed	–	–	–	Wavy, wide	2.89 ± 0.69a (2.14~3.92)	–	–	Coarsely reticulate

No.	Taxon	Observations	Pores	Presence	Shape	Size	Wall	Measurement 1	Measurement 2	Measurement 3	Sculpture
16	Tobacco pipe Dutchman's pipe *A. gibertii*	Not observed	Present	Subround	Smaller	Smooth, wide	1.64 ± 0.32cd (1.34~2.01)	0.23 ± 0.06de (0.14~0.29)	0.19 ± 0.05cde (0.13~0.27)	Coarsely reticulate
17	Giant-flowered Dutchman's pipe *A. gigantea*	Not observed	—	—	—	Smooth, wide	1.23 ± 0.29de (0.90~1.61)	—	—	Cerebroid
18	Elegant Dutchman's pipe *A. littoralis*	Not observed	Present	Subround	Smaller	Wavy, wide	1.75 ± 0.24c (1.46~1.98)	0.32 ± 0.08b (0.19~0.42)	0.22 ± 0.04bc (0.17~0.27)	Coarsely reticulate
19	Pelican flower *A. ringens*	Not observed	Present	Subround	Smaller	Wavy, wide	2.07 ± 0.22b (1.65~2.44)	0.21 ± 0.01de (0.19~0.22)	0.14 ± 0.03e (0.10~0.18)	Coarsely reticulate

Note: Lumen diameter < 0.70 μm indicates a small lumen.

1. *Isotrema austroyunnanense*; 2. *I. cangyuanense*; 3. *I. caulialatum*; 4. *I. fulvicomum*; 5. *I. hainanense*; 6. *I. hainanense* subsp. *yingjiangense*. **A.** General pollen morphology; **B.** Polar view; **C.** Exine ornamentation. The same below.

Fig. 1 Pollen morphology of six species of *Isotrema* under SEM (I)

7. *Isotrema howii*; 8. *I. petelotii*; 9. *I. sanyaense*; 10. *I. tongbiguanense*; 11. *I. hainanense*; 12. *I. yangii*.

Fig. 2 Pollen morphology of six species of *Isotrema* under SEM (II)

13. *Aristolochia anguicida*; 14. *A. chlamydophylla*; 15. *A. galeata*; 16. *A. gibertii*; 17. *A. gigantea*; 18. *A. littoralis*; 19. *A. ringens*.

Fig. 3 Pollen morphology of seven species of *Aristolochia* under SEM (III)

2.2 Multivariate Statistical Analysis

2.2.1 Principal Component Analysis (PCA)

Principal component analysis (PCA) was performed on five key morphological indicators of pollen from 19 species of Aristolochiaceae plants (polar-axis length, equatorial-axis length, P/E value, pollen size, and colpus width). The KMO measure of sampling adequacy was 0.495, slightly lower than the conventional recommended threshold of 0.6, but Bartlett' s test of sphericity reached an extremely significant level ($P < 0.001$), indicating significant correlations among the morphological indicators and satisfying the basic requirements for principal component analysis. The results showed that the cumulative contribution rate of the first three principal components reached 99.935% (Table 4), indicating that these three principal components contained almost all the information of the five pollen morphological indicators and could effectively represent the original variables for subsequent analysis. After Varimax rotation, the loading matrix of each principal component (Table 5) revealed a significant distribution of feature weights:

The contribution rate of the first principal component (PC1) was 66.203%. It was mainly dominated by polar-axis length (loading 0.983), equatorial-axis length (loading 0.967), and pollen size (loading 0.984), all of which had extremely high positive loadings, whereas the contributions of P/E value (loading -0.167) and colpus width (loading 0.180) were relatively small. This indicates that PC1 primarily reflects variation in overall pollen size and can be defined as the "pollen size factor." Judging from the scores, the PC1 scores of species of the genus *Aristolochia* were concentrated between -0.92 and 0.05, all being negative or close to 0; species of the genus *Thottea* had a wider score span, among which *Thottea championii* had the highest score (2.97), followed by *T. tomentosa* (1.40) and *T. hainanensis* (1.06). Only *T. parviflora* (-0.74) and *Asarum* [[unclear: species name]] (0.01) overlapped with the genus *Aristolochia* in scores. The score ranges of the two genera as a whole showed an obvious separation trend, verifying that "pollen size is the most core differentiating indicator between the two genera." The contribution rate of the second principal component (PC2) was 19.698%. It was mainly dominated by the polar-equatorial ratio (P/E), with the highest loading of 0.976, while the absolute values of the loadings of the other indicators were all less than 0.2. This indicates that PC2 mainly reflects pollen shape characteristics and can be defined as the "pollen shape factor." Differentiation on this principal component is mainly manifested among closely related species within genera: among the genus *Aristolochia*, *Aristolochia versicolor* had the highest P/E value (1.13), and its PC2 score (3.30) was significantly higher than those of

other species in the same genus; among the genus *Thottea*, *T. hainanensis* had the lowest P/E value (1.02), and its PC2 score (-1.22) was the lowest, followed by *T. siliquosa* (-0.61), forming differences from other species of the same genus. This suggests that PC2 can serve as an auxiliary factor for identifying closely related species. The contribution rate of the third principal component (PC3) was 14.033%. The core driving indicator was colpus width (loading 0.974), indicating that PC3 mainly reflects the ornamentation characteristics of the pollen outer wall (colpus width) and can be defined as the "pollen ornamentation factor." PC3 scores of species of the genus *Aristolochia* were concentrated between -0.69 and 1.22 (with *A. austroguangdongensis* having the highest score), whereas scores of species of the genus *Thottea* had a wider span (-1.58–2.77). *T. siliquosa* (with the widest colpus) and *T. championii* (with the narrowest colpus) formed extreme differentiation, strengthening the taxonomic value of ornamentation.

The distribution of principal component scores showed that the 12 species of the genus *Aristolochia* were concentrated in the low-score region of PC1, while the 7 species of the genus *Thottea* were concentrated in the high-score region of PC1. Only *T. parviflora* and *Asarum* [[unclear: species name]] were close to the genus *Aristolochia* in pollen size and were distributed in the overlapping region of the two genera. This is consistent with the result of "cross-boundary clustering" in cluster analysis. Overall, pollen size (dominated by PC1) and colpus width (dominated by PC3) are the core driving factors for morphological differentiation of pollen between the two genera, whereas the polar-equatorial ratio (dominated by PC2) provides a key supplement for identifying closely related species within genera. Together, the three constitute an indicator system for identifying pollen morphology among the two genera of Aristolochiaceae and their closely related species.

Table 4 Contribution ratio of the first three major components of pollen characteristics from 19 Aristolochiaceae plants

Principal component	PC1	PC2	PC3
Eigenvalue	3.310	0.985	0.702
Contribution ratio (%)	66.203	19.698	14.033
Total contribution ratio (%)	66.203	85.901	99.935

Table 5 Principal component matrix of pollen characteristics of 19 Aristolochiaceae plants

Principal component	PC1	PC2	PC3
Polar axis length (P)	0.983	-0.093	0.158
Equatorial axis length (E)	0.967	-0.186	0.173
P/E	-0.167	0.976	-0.138
Pollen size (P × E)	0.984	-0.134	0.106

Principal component	PC1	PC2	PC3
Width of muri	0.180	-0.139	0.974

2.2.2 Cluster analysis

Based on the standardized data of the above five morphological indicators, a tree diagram (Fig. 4) was constructed using the Euclidean distance coefficient and the unweighted pair-group method with arithmetic mean (UPGMA). The results showed that, at the Euclidean distance level of 15, all species were clearly divided into four types. The clustering results not only clearly presented the pattern of intergeneric differentiation, but also revealed the close relationships among closely related species within genera. They were highly consistent with the PCA results, both showing intergeneric differentiation and revealing close relationships among congeneric or closely related species.

Type I: This type included 11 species of *Isotrema* and 2 species of *Aristolochia* (*Aristolochia kaempferi* and *A. contorta*). At Euclidean distances of 5–10, it could be further divided into two subtypes. In subtype Ia (10 species of *Isotrema* and *Aristolochia kaempferi*), the interspecific genetic distances between *Isotrema fulvicomum* and *I. hainanense*, and between *I. fargesii* and *I. sanyaense*, were the smallest; their pollen morphology was highly similar, making them the species combinations with the closest relationships. In addition, *Aristolochia kaempferi* clustered within this branch because its pollen size (622.83 μm^2) and muri width (0.92 μm) were highly consistent with the characteristics of the species in this subtype of *Isotrema*, reflecting pollen morphological similarity caused by convergent evolution. Subtype Ib (*Isotrema austroyunnanense* and *Aristolochia contorta*) clustered across boundaries because of convergent characteristics in muri width and pollen size, reflecting the influence of convergent evolution.

Type II: This type contained only one species, *Isotrema versicolor*. It stood alone because it had the highest P/E value (1.13), and it was genetically distant from the core group, supporting its status as an independent close species.

Type III: This type included four *Aristolochia* species (*A. debilis*, *A. mollissima*, *A. gigantea*, and *A. tubiflora*). At Euclidean distances of 5–10, it was divided into two subtypes, reflecting intrageneric proximity and differentiation. Subtype IIIc (3 species): *A. debilis*, *A. mollissima*, and *A. gigantea* were most closely related and morphologically most similar. Differences in pollen size and muri width were small, and only the ornamentation type was differentiated; pollen size (1 289.14 μm^2–1 478.42 μm^2) and muri width (1.23 μm–1.75 μm) differed little, and only the ornamentation type (coarsely reticulate vs. brain-striate) showed differentiation. Subtype IIId (*A. tubiflora*) stood alone because it had the greatest muri width (2.89 μm).

Type IV: This type contained only one species, *Aristolochia delavayi*. It stood alone because of its very large pollen volume (2 026.67 μm^2), specialized fine-

Fig. 4 Cluster analysis of pollen morphology of 19 species of Aristolochiaceae

Figure 1: Fig. 4 Cluster analysis of pollen morphology of 19 species of Aristolochiaceae

reticulate ornamentation, and germinal-furrow structure. It was markedly separated from the other congeneric species, reflecting highly specialized evolutionary characteristics.

Fig. 4 Cluster analysis of pollen morphology of 19 species of Aristolochiaceae

3 Discussion

3.1 Taxonomic value of pollen morphological characters

By combining improved electron-microscope sample preparation, high-resolution scanning observations, and multivariate statistical analysis, this study systematically revealed the stratified morphological patterns of pollen in Aristolochiaceae at the levels of family-level conservation, generic differentiation, and species-level specificity, providing stable palynological evidence for taxonomic revision of this family. At the family level, the pollen of all species in this family is monad, subspheroidal, and does not form compound pollen, which is consistent with the classical palynological conclusions established by Erdtman (1952), and also corroborates the results of family-level delimitation in recent molecular systematics (Nakonechnaya & Kalachev, 2018). Intergeneric differentiation is mainly reflected in the combined characters of pollen size and exine ornamentation: *Isotrema* is characterized stably by small to medium-sized pollen combined with fine reticulate ornamentation, whereas *Aristolochia* mostly has medium to large pollen, with the exine coarse-reticulate or cerebriform; the boundaries of pollen characters between the two genera are clear. This differentiation pattern is consistent with the traditional morphological taxonomic view, and is also highly consistent with the generic phylogenetic branching relationships revealed by chloroplast genomes (Ohi-Toma et al., 2006; Zhu Xinxin et al., 2019). Within species and among closely related species, fine delimitation can be achieved by quantitative parameters such as reticulum lumen diameter, murus width, and the polar/equatorial ratio (P/E). For example, the reticulum lumen diameter of *Isotrema yingjiangense* is (0.17 ± 0.03) μm, whereas that of *I. hainanense* reaches (0.27 ± 0.02) μm, showing significant differences in ornamentation pore diameter; *Aristolochia galeata* has a distinctive broad-reticulate murus structure of (2.89 ± 0.69) μm. These quantitative palynological characters can provide observable and repeatable reference standards for the precise identification of closely related species.

The three-level quantitative discrimination framework of size–shape–ornamentation constructed in this study screened key classification indicators such as pollen size, ornamentation complexity, and germination-aperture morphology

through principal component analysis (PCA), and then combined systematic cluster analysis (UPGMA) to visually verify the phylogenetic relationships among groups, thereby remedying the shortcomings of traditional palynology, which relies only on qualitative description and subjective human judgment. This research approach is similar to the quantitative palynological research models for subgroups in *Salix* (Gu Yanan et al., 2025), *Fagopyrum* (Hu Meng et al., 2025), and *Aeschynanthus* (Zhu Ying et al., 2025), and can provide reference for similar studies and promote the transformation of pteridophyte palynology toward quantitative identification. At the same time, this study also found that the pollen morphology of species such as *Aristolochia anguicida* and *A. ringens* shows intergeneric cross-clustering in the clustering results. This indicates that pollen, as an evolutionarily conserved microscopic character, often has a rate of morphological differentiation that lags behind the rhythm of molecular phylogenetic divergence; relying solely on single pollen evidence makes it difficult to fully clarify disputes over generic delimitation, and taxonomic revision requires the integration of multidimensional information from morphology, molecules, and ecology (González et al., 2001).

3.2 Systematic Evolution and Ecological Adaptation of Pollen Morphology

With reference to the evolutionary pattern proposed by Walker & Skvarla (1975), in which pollen of angiosperms evolves from simple structures toward complex ornamentation, and in combination with the types of exine ornamentation and the results of cluster analysis, this study preliminarily clarifies the main evolutionary trajectory of pollen ornamentation in the Lamiaceae: granular → finely reticulate → coarsely reticulate. Among them, the cerebriform ornamentation of *Salvia rosmarinus* represents an independently specialized type formed as an adaptation to a special mode of pollen dispersal. Groups characterized by granular or finely reticulate ornamentation and small pollen occupy relatively primitive evolutionary positions; the core Lamiaceae groups possess coarsely reticulate ornamentation and larger pollen size and are therefore relatively derived; *Salvia rosmarinus*, with its extremely large pollen volume, specialized germinal furrows, and complex exine ornamentation, constitutes an independently specialized lineage. The differentiation of its pollen morphology is evidently co-evolved with pollen dispersal mode and habitat adaptation, reflecting a high degree of correspondence between microstructure and ecological function.

The Lamiaceae are pollinated mainly by bees and dipteran insects. Nearly spherical pollen can improve adhesion efficiency on the insect body surface (Matallana-Puerto et al., 2024). Differentiation in pollen size between the two genera is closely related to pollination strategy: the smaller pollen of *Teucrium* is more suitable for generalized pollinators to disperse over long distances, whereas the larger pollen of *Salvia* is better adapted to the fixed-point pollination process of specialized pollinating insects. Micro-morphological differences in exine ornamentation further reflect habitat-adaptive characteristics of the groups: the

dense finely reticulate ornamentation on the pollen surface of *Teucrium* can reduce retention of moisture on the pollen surface in humid understory environments, thereby lowering the risk of pollen inactivation; the coarsely reticulate or cerebriform ornamentation of *Salvia* enhances the mechanical strength of the pollen wall, improving pollen resistance in open habitats or under drought conditions (Walker, 1971). In addition, some species such as variegated *Teucrium* show a pattern in which morphological traits are not fully coupled with the molecular phylogeny, demonstrating the evolutionary conservatism of pollen morphology and indicating that morphological differentiation proceeds more slowly than genetic differentiation; this further illustrates the necessity of multi-evidence integrated analysis in studies of evolutionary mechanisms.

3.3 Optimization Effects of the Improved DMP Sampling Technique and Methodological Comparative Analysis

Pollen of the Lamiaceae has thin walls and high water content. Conventional critical-point drying with tertiary butyl alcohol or natural air drying easily causes shrinkage, collapse of the exine reticulum, blurred ornamentation boundaries, and large errors in morphological measurement; this has also been a key technical bottleneck that previously made it difficult to carry out quantitative palynological analysis in this group (Nakonechnaya & Kalachev, 2018). Based on the DMP dehydration system established by Halbritter (1998), this study optimized the dehydration-solution ratio, soaking duration, rinsing procedure, and critical-point drying parameters for Lamiaceae materials, establishing a standardized pretreatment workflow suitable for thin-walled and delicate pollen. Pollen processed by traditional sampling methods generally shows morphological distortion and twisting or loss of fidelity in the exine microstructure, making it difficult to accurately measure fine features such as the lumina and muri of the reticulum. By contrast, pollen treated using the improved DMP procedure retains a complete overall outline, shows good repeatability in all morphological indicators, and allows fine microstructures such as lumina, muri, and specialized germinal furrows to be clearly and stably quantified. The complete preservation of exine ornamentation details provides the data foundation for constructing a three-level discrimination system and conducting principal component and cluster analyses. If traditional sampling methods were used, distortion of pollen structure would directly interfere with the determination of generic and species-level differentiation traits.

The improved procedure does not require highly toxic fixatives such as osmium tetroxide, is easy to operate, and can be widely applied to SEM observation of other thin-walled pollen groups in Lamiales. However, the method has limitations: it is applicable only to fresh pollen at anthesis and cannot repair irreversible deformation of herbarium specimens after drying; if a large amount of glandular secretion adheres to the pollen surface, additional degreasing pretreatment is required before observation; and the procedure is sufficient only for surface morphological observation by scanning electron microscopy, while

analysis of exine ultrastructure still requires specialized fixation and embedding systems for transmission electron microscopy. This standardized pretreatment scheme can unify pollen observation standards across different laboratories, reduce human measurement bias introduced by pretreatment, fill the domestic technical gap in specialized pollen sampling methods for the Lamiaceae, and also provide a technical paradigm for quantitative palynological studies of other thin-walled medicinal plants, thereby helping to standardize the development of quantitative palynological analysis systems.

3.4 Conclusion

This study presents innovations in three aspects. First, it improves the complete operating workflow for DMP electron-microscope specimen pretreatment, solving the problem that the thin-walled, high-water-content pollen of the Lamiaceae is easily deformed during slide preparation and preserving, to the greatest extent, the details of exine surface ornamentation. Second, it integrates SEM morphological observation, quantitative pollen measurement, and multivariate statistical analysis to construct a species-identification system, effectively improving the discrimination accuracy for closely related species. Third, it completes the determination of quantitative indices of pollen morphology for 19 species of Lamiaceae, records for the first time two features—cerebriform exine of *Salvia rosmarinus* and specialized germinal furrows of *Salvia przewalskii*—and supplements and improves the basic palynological data for the Lamiaceae in this region.

In summary, pollen traits at the family level in the Lamiaceae are generally stable and conserved, whereas differences in pollen morphology among genera are pronounced, and species can be clearly distinguished by subtle pollen characters. Pollen size as a whole, polar/equatorial ratio, exine ornamentation morphology, and the dimensional parameters of lumina and muri can all serve as core palynological evidence for species identification and phylogenetic inference. The quantitative discrimination constructed in this paper

classification framework can avoid the subjective bias produced by traditional descriptions that rely only on the naked eye, making the identification results of closely related and difficult-to-distinguish species more stable and reliable. Evidence from pollen morphology shows that *Isotrema* and *Aristolochia* are closely related, and that the basic pollen characters shared by the two derive from a common ancestor; meanwhile, the pollen of the two groups shows clear differentiation in volume and exine ornamentation, which was caused by their radiative evolution in adaptation to different living environments. Special microstructures such as reticulate exine ornamentation, specialized germinal apertures, and extremely large pollen can serve as diagnostic markers for the independent evolution of some species, and also provide key microscopic evidence for reconstructing the systematic development and evolution of Aristolochiaceae. The conclusions obtained from principal component analysis and systematic clustering can corroborate each other, clarifying the long-standing controversy over

the delimitation of *Isotrema* and *Aristolochia*, refining the relationships among closely related species within the genera, and providing theoretical support at the palynological level for revision of the classification system of Aristolochiaceae, correction of species names, and conservation of rare wild germplasm resources. At the same time, the standardized pretreatment method for thin-walled pollen established in this study, together with the analytical approach combining qualitative observation, quantitative measurement, and statistical verification, expands the research methods of palynology in angiosperms and has reference and promotional value for studies of the micromorphology of pollen in other thin-walled pollen plants.

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