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Anatomical features of pollinia and caudicle in *Epidendrum* (Orchidaceae; Epidendroideae)

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Abstract
The structural diversity of pollinia is one of the most relevant characters for the species classification in Orchidaceae. In this study, we analyzed the development of the pollinia and caudicle in *Epidendrum* species belonging to the subgenus *Amphyglottium*, one of the groups with the most variable morphology of the genus. These species have different pollination strategies (e.g., presence vs. absence of nectar) and grow in different types of habitat. Floral buds and flowers at anthesis were collected, and the usual methodology for performing anatomical studies, scanning microscopy, and confocal microscopy following established protocols. Our investigations revealed that the studied species have bithecal anthers and each theca has two pollinia and two caudicles. No interspecific variation was observed regarding the formation of the male gametophyte, indicating that is a stable character for the genus: the archesporial cells undergo a meiotic process that originates microspores, which remain united in tetrad and pass through asymmetric mitosis to form viable pollen grains. The caudicle is of the appendicular type and shows the male gametophyte formation.

Keywords Microgametogenesis, Microsporogenesis · Orchid · Pollen grain

1 Introduction

Orchidaceae, one of the largest families of angiosperms, is recognized for its morphologically diverse and complex flowers (Dressler 1981; Chase et al. 2015). One of the diagnostic characteristics of the family is the presence of anthers that have pollen grains grouped in pollinia or in masses, which are removed as a single unit from the flower during the pollination process (Dressler 1981). Pollinia are considered a key innovation in Orchidaceae and may have played an important role in promoting the group's enormous radiation (Dressler 1993). According to Freudenstein and Rasmussen (1996), pollinium can be defined as a cohesive mass of pollen that is separated from other masses of pollen by sterile tissue and may be connected to others by extensions of modified tissue. Pollinia may be accompanied by accessory structures (e.g., caudicles, stipes, or viscidia), forming a unit called a pollinarium. The main function of these structures is adhering the pollinia to the body of the pollinator, thus facilitating the pollination process (Dressler 1993).

Variation in pollinia structure is one of the most important characters for the classification of species in the family. This diversity is present at different levels, such as the number of pollinia per anther, the nature of pollen aggregation, and differences in pollen wall structure (Freudenstein and Rasmussen 1996; Freudenstein et al. 2002; Pacini and Hesse 2002). Among the five currently recognized subfamilies, only Orchidoideae and Epidendroideae have authentic pollinia and pollinarium (Singer et al. 2008; Chase et al. 2015). Epidendroideae, the largest of the subfamilies, shows great diversification in the structure of the pollinia and pollinarium. The number of pollinia per anther varies between two and eight and is generally a uniform number within subtribes (Freudenstein and Rasmussen 1996). The texture of the pollinia also varies and can be granular, septate (subdivided into masses), or compact (high degree of cohesion) (Johnson and Edwards 2000).

Different pollinarium traits among the Epidendroideae have been analyzed, and the evolution of this structure has been evaluated in many previous studies (Dressler

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1993; Freudenstein and Rasmussen 1996, 1997; Johnson and Edwards 2000; Hidayat et al. 2006; Nieto and Damon 2008; Singer et al. 2008; Mosquera-Mosquera et al. 2019). However, information regarding pollinia development and accessory structures of pollinia has been recorded for only a few number of species. For *Epidendrum* L. (subtribe Laeliinae), the most diverse genus of the subfamily, records of male gametophyte development are restricted only to *E. ibaguense* H.B.K. and *E. scutella* Lindl. (Cocucci and Jensen 1969; Blackman and Yeung 1983a, b; Yeung 1987). *Epidendrum* presents wide morphological variation, especially in relation to habit, morphology, and flower color; consequently, several species complexes (Pinheiro et al. 2010). The genus also presents variation in reproductive strategies, with flowers that have nectar as a floral reward and others that simulate rewards, thereby being pollinated by deceit (Cardoso-Gustavson et al. 2018). In the present study, we selected six species of *Epidendrum* subg. *Amphyglottium* Lindl. that present two distinct pollination strategies: floral rewards in the form of nectar and rewardless species. Our aim were verify if pollinia development differ between species, and it can be used as a distinctive character for groups that present species with distinct pollination systems. The development of the pollinia and its accessory structures were described, contributing with information about the development of the male gametophyte in species with variations in reproductive strategies.

2 Material and methods

Species analyzed and study area Six species of *Epidendrum* that occur in different regions of Brazil and in distinct habitats were selected (Fig. 1a–f). *Epidendrum orchidiflorum* Salzm. occurs in dry vegetation communities that are found in coastal and *Caatinga* vegetation in northeastern Brazil (Pachon 2016). Among the species analyzed, only this species presents nectar as a floral reward while the others are rewardless species, presenting pollination by deceit (Cardoso-Gustavson et al. 2018). *Epidendrum denticulatum* Barb. Rodr. occurs in the *Cerrado* and coastal vegetation of southeastern Brazil (Pinheiro et al. 2015). *Epidendrum fulgens* Brongn and *E. puniceoluteum* F. Pinheiro & F. Barros occur in *restinga* vegetation, which is composed

Fig. 1 *Epidendrum* flowers. **a** *E. orchidiflorum*; **b** *E. denticulatum*; **c** *E. fulgens*; **d** *E. puniceolutum*; **e** *E. secundum*; **f** *E. xanthinum.* Scale bars: 1 cm

of a mosaic of different coastal plant communities along the Brazilian coast (Araujo 1992). *Epidendrum secundum* Jacq has a very wide distribution, occurring in mountains of the Brazilian plateau, the Serra do Mar, the Andes, and the Guiana Plateau (Hágsater and Soto-Arenas 2005). *Epidendrum xanthinum* Lindl. is restricted to high mountainous altitudes of southeastern Brazil, mainly in the Serra dos Órgãos and Cadeia do Espinhaço (Pinheiro et al. 2016). All species were collected and cultivated in the orchidarium of the Department of Plant Biology of the Universidade Estadual de Campinas, São Paulo.

Anatomical analyses To analyze the pollinia and accessory structures, floral buds and flowers in anthesis were collected. Samples were fixed in 10% neutral buffered formalin solution (Lillie 1965), dehydrated in increasing ethylic series, and infiltrated in hydroxyethylmethacrylate (Gerrits and Smid 1983). Subsequently, the samples were sectioned at 3µm thickness on a Leica RM2245 rotary microtome and stained with 0.05% toluidine blue in phosphate buffer pH 4.5, and mounted using water (Sakai 1973). The slides were analyzed under an Olympus BX50 optical microscope and photographed using an Olympus DP73 digital camera. Photomacrographs were obtained using a stereomicroscope Leica M80 coupled with a Leica DFC 295 digital camera using LAS software (version 4.1).

Scanning electron microscopy Botanical material was fixed in 10% neutral buffered formalin solution (Lillie 1965), dehydrated in increasing ethylic series and critical point dried under carbon dioxide (CO_2) in a Balzers model CPD 030 Critical Point Dryer. The material was then mounted on metal supports and coated with colloidal gold for 220 s on the Bal-Tec model SCD 050 equipment (Bozzola and Russel 1992). Analysis and electron micrograph recordings were performed using a LEO VP 435 scanning electron microscope at 20 kV.

Laser scanning confocal microscopy Thick sections of the samples, obtained using the rotary microtome as described above, were analyzed using a laser scanning confocal microscope (Leica TCS SPE) with a 405nm laser channel to excite the following fluorochromes: 0.5% aniline blue (Oparka and Read 1994), emitting at 490–520 nm; 0.01% auramine O (Heslop-Harrison 1977), emitting at 540–656 nm; 0.1% calcofluor white (O'Brien and McCully 1981), emitting at 480–560 nm; and 6-diamidino-2-phenylindole – DAPI (Coleman and Goff 1985), emitting at 401–445 nm to detect callose, phenolic compounds (sporopollenin), cellulose, and nuclear DNA, respectively. Images were captured by direct acquisition with Z-pitch ranging from 0.13 to 0.27 mm, which generated 30 to 40 optical sections in LAS AF Lite 2.6.0 software (Leica Microsystems).

3 Results

Pollinia development All species analyzed presents similar pollinia development. The anthers are bithecal and each theca has two pollinia and two caudicles (Figs. 2a–f and 3a). In the early stages, the anther primordium is composed of meristematic tissue (Fig. 3a). In young bud stamens, the anther wall consists of an epidermis, an endothecium, two middle layers, and one tapetum layer. In subsequent stages, the epidermis and endothecium are formed by periclinally flattened cells and remain intact throughout the development of the male gametophyte. Part of the middle layer can be compressed during anther development. The tapetum divides to form up to three layers (Fig. 3b). The tapetum is glandular and composed of cells with dense cytoplasm and evident nuclei.

In the young anther, the sporogenous cells differentiate into microspore mother cells (MMCs), which have an evident nucleus. At this stage of development, intine is deposited in the MMCs cell wall (Fig. 3c). The MMCs undergo the first stage of meiosis (Fig. 3c) resulting in a microspore dyad (Fig. 3d), and then, the cells of the dyad undergo the second stage of meiosis, originating a tetrad of microspores, with cell wall formation only at the end of the meiotic process (Fig. 3e–g). The tetrads remain united due to the presence of callose that surrounds them (Fig. 3f). Occasionally, incomplete cell walls are formed and remain until the end of male gametophyte development (Fig. 3h). The tetrads of the periphery of the pollinia are linear, while those of the central portion have a tetrahedral arrangement. At the end of the second meiotic stage, exine is deposited on the microspores cell walls, as well as of the pollinia periphery (Fig. 3e, g).

The microspores then undergo an asymmetric mitotic division and, after cytokinesis, originate the male gametophyte (Fig. 3g, h). The male gametophyte is formed by a larger cell, the vegetative cell, and a smaller cell, the generative cell in a parietal position (Fig. 3h). At this stage of development, callose is no longer detected, and only sporopollenin can be observed occurring unevenly, being thicker in the male gametophyte of the pollinia periphery (Figs. 3i, j). The generative cell migrates into the vegetative cell's cytoplasm and starts to present a condensed nucleus (Fig. 3k, l). The bicellular male gametophyte remain united in a tetrad until the time of pollinia dispersal by the pollinator.

Caudicle development All species analyzed presents similar caudicle development. The caudicle is visible from the first stages of anther development. In its early stage, it is represented by a mass of small meristematic cells, situated just below the epidermis of the microsporangium (Figs. 3a

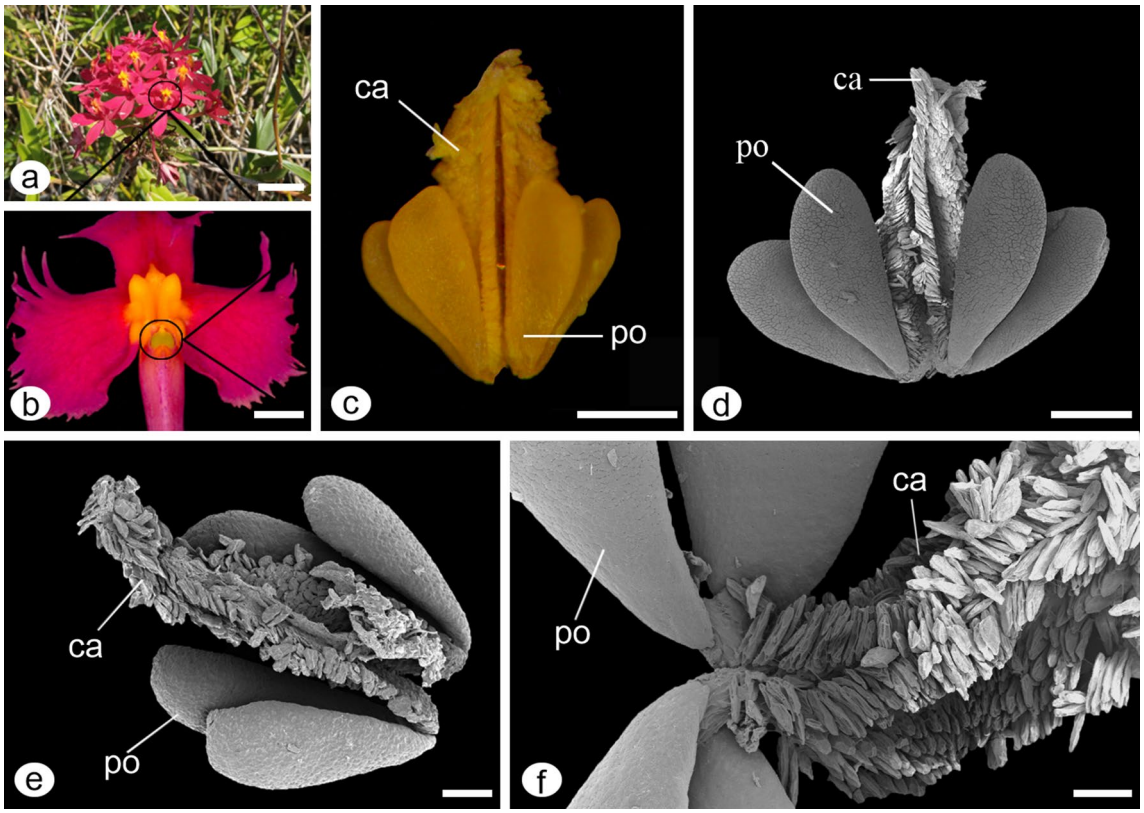

Fig. 2 Pollinia and caudicle structure in *Epidendrum* species. a-d E. *puniceolutum* **a-d** *E. puniceolutum*; **e** *E. xanthinum*; **f** *E. fulgens*. **a** Flowers of *E. puniceolutum*; **b** Detail of the labellum of *E. puniceolutum;* **c** Detail of the pollinia and caudicle; **d-f** Scanning electron micrograph showing details of the pollinia and caudicle; ca = caudicle; po = pollinia. Scale bars: *a* = 1 cm; *b* = 3 mm; *c* = 0,5 mm; *d* = 500 µm; *e*, *f* = 100 µm

and 4a). These cells are small and have thin cell walls with a large nucleus located in the center (Fig. 4a, b). Part of the caudicle's small cells differentiate, increase considerably in size, and start to present a cell wall with greater cellulose deposition, while the other parts remain undifferentiated with cells similar to those of the tapetum (Fig. 4b–d).

At the next stage of development, while sporogenesis occurs in the sporogenous tissue of the pollinia, changes are also observed in the caudicle (Fig. 4d). The caudicle cells, which have increased in size, undergo the first stage of meiosis, giving rise to a dyad (Fig. 4e, f). The cells of the dyad then undergo second stage of meiosis, giving rise to a tetrad, which may have different formats (Fig. 4g, h). During the meiotic division, intine is deposited in the caudicle cells walls. (Fig. 4e). Soon after the formation of the tetrad, a mitotic cell division is observed, similar to that described for male gametophyte (Fig. 4i, j). After this stage, it is observed that the cells present a thick cell wall due to the exine deposition around each cell of the caudicle (Fig. 4i, k). These cells remain united until pollinia dispersal by the pollinator.

4 Discussion

Herein, we describe male gametophyte formation and caudicle development in six species of *Epidendrum* that present two different pollination strategies. The rewarding species present nectar, while rewardless species present pollination by deceit. The formation of the male gametophyte is similar in the species studied independently of the reproductive strategy: the archesporial cells undergo a meiotic process that originates microspores, which remain united in tetrad then undergo mitosis, originating viable male gametophyte. The male gametophyte remain grouped in tetrads, constituting a unit called pollinium. The development of the caudicle occurs concomitantly with the development of the pollinia. Two cell types are observed in the structure: cells that differentiate and form male gametophyte and cells that remain undifferentiated, exhibiting characteristics of tapetum cells.

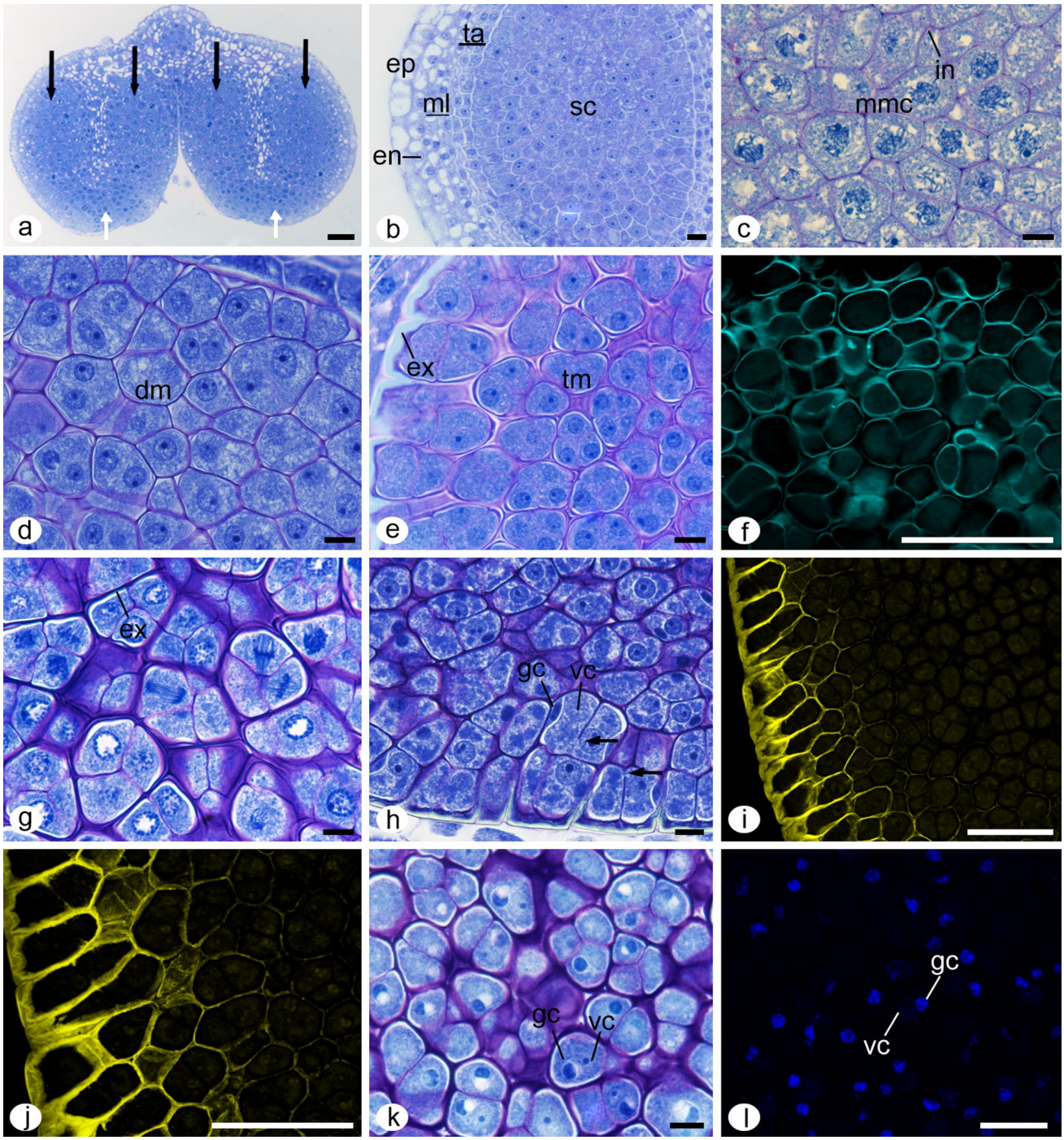

Fig. 3 Longitudinal sections of pollinia and caudicle in *Epidendrum* species. **a, c** *E. denticulatum*; **b, d** *E. puniceolutum*; **e** *E. xanthinum*; **f, i, j, l** *E. fulgens*; **g, h** *E. orchidiflorum*; **k** *E. secundum*. **a** Overview of the bitecae anther. Black arrows indicate the microsporangia and white arrows indicate the caudicle; **b** Anther wall formed by epidermis, endothecium, middle layer and tapetum. The pollinia presents sporogenous cells; **c** Microspore mother cells; **d** Dyad of microspores; **e** Tetrad of microspores; **f** Callose deposition in microspores cell wall evidenced by aniline blue; **g** Microspores in mitotic cycle; **h** Male gametophyte showing vegetative cell and generative cell. Black arrows indicate incomplete cell wall; **i** Unequal deposition of sporopollenin on the male gametophyte evidenced by auramine; **j** Detail of sporopollenin deposition in the male gametophyte on the periphery of the pollinia evidenced by auramine; **k** Male gametophyte with vegetative cell and generative cell; **l** Vegetative cell and generative cell evidenced by DAPI. **f, i, j, l** Laser scanning confocal microscopy. dm = dyads of microspores e = epidermis; en = endothecium; ex = exine; gc = generative cell; in = intine; ml = middle layer; mmc = microspore mother cell; sc = sporogenic cells; ta = tapetum; tm = tetrad of microspores; vc = vegetative cell. Scale bars: *a*, *f*, *i*, *j* = 50 µm; *b*, *l* = 20 µm; *c*, d, *e*, *g*, *h*, *k* = 100 µm

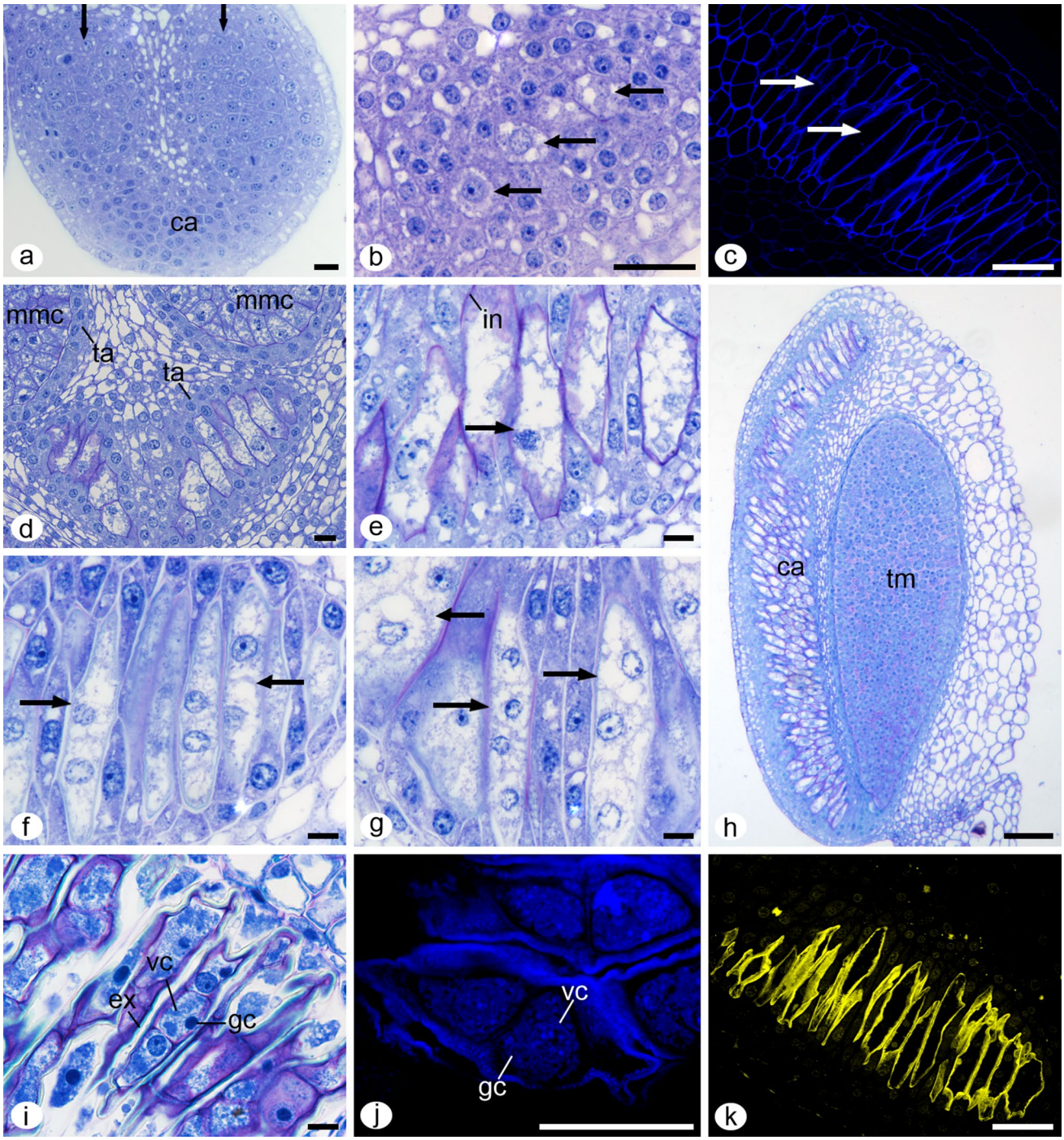

Fig. 4 Longitudinal and transverse sections of pollinia and caudicle in *Epidendrum* species. **a, i** *E. xanthinum*; **b** *E. denticulatum*; **c, j, k** *E. fulgens*; **d, f** *E. puniceolutum*; **e** *E. orchidiflorum*; **h** *E. secundum*. **a** Detail of one of the thecae of the anther. Black arrows indicate the microsporangia; **b** Caudicle presenting two types of cells. Black arrows indicate differentiating cells; **c** Cell walls of the caudicle evidenced by calcofluor; **d** Caudicle cells and sporangium cells of pollinia in meiosis; **e** Caudicle cell in meiosis indicated by the black arrow; **f** Dyad of cells in the caudicle indicated by the black arrow; **g** Tetrad of cells in the caudicle indicated by the black arrow; **h** Tetrad of cells in caudicle and tetrad of microspores in pollinia; **i** Pollen grains showing two cells; **j** Vegetative cell and generative cell evidenced by DAPI; **k** Deposition of sporopollenin in pollen grain evidenced by auramine. **c, j, k** Laser scanning confocal microscopy. ca = caudicle; ex = exine; gc = generative cell; in = intine; mmc = microspore mother cell; ta = tapetum; tm = tetrad of microspores; vc = vegetative cell. Scale bars: *a*, *d*, *j* = 20 µm; *b*, *e*, *f*, *g*, *i* = 10 µm; *c*, *k* = 50 µm; *h* = 100 µm

Pollinia development The species analyzed present an anther wall structure formed by six or seven layers: the epidermis, the endothecium, two middle layers, and the tapetum, which has two or three layers. In general, in Orchidaceae, the anther wall is composed of four to five layers, and some species with only a single middle layer have been reported in species of the genera *Paphiopedilum* Pfitzer, *Vanda* Jones ex R. Br., *Epipactis* Seg. and *Habenaria* Willd. (Swamy 1949; Rao and Sood 1979; Sood 1986, 1997). However, presence of anthers with more parietal layers has been described for species from different genera, such as *Ophrys* L., *Cypripedium* L., and *Goodyera* R. Br. (Swamy 1949; Sood and Rao 1988; Aybeke 2012; Kant and Goel 2013; Ghimire et al. 2020), making this feature an important embryological and taxonomic character. The tapetum is uninucleate and of the secretory type, as in most species of the family studied to date (Swamy 1949; Sood and Rao 1988; Ghimire et al. 2020). Most species of the family present only one tapetum layer; however, species with up to three tapetum layers have been recorded in *Ophrys, Cypripedium* and *Goodyera* (Swamy 1949; Sood and Rao 1988; Aybeke 2012; Ghimire et al. 2020). The presence of more than one tapetum layer may be related to the secretory functions of the tissue, enabling the substances secreted by the tapetum to reach the microspores/male gametophyte arranged in the center of the pollinia (Aybeke 2012).

The stages of microsporogenesis occur regularly in all species analyzed, as dyads and tetrads of microspores were observed. Microsporogenesis is of the simultaneous type, corroborating what has been described for most species of the family (Swamy 1949; Johri 1992). In all species analyzed, formation of the cell wall that individualizes microspores was incomplete; a structural feature that remains in mature male gametophyte. This characteristic was previously described for *E. ibaguense*, and the presence of cytoplasmic bridges was observed in this region (Yeung 1987). The microspores can be arranged in a tetrahedral form, but those at the periphery of the pollinia are arranged in linear form, mirroring the description for *E. ibaguense,* for species of other genera of Epidendroideae as *Dendrobium* Sw. and *Bulbophyllum* Thouars, for species from other subfamilies, such as *Habenaria* and *Peristylus* Blume from Orchidoideae (Swamy 1946, 1949; Blackman and Yeung 1983a; Yeung 1987).

In the species analyzed, callose deposition occurs during the meiotic process, remaining in the newly formed male gametophyte, correlating with the findings for *E. ibaguense* (Blackman and Yeung 1983a). Recent studies have identified variation in callose deposition in species of the family. In *Dendrobium officinale* Kimura and Migo, callose formation occurred after meiosis of microspore mother cells, being deposited in the tetrads and on the surface of the pollinia (Deng et al. 2023). In *Anoectochilus roxburghii* (Wall.) Lindl. ex Wall., callose deposition occurs before meiosis, involving the entire pollinium, but not the microspore mother cells inside the pollinium (Chen et al. 2021). This variation motivates the need for further studies on callose deposition in Orchidaceae pollinia. Callose deposition in microspores acts as a barrier that prevents the uneven movement of genetic material between cells, ensuring genetic autonomy of microspores as well as being important in exine formation (Heslop-Harrison 1968; Bhandari 1984; Deng et al. 2023).

The deposition of sporopollenin occurs during the meiotic and mitotic process, resulting in the formation of male gametophytes that present differences in cell walls. Male gametophyte at the periphery show higher exine deposition and consequently thicker cell walls when compared that found in the center of the pollinium, which show thinner cell walls. This event occurs because the male gametophyte of the pollinium that are in the periphery present direct contact with the tapetum cells and the inners present contact only with other male gametophyte, as described by Cocucci and Jensen (1969). Differences in sporopollenin deposition seem to be a pattern in the family, and are probably related to the pollen aggregation process in the pollinia (Cocucci and Jensen 1969; Blackman and Yeung 1983a; Schlag and Hesse 1993; Pandolfi and Pacini 1995; Chen et al. 2021; Deng et al. 2023). In *E. scutella*, the exine is present around all tetrads, but only the outers have a thick exine and ornamentation (Cocucci and Jensen 1969). In contrast, in *E. ibaguense* and *A. roxburghii*, the exine is deposited only on the outer surface of the pollinium (Yeung 1987; Chen et al. 2021). In the pollinia of *Polystachia pubescens* (Lindley) Rchb.f., three types of exine have been observed: one that forms on the surface of the pollinia, second type that forms between the tetrads, and one that forms inside the tetrads, consisting of two layers of intine (Schlag and Hesse 1993).

Caudicle development The caudicle is a structure unique to Orchidaceae, derived from anther tissue and considered the only example of haploid tissue with a nonsexual function (Dressler 1981; Blackman and Yeung 1983b; Johnson and Edward 2000). Its structure may vary among the different species of the family. In the species analyzed here, the development of the caudicle was very similar to that of the pollinia. At the beginning of caudicle differentiation it was possible to observe two groups of cells: ones with a thick cell wall and ones with thin cell walls. These results corroborate those described for caudicle development in *E. ibaguense*, in which cells undergoing meiosis are named structural cells (Blackman and Yeung 1983b). The thin-walled cells are similar to the pollinium tapetum cells and are responsible for the deposition of sporopollenin in the newly formed male gametophyte, as well as the deposition of elastoviscin in the caudicle (Dressler 1981; Blackman and Yeung 1983b; Yeung 1987). These substances are responsible for the

mature caudicle's elastic properties (Blackman and Yeung 1983b; Johnson and Edwards 2000; Zhang et al. 2020).

The presence of male gametophyte and the deposition of elastoviscin characterize the caudicle as an appendicular structure (Freudenstein and Rasmussen 1997). In *E. ibaguense*, ultrastructural analysis revealed the presence of lipids in the elastoviscin secreted by cells of the caudicle (Blackman and Yeung 1983b). This seems to be a pattern of secretion, since it has previously been described for caudicles of other genera, such as *Phalaenopsis* Blume and *Oncidium* Sw. (Yeung and Law 1987; Zhang et al. 2020). The six *Epidendrum* species studied here present only the caudicle as an accessory structure and is the only structure which provides weak points in the pollinarium, allowing the pollinia to detach from the pollinator, favoring its deposition on the stigma (Dressler 1981).

Disagreements remains regarding the evolution of the pollinia and the caudicle in Orchidaceae. Due to the ontogenetic similarity between pollinia and the caudicle, Blackman and Yeung (1983b) suggested that the caudicle in *E. ibaguense* would have evolved partially or totally from a pollinia. Thus, for these authors, the species originally presented eight pollinia, correlating with the proposal from Dressler (1981), who suggested that eight is the plesiomorphic number of pollinia for the tribe Epidendreae. However, Freudenstein and Rasmussen (1996) and Cameron et al. (1999) argued that the anther formed by four pollinium in Orchidaceae seems to be the plesiomorphic state, and anthers with two or eight pollinia the derived states. Recently, Mosquera-Mosquera et al. (2019) analyzed the morphological variation of the pollinarium of Epidendroideae and reconstructed the ancestral states of the pollinarium characters. These authors emphasize that, in the subfamily, the evolution of pollinarium and pollinia did not occur in a linear form, with several transitions having occurred during the diversification of Epidendroideae. The reconstruction of character states suggests, as the most parsimonious state, that the common ancestor would have a complete pollinarium and probably constituted four juxtaposed pollinia, a caudicle, a stipe, and a viscidia. This is also observed for the whole subtribe Laeliinae, for which there are many examples of transitions between four and eight pollinia (Van den Berg et al. 2009). Based on these arguments and on the characteristics of the structures observed in the species of *Epidendrum* studied herein, it can presume that the pollinarium of the species of this genus would have been derived from a structure with four, not eight, pollinia as previously postulated.

Our results show that there is no variation in the development of pollinia and caudicle among the species. The different reproductive strategies presented by the species are not related to the development of the pollinia, indicating that these characteristics are conserved and could be potential synapomorphies for the group. Differences in deposition of sporopollenin between male gametophyte from the periphery and also center of pollinium were observed. This deposition is directly related to the type of aggregation of the male gametophyte in the pollinia. In this sense, studies are needed to elucidate the pattern of this substance in the male gametophyte of the species. During the development of the caudicle, male gametophytes are formed, which are initially viable. Further research is needed to confirm this viability, providing data that will help to understand the evolution of this structure in the family.

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Author contributions MFA collected the materials, conducted the laboratory work, carried out analyses and wrote the manuscript. KLGT conducted confocal microscopy analysis, supervised the laboratory work and revised the manuscript. FP and JFAB supervised the laboratory work and revised the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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