Pollinium Development and Taxonomic Significance of Arundina graminifolia, a Traditional Dai Medicine (Postprint)

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Abstract

The development of pollinia in the monotypic orchid genus Arundina was observed using paraffin sectioning technique, encompassing floral morphological and anatomical characteristics, the mechanism of formation of eight pollinia, anther wall development patterns, microsporogenesis, and male gametophyte development, thereby providing embryological evidence for the complex phylogenetic relationships of this genus. The results are as follows. Mature anthers possess two locules, each containing four golden pollinia clustered together and covered by a white anther cap. In early anther primordia, a pair of juxtaposed lateral locules differentiated; within each locule, a pair of intersecting sterile septal tissues differentiated from the central microsporangium, partitioning it into four secondary microsporangia that ultimately developed into four pollinia. At anther maturity, incomplete degradation of the septal tissues between pollinia within each locule resulted in the four pollinia cohering into a cluster at the dehiscence site. The well-developed anther wall comprises 6–7 layers, from outermost to innermost: epidermis, 3–4 layers of endothecium, middle layer, and binucleate tapetum, conforming to the developmental pattern of a multilayered anther wall. Upon anther maturation, the epidermis degenerated, fibrous thickening occurred in the 3–4 endothecial layers, and the middle layer and tapetum underwent complete degradation. Microspore mother cells produced microspore tetrads arranged in tetrahedral, bilaterally symmetrical, and decussate configurations through simultaneous cytokinesis. The microspore tetrads remained enclosed within the same callose mass to complete male gametophyte development, forming 2-celled tetrads. These tetrads were arranged in pairs, either loosely or tightly, constituting mealy pollinia. Building upon previous research, this study confirms, supplements, and analyzes the developmental characteristics of pollinia in Arundina, providing embryological evidence for the phylogenetic relationships of this genus.

Full Text

Pollinia Development in *Arundina graminifolia* (Orchidaceae) with Taxonomic Implications

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Abstract

This study investigated the developmental process of pollinia in the monotypic genus *Arundina* (Orchidaceae) using paraffin sectioning techniques, examining floral morphological characteristics, the formation mechanism of eight pollinia, anther wall development patterns, microsporogenesis, and male gametophyte development to provide embryological evidence for the genus' s complex phylogenetic relationships. The mature anther contains two thecae, each housing four golden pollinia clusters enveloped by a white anther cap. During early development, the anther primordium differentiates into a pair of juxtaposed lateral thecae. In each theca, a pair of intersecting sterile septa differentiates within the central microsporangium, dividing it into four secondary microsporangia that ultimately develop into four pollinia. At anthesis, incomplete degradation of the septal tissue between pollinia within each theca causes the four pollinia to cohere into a cluster at the dehiscence site. The well-developed anther wall comprises 6–7 layers, from outermost to innermost: epidermis, 3–4 layers of endothecium, middle layer, and binucleate tapetum, conforming to the massive type development pattern. At maturity, the epidermis degenerates, fibrous thickenings develop in 3–4 endothecial layers, and the middle layer and tapetum degrade completely. Microspore mother cells undergo simultaneous cytokinesis to produce microspore tetrads arranged in tetrahedral, isobilateral, and decussate patterns. The tetrads remain within a common callose wall to complete male gametophyte development, forming 2-celled pollen tetrads. These tetrads associate either loosely or tightly in pairs to constitute granular pollinia. Building upon previous research, this study confirms, supplements, and analyzes the pollinia development characteristics of *Arundina*, providing embryological evidence for the genus' s phylogenetic relationships.

Keywords: *Arundina graminifolia*, pollinia development, microsporogenesis, massive type anther wall, taxonomy

Introduction

Arundina Blume is a rare "weed orchid" in the subfamily Epidendroideae (Orchidaceae), widely distributed across tropical and subtropical Asia at elevations of 400–2800 m in grasslands, stream valleys, shrub understories, and forests. In China, the genus occurs in southern tropical and subtropical regions. Taxonomically, *Arundina* has been variously treated as comprising one species, two species, or two subspecies, a controversy attributed to its broad distribution, diverse habitats, and considerable variation in leaf morphology and flower color. Subsequent anatomical and molecular evidence has confirmed this morphological variability. Chinese botanists initially cautiously recognized 1–2 species in the genus before definitively concluding that only one highly variable species occurs in China. This study adopts the monotypic genus concept, considering *Arundina graminifolia* as a broadly defined species adapted to different habitats.

However, the systematic position and phylogenetic relationships of *Arundina* at the subtribal level remain unclear. The genus has been assigned to various subtribes including Thuniinae, Coelogyninae, and Arundininae. Traditional morphological classification placed it in Thuniinae, later moving it to Arundininae, where it was considered closely related to *Thunia*, *Bletilla*, and several small extralimital genera (*Bromheadia*, *Claderia*, *Dilochia*, *Trichoma*). Conversely, molecular systematics has positioned it within Coelogyninae or Arethusinae, suggesting affinities with *Coelogyne*, *Pleione*, *Dendrochilum*, and two small extralimital genera (*Dilochia* and *Glomera*). Despite molecular systematics having clearly divided Orchidaceae into five subfamilies, the delimitation of tribes and subtribes within subfamilies and their phylogenetic relationships remain confused, requiring additional evidence for clarification—*Arundina* being no exception.

Orchidaceae exhibits rich diversity and specialization in anther developmental characteristics, with embryological features such as pollinium number and formation mechanism, anther wall development patterns, microsporogenesis, and male gametophyte formation showing significant variation among subfamilies, subtribes, and genera, thus holding important taxonomic significance. Early studies examined floral and seed development in *Arundina graminifolia* and the related genus *Bromheadia*, revealing anther walls composed of 5–6 cell layers, glandular tapetum, and microspore tetrad arrangements. Notably, *Arundina* possesses binucleate tapetal cells whereas *Bromheadia* has uninucleate cells. However, these previous works lacked detailed descriptions of crucial embryological features such as pollinium formation mechanisms, anther wall development types, cytokinesis patterns during microspore mother cell meiosis, and pollen abortion phenomena.

Due to its ornamental and medicinal value, research on *Arundina* has primarily focused on seed germination, protocorm induction, cytology, reproductive ecology, and chemical composition analysis. With increasing human disturbance, *Arundina* has been listed as a rare and endangered species in its native Asian range, yet has become a dominant naturalized species in Central and South America and Pacific islands, regarded as a potentially invasive alien species. Therefore, this study investigates pollinia development in *Arundina* using paraffin sectioning to provide embryological evidence for understanding its systematic relationships and reproductive biological mechanisms relevant to in-situ conservation and invasion biology.

Materials and Methods

Experimental materials were collected from cultivated plants at the South Medicine Garden, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, which flower normally. Flower buds and open flowers at different developmental stages were collected during 2017 and 2018. Fresh material was observed and photographed under a Leica stereomicroscope to document mature floral morphological characteristics. Additional fresh material was fixed in 50% ethanol for 24 hours, then transferred to 70% ethanol for storage at room temperature. After removing excess sepals and petals under a dissecting microscope, only the columns were retained for paraffin sectioning. Samples were dehydrated through an ethanol series, cleared in xylene, and embedded in paraffin. Sections of 5–7 m thickness were cut using a Leica RM2235 microtome, stained with iron-vanadium-hematoxylin, mounted in neutral resin, and observed under a Nikon E600 optical microscope. Images were compiled and analyzed using Adobe Photoshop 7.0.

Results

2.1 Floral Morphological Characteristics

The racemose inflorescence is terminal, bearing 2–10 flowers with only one opening at a time. Flowers are large, pink or purplish, with three narrowly elliptic-lanceolate sepals and two petals that are ovate-elliptic, subequal to the sepals but distinctly broader, attached to the column base. The labellum is oblong-ovate, basally incurved and embracing the column, with irregular folds at the apex [FIGURE:1,2]. The column measures 1.5–2 cm long, pale purple, with wing-like extensions on both sides at the apex that continue onto the anther dorsum. The column lacks a distinct foot, but possesses a well-developed white rostellum [FIGURE:3-5]. The anther comprises two thecae [FIGURE:6,9], each containing four golden pollinia that cohere into a cluster. The pollinia are granular [FIGURE:7,8]. The white anther cap is helmet-shaped, with a groove at the top resulting from anther dehiscence, a smooth dorsal surface, shallow teeth along the bottom margin, and conspicuous anther wall remnants on the inner wall [FIGURE:9-11].

2.2 Formation Mechanism of Eight Pollinia

The early anther primordium consists of actively dividing meristematic cells. As the central connective tissue differentiates, a pair of juxtaposed lateral thecae forms, bending toward the labellum and enclosing the differentiated archesporial tissue. Subsequently, archesporial cells in different regions follow three developmental pathways: central archesporial cells differentiate into sterile septal tissue; a subepidermal layer of archesporial cells functions as primary parietal cells; and remaining archesporial cells develop directly into microspore mother cells [FIGURE:1,2]. During microspore mother cell meiosis, each theca differentiates two intersecting sterile septa along the polar axis [FIGURE:2-8] and equatorial axis [FIGURE:9,10], dividing the microsporangium into four secondary microsporangia in a cruciate pattern that develop into four pollinia at maturity. Throughout pollinium development, the four secondary microsporangia in each theca remain tightly connected to the anther wall before microspore mother cell meiosis [FIGURE:1-4]. At the microspore tetrad stage, obvious gaps appear between secondary microsporangia and the anther wall as the middle layer and tapetum degrade [FIGURE:5,6]. Concurrently, septal tissue within each theca undergoes differential degradation, with tissue near the dehiscence site degrading more rapidly and completely than tissue near the connective [FIGURE:6-8], resulting in partial fusion and adhesion of the four pollinia within each theca [Figure 8: see original paper] to form a cluster of four [FIGURE:1:7,8].

2.3 Anther Wall Development Pattern

The early anther primordium consists of an outer layer of regularly arranged epidermal cells and inner deeply stained archesporial cells. A subepidermal layer of archesporial cells then functions as primary parietal cells, dividing periclinally into outer secondary parietal cells and inner secondary parietal cells [Figure 1: see original paper]. The former directly develops into the first endothecial layer, while the latter undergoes several periclinal divisions to produce 2–3 additional endothecial layers, a middle layer, and tapetum [FIGURE:2,3], completing anther wall development by the microspore mother cell stage [Figure 4: see original paper]. The mature anther wall comprises 6–7 layers [FIGURE:4,6]: epidermis, 3–4 endothecial layers, middle layer, and tapetum, conforming to the massive type development pattern [Figure 9: see original paper]. During microspore mother cell meiosis, the middle layer and tapetum begin degenerating, creating obvious gaps between sporangia and anther wall [FIGURE:6-9]. Tapetal cells are small, round, and binucleate [FIGURE:4,5], representing the glandular tapetum type. Epidermal cells also degrade, becoming irregularly toothed [Figure 6: see original paper] and gradually reducing to remnants by the 2-celled pollen stage [FIGURE:7-12]. During male gametophyte formation, 3–4 endothecial layers enlarge, become broadly ovate, and develop uneven fibrous thickenings. By the 2-celled pollen stage [FIGURE:7-12], only 1–2 endothecial layers remain, with ruptures appearing at the theca apex [Figure 12: see original paper] in preparation for pollen release.

2.4 Microsporogenesis and Male Gametophyte Development

Microspore mother cells are irregularly polygonal with enlarged cell outlines, high nuclear-cytoplasmic ratios, and compact arrangement [Figure 1: see original paper]. Meiosis I produces two daughter nuclei without cell plate formation [FIGURE:2,3], and meiosis II completes within the same cell wall, yielding pre-tetrads with indistinct cell walls [Figure 4: see original paper]. The four microspores then develop distinct walls to form callose-enclosed microspore tetrads [FIGURE:5,6], demonstrating simultaneous cytokinesis [Davis, 1966]. During male gametophyte development, microspores are not released individually from callose but complete mitosis as tetrads, forming a small generative cell and large vegetative cell to produce 2-celled pollen tetrads [FIGURE:8,9]. These tetrads associate in pairs, either tightly or loosely, forming granular pollinia [FIGURE:1:7,8]. Some peripheral microspore tetrads fail to undergo mitosis, exhibiting pollen abortion [Figure 12: see original paper]. Microspore tetrads display diverse arrangements, primarily tetrahedral [FIGURE:6-9] and decussate [Figure 11: see original paper], with rare isobilateral patterns [Figure 10: see original paper].

Discussion

3.1 Confirmation, Clarification, and Supplementation of Embryological Features in *Arundina*

Comparative analysis with previous studies on *Arundina* anther development [Rao, 1967] confirms several stable characteristics: two anther thecae, 6–7-layered anther wall, binucleate secretory tapetum, diverse microspore tetrad arrangements (tetrahedral, isobilateral, decussate), and 2-celled pollen tetrads. This study clarifies anther wall composition, identifying 6–7 layers including 3–4 endothecial layers and one middle layer, contrasting with previous reports of 5–6 layers with 1–2 endothecial layers and 2–3 middle layers. This discrepancy stems from differential identification of endothecial versus middle layer cells. Based on the characteristic large, broadly ovate shape and later fibrous thickening of endothecial cells versus the slender profile of middle layer cells [Davis, 1966; Johri et al., 1992; Hu, 1982, 2005], we interpret the 2–3 subepidermal layers of similar, broadly ovate cells as endothecium. Furthermore, this study systematically reveals the formation mechanism of eight pollinia, massive anther wall development, and simultaneous cytokinesis, analyzing their taxonomic significance through comparative evaluation with other orchids.

3.2 Taxonomic Significance of the Eight-Pollinia Formation Mechanism in *Arundina*

Our results confirm that the eight-pollinia condition in Epidendroideae arises from the differentiation of two intersecting sterile septa within the central region of each theca during early anther development [Freudenstein & Rasmussen, 1996]. Over 90% of orchid species possess pollinia of varying numbers and forms,

concentrated in Epidendroideae and Orchidoideae [Cameron et al., 1999; Chase et al., 2003, 2015]. Developmental anatomical studies demonstrate that different pollinium numbers (2, 4, 6, 8) result from varying numbers and orientations of sterile septa that differentiate within the paired lateral thecae of early anther primordia and degrade at maturity to create spaces [Freudenstein & Rasmussen, 1996]. This mechanism has been confirmed and supplemented by subsequent research. For example, *Cymbidium* with two deeply cleft pollinium pairs exhibits incomplete septal penetration of microsporangia [Bhanwra et al., 2006; Kant & Goel, 2013], while *Malaxis* with four pollinia develops from complete septal penetration and degradation [Kant & Hossain, 2010; Gurudeva, 2015]. Subtribe Aeridinae shows 2–4 pollinium clusters with varying degrees of clefting, entire margins, or stickiness resulting from different septal orientations and penetration depths [Zhang et al., 2019; Li et al., 2019]. However, few developmental data exist for eight-pollinia taxa in Epidendroideae, with two hypothesized formation pathways [Freudenstein & Rasmussen, 1996]. The first involves differentiation of longitudinal and transverse septa in each theca during early development, producing four short, club-shaped pollinia, as observed in *Calanthe rubens*, *Eria javanica*, and *Thelasis pygmaea*. The second hypothetical pathway involves differentiation of two cross-shaped longitudinal septa along the polar axis, potentially producing four pairs of long, club-shaped pollinia, though lacking anatomical confirmation. Our results confirm that *Arundina* follows the first pathway.

Additionally, we reveal that the clustered arrangement of four pollinia in *Arundina* results from more rapid and complete degradation of septal tissue near the theca dehiscence site than near the connective at anthesis, a phenomenon not previously reported [Freudenstein & Rasmussen, 1996]. Among eight-pollinia taxa in Epidendroideae, particularly within subtribes related to *Arundina* (Bletillinae, Bletiinae, Anthogoniinae), descriptions of pollinium clusters remain ambiguous regarding presence/absence of caudicles or sticky substances [Chen et al., 1999]. For instance, *Laelia* with eight pollinia may be described as either eight independent pollinia or two clusters of four deeply cleft pollinia, requiring internal anatomical examination for accurate determination [Freudenstein & Rasmussen, 1996]. In *Arundina*, the four-pollinia cluster forms not through deep clefting of microsporangial tissue but via secondary fusion at the base after septal segmentation, representing partial degradation and refusion of the four secondary microsporangia. Thus, comparative developmental morphological studies of pollinium formation mechanisms across orchids provide substantial taxonomic significance for delimiting tribes, subtribes, and genera.

3.3 Taxonomic Significance of the Massive Anther Wall Development Pattern in *Arundina*

In angiosperms, anther wall layer number and origin are stable at suprageneric taxonomic levels, holding important systematic value [Davis, 1966; Johri et al.,

1992]. Based on layer number and middle layer origin, four basic types are recognized: the basic type with five layers including two middle layers; monocot and dicot types with four layers but different middle layer origins; and the reduced type with only three layers lacking a middle layer [Davis, 1966; Johri et al., 1992; Hu, 1982, 2005]. The dicot type occurs in approximately 80% of studied angiosperms, predominantly in dicots, while the monocot type appears in most monocots and occasionally in some dicots such as *Sladenia* [Li et al., 2003]. The basic and reduced types are rare, occurring only in select taxa.

The 6–7-layered anther wall in *Arundina* represents the massive type, confirming and supplementing that Orchidaceae exhibits two anther wall development patterns: monocot and massive types. Traditionally, orchid anther walls have been described as consistently monocot-type with four cell layers, considered a stable familial characteristic [Davis, 1966; Johri et al., 1992; Hu, 1982, 2005]. This pattern occurs widely across subfamilies, including *Goodyera* in Orchidoideae [Sood, 1988; Kant & Goel, 2013], *Habenaria* [Sood, 1986], and Epidendroideae genera such as *Microstylis* [Sood, 1985, 1986], *Malaxis* [Sood, 1992; Kant & Hossain, 2010; Gurudeva, 2015], and *Pennilabium yunnanense* [Li et al., 2019]. However, some orchids possess 5–7+ layered anther walls, termed massive type [Sood, 1989], unique to Orchidaceae. Early descriptions of massive anther walls appeared in Epidendroideae genera *Dendrobium* and *Vanda* [Swamy, 1949], *Arundina* [Rao, 1965], and the related genus *Bromheadia* [Jeyanayaghy & Rao, 1966], and later in Cypripedioideae (*Cypripedium cordigerum*) [Sood & Rao, 1988; Kant, 2011]. Following its initial naming and description in *Liparis* [Sood, 1989], massive anther walls have been reported in Epidendroideae genera *Cymbidium* [Kant et al., 2013], *Vanda* [Zhang et al., 2019], and *Spathoglottis* [Sriyot et al., 2015]. Although both monocot and massive types occur across Cypripedioideae, Epidendroideae, and Orchidoideae, each genus maintains a stable anther wall type. Continued accumulation of new data will enhance understanding of the systematic significance of anther wall development patterns in Orchidaceae.

3.4 Systematic Significance of Simultaneous Cytokinesis and Microspore Tetrad Arrangement in *Arundina*

Arundina exhibits simultaneous cytokinesis producing tetrahedral, isobilateral, and decussate microspore tetrads, similar to most studied orchids [Swamy, 1949; Johri et al., 1992; Sriyot et al., 2015; Zhang et al., 2019; Li et al., 2019]. Three viewpoints address the systematic significance of cytokinesis patterns and tetrad arrangements in angiosperms. First, early research recognized two cytokinesis types—simultaneous and successive—as characteristic of dicots and monocots, respectively [Johri et al., 1992]. However, although Orchidaceae represents typical monocots, most studied orchids exhibit simultaneous cytokinesis [Swamy, 1949; Johri et al., 1992; Liang, 1981; Xu & Yang, 1986; Sood, 1985, 1986, 1988; Sood & Rao, 1986; Sriyot et al., 2015], with only a few genera such as *Ophrys* [Aybeke, 2012] and *Vanda* [Zhang et al., 2019] showing successive cytokinesis. Thus, orchid cytokinesis patterns hold significant evolutionary importance warranting

further investigation.

Second, some studies suggest correlations between cytokinesis type and tetrad arrangement in basal angiosperms and monocots [Nadot et al., 2008]. Successive cytokinesis reportedly produces only tetragonal (or decussate, T-shaped, linear) tetrads, whereas simultaneous cytokinesis yields more diverse arrangements including tetragonal, tetrahedral, and rhomboidal types [Nadot et al., 2008]. Our results and existing orchid data do not support this correlation. Third, the relationship between cytokinesis pattern and pollen aperture type requires further exploration. While no direct correlation exists in basal angiosperms and monocots [Nadot et al., 2008], studies on *Arabidopsis* indicate that successive cytokinesis influences aperture patterning [Albert et al., 2011]. For most orchids lacking apertures [Johri et al., 1992], the relationship between cytokinesis and pollen morphology merits further investigation. In summary, the embryological features exhibited during *Arundina* pollinia development contribute importantly to understanding anther developmental diversity in Orchidaceae.

3.5 Comparative Anther Development Between *Arundina* and the Related Genus *Bromheadia*

Potential relatives of *Arundina* include *Thunia*, *Bletilla*, *Coelogyne*, and *Pleione*, but embryological data are lacking for most, permitting comparison only with the Malaysian genus *Bromheadia* [Jeyanayaghy & Rao, 1966]. Both genera share several anther developmental features consistent with most orchids [Johri et al., 1992]: simultaneous cytokinesis, glandular tapetum, fibrous endothecial thickenings, and 2-celled pollen tetrads. These similarities, particularly the massive anther wall, support their status as related genera. However, *Arundina* and *Bromheadia* differ markedly in tapetal cell nuclear number and tetrad arrangement. *Arundina* possesses the rare binucleate tapetum for Orchidaceae and shows tetrahedral, isobilateral, and decussate tetrad arrangements, whereas *Bromheadia* has the common uninucleate tapetum and tetrahedral, isobilateral, and linear tetrads. Thus, available embryological data support a phylogenetic relationship between *Arundina* and *Bromheadia*, while highlighting distinct characteristics.

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