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POLLEN ONTOGENY IN *BRASENIA* (CABOMBACEAE, NYMPHAEALES)[1]

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Brasenia is a monotypic genus sporadically distributed throughout the Americas, Asia, Australia, and Africa. It is one of eight genera that comprise the two families of Nymphaeales, or water lilies: Cabombaceae (*Brasenia*, *Cabomba*) and Nymphaeaceae (*Victoria*, *Euryale*, *Nymphaea*, *Ondinea*, *Barclaya*, *Nuphar*). Evidence from a range of studies indicates that Nymphaeales are among the most primitive angiosperms. Despite their phylogenetic utility, pollen developmental characters are not well known in *Brasenia*. This paper is the first to describe the complete pollen developmental sequence in *Brasenia schreberi*. Anthers at the microspore mother cell, tetrad, free microspore, and mature pollen grain stages were studied using combined scanning electron, transmission electron, and light microscopy. Both tetragonal and decussate tetrads have been identified in *Brasenia*, indicating successive microsporogenesis. The exine is tectate-columellate. The tetrad stage proceeds rapidly, and the infratectal columellae are the first exine elements to form. Development of the tectum and the foot layer is initiated later during the tetrad stage, with the tectum forming discontinuously. The endexine lamellae form during the free microspore stage, and their development varies in the apertural and non-apertural regions of the pollen wall. Degradation of the secretory tapetum also occurs during the free microspore stage. Unlike other water lilies, *Brasenia* is wind-pollinated, and several pollen characters appear to be correlated with this pollination syndrome. The adaptive significance of these characters, in contrast to those of the fly-pollinated genus *Cabomba,* has been considered. *Brasenia* does not produce pollenkitt nor develop tectal microchannels as does *Cabomba.* Instead, the discontinuity of the tectum reduces the amount of sporopollenin in the wall, which may allow for more effective wind dispersal. The importance of reassessing palynological characters in light of new ontogenetic data and the phylogenetic implications of this reevaluation are also discussed.

Key words: anther; *Brasenia*; Cabombaceae; development; Nymphaeales; ontogeny; pollen; ultrastructure.

Brasenia is one of eight genera comprising the two families of Nymphaeales, or water lilies: Cabombaceae (*Brasenia*, *Cabomba*) and Nymphaeaceae (*Victoria*, *Euryale*, *Nymphaea*, *Ondinea*, *Barclaya*, *Nuphar*). The intergeneric relationships within Nymphaeales have been elucidated in recent years, supporting the position of *Brasenia* and *Cabomba* as divergent sister taxa within the monophyletic family Cabombaceae (Les et al., 1999; Liu et al., 2005).

Brasenia is sporadically distributed in freshwater ponds and lakes in temperate and tropical regions of the Americas, eastern Asia, Australia, Africa, and the West Indies. Although no extant populations occur in Europe, Tertiary and Quaternary fossils have been recovered from several European localities (see Williamson and Schneider, 1993). The sole species of the genus, *Brasenia schreberi* J.F. Gmelin, is characterized by bright green, floating, peltate leaves and small, purple flowers. The plant produces thick mucilage that covers all of the underwater organs, including the underside of leaves, petioles, stems, and developing floral buds (Osborn and Schneider, 1988; Williamson and Schneider, 1993). This mucilage has anti-algal and anti-bacterial properties and may function in alleopathic weed control (Elakovich and Wooten, 1987).

Brasenia is unique among water lilies in being the only genus that exhibits anemophily, a condition reflected in many of its floral and pollen characters. Anthesis generally occurs from June through September, and the flowers have a 2-day blooming period, with anther dehiscence occurring on the morning of the second day (Osborn and Schneider, 1988; Osborn et al., 1991).

There has been an increased focus on Nymphaeales in recent years, as evidence from a broad spectrum of phylogenetic studies indicates that water lilies are among the most primitive of flowering plants. Studies using both molecular and morphological characters have consistently placed *Amborella,* or *Amborella* plus Nymphaeales, as the sister group to the remaining angiosperms (e.g., Qiu et al., 1999; Barkman et al., 2000; Doyle and Endress, 2000; Graham and Olmstead, 2000; Soltis et al., 2000; Borsch et al., 2003; Hilu et al., 2003; Aoki et al., 2004; see also Soltis and Soltis, 2004, and references therein).

Investigations of pollen structure and development, and the accompanying changes within the anther, yield an array of characters potentially useful for assessing phylogenetic relationships (Blackmore and Crane, 1988; Blackmore and Barnes, 1990; Kreunen and Osborn, 1999). However, relatively little is known about the pollen of Nymphaeales, including *Brasenia*. In the majority of studies, light microscopy (LM) was used exclusively to investigate mature pollen grains (e.g., Erdtman, 1954; Snigerevskaya, 1955). Scanning electron microscopy (SEM) has also been used to study the mature pollen of several Nymphaealean genera, including *Brasenia* (e.g., Walker, 1976; Zhang, 1984; Osborn and Schneider, 1988). Fewer studies have employed the use of transmission electron microscopy (TEM) to investigate the mature pollen wall in *Brasenia* (e.g., Ueno and Kitaguchi, 1961; Osborn et al., 1991; Meyer-Melikyan and Diamandopulu, 1996).

Previous studies of mature pollen of *Brasenia* have

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consistently characterized the grains as monosulcate and as having scabrate or indistinct surface ornamentation (e.g., Zhang, 1984; Osborn et al., 1991). The exine, however, has been contradictorily characterized as having a granular infratectum by Snigerevskaya (1955), as echinate-intectate with whole or partial columellae by Ueno and Kitaguchi (1961), and as tectate-columellate by both Osborn et al. (1991) and Meyer-Melikyan and Diamandopulu (1996). Because tectal and infratectal architecture are potentially valuable phylogenetic characters, it is necessary to resolve these conflicting descriptions in order to accurately assess these characters in *Brasenia*.

These characters, and others, can be better understood in the context of their ontogeny; however, pollen development has not been extensively studied in Nymphaeales, with the exception of *Nymphaea* (Gabarayeva and Rowley, 1994; Gabarayeva and El-Ghazaly, 1997; Gabarayeva et al., 2001). Pollen ontogeny has never been investigated at the ultrastructural level for five of the eight genera, including *Brasenia*. One study by Khanna (1965) did investigate pollen formation in *Brasenia* using LM and anther smears. This study, however, was limited in its scope and focused on the cytological (chromosomal) aspects of microsporogenesis.

The objective of this paper was to comprehensively document pollen wall ontogeny in *Brasenia* using combined LM, SEM, and TEM. The details of pollen wall development and the accompanying changes within the anther in *Brasenia* were then compared to those of the other Nymphaealean genera, primarily *Cabomba*, in an effort to elucidate the phylogenetic implications of pollen development within the water lilies.

MATERIALS AND METHODS

Plant material—Floral buds of *Brasenia schreberi* are submerged and located in clusters just below the surface of the water. Seventy-six submerged buds (Fig. 1) were collected at various stages of development in June and July of 2003, 2004, and 2005 from the Atlanta/Long Branch Conservation area near Macon, Missouri, USA (39°52′ N, 92°30′ W). In addition, five emergent buds (Fig. 2), 10 first-day flowers (Fig. 3), five second-day flowers (Fig. 4), and one third-day flower (Fig. 5) were collected. Five additional first-day flowers were hand-pollinated and set aside for 1–2 h to allow pollen germination to occur.

Fresh submerged buds and flowers were taken back to the laboratory and sorted into nine size categories based on bud length. The thick layer of mucilage enveloping the submerged buds was removed with paper towels. The buds and flowers were dissected, and whole anthers were removed. Anthers were measured from the distal tip of the anther to the top of the filament. To facilitate fixation, both ends of each anther were sliced open using a double-edge razor blade, whereas the smallest anthers (<1.0 mm) were sliced in half. Anthers were chemically fixed for 24 h in Karnovsky's fixative. The anthers were then buffer-washed in 0.2 M phosphate buffer (pH 7.4), postfixed for 2 h in 2% osmium tetraoxide buffered in 0.2 M phosphate, and buffer-washed again at least four times. First-day and second-day flowers were also cut into quarters and fixed using the same procedure. Hand-pollinated, first-day flowers were dissected further, with whole stigmata removed and fixed. All anthers, stigmata, and flowers were then dehydrated in a graded ethanol series and stored in 75% ethanol.

Microscopy—For LM and TEM, two to nine anthers from each bud were further dehydrated, then infiltrated and embedded in Spurr's epoxy resin (Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA) following standard protocols. To examine overall pollen and anther histology and identify specimens suitable for ultrastructural studies, the embedded anthers were thick-sectioned with a Leica (Bannokburn, Illinois, USA) Ultracut UCT Ultramicrotome using glass and diamond knives. Thick-sections (850 nm) were collected on glass slides, stained with Richardson's stain (methylene blue and azure II), and imaged with an Olympus (Lake Success, New York, USA) BHS compound microscope. Well-preserved anthers at a range of developmental stages were then thin-sectioned using a diamond knife. Thin-sections (70 nm) were collected and dried on formvar-coated copper slot grids and were stained with uranyl acetate (8 min) and lead citrate (6 min), then imaged with a JEOL (Peabody, Massachusetts, USA) JEM-100SX transmission electron microscope at 80 kV.

For SEM, anthers, buds, and stigmata were further dehydrated in a graded series of ethanol and critical point dried, following standard procedures. Individual anthers were either cut in cross-section using a double-edge razor

Figs. 1–5. Floral cycle. **1**. Cluster of submerged buds at various stages of development surrounded by substantial mucilage. Note immature, coiled leaf and thumb for scale. Bar = 2.0 cm. **2.** Emergent bud showing residual mucilage. Bar = 1.0 cm. **3.** First-day flower showing reflexed perianth and exposed stigmata. Bar = 10.0 cm. **4.** Second-day flower showing dehiscent anthers borne upon elongated filaments. Note abundant pollen within each anther and the now centrally aggregated cluster of stigmata. Bar = 10.0 cm. **5.** Third-day flower with non-reflexed perianth and dehisced anthers. Bar = 10.0 cm.

blade or macerated using a syringe needle, and mounted onto aluminum stubs with double-sided tape. The edges of the tape were affixed to the stub with colloidal graphite. Stubs were then sputter-coated with gold-palladium and imaged with a JEOL JSM-6100 scanning electron microscope at 5 kV.

Sizes of pollen characters were determined by measuring the characters on micrographs of at least five different microspores/pollen grains.

RESULTS

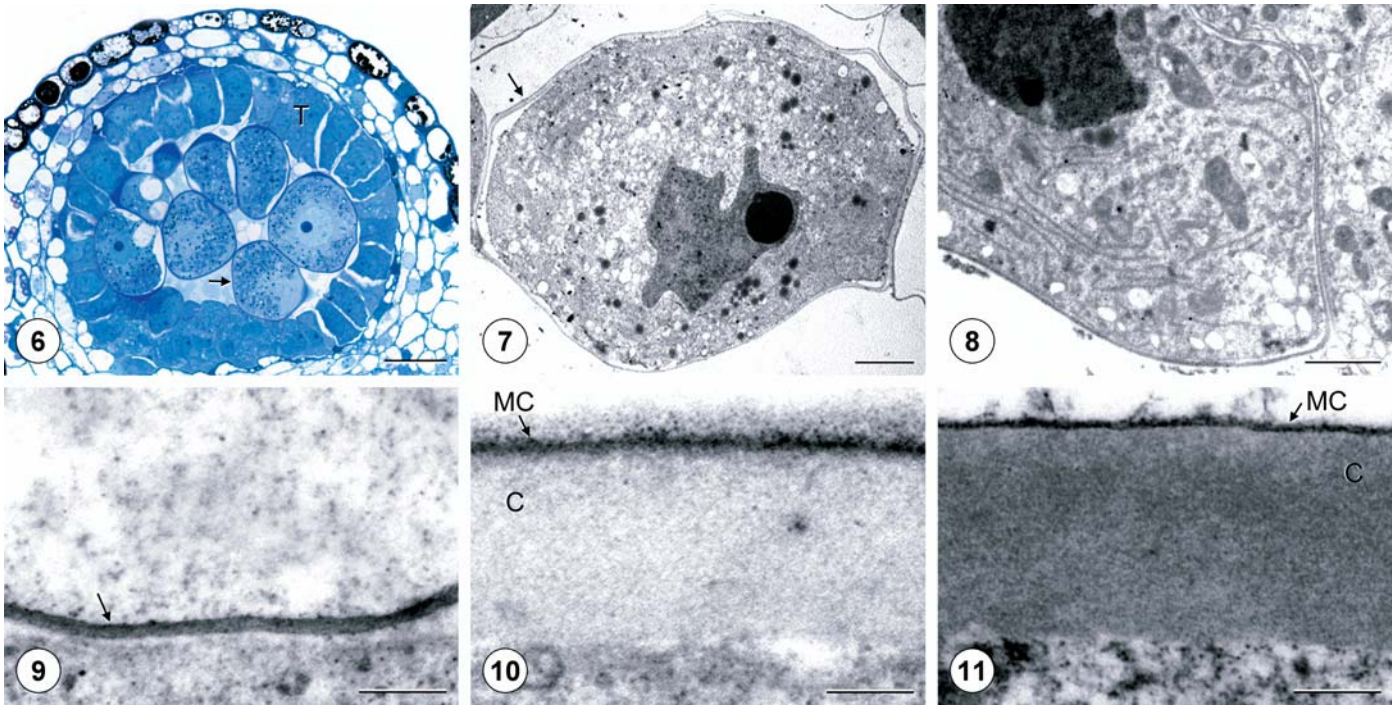
Timing of anther and pollen development is fairly consistent among the anthers within a single floral bud and is nearly synchronous within individual anthers. Each anther contains abundant pollen. The results are divided into sections based on major ontogenetic stage, including the microspore mother cell, tetrad, free microspore, and mature pollen grain stages.

Microspore mother cell stage—Microspore mother cells are present in anthers that range in length from 0.7 to 1.8 mm. Early microspore mother cells are fairly large and appress against one another and against the tapetum (Fig. 6). The nuclei and nucleoli are distinct, and the plasmalemma of each microspore mother cell is surrounded by an electron-dense microspore mother cell coat (Figs. 6, 7). As microspore mother cells develop, they become further separated from one another, and a layer of callose is deposited between the plasmalemma and the microspore mother cell coat. This callose layer is initially thin, but as development proceeds, the callose thickens to reach a maximum thickness of 0.45 μm (Figs. 9–11). The microspore mother cell coat persists outside of the callose layer throughout the microspore mother cell stage (Figs. 9–11).

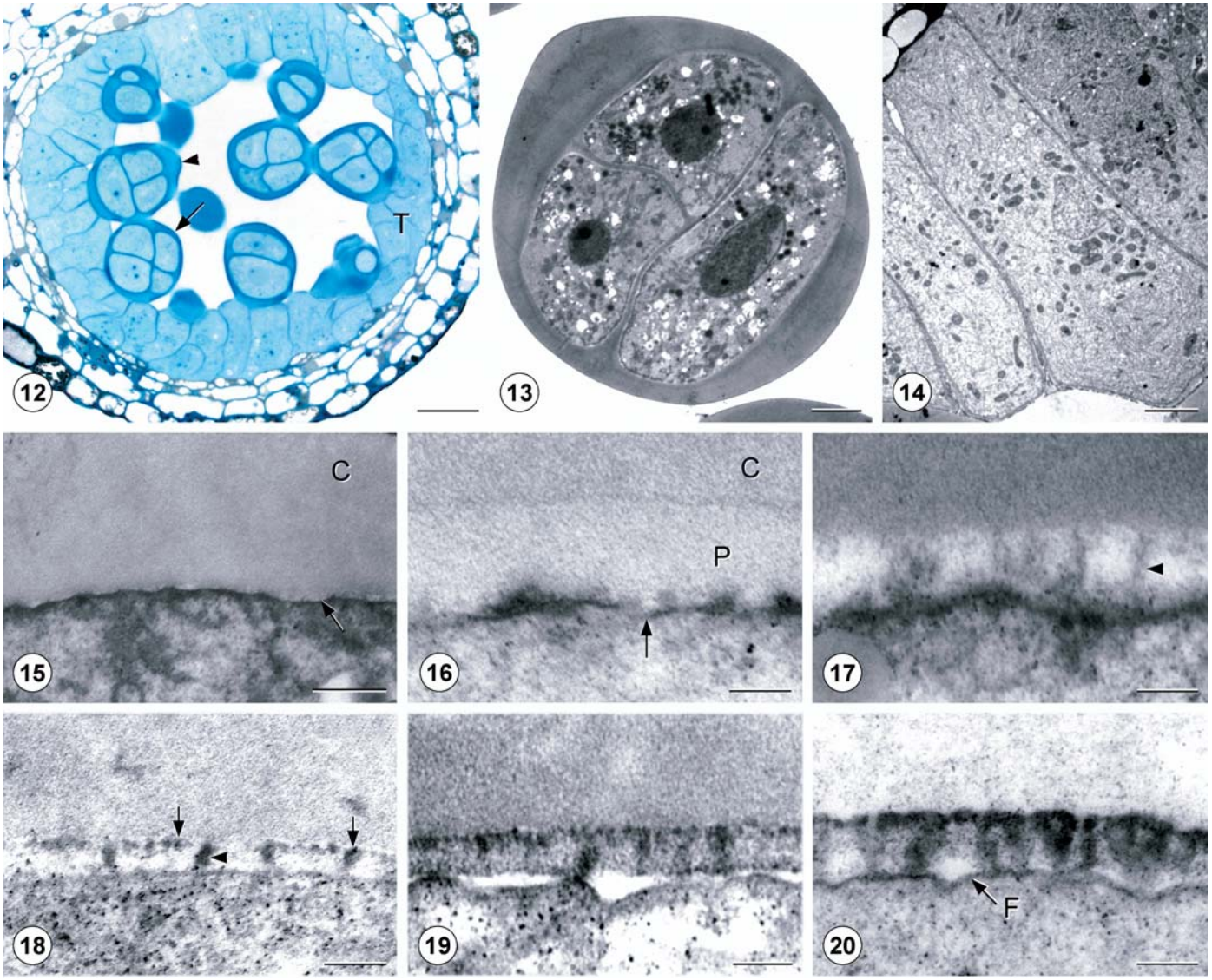
The tapetum is well differentiated throughout the microspore mother cell stage (Figs. 6, 8). Tapetal cells are large, tightly adjoined, and multinucleate with abundant endoplasmic reticulum.

Tetrad stage—Tetrads are present in anthers that range from 1.5 to 2.1 mm in length. Each microspore mother cell has undergone meiosis to form a tetrad of microspores. Thick callose surrounds the entire tetrad, while thinner callose septae extend between the individual microspores (Figs. 12, 13). Tetrads occur regularly in both tetragonal and decussate configurations, with both tetrad geometries consistently occurring within the same anther locules (Fig. 12). In early tetrads, the microspore plasmalemma is appressed against the callose (Fig. 15). As development proceeds, the plasmalemma pulls away from the callose, and an electron translucent primexine forms between the callose and the microspore plasmalemma (Fig. 16). The microspore mother cell coat is still present in the early tetrad stage.

In the middle-tetrad stage, a thin, electron-dense layer is laid down within the primexine, adjacent to the plasmalemma. Electron-dense tufts form along this layer. These tufts develop into the procolumellae, which subsequently elongate and extend through the primexine (Fig. 17). These thin, fibrillar elements are the precursors to the columellae that will comprise the infratectum. After the procolumellae have elongated, the tectum begins to form at the outer edge of the primexine.



Figs. 6–11. Microspore mother cell stage. **6.** Section of a single anther locule showing microspore mother cells and differentiated tapetum. Note that the microspore mother cells appress against one another and are surrounded by a thin callose layer (arrow). LM, bar = 25.0 μm. **7.** Section through a single microspore mother cell showing nucleus, nucleolus, and thin callose layer (arrow). TEM, bar = 4.0 μm. **8.** Section through a portion of a tapetal cell showing abundant endoplasmic reticulum. TEM, Bar = 2.0 μm. **9.** Detail of an early microspore mother cell wall showing thin, initial layer of callose (arrow). TEM, bar = 0.2 μm. **10.** Detail of an intermediate microspore mother cell wall showing microspore mother cell coat surrounding a thick layer of callose. TEM, bar = 0.2 μm. **11.** Detail of a late microspore mother cell wall showing thickened callose layer and well-defined microspore mother cell coat. TEM, bar = 0.2 μm. C, callose; MC, microspore mother cell coat; T, tapetum.

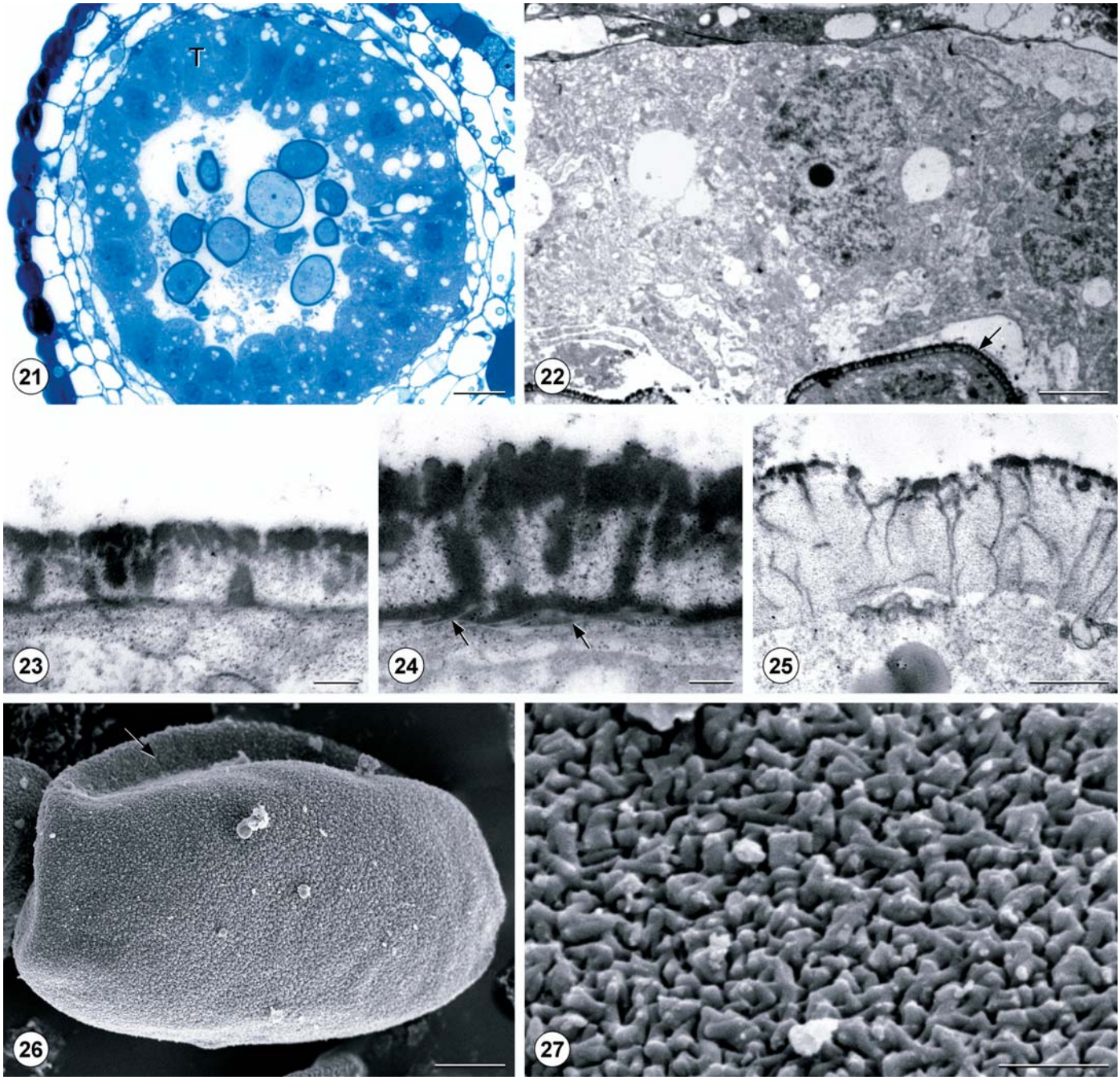


Figs. 12–20. Tetrad stage. **12.** Section of a single anther locule showing tetrads with both tetragonal (arrow) and decussate (arrowhead) types. Note tapetum. LM, bar = 25.0 μm. **13.** Detail of a single decussate tetrad showing thick layer of callose surrounding the entire tetrad and thinner callose septae between the individual microspores. TEM, bar = 4.0 μm. **14.** Section of tapetal cells. TEM, bar = 4.0 μm. Figs. 15–20. Progressively older microspore walls. **15.** Detail of early outer tetrad wall showing callose directly above plasmalemma (arrow). TEM, bar = 0.5 μm. **16.** Detail of outer tetrad wall with developing primexine between callose and plasmalemma (arrow). TEM, bar = 0.1 μm. **17.** Detail of outer tetrad wall showing procolumellae (arrowhead) extending into the primexine. TEM, bar = 0.1 μm. **18.** Detail of outer tetrad wall showing primary tectal elements (arrows) and columellae (arrowhead). TEM, bar = 0.1 μm. **19.** Detail of later outer tetrad wall showing further deposition of tectum and columellae. TEM, bar = 0.1 μm. **20.** Detail of late outer tetrad wall showing initial foot layer. TEM, bar = 0.1 μm. C, callose; F, foot layer; P, primexine; T, tapetum.

Initially, the tectum is laid down as small, globular elements (Fig. 18), which remain as distinct elements throughout ontogeny.

As the tetrad stage continues, the columellae and tectal elements become more robust and more electron dense (Fig. 19). The microspore mother cell coat disintegrates in the middle tetrad stage and the plasmalemma separates slightly from the developing wall (Fig 19). In the late-tetrad stage, a thin foot layer forms in this space at the base of the developing columellae (Fig. 20). The tapetal cells are intact throughout the tetrad stage. They remain tightly appressed, as in the microspore mother cell stage, and contain abundant endoplasmic reticulum (Figs. 12, 14).

Free microspore stage—Free microspores are found in anthers ranging in length from 2.1 to 2.9 mm. This stage initiates when the callose layer surrounding the tetrad breaks apart and the four microspores separate from one another (Fig. 21). During the early-free microspore stage (Figs. 21–27), the tectum, columellae, and foot layer become more electron dense than in the tetrad stage. Throughout the early-free microspore stage, these layers continue to develop and thicken. In the earliest free microspores, the columellae are 50 nm wide and 100 nm tall. As the early-free microspore stage progresses, the width of the columellae increases to 75 nm and the height doubles to 200 nm. Similarly, the tectal elements expand both laterally and in thickness. The overall thickness of the tectum



Figs. 21–27. Early-free microspore stage. **21.** Section of a single anther locule showing free microspores and early vacuolization of tapetal cells. Note tapetum sectioned slightly obliquely. LM, bar = 25.0 μm. **22.** Detail of a tapetal cell with an adjacent microspore (arrow). TEM, bar = 4.0 μm. **23.** Detail of early-free microspore wall showing well-defined columellae, discontinuous tectum, and thin foot layer. TEM, bar = 0.1 μm. **24.** Detail of later early-free microspore wall showing endexine lamellae (arrows). Note elongation of columellae and thickened tectum. TEM, bar = 0.1 μm. **25.** Section through apertural region of early-free microspore wall showing less organized exine. TEM, bar = 0.5 μm. **26.** Single free microspore showing elongate apertural groove (arrow). SEM, bar = 5.0 μm. **27.** Detail of early-free microspore surface showing initial scabrate elements. SEM, bar = 1.0 μm. T, tapetum.

increases almost three-fold throughout the early-free microspore stage. The thickness of the foot layer doubles during the early free microspore stage to 30–40 nm (Figs. 23, 24).

The endexine is initiated in the early-free microspore stage. The endexine is laid down in thin, discontinuous plates, or lamellae in both non-apertural regions of the wall and regions directly adjacent to the developing aperture (Fig. 24). Formation of the aperture also becomes apparent during the early-free microspore stage, with initiation as a groove running the length of the free microspore (Fig. 26). This region is characterized by a thin to absent foot layer that is pulled away from the plasmalemma. The tectum and infratectum in the apertural region are less organized than in the non-apertural regions of the wall, and the tectal elements are highly

discontinuous (Fig. 25). The endexine lamellae are sometimes present directly adjacent the plasmalemma, with some lamellae extending up into the space between the plasmalemma and the exine (Fig. 25). The surface of the early-free microspore is scabrate, with discontinuities in the tectum apparent as gaps in the surface of the free microspore (Fig. 27). The tapetum remains intact throughout the early-free microspore stage, but the tapetal cells become vacuolated (Figs. 21, 22).

During the middle-free microspore stage (Figs. 28–33), the thickness of the foot layer increases more than four-fold, reaching a thickness of 140 nm. As the foot layer thickens, the endexine becomes compressed in non-apertural regions until the individual lamellae are difficult to distinguish (Figs. 30, 31). Individual lamellae do remain apparent in regions of the wall adjacent to the aperture, and some extend into the apertural region (Figs. 32, 33). The columellae no longer increase significantly in width and height during the middle-free microspore stage, but the tectum does increase in thickness. New, larger tectal elements develop above the first-formed elements, filling in gaps in the tectum (Figs. 30, 31). However, many discontinuities in the tectum remain, and several of the first-formed, small tectal elements are still visible on the lower side of the tectum. These elements give the inner tectum a slightly granular appearance (Figs. 30, 31). The outermost, secondary tectal elements are also apparent on the surface of the free microspore as developing rodlets. The rodlets are initially small, measuring 0.5 μm in length and 0.2 μm in width, and appear to be randomly organized (Fig. 29).

The aperture continues to develop during the middle-free microspore stage. The tectum and columellae in the apertural wall are still much less organized than those in the non-apertural regions, but the tectum covers the entire apertural region (Fig. 33). In the middle-free microspore stage, the plasmalemma invaginates away from the exine in the apertural region and deposition of the intine begins (Fig. 33). The intine does not yet begin to form in the non-apertural regions (Figs. 30, 31). The microspores begin to become vacuolated, and the tapetum begins to disintegrate during the middle-free microspore stage (Fig. 28).

In the late-free microspore stage (Figs. 34–39), the plasmalemma pulls away from the developing pollen wall, and the intine begins to develop in this space in the non-apertural wall (Figs. 37, 38). The intine increases in thickness throughout the late-free microspore stage, becoming thickest beneath the aperture (Fig. 36). The endexine lamellae are not distinguishable in the non-apertural regions of the wall (Figs. 37–39). More secondary tectal elements are deposited, but the tectum remains discontinuous, and the primary tectal elements remain visible (Figs. 37–39). The tapetum continues to undergo programmed cell death throughout the late-free microspore stage (Figs. 34, 35), and a small number of orbicules are released from the dissociating tapetal cells (Fig. 35). By the end of the late-free microspore stage, the tapetum is completely disintegrated; however, remnants of the tapetal cells remain attached to the middle layers (Fig. 34). The free microspores become increasingly vacuolated in the late-free microspore stage (Fig. 34).

Mature grain stage—Mature pollen grains of *Brasenia* (Figs. 40–44) are oblate, monosulcate, and measure 45–50 μm in length and 30–35 μm in width. Mature pollen is present in anthers ranging from 2.5 to 3.1 mm, which occur in emergent first-day and second-day flowers. The mature pollen wall is characterized by robust columellae; a thick, discontinuous tectum; and a thick foot layer (Figs. 42, 43). In the non-apertural region, the lamellate endexine has become highly compressed such that individual lamellae are no longer apparent (Fig. 42), while in the regions of the wall adjacent to the aperture, the lamellations remain visible (Figs. 43, 47M). The substantial intine is fully developed and bi-layered in mature pollen grains (Fig. 44). Rodlets on the surface of the pollen grain are 1.0 μm in length and are randomly organized on the non-apertural pollen wall (Fig. 41). The sculptural rodlets are not present on the apertural surface, as a gap in the exine forms over the aperture, leaving the intine as the only remaining component of the wall (Figs. 40, 44). In pollen that has dehisced and germinated (Figs. 45, 46), the aperture is broad and opens along the entire length of the grain (Figs. 45, 46). The pollen tube bulges out along the length of the aperture and may emerge from one or both ends (Figs. 45, 46).

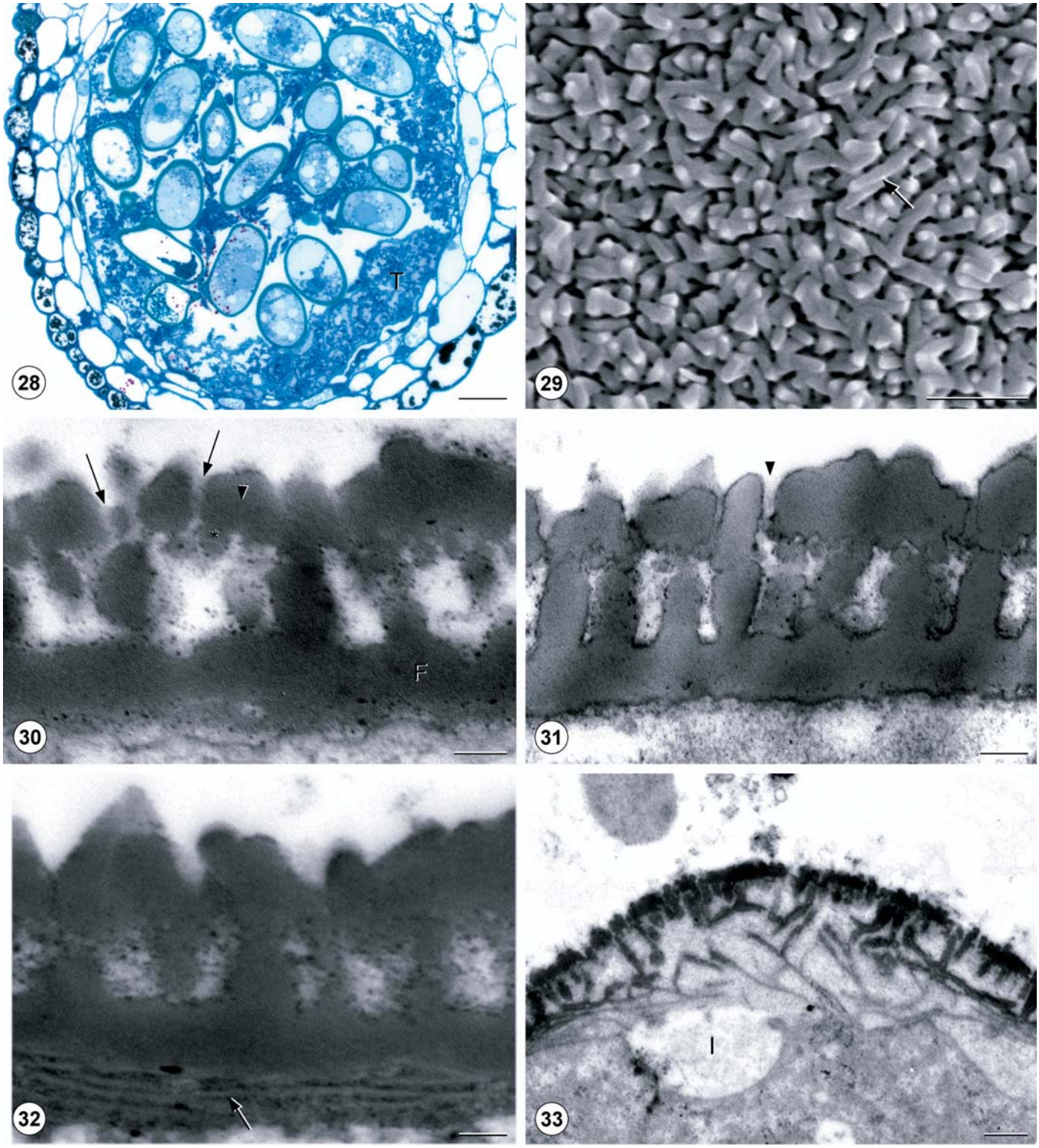
DISCUSSION

This is the first study to comprehensively study pollen and anther ontogeny in *Brasenia*. Only one published study (Khanna, 1965) has examined pollen development in *Brasenia*, and this study was based exclusively on LM. Several key developmental characters are discussed next and summarized in Fig. 47. These characters are interpreted in light of current phylogenetic hypotheses.

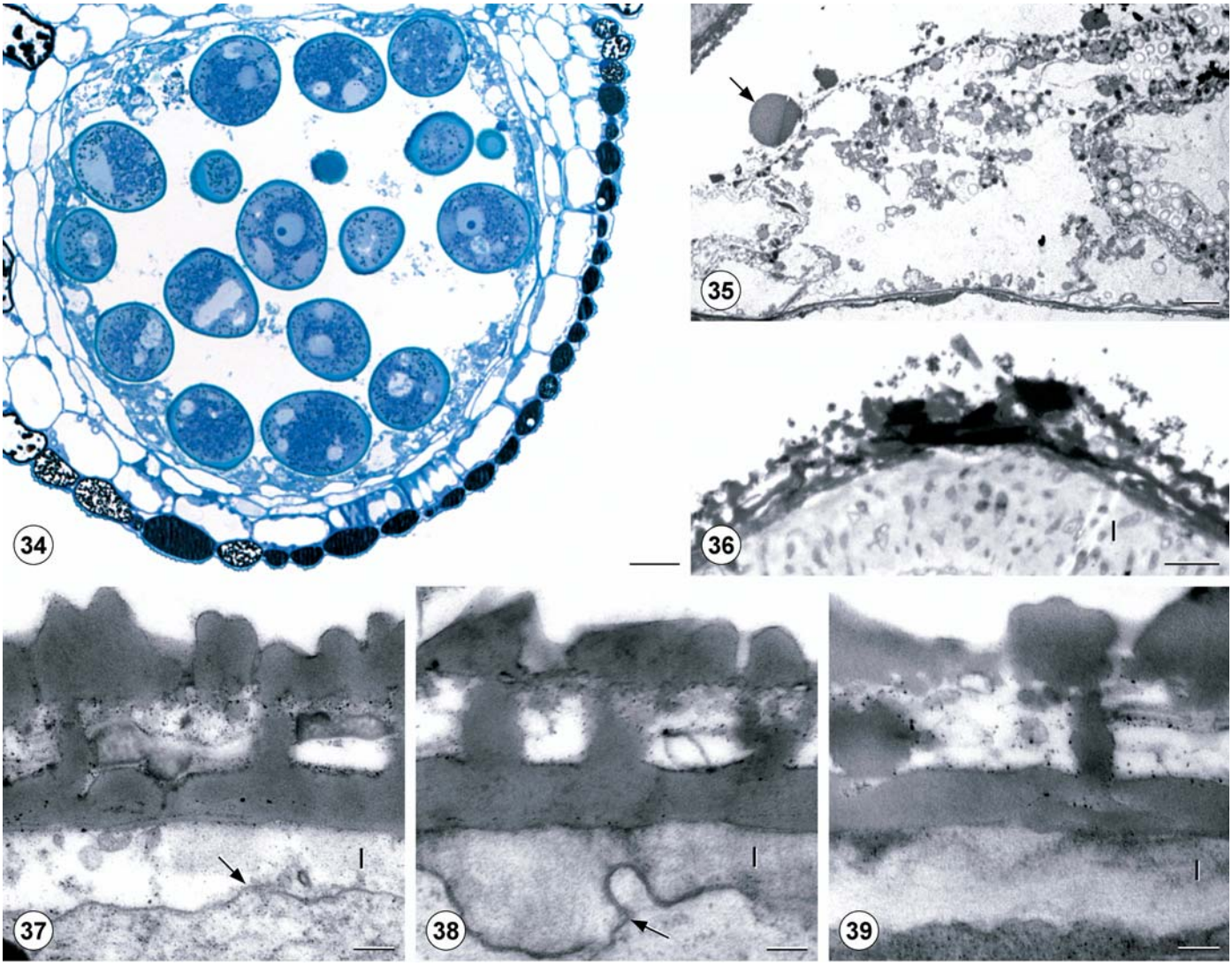
Ontogenetic timing—Microspore mother cells become differentiated very early in bud development and anthers more than double in length before meiosis occurs. In contrast, the tetrad stage occurs rapidly. The range of anther sizes that contain tetrads is smaller (1.5–2.1 mm) and overlaps extensively with the contiguous stages. Only anthers ranging from 1.8 to 2.0 mm exclusively contain tetrads. Free microspores are also found within a relatively narrow range of anther sizes (2.1–2.9 mm). During the free microspore stage, the microspore wall undergoes a substantial increase in size. Most of pollen development occurs in submerged buds; however, the latest free microspores occur in emergent buds. The free microspores in emergent buds have a nearly fully developed intine, but the intine is not yet bi-layered. Fully mature pollen grains are found in the nondehiscent, aggregated anthers of first-day flowers and the pre-dehiscent anthers of second-day flowers. Mature pollen is dehisced early in the morning on the second-day of anthesis.

Tetrad geometry and microsporogenesis—Both tetragonal and decussate tetrad configurations were found regularly in anthers of *Brasenia*, with both types consistently occurring in the same anther locules. This indicates the occurrence of successive microsporogenesis and contradicts Khanna's (1965) report of simultaneous microsporogenesis.

Two major patterns of microsporogenesis, simultaneous and successive, have been recognized and tetrad geometry reflects which pattern occurs (Furness et al., 2002). In simultaneous microsporogenesis, the first meiotic division is immediately followed by the second meiotic division. Cytokinesis and callose deposition occur after both divisions have occurred. Simultaneous microsporogenesis results in tetrahedral tetrad configurations. In contrast, during successive microsporogenesis, callose is deposited after the first meiotic event, forming a septum, thereby separating the two haploid nuclei. These nuclei then undergo the second meiotic division. Successive microsporogenesis results in a variety of tetrad geometries,



Figs. 28–33. Middle-free microspore stage**. 28.** Section through a single anther locule showing numerous free microspores. Note commencement of tapetal disintegration and increased vacuolization of the microspores. LM, bar = 25.0 μm. **29.** Detail of middle-free microspore surface showing initial deposition of rodlets (arrow). SEM, bar = 1.0 μm. **30.** Detail of middle-free microspore wall showing robust columellae and thick foot layer. Note primary tectal elements (asterisk) beneath secondary tectal elements (arrowhead) and the discontinuities within the tectum (arrows). TEM, bar = 0.1 μm. **31.** Detail of the non-apertural region of a later middle-free microspore wall showing absence of apparent endexine lamellae and distinct channels through the tectum (arrowhead). TEM, bar = 0.1 μm. **32.** Detail of later middle-free microspore wall directly adjacent to the aperture showing well-defined endexine lamellae (arrow). TEM, bar = 0.1 μm. **33.** Detail of aperture region of a middle-free microspore. Note developing intine and disorganization of endexine lamellae. TEM, bar = 0.5 μm. F, foot layer; I, intine; T, tapetum.



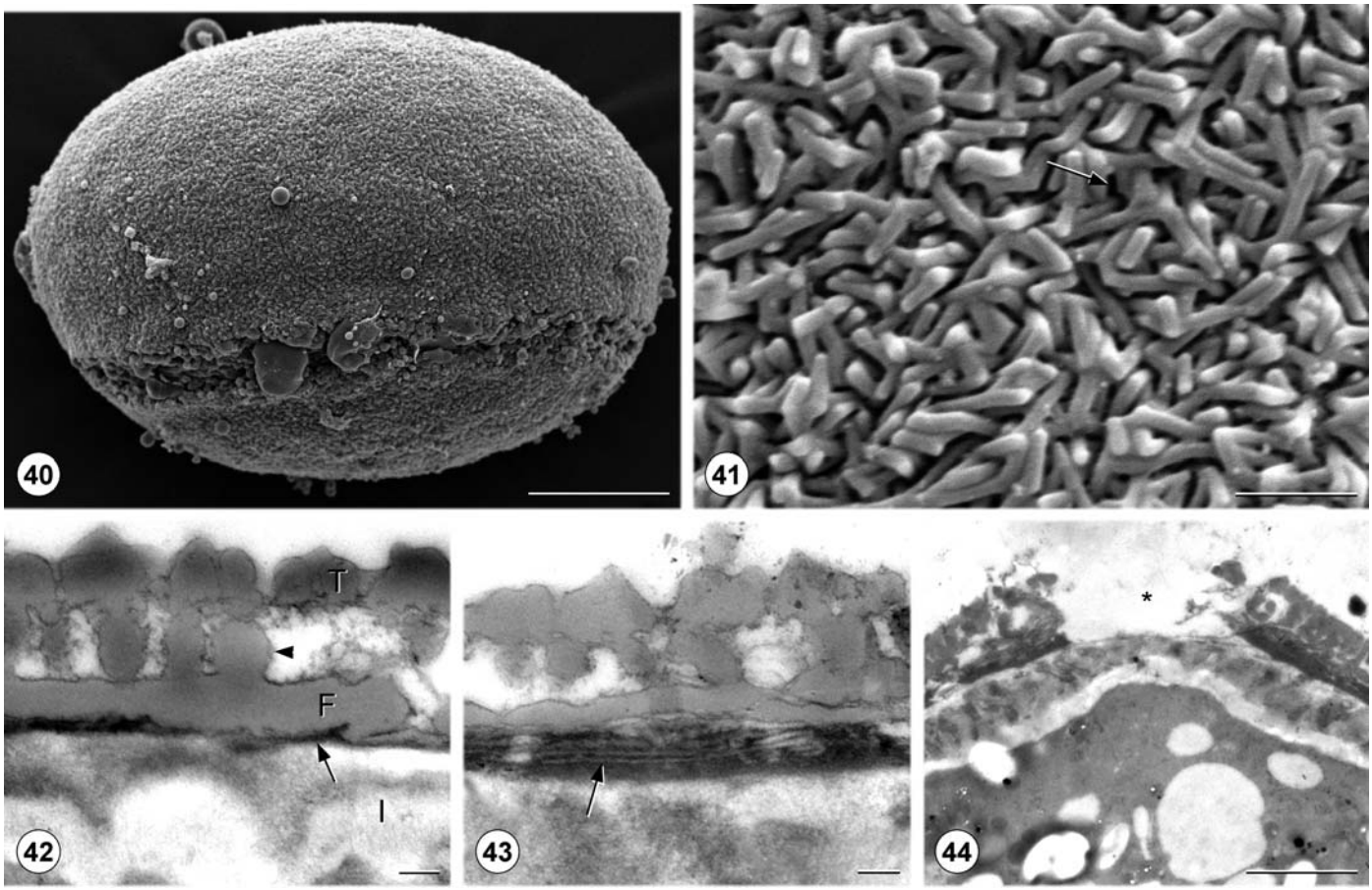
Figs. 34–39. Late-free microspore stage. **34.** Section through a single anther locule showing late-free microspores that are now vacuolated. Note disintegration of tapetum. LM, bar = 25.0 μm. **35.** Detail of a dissociated tapetal cell showing orbicule (arrow). TEM, bar = 2.0 μm. **36.** Detail of apertural wall showing significant intine. TEM, bar = 1.0 μm. **37.** Detail of free microspore wall. Note plasmalemma (arrow) has pulled away from the endexine and the intine has begun to develop. TEM, bar = 0.1 μm. **38.** Detail of later free microspore wall showing developing intine. Note strong undulations of the plasmalemma (arrow). TEM, bar = 0.1 μm. **39.** Detail of late free microspore showing well-developed intine. Note that the undulations of plasmalemma have become less pronounced. TEM, bar = 0.1 μm. I, intine.

including tetragonal and decussate configurations (Furness et al., 2002).

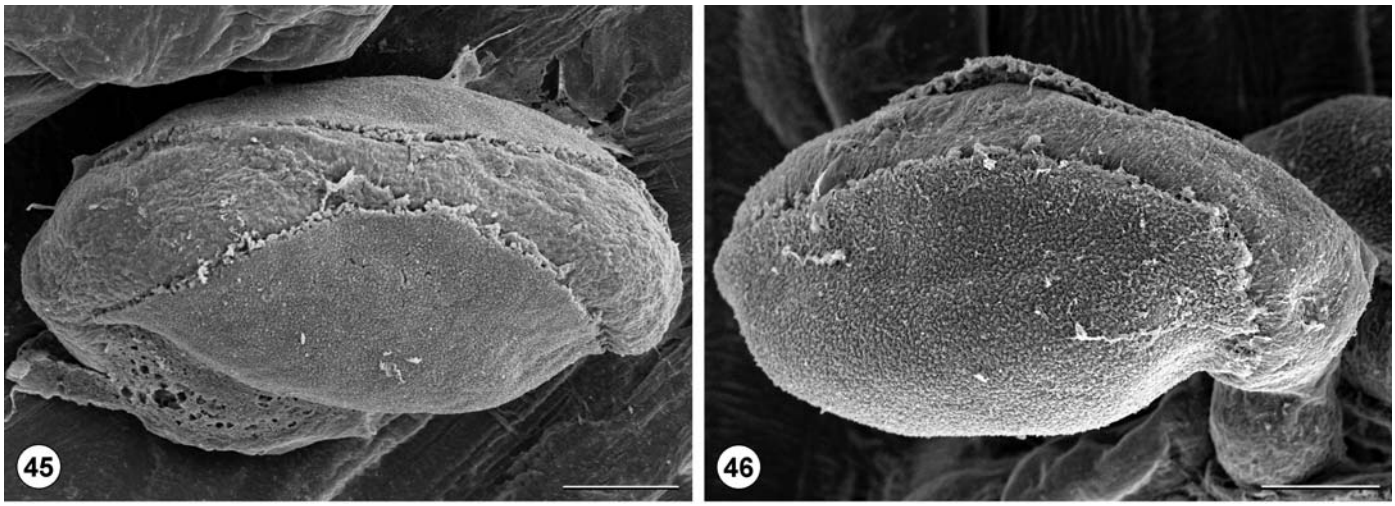
The pattern of microsporogenesis is considered labile in basal angiosperms; however, the simultaneous type has been considered plesiomorphic in flowering plants (Furness et al., 2002). This characterization was based, in part, on Khanna's (1965) study of *Brasenia* that had reported simultaneous microsporogenesis in the genus. Khanna (1965) described the tetrads of *Brasenia* as tetrahedral with occasional tetragonal forms. However, in the present study tetrahedral tetrads were never observed, indicating that microsporogenesis is successive in *Brasenia*.

Recent investigations of *Cabomba* have indicated that successive microsporogenesis also occurs in that genus (Osborn et al., 2003). Moreover, *Amborella* has been described has having successive microsporogenesis (Furness et al., 2002). The tetrads of *Nymphaea* (Gabarayeva and Rowley, 1994) and permanent tetrads of *Victoria* (Roland, 1965; Osborn et al., 2004), however, are found in tetrahedral arrangements, indicating simultaneous microsporogenesis. As successive microsporogenesis is more prevalent in basal angiosperms than previously thought, the phylogenetic status of this character should be reassessed.

Tectum ultrastructure—The exine of *Brasenia* is relatively thick, with well-defined layers. This study delineates the pattern of ontogeny of these layers and clarifies the ultrastructure of the mature exine. The mature tectum of *Brasenia* is bi-layered, with an outer homogeneous layer and an inner granular-like layer. The presence and



Figs. 40–44. Mature pollen grains. **40.** Mature pollen grain showing single, elongate aperture. SEM, bar = 25.0 μm. **41.** Detail of grain surface showing sculptural rodlets. Note discontinuities that penetrate into the tectum (arrow). SEM, bar = 1.0 μm. **42.** Detail of mature non-apertural wall showing discontinuous tectum, robust columellae (arrowhead), thick foot layer, compressed endexine (arrow), and bi-layered intine. TEM, bar = 0.1 μm. **43.** Detail of mature pollen wall adjacent to the aperture showing well-defined, noncompressed endexine lamellae (arrow). TEM, bar = 0.1 μm. **44.** Detail of aperture in transverse section showing absence of exine (asterisk). Bar = 2.0 μm. F, foot layer; I, intine; T, tectum.



Figs. 45–46. Dehisced pollen grains. **45.** View of mature pollen grain on receptive stigma showing broad aperture extending the entire length of the grain and pollen tube emerging from both ends of pollen grain. SEM, bar = 10.0 μm. **46.** Germinating pollen grain showing pollen tube emerging from the aperture and contacting stigmatic surface. SEM, bar = 10.0 μm.

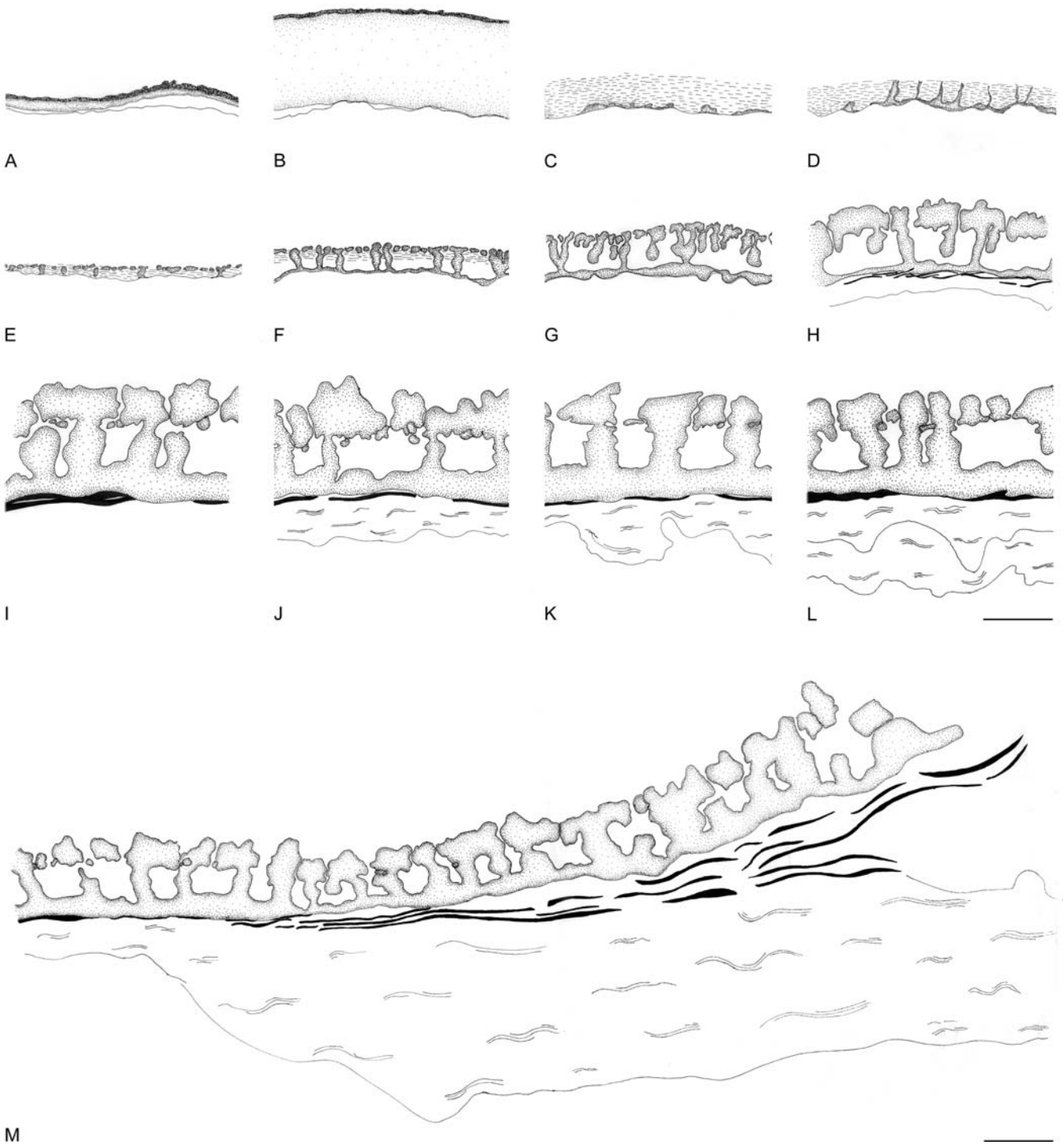


Fig. 47. A–L. Summary of major ontogenetic events during sporoderm development. (A–B) Microspore mother cell stage. (C–F) Tetrad stage. (G–K) Free microspore stage. (L) Mature grain stage. Bar = 0.3 μm. (M) Mature pollen wall showing transition between non-apertural wall and the aperture region. Non-apertural wall at left. Bar = 0.4 μm. Characters illustrated include callose (lightly stippled), primexine (horizontal long dashes), ectexine (densely stippled), endexine (solid black), and intine (discontinuous, wavy lines).

ultrastructure of this inner layer make it somewhat difficult to interpret the structure of the mature exine, especially in oblique section. From the new ontogenetic data, it is clear that the inner layer consists of small, primary tectal elements that are initially deposited in the middle-tetrad stage. The outer, more homogenous layer is composed of secondary tectal elements that are deposited later in the free microspore stage (Fig. 47).

The tectum in *Brasenia* is laid down discontinuously throughout ontogeny and remains discontinuous in the mature

pollen wall. The mature tectum of *Cabomba* is also discontinuous, although the origin of the discontinuities is ontogenetically different between the genera. In both *Cabomba* and *Brasenia,* the tectum initiates as unfused elements. In *Cabomba,* the tectal elements partially fuse and form a more continuous tectum during the tetrad stage; the discontinuities secondarily arise from the formation of microchannels during the free microspore stage (Osborn et al., 2003; Gabarayeva et al., 2003). These microchannels dissect the entire tectum and have been hypothesized to be reservoirs for the copious pollenkitt in *Cabomba,* which is correlated with fly pollination (Osborn et al., 1991). *Brasenia* is wind-pollinated and does not produce pollenkitt, nor are microchannels present in the tectum. In *Brasenia,* the discontinuities in the tectum occur because neither the primary nor the secondary, sculptural tectal elements become fused during development. Osborn et al. (1991) have hypothesized that the discontinuous nature of the tectum in *Brasenia* may reduce the amount of wall material in the tectum. This would reduce the density and the settling speed of the grains, allowing for more effective wind pollination.

Infratectum ultrastructure—The present study indicates that the exine of *Brasenia* is tectate-columellate, which supports the description by Osborn et al., (1991). The new ontogenetic data unequivocally indicate that the granular-like elements found in *Brasenia* are part of the tectum and not the infratectum. The columellae of *Brasenia* are the first elements of the exine that form, first appearing in the middle tetrad stage (Fig. 47). The well-defined columellae are similar in structure to those of *Cabomba* (Osborn et al., 1991, 2003; Gabarayeva et al., 2003), but are much more robust than those of the six genera of Nymphaeaceae.

The inner granular-like layer of the tectum has created some difficulty in accurately describing infratectum ultrastructure in *Brasenia*. Ueno and Kitaguchi (1961) characterized mature pollen grains of *Brasenia* as intectate with partial or whole columellae, while Walker (1976), using Ueno and Kitaguchi's (1961) micrographs, interpreted the infratectum as granular, lacking well-defined columellae. Furthermore, the pollen of all eight Nymphaealean genera has historically been described as having a granular infratectum (Ueno and Kitaguchi, 1961; Ueno, 1962; Walker, 1974, 1976). This character state has been considered plesiomorphic among angiosperms (Doyle et al., 1994), and a shared state with gymnospermous anthophytes (e.g., Osborn et al., 1991, 1993; Osborn and Taylor, 1995; Osborn, 2000). This interpretation has changed in recent years, as phylogenetic studies have called into question the validity of the "anthophyte clade" (Bowe et al., 2000; Chaw et al., 2000; Donoghue and Doyle, 2000 and references therein). Moreover, a columellar infratectum has more recently been documented in *Cabomba* and *Brasenia* (Osborn et al., 1991, 2003; Gabarayeva et al., 2003), as well as in *Nymphaea* (Rowley et al., 1992; Gabarayeva and Rowley, 1994; Gabarayeva and El-Ghazaly, 1997; Gabarayeva et al., 2001), and all other genera of Nymphaeaceae (Osborn et al., 2004). Columellar pollen is also now recognized in other basal angiosperms, including Illiciales, Trimeniaceae, Austrobaileyaceae, and Schisandraceae (see Doyle and Endress, 2000).

In addition to granular and columellar states, Doyle and Endress (2000) recognized an "intermediate" state for the infratectum, and they determined this as the ancestral character state in angiosperms. Doyle and Endress (2000) scored *Amborella* as intermediate, whereas they scored Nymphaeales as both intermediate (Cabombaceae and *Barclaya*) and granular (the five other genera of Nymphaeaceae). The current authors disagree with this scoring of Nymphaeales, but recognize that defining an "intermediate" state for the infratectum is a prudent step and indicates that this trait, as well as other palynological characters, is in need of detailed ontogenetic study.

Endexine lamellations—The endexine in *Brasenia* has not been well described or well documented prior to this study. Thanikaimoni (1985) interpreted Ueno and Kitaguchi's (1961) micrographs and described the endexine in *Brasenia* as lamellate. Osborn et al. (1991) regarded these structures as either a component of the intine or as an artifact, while Walker (1976) did not mention the presence of an endexine in *Brasenia*.

In mature pollen, the endexine appears to be lamellate only in the regions of the wall immediately adjacent to the aperture (Fig. 47M). These lamellations are thin and the endexine, as a whole, is not substantially thick. However, from the developmental data it is clear that the endexine is laid down in well-defined plates, both in regions of the wall adjacent to the aperture and in non-apertural regions, initiating during the early-free microspore stage. As the foot layer thickens significantly throughout the early- and middle-free microspore stages, the endexine becomes compressed in the non-apertural wall. By the middle-free microspore stage, the lamellations have become compressed such that they are no longer apparent.

Surface ornamentation—The pollen surface of *Brasenia* has been described as scabrate, indistinct, or shapeless (Snigerevskaya, 1955; Walker, 1976; Osborn et al., 1991). In the present study, however, small cylindrical elements, or rodlets, were consistently observed. The sculptural rodlets correspond to the secondary tectal elements. At the early-free microspore stage, the surface does appear scabrate, because the tectum is composed only of small, primary elements. The rodlets appear later in development, after the secondary tectal elements begin to be deposited in the middle-free microspore stage. Initially, these rodlets are small and sparsely cover the microspore. As development proceeds, the number and size of the rodlets increase such that the non-apertural surface of the mature pollen grain becomes completely covered by rodlets. Rodlets are not present on the mature apertural wall.

The pollen of only two genera of Nymphaeales have significant surface ornamentation, *Nuphar* and *Cabomba*. The surface of mature pollen of *Nuphar* is characterized by large, vertical spines (Ueno and Kitaguchi, 1961; Walker, 1976; Takahashi, 1992), whereas the mature pollen of *Cabomba* has parallel rods that run the length of the grain (Walker, 1976; Osborn et al., 1991). The apertural region of *Cabomba,* however, lacks these parallel rods and is instead characterized by smaller rods that are randomly oriented (Osborn et al., 2003). These apertural rods in *Cabomba* resemble the rodlets found on the surface of mature *Brasenia* pollen.

Tapetal ontogeny—The tapetum in *Brasenia* first becomes distinguishable in the microspore mother cell stage, when the cells are multinucleate and have abundant endoplasmic reticulum. Tapetal cells persist through the tetrad stage and undergo vacuolization in the early-free microspore stage. In the middle-free microspore stage, the tapetal cells begin to

disintegrate, and only a remnant of the tapetum remains by the late-free microspore stage. The cells never become detached from the anther wall and, as such, the tapetum in *Brasenia* is of the secretory type.

The secretory type of tapetum is predominant in land plants and is found in most gymnosperms (Pacini, 1997; Furness and Rudall, 2001 and references therein). All Nymphaealean genera, as well as *Amborella,* have been previously described as having a secretory tapetum, which has been considered the plesiomorphic type within angiosperms (Furness and Rudall, 2001). However, other investigations have indicated the presence of an amoeboid tapetum in *Cabomba* (Osborn et al., 2003) and a cyclic-invasive tapetum in *Nymphaea* (Rowley et al., 1992).

Orbicules, or Ubisch bodies, are small bodies composed of sporopollenin and are almost always associated with a secretory tapetum. Their function is unclear, although they may serve to transport sporopollenin from the tapetum to the developing microspore (Huysmans et al., 1998; Furness and Rudall, 2001) or as an aid in pollen dispersal. This latter function may play an especially important role in wind pollination (Pacini and Franchi, 1993). Orbicules are present in *Brasenia*, but even though *Brasenia* has a secretory tapetum and is wind-pollinated, they occur in small numbers and appear only in the late-free microspore stage.

Conclusion—As a member of Nymphaeales, *Brasenia* is among the most primitive of flowering plants. Because of their position near the base of the angiosperm tree, water lilies are of considerable interest. Despite their utility in phylogenetic analysis, pollen characters, especially ontogenetic characters, are not well known in Nymphaeales. The present study elucidated salient pollen developmental characters of *Brasenia* and reexamined the ultrastructure of the mature pollen wall. Several key pollen characters for *Brasenia*, such as tetrad geometry and infratectum ultrastructure, have been mischaracterized in the literature, and the subsequent scoring of those characters in a range of phylogenetic studies has been impacted. Other characters, such as the presence of endexine lamellae in the non-apertural wall, the granular-like nature of the inner tectum, and the formation of sculptural rodlets, are only revealed upon investigation of the complete developmental sequence. This emphasizes the need for careful and consistent scoring of pollen characters, the importance of including ontogenetic data in phylogenetic analyses, and the re-evaluation of how a range of traditional characters are interpreted in a phylogenetic context.

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