Floral Morphology and Diandrous Anther Development of Paphiopedilum purpuratum and Their Taxonomic Significance (Postprint)

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Abstract

The taxonomic systematics of the genus Paphiopedilum remains highly controversial and urgently requires additional data for clarification. This study employed stereomicroscopy and paraffin sectioning techniques to observe the floral structure and diandrous anther developmental characteristics of Paphiopedilum purpuratum, providing new data for the taxonomy of Paphiopedilum and its subfamily Cypripedioideae. The results indicate: (1) Floral morphological features support the placement of Paphiopedilum purpuratum in the section Pardalopetalum of subgenus Paphiopedilum, including sepals with venation, oblong petals with dark brown spots, a labellum with erect auriculate structures, and a crescent-shaped staminode, among others. (2) The anther primordium differentiates into a pair of laterally juxtaposed locules, with an incompletely traversing sterile septal tissue differentiating centrally within the locule, dividing it into two microsporangia. At anther maturity, the sterile septal tissue is degraded and absorbed, and the two pollen sacs undergo secondary fusion to form a saddle-shaped viscid pollinium. (3) The fully developed anther wall comprises four layers, from outer to inner: epidermis, endothecium, middle layer, and tapetum, conforming to the monocot-type anther wall. The anther wall possesses a tapetum and inner tapetum, both binucleate. At the 2-celled pollen stage, the middle layer and tapetum undergo degradation, while the endothecium develops fibrous thickenings. (4) Microspore mother cells undergo simultaneous cytokinesis to form microspore tetrads in various arrangements, including tetrahedral, bilaterally symmetrical, and decussate types, with conspicuous asynchrony in meiosis among microspore mother cells within the same locule. (5) During the male gametophyte developmental stage, microspores either remain within the tetrad or become liberated from it, developing into 2-celled pollen through mitosis, forming viscid tetrads or individual pollen grains.

In combination with existing data, this study comparatively analyzes the taxonomic significance of characteristics such as the incompletely traversing sterile septal tissue, monocot-type anther wall, binucleate tapetum and inner tapetum, simultaneous cytokinesis, and viscid anther in Paphiopedilum purpuratum, providing new data for understanding the taxonomy and conservation biology of Paphiopedilum and Cypripedioideae.

Full Text

Preamble

Floral Morphology and Anther Development in Diandrous Orchid *Paphiopedilum purpuratum* with Their Taxonomic Implications

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Abstract: The taxonomy of *Paphiopedilum* Pfitz. has long been disputed and requires additional data for clarification. We investigated the floral morphology and anther development of *P. purpuratum* using stereomicroscopy and paraffin sectioning to provide new insights into the systematics of *Paphiopedilum* and the subfamily Cypripedioideae. Our results reveal: (1) Floral morphological features support the placement of *P. purpuratum* in section *Barbata* of subgenus *Paphiopedilum*, including veined sepals, oblong petals with blackish-maroon spots, a lip with erect auriculate structures, and a lunate staminode. (2) The anther primordium differentiates into a pair of laterally juxtaposed thecae, with an incomplete sterile septum differentiating centrally within each theca, dividing it into two microsporangia. At anthesis, the sterile septum degrades, and the two pollen sacs undergo secondary fusion to form a saddle-shaped mass of sticky pollen. (3) The mature anther wall comprises four layers—epidermis, endothecium, middle layer, and tapetum—conforming to the monocotyledonous type. Both tapetum and inner tapetum are binucleate. At the 2-celled pollen stage, the middle layer and tapetum degrade while the endothecium develops fibrous thickenings. (4) Microspore mother cells undergo simultaneous cytokinesis, forming microspore tetrads with varied arrangements: tetrahedral, isobilateral, and decussate, with asynchronous meiosis evident within the same theca. (5) During male gametophyte development, microspores either remain within tetrads or are released, developing into 2-celled pollen through mitosis, forming either tetrads or monads of sticky pollen. Based on these findings, we discuss the taxonomic significance of key features including the incomplete sterile septum, monocotyledonous anther wall type, binucleate tapetum and inner tapetum, simultaneous cytokinesis, and sticky pollen, providing new data for understanding the taxonomy and conservation biology of *Paphiopedilum* and

Cypripedioideae.

Keywords: *Paphiopedilum*, Cypripedioideae, anther wall development, microsporogenesis, gametogenesis, pollen dispersal unit, taxonomy

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Introduction

The subfamily Cypripedioideae Lindl. exhibits distinctive floral morphology within Orchidaceae, clearly differing from the other four subfamilies, most notably in possessing two fertile stamens and releasing sticky pollen masses at anther dehiscence (Dressler, 1986; Kocyan & Endress, 2001; Singer et al., 2008). As a basal lineage in Orchidaceae, the diandrous Cypripedioideae is closely related to the triandrous Apostasioideae Rchb., comprising five genera and approximately 180 species, each with distinct geographic distributions (Dressler, 1986; Cox et al., 1997; Pridgeon et al., 1999). Apart from three small genera endemic to South America, the remaining two genera occur in China: *Cypripedium* L. and *Paphiopedilum* Pfitz. (Cribb, 1998; Lang, 1999; Liu et al., 2009). Cypripedioideae species possess high ornamental value (Cribb, 1998; Lang, 1999), and their wild resources have received considerable attention, with both Chinese *Paphiopedilum* and *Cypripedium* species listed in the National Key Protected Wild Plants of China (Ministry of Agriculture and Rural Affairs and State Forestry and Grassland Administration, 2021).

Taxonomic systems for *Paphiopedilum* have long been controversial, primarily concerning the treatment of subgenera and sections within the genus. Early classifications divided *Paphiopedilum* into two or six subgenera based on lip morphology (Atwood, 1984; Cribb, 1987) and cytology (Karasawa, 1980, 1982). Some studies advocated for three subgenera (Cribb, 1998; Pridgeon et al., 1999), a concept supported by molecular phylogenetic evidence (Cox et al., 1997; Chochai et al., 2012; Tsai et al., 2020), yet current Chinese classification systems for *Paphiopedilum* still adopt the two-subgenus concept (Lang, 1999; Liu et al., 2009). In studies reconstructing the classification system, leaf mottling patterns and lip morphology have commonly been used for species identification (Cribb, 1998; Pridgeon et al., 1999; Lang, 1999; Liu et al., 2009), while the taxonomic value of floral structure has received minimal attention. Cribb (1998) noted that section *Barbata* is distinguished from other sections in subgenus *Paphiopedilum* by its solitary flowers, erect auriculate lips, and mottled leaves with light and dark patches, whereas section *Coryopedilum* typically has multi-flowered inflorescences, non-auriculate lips, and smooth, unspotted leaves.

Paphiopedilum purpuratum (Lindl.) Stein, the earliest Chinese *Paphiopedilum* species to be cultivated and described, has type specimens from Hong Kong and is distributed in Fujian, Guangdong, Guangxi, and Yunnan provinces, as well as in Vietnam (Liu et al., 2009). Based on its lip being smaller than the dorsal sepal, it has been placed in subgenus *Paphiopedilum* (Cribb, 1998; Pridgeon et al., 1999; Lang, 1999). Recent molecular phylogenetic studies have further assigned it to section *Barbata* (Cox et al., 1997; Chochai et al., 2012; Tsai et al., 2020), yet morphological and anther development data remain scarce (Liu et al., 2009).

Floral morphology and anther developmental embryology play crucial roles in orchid systematics, including features such as lip and gynostemium morphology, number of fertile stamens and anther locules, anther wall development type, tapetum characteristics, microspore mother cell division patterns, and pollen dispersal units at maturity (Burns et al., 1985; Dressler, 1986, 1993; Johri et al., 1992; Johnson & Edwards, 2000; Harder & Johnson, 2008; Li, 2023). However, such studies remain limited (Sood, 1989; Tan et al., 2020). The latest Orchidaceae classification system provides a framework for understanding the systematic significance of floral and embryological features across the five subfamilies (Chase et al., 2015). Existing anther development data primarily focus on the triandrous Apostasioideae (Kocyan & Endress, 2001), and the monandrous subfamilies Orchidoideae (Swamy, 1946; Maheshwari & Narayanaswami, 1952; Kant & Bhanwra, 2010) and Epidendroideae (Bhanwra & Vij, 2003; Kant, 2019, 2023; Tan et al., 2020). Within the diandrous Cypripedioideae, detailed anther development data are available for *Cypripedium* (Swamy, 1949; Brown & Lemmon, 1996; Sood & Rao, 1988; Kant, 2011), but relatively scarce for *Paphiopedilum* (Swamy, 1949; Ren & Wang, 1987), and even more limited for the remaining three genera (Johri et al., 1992; Cribb, 1998). This study investigates the floral structure and anther development of *P. purpuratum*, focusing on sterile septum distribution and differentiation, anther wall type, cytokinesis patterns, and anther morphology, to provide new data for understanding the taxonomy and conservation biology of *Paphiopedilum* and Cypripedioideae.

1. Materials and Methods

Paphiopedilum purpuratum plants cultivated at the Southwest Forestry University Research and Teaching Practice Base (Yunnan Fengchunfang Biotechnology Co., Ltd. Orchid Resource Garden) were grown for multiple years and flowered normally. From 2017 to 2020, flower buds at different developmental stages and open flowers were collected for four consecutive years. Approximately 15 fresh open flowers were observed under a Leica stereomicroscope (Leica M165 FC) to document morphological features of the lip, gynostemium, and pollinia, with photographs taken. Remaining materials were fixed in 50% ethanol for 24 hours, then transferred to 70% ethanol for storage at room temperature.

Fixed materials were categorized into three developmental stages (young bud stage, bud stage, and anthesis), with 5–10 flowers sampled per stage. Materials underwent ethanol gradient dehydration, xylene clearing, paraffin infiltration and embedding, and conventional paraffin sectioning (Leica RM 2235, 8 m thickness). Sections were dried, dewaxed, rehydrated, stained with Ehrlich's hematoxylin, and mounted with neutral resin to create permanent slides. Sections were observed and photographed under a Nikon microscope (Nikon E100), with plates prepared using Adobe Photoshop CS6. Orchid structural terminology followed *A Field Guide to the Orchids of China* (Chen et al., 2009).

2.1 Floral Morphology and Anatomical Characteristics

Paphiopedilum purpuratum exhibits the following typical floral structural features (Plate I: 1): solitary flowers with scapes reaching over 30 cm; large, deep purple flowers with distinct stripes; white, broadly ovate dorsal sepals with revolute margins and abruptly narrowed apical horns; synsepal narrowly lanceolate, growing downward alongside the ovary; lateral petals oblong with purplish-red venation and shallowly undulate, revolute margins; lip deeply helmet-shaped with a broad square mouth, lacking apical notches, and bearing erect auriculate structures on both sides; basal lateral lobes involute with verrucose projections.

Under the dissecting microscope, the stamen and pistil are observed to be fused only at the base, forming a semi-confluent gynostemium that is flattened and semi-cylindrical, with dorsal and ventral surfaces densely covered with purple pubescence. Two fertile stamens are present on each side with fleshy, short, thick filaments; above these are independently growing stigma and staminode, fleshy and flattened, shield-shaped, bending 90° toward the adaxial surface (Plate I: 2–4). The staminode is inverted heart-shaped and semilunar (Plate I: 2, 3); the stigma is disc-shaped with a smooth surface and three shallow lobes (Plate I: 3–5). During early anther development, the two fertile stamens are nearly equal in height to the stigma (Plate I: 5), with anthers composed of a pair of lateral thecae (Plate I: 6), each theca bearing a longitudinal shallow groove at the apex (Plate I: 5, 6). At later developmental stages, thecae dehisce along these grooves (Plate I: 7), exposing smooth, curved, rod-shaped pollen that adheres into a mass (Plate I: 8).

Plate I: Floral Morphology in *Paphiopedilum purpuratum*

1. Flower. 2–4. Mature gynostemium in front (2), side (3), and back (4) views, showing the columnar gynostemium, fleshy shield-shaped stigma, flattened semilunar staminode, and two fertile stamens. 5, 6. Early-stage gynostemium in top (5) and side (6) views, showing the three-lobed stigma surface and fertile stamens composed of two lateral thecae with longitudinal shallow grooves (arrow indicated). 7. Ripened anther showing dehiscence of each theca (arrow indicated). 8. Ripened anther with degraded anther wall exposing smooth sticky pollen mass. a. Anther; aw. Anther wall; d. Dorsal sepal; f. Filament; g. Gynostemium; l. Lip; p. Petal; sp. Sticky pollen mass; t. Theca; sta. Staminode; sti. Stigma.

2.2 Anther Wall Development

In cross-sections of early-stage anthers, two fertile stamens are observed at the gynostemium base, each anther composed of a pair of lateral thecae curving toward the adaxial surface in a manner encircling the gynostemium (Plate II: 1). The thin-walled cells at the junction of each theca pair are lightly stained, with a prominent main vascular bundle forming a relatively wide connective tissue (Plate II: 1).

A group of archesporial cells in the anther locule center differentiates into lightly stained thin-walled cells that do not participate in sterile septum formation, with the outermost 1–2 layers radially elongating to develop into binucleate inner tapetum (Plate II: 2, 3). During the microspore mother cell stage, a single archesporial cell beneath the anther epidermis undergoes two periclinal divisions to form a mature four-layered anther wall, from outermost to innermost: epidermis, endothecium, middle layer, and tapetum (Plate II: 4, 5). Thus, the anther wall development pattern conforms to the monocotyledonous type. During microspore mother cell meiosis, epidermal cells are mostly regular rectangles (Plate II: 1, 7), though some appear wavy or toothed (Plate II: 4–6); endothecial cells enlarge to become square to rectangular (Plate II: 4–10); middle layer cells are small and uninucleate; tapetal cells enlarge and are distinctly binucleate (Plate II: 4–8). At the microspore tetrad or monad stage (Plate II: 6–9), the middle layer and tapetum show obvious degradation, endothecial cells continue to enlarge, and epidermal cells become narrower. At anther maturity, only epidermal remnants and a band of fibrously thickened endothecium remain (Plate II: 10). In both longitudinal and cross-sections of mature anthers, the sterile septum shows significant degradation, with the anther wall thinning at the apical depression (Plate II: 11) and rupturing to release pollen (Plate II: 12).

Plate II: Development of Anther Wall in *Paphiopedilum purpuratum*

1. Early anther primordium differentiating into a pair of lateral juxtaposed thecae, with central differentiation of sterile septum tissue and shallow U-shaped microsporangia. 2, 3. Sterile septum margin differentiating 1–2 layers of binucleate inner tapetum cells (3). 4, 5. Four-layered anther wall during microspore mother cell meiosis, note binucleate tapetum (arrow indicated). 6–9. Anther wall at microspore tetrad stage, showing toothed epidermis, enlarged endothecium, degraded middle layer and tapetum; note binucleate inner tapetum (white arrow in 8). 10. Anther wall at 2-celled pollen stage, showing degraded epidermis and fibrously thickened endothecium. 11, 12. Longitudinal (11) and cross (12) sections of ripened anther, showing degraded central sterile septum and thinning and rupture of anther wall at theca apex (arrow indicated in 11). cn. Connective tissue; ep. Epidermis; en. Endothecium; it. Inner tapetum; m. Middle layer; sp. Sporangium; ss. Sterile septum; st. Stigma; t. Tapetum.

2.3 Microsporogenesis and Male Gametophyte Development

During early development, meristematic tissue beneath the anther locule epidermis undergoes mitotic division to form archesporial cells; central locular archesporial cells differentiate into lightly stained sterile septum tissue, while outer archesporial cells differentiate into deeply stained sporogenous cells with high nuclear-cytoplasmic ratios (Plate III: 1, 2), which subsequently divide to form tightly arranged microspore mother cells with high nuclear-cytoplasmic ratios (Plate III: 1). Microspore mother cells undergo meiosis I prophase (Plate III: 2, 3), metaphase (Plate III: 4), anaphase (Plate III: 5), and meiosis II, forming four daughter nuclei arranged in a tetrahedral pattern within a common cytoplasm (Plate III: 5). Subsequently, distinct cell walls appear between the four nuclei, producing microspore tetrads with three arrangement patterns: tetrahedral (Plate III: 7), isobilateral (Plate III: 8), and decussate (Plate III: 9). Therefore, *P. purpuratum* exhibits simultaneous cytokinesis during microspore mother cell meiosis (Johri et al., 1992). Following this, some microspores are released from tetrads (Plate III: 10), while others remain within tetrads (Plate III: 11), both developing into 2-celled pollen through mitosis, forming either tetrads or monads of sticky pollen (Plate III: 12).

Plate III: Microsporogenesis and Gametogenesis in *Paphiopedilum purpuratum*

1. Microspore mother cells. 2–5. Microsporogenesis: Meiosis prophase I (2, 3), metaphase I (4), anaphase I (5). 6. Anaphase II showing tetrahedrally arranged four daughter nuclei without obvious cell walls. 7–9. Varied arrangements of microspore tetrads: tetrahedral (7), isobilateral (8), and decussate (9). 10. Microspores released from tetrads. 11, 12. Some microspores retained within tetrads developing into 2-celled pollen tetrads (11); others released as monads developing into 2-celled single pollen grains (12). gc. Generative cell; vc. Vegetative cell. (Scale bars = 2 m)

3. Discussion and Conclusion

Stamen number and anther developmental embryology—including locule number, anther wall development type, microsporogenesis cytokinesis pattern, tetrad arrangement, and pollen dispersal unit type—hold significant systematic value in angiosperms, particularly in Orchidaceae (Johri et al., 1992; Dressler, 1993; Hu, 2005; Singer et al., 2008; Li, 2023). Current research on orchid anther development has concentrated on the triandrous Apostasioideae and monandrous Orchidoideae and Epidendroideae, with limited data for the diandrous Cypripedioideae, and virtually no studies on the monandrous Vanilloideae (Li, 2023). This study examines the floral morphology and anther developmental embryology of *P. purpuratum*, comparing these features with existing data to analyze their taxonomic significance.

3.1 Taxonomic Significance of Lunate Staminodes and Veined Sepals

Paphiopedilum purpuratum exhibits distinctive floral morphological features—purple-striped dorsal sepals and a lunate staminode—that clearly differentiate it from most *Paphiopedilum* species and provide high species recognition value. Notably, these two features also occur in the other five species of section *Barbata* and three species of section *Coryopedilum* within the same subgenus. The former includes three close relatives of *P. purpuratum* [*P. callosum*, *P. barbatum*, *P. lawrenceanum*] (Cox et al., 1997; Chochai et al., 2012; Tsai et al., 2020) and two Chinese species [*P. venustum*, *P. wardii*] (Liu et al., 2009). The latter includes *P. rothschildianum*, *P. sanderianum*, and *P. philippinense*, all sharing these taxonomically significant morphological features (Cribb, 1998). This suggests potential phylogenetic connections between sections *Barbata* and *Coryopedilum* warranting further investigation. Given that staminode morphology shows clear interspecific variation in *Paphiopedilum* (Cribb, 1998), its taxonomic significance deserves continued attention.

3.2 Pollen Dispersal Unit Type in Mature Anthers of *P. purpuratum*

Upon anther dehiscence, *P. purpuratum* exposes a mass of smooth yet sticky pollen dispersal units (PDUs), where pollen grains are adhered into a cohesive mass resembling the pollinia of Epidendroideae. Previous studies considered monad pollen as a diagnostic feature of *Paphiopedilum* (Swamy, 1949; Poddubnaya-Arnoldi, 1960), with *Cypripedium* pollen also described as 2-celled monads (Sood & Rao, 1988). However, subsequent research confirmed that mature PDUs in both *Paphiopedilum* (Ren & Wang, 1987) and *Cypripedium* (Kant, 2011) are sticky pollen masses, with the adhesive substance derived from tapetal degradation products containing yellow carotenoid pigments, termed pollenkitt, which coheres all pollen within an anther to enhance pollination efficiency (Ren & Wang, 1987). This differs markedly from PDU types in the other four subfamilies (Singer et al., 2008). Our observations reveal relatively sparse pollen cells in mature *P. purpuratum* anthers, containing both monads and tetrads, unlike the tightly arranged pollinia of *Vanda* (Zhang et al., 2019) or *Pennilabium* (Li et al., 2020). Such “sticky pollen” is clearly visible on pollinators of *P. dianthum* (Shi et al., 2007) and *Cypripedium henryi* (Li et al., 2008), supporting previous conclusions (Ren & Wang, 1987) and providing morphological and embryological data for understanding pollination biology in Cypripedioideae.

3.3 Importance of Anther Locule Number and Sterile Septum Differentiation

Existing data demonstrate that sterile septum differentiation and degradation during orchid anther development are crucial for understanding PDU formation across the five subfamilies (Li, 2023). In monandrous Orchidoideae and Epidendroideae, the number, orientation, and position of early-differentiated sterile septa determine the type and number of massulae and pollinia (Freudenstein & Rasmussen, 1996, 1997; Freudenstein et al., 2002; Kant & Goel, 2014), as these septa later degrade to partition mature pollen sacs into PDUs of varying sizes (equal or unequal), numbers (2, 4, 8), and morphologies (entire, poricidal, shallowly or deeply lobed) (Freudenstein & Rasmussen, 1996; Singer et al., 2008; Kant & Hossain, 2010; Kant et al., 2013; Zhang et al., 2019; Li et al., 2020; Tan et al., 2020). This study observed that early *P. purpuratum* anthers differentiate into a pair of lateral thecae, with central archesporial cells in each theca differentiating an incomplete sterile septum forming a shallowly concave, arc-shaped microsporangium. This aspect has been overlooked in previous studies, causing confusion regarding the relationship between locule number and sterile septum differentiation, as seen in descriptions of *Cypripedium cordigerum* and *C. japonicum* as having four-loculed anthers and four microsporangia (Sood & Rao, 1988; Ghimire et al., 2020). Therefore, the relationship between anther locule number and sterile septum differentiation warrants in-depth investigation (Kant & Goel, 2014).

3.4 Taxonomic Significance of Monocotyledonous Anther Wall Development in *P. purpuratum*

The four-layered anther wall of *P. purpuratum* conforms to the monocotyledonous type, differing from existing reports in Cypripedioideae and thus possessing taxonomic value. For example, congeneric *P. druryi* (Swamy, 1949) and *P. godefroyae* (Ren & Wang, 1987) have five-layered basic-type anther walls, while *Cypripedium cordigerum* (Sood & Rao, 1988; Kant, 2011) and *C. japonicum* (Ghimire et al., 2020) have 6–8 layers, representing the multi-layered type. In Orchidaceae, the monocotyledonous type predominantly occurs in Orchidoideae, such as *Goodyera* (Sood, 1988), *Ophrys* (Aybeke, 2012), *Habenaria* (Vij & Sharma, 1987; Sood, 1986), *Herminium* (Sood & Rao, 1986), and *Satyrium* (Rao & Sood, 1979), as well as in Epidendroideae (*Pennilabium*; Li et al., 2020). Multi-layered anther walls are common in Epidendroideae, including *Vanda* (Zhang et al., 2019) and *Arundina* (Tan et al., 2020). Additionally, Apostasioideae mature anther walls typically have 4–5 layers, sometimes with two-layered thickened endothecium, though the characteristics and taxonomic significance of their anther wall types remain unaddressed (Kocyan & Endress, 2001; Singer et al., 2008). We conclude that anther wall layer number and type show remarkable diversity in Orchidaceae, uncommon in other angiosperms, and deserve greater attention.

Tapetal cells are typically uninucleate in angiosperms, though some species have binucleate cells that are relatively stable within genera and possess systematic significance (Johri et al., 1992). Both tapetum and inner tapetum of *P. purpuratum* are binucleate, consistent with observations in *P. druryi* (Swamy, 1949) and *P. godefroyae* (Ren & Wang, 1987), but differing from the uninucleate tapetum reported in *Cypripedium cordigerum* (Sood & Rao, 1988; Kant, 2011) and *C. japonicum* (Ghimire et al., 2020). This suggests that tapetal cell nuclear number may serve as a criterion for delimiting *Paphiopedilum* and *Cypripedium*. Interestingly, Swamy (1949) systematically examined gametophyte development in 16 orchid genera (28 species), noting that binucleate tapetum in *P. druryi* distinguished it from other monandrous orchids, leading to its proposal as a diagnostic feature for Cypripedioideae. However, Sood (1988) later discovered binucleate tapetum in *Goodyera repens* (Orchidoideae), suggesting this feature is not exclusive to diandrous Cypripedioideae—a conclusion supported by subsequent studies. Current orchid anther development data indicate uninucleate tapetum is widespread in Apostasioideae, Orchidoideae, and Epidendroideae (Vij & Sharma, 1987; Singer et al., 2008; Zhang et al., 2019), while binucleate tapetum occurs relatively rarely in Cypripedioideae (*Paphiopedilum*) and some Orchidoideae and Epidendroideae species, such as *Epipactis* (Sood, 1997; Bhanwra et al., 2006a), *Cymbidium pendulum*, *Smitinandia micrantha* (Bhanwra et al., 2006b), and *Spathoglottis plicata* (Sriyot et al., 2015). Additionally, in *Spiranthes sinensis*, tapetal cells are uninucleate during early differentiation but become binucleate at maturity (Sood, 1989), a process not observed in *P. purpuratum*. Therefore, comprehensive observation of anther wall structure throughout development is needed to determine tapetal cell nuclear number (Johri et al., 1992).

3.5 Taxonomic Significance of Microsporogenesis and Male Gametophyte Development in *P. purpuratum*

Microspore mother cells in *P. purpuratum* undergo simultaneous cytokinesis to form microspore tetrads, which develop into 2-celled pollen through mitosis, with co-occurring monad and tetrad pollen—consistent with existing Cypripedioideae data (Poddubnaya-Arnoldi, 1960; Sood & Rao, 1988; Brown & Lemmon, 1996; Kant, 2011; Ghimire et al., 2020) and serving as an embryological feature distinguishing this subfamily from others. Although early studies suggested successive cytokinesis in *Cypripedium* (Guignard, 1882), this has not been supported by subsequent research. Simultaneous cytokinesis is common in Orchidaceae, whereas successive cytokinesis is relatively rare, occurring in *Vanda* (Zhang et al., 2019), *Ophrys* (Aybeke, 2012), and *Spathoglottis* (Sriyot et al., 2015). Thus, the evolutionary trends of microsporogenesis cytokinesis patterns in orchid classification warrant focused attention (Furness & Rudall, 1999; Furness et al., 2002).

The diverse tetrad arrangements in *P. purpuratum*—tetrahedral, isobilateral, and decussate—provide new data for understanding the taxonomic significance of tetrad configuration. Studies show orchid microspore tetrads exhibit up to five arrangement types: tetrahedral, isobilateral, decussate, T-shaped, and linear (Hu, 1982; Johri et al., 1992). Tetrahedral is most common, followed by isobilateral, with other types being relatively rare. Notably, tetrad arrangement generally corresponds to the internal arrangement of tetrads within PDUs, confirmed in Orchidoideae massulae (Kant et al., 2013) and Epidendroideae compact pollinia (Zhang et al., 2019; Tan et al., 2020; Li et al., 2020), which is important for understanding mature pollen morphology in Orchidaceae.

3.6 Conclusions

Based on this study, we propose the following conclusions: (1) The striped dorsal sepal and lunate staminode of *P. purpuratum* are rare in *Paphiopedilum* and may serve as a link between sections *Barbata* and *Coryopedilum* within subgenus *Paphiopedilum*, warranting further phylogenetic investigation. (2) Structural differences in the gynostemium between bud and anthesis stages indicate that morphogenesis of fertile stamens, staminodes, and the stigma occurs during later floral development (bud stage), with distinct differentiation in growth rates among these organs, where anther development precedes that of staminodes and stigmas. (3) *Paphiopedilum purpuratum* anthers possess a pair of lateral thecae with sterile septum differentiation, differing from previous records of four-loculed anthers and four microsporangia in congeneric species and deserving attention. (4) Tapetal cell nuclear number may serve as a criterion for delimiting *Paphiopedilum* (binucleate) from *Cypripedium* (uninucleate). (5) Embryological features of *P. purpuratum* anther development show both similarities and differences with related orchid groups, holding taxonomic significance regarding locule number, anther wall development type, tapetal nuclear number, cytokinesis type, and tetrad arrangement patterns. (6) Current data demonstrate clear differences in floral morphology and anther developmental embryology among orchid taxa (Aybeke, 2012; Sriyot et al., 2015; Tan et al., 2020), yet fewer than 200 species have been studied (author's preliminary estimate). Therefore, representative studies at supraspecific levels (section, subgenus, genus, subtribe, tribe, subfamily) are necessary to gradually accumulate data and provide floral and embryological evidence for resolving controversial systematic issues (Swamy, 1949; Kant, 2010; Kant & Goel, 2013; Valencia-Nieto et al., 2016).

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References

Chen XQ, Liu ZJ, Luo YB, 2009. *A Field Guide to the Orchids of China* [M]. Beijing: China Forestry Publishing House.

Hu SY, 1982. *Embryology of Angiosperms* [M]. Beijing: Higher Education Press.

Hu SY, 2005. *Reproductive Biology of Angiosperms* [M]. Beijing: Higher Education Press: 25-92.

Lang KY, 1999. *Flora of China (Orchidaceae)*, Vol. 17 [M]. Beijing: Science Press: 54-71.

Liu ZJ, Chen SC, Chen LJ, et al., 2009. *The Genus Paphiopedilum in China* [M]. Beijing: Science Press.

Li L, Zhang J, Li CR, et al., 2020. Pollinia development in a new species of *Pennilabium yunnanense* (Orchidaceae) [J]. *Bulletin of Botanical Research*, 40(2): 161-171.

Li L, 2023. Research progress on diversity of androecium development of Orchidaceae [J]. *Guihaia*, 43(8): 1537-1552.

Ministry of Agriculture and Rural Affairs and State Forestry and Grassland Administration, 2021. *List of National Key Protected Wild Plants in China* [R]. Beijing: Ministry of Agriculture and Rural Affairs and State Forestry and Grassland Administration.

Ren L, Wang FX, 1987. Embryology of *Paphiopedilum* [J]. *Journal of Integrative Plant Biology*, 29(1): 14-22.

Shi J, Cheng J, Luo D, et al., 2007. Pollination syndromes predict brood-site deceptive pollination by female hoverflies in *Paphiopedilum dianthum* (Orchidaceae) [J]. *Acta Phytotaxonomica Sinica*, 45(4): 551-560.

Tan QQ, Li L, Zhang J, et al., 2020. Pollinia development in *Arundina graminifolia* (Orchidaceae) with taxonomic implications [J]. *Guihaia*, 40(1): 83-94.

Zhang J, Zhang H, Li L, et al., 2019. Pollinia development in rare orchid *Vanda coerulea* with its taxonomic implications [J]. *Acta Botanica Boreali-Occidentalia Sinica*, 39(7): 1143-1153.

Atwood J, 1984. The relationships of the slipper orchids (Subfamily Cypripedioideae) [J]. *Selbyana*, 7: 129-247.

Aybeke M, 2012. Anther wall and pollen development in *Ophrys mammosa* L. (Orchidaceae) [J]. *Plant Systematics and Evolution*, 298(6): 1015-1023.

Burns Balogh P, Bernhardt P, 1985. Evolutionary trends in the androecium of the Orchidaceae [J]. *Plant Systematics and Evolution*, 149(1): 119-134.

Brown RC, Lemmon BE, 1996. Nuclear cytoplasmic domains, microtubules and organelles in microsporocytes of the slipper orchid *Cypripedium californicum* A. Gray dividing by simultaneous cytokinesis [J]. *Sexual Plant Reproduction*, 9(3): 145-152.

Bhanwra RK, Vij SP, 2003. The development of anther and male gametophyte in *Eulophia hormusjii* Duthie [J]. *Journal of the Orchid Society of India*, 17(1-2): 87-91.

Bhanwra RK, Vij SP, Kant R, et al., 2006a. Pollinium development in *Epipactis veratrifolia* [J]. *Journal of the Orchid Society of India*, 20(1-2): 1-6.

Bhanwra RK, Vij SP, Chandel V, et al., 2006b. Development of pollinium in two epidendroid orchids [J]. *Current Science*, 90: 1384-1387.

Cribb PJ, 1987. *The Genus Paphiopedilum* [M]. London: Collingridge.

Cox AV, Pridgeon AM, Albert VA, et al., 1997. Phylogenetics of the slipper orchids (Cypripedioideae, Orchidaceae): nuclear rDNA ITS sequences [J]. *Plant Systematics and Evolution*, 208(3): 197-223.

Cribb P, 1998. *The Genus Paphiopedilum* [M]. Kota Kinabalu: Natural History Publications (Borneo) in association with Royal Botanic Gardens Kew.

Chochai A, Leitch IJ, Ingrouille MJ, et al., 2012. Molecular phylogenetics of *Paphiopedilum* (Cypripedioideae; Orchidaceae) based on nuclear ribosomal ITS and plastid sequences [J]. *Botanical Journal of the Linnean Society*, 170: 176-196.

Chen SC, Liu ZJ, Zhu GH, et al., 2009. *Flora of China* Vol. 25 [M]. Beijing: Science Press.

Chase MW, Cameron KM, Freudenstein JV, et al., 2015. An updated classification of Orchidaceae [J]. *Botanical Journal of the Linnean Society*, 177(2): 151-174.

Dressler RL, 1986. Features of pollinaria and orchid classification [J]. *Lindleyana*, 1(2): 95-97.

Dressler RL, 1993. *Phylogeny and Classification of the Orchid Family* [M]. Cambridge: Cambridge University Press.

Freudenstein JV, Rasmussen FN, 1996. Pollinium development and number in the Orchidaceae [J]. *American Journal of Botany*, 83(7): 813-824.

Freudenstein JV, Rasmussen FN, 1997. Sectile pollinia and relationships in the Orchidaceae [J]. *Plant Systematics and Evolution*, 205: 125-146.

Freudenstein JV, Harris EM, Rasmussen FN, 2002. The evolution of anther morphology in orchids: incumbent anthers, superposed pollinia, and the vandoid complex [J]. *American Journal of Botany*, 89(11): 1747-1755.

Furness CA, Rudall PJ, 1999. Microsporogenesis in monocotyledons [J]. *Annals of Botany*, 84(4): 475-499.

Furness CA, Rudall PJ, Sampson FB, 2002. Evolution of microsporogenesis in angiosperms [J]. *International Journal of Plant Sciences*, 163(2): 235-260.

Guignard L, 1882. Recherches sur le développement de l'anthère et du pollen chez les Orchidées [J]. *Annales des Sciences Naturelles-Botanique*, 6. sér, 14: 26-45.

Ghimire B, Son SW, Kim JH, et al., 2020. Gametophyte and embryonic ontogeny: understanding the reproductive calendar of *Cypripedium japonicum* Thunb. (Cypripedoideae, Orchidaceae), a lady's slipper orchid endemic to East Asia [J]. *BMC Plant Biology*, 20(20): 426.

Harder LD, Johnson SD, 2008. Function and evolution of aggregated pollen in angiosperms [J]. *International Journal of Plant Sciences*, 169(1): 59-78.

Johri BM, Ambegaokar KB, Srivastava PS, 1992. *Comparative Embryology of Angiosperms* [M]. Springer Berlin Heidelberg.

Johnson SD, Edwards TJ, 2000. The structure and function of orchid pollinaria [J]. *Plant Systematics and Evolution*, 222: 243-269.

Karasawa KA, 1980. Karyomorphological studies on two species of *Paphiopedilum* [J]. *Bulletin of the Hiroshima Botanical Garden*, 3: 69-74.

Karasawa KA, 1982. Revision of the genus *Paphiopedilum* (Orchidaceae) [J]. *Bulletin of the Hiroshima Botanical Garden*, 5: 1-69.

Kocyan A, Endress PK, 2001. Floral structure and development and systematic aspects of some 'lower' Asparagales [J]. *Plant Systematics and Evolution*, 229(3): 187-216.

Kant R, Hossain MM, 2010. Development of pollinium in *Malaxis muscifera* (Lindl.) Kuntze [J]. *Bangladesh Journal of Botany*, 39(2): 193-198.

Kant R, Bhanwra RK, 2010. Development of anther in relation to sectile pollinium in *Zeuxine strateumatica* (Lindl.) Schltr [J]. *Journal of Biological and Life Sciences*, 1(1): 5-12.

Kant R, 2011. Development of monads and associated changes in anther in *Cypripedium cordigerum* D. Don: a rare orchid [J]. *Vegetos*, 24(1): 103-110.

Kant R, Hossain MM, Attri LK, 2013. Pollinium development in *Spiranthes sinensis* (Pers.) Ames. and *Cymbidium pendulum* SW: a comparative study [J]. *Bangladesh Journal of Botany*, 42(2): 179-186.

Kant R, Goel S, 2014. Development of anther in *Goodyera repens* (L.) R. Br.: a massulate orchid [J]. *Vegetos*, 26(1): 9-18.

Kant R, 2019. Development of pollinium and associated changes in anther of *Calanthe tricarinata* Lindl., an epidendroid orchid [J]. *Taiwania*, 64(3): 269-279.

Kant R, 2023. Development of anther and sectile pollinium in *Herminium lanceum* (Thunb. ex Sw.) Vuijk—a massulate orchid [J]. *Israel Journal of Plant Sciences*, 1(aop): 1-8.

Li P, Luo YB, Deng YX, Kou Y, 2008. Pollination of the lady's slipper *Cypripedium henryi* Rolfe (Orchidaceae) [J]. *Botanical Journal of the Linnean Society*, 156: 491-499.

Maheshwari P, Narayanaswami S, 1952. Embryological studies on *Spiranthes australis* Lindl [J]. *Botanical Journal of the Linnean Society*, 53(355): 474-486.

Poddubnaya-Arnoldi VA, 1960. Study of fertilization in the living material of some angiosperms [J]. *Phytomorphology*, 10(1): 185-198.

Pridgeon AM, Cribb PJ, Chase MW, et al., 1999. *Genera Orchidacearum: Volume 1: Apostasioideae and Cypripedioideae* [M]. Oxford University Press on Demand.

Rao PRM, Sood SK, 1979. Life history of *Satyrium nepalense* (Orchidaceae) [J]. *Norwegian Journal of Botany*.

Swamy BGL, 1946. The embryology of *Zeuxine sulcata* Lindl [J]. *New Phytologist*, 45(1): 132-136.

Swamy BGL, 1949. Embryological studies in Orchidaceae, I: Gametophytes [J]. *American Midland Naturalist*, 41: 184-201.

Sood SK, 1986. Gametogenesis, integuments initiation and embryogeny in three species of *Habenaria* (Orchidaceae, Orchideae) [J]. *Proceedings of the National Academy of Sciences, USA*, 96(6): 487-494.

Sood SK, Rao PRM, 1986. Development of male and female gametophytes in *Herminium angustifolium* (Orchidaceae) [J]. *Phytomorphology.*

Sood SK, Rao PRM, 1988. Studies in the embryology of the diandrous orchid *Cypripedium cordigerum* (Cypripedieae, Orchidaceae) [J]. *Plant Systematics and Evolution*, 160(3-4): 159-168.

Sood SK, 1988. Development of gametophytes, embryogeny and pericarp in *Goodyera repens* (Orchidaceae, Neottieae) [J]. *Proceedings of the National Academy of Sciences, USA*, 98(2): 149-156.

Sood SK, 1989. Embryology and systematic position of *Liparis* (Orchidaceae) [J]. *Plant Systematics and Evolution*, (166): 1-9.

Sood SK, 1997. Gametogenesis, seed development and pericarp in *Epipactis latifolia* L [J]. *Journal of the Indian Botanical Society*, 76: 11-15.

Singer RB, Barbara G, Cross H, et al., 2008. The use of orchid pollinia or pollinaria for taxonomic identification [J]. *Selbyana*, 29(1): 6-19.

Sriyot N, Thammathaworn A, Theerakulpisut P, 2015. Embryology of *Spathoglottis plicata* Blume: a reinvestigation and additional data [J]. *Tropical Natural History*, 15(2): 97-115.

Tsai CC, Liao PC, Ko YZ, et al., 2020. Phylogeny and historical biogeography of *Paphiopedilum* Pfitzer (Orchidaceae) based on nuclear and plastid DNA [J]. *Frontiers in Plant Science*, 11: 126.

Vij SP, Sharma M, 1987. Embryological studies in Orchidaceae. V. *Epipactis* Adams [J]. *Phytomorphology.*

Valencia-Nieto B, Sosa V, Marquez-Guzman J, 2016. Late stages of anther development and anther attributes in *Microepidendrum* differ from other genera of Epidendrum alliance (Orchidaceae) [J]. *Flora*, 218: 35-43.

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